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Experimental and Computational Investigation into Mechanobiology of Osteocytes

Conleth A. Mullen B.E. (2009)

A thesis submitted to the National University of Ireland as fulfilment of the requirements for the degree of Doctor of Philosophy

October 2014

Biomedical Engineering
College of Engineering and Informatics
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Supervisor of Research: Dr. Laoise M. McNamara
Abstract

Osteocytes are terminally differentiated bone cells, derived from osteoblasts, which comprise over 90% of the cells in mature bone tissue and are known to be highly sensitive to extracellular mechanical cues. The effect of extracellular mechanical stimuli on osteoblast differentiation in particular has been well studied, with both mesenchymal and embryonic stem cells shown to differentiate into the osteoblast phenotype when cultured on substrates that mimic the mechanical properties of developing bone tissue, osteoid. However, relatively little attention has been paid thus far to the effects of extracellular mechanics on the differentiation of osteocytes, despite their clear importance to the function of bone tissue as a whole. The aim of this thesis is to uncover the effects of extracellular mechanical stimuli on osteocyte differentiation, and thus inform future bone tissue engineering strategies.

The first study of this thesis examined the effects of passive substrate stiffness and intercellular separation on osteocyte differentiation of the pre-osteoblast MC3T3-E1 cell line. Cells were cultured on Type 1 collagen based substrates of different stiffnesses while seeding density was used to control intercellular separation. It was found that osteocyte differentiation, as measured by morphological analysis, alkaline phosphatase (ALP) activity, substrate mineralisation and gene expression profile, occurred on substrates of approximately 300 Pa at an initial seeding density of $10^3$ cells/cm$^2$. Interestingly, this stiffness was much lower than those previously found to induce osteoblast differentiation.

The second study of this thesis used finite element modelling to examine the isometric stress generated in the cell body when cultured on the substrates investigated in the first study. Cell and substrate mechanical properties were
measured through atomic force microscopy, while cell morphologies and the 
locations of focal adhesion complexes were determined through confocal 
microscopy. The results demonstrated that intracellular tension was influenced by 
cell morphology, focal adhesion location and density as well as substrate stiffness, 
suggesting a role for isometric tension in osteocyte mechanobiology.

The third study presented here investigated the combined effects of substrate 
stiffness, thickness, fibrosity and crosslinking density on cell spread area, a known 
indicator of osteogenic differentiation potential. MC3T3-E1 cells were cultured on 
flat and wedge shaped collagen and polyacrylamide (PA) gels, with stiffness being 
controlled through the polymerisation process of PA gels or 1-Ethyl-3-(3-
dimethylaminopropyl)carbodiimide crosslinking of collagen. It was found that cells 
on the fibrous collagen substrates adopt the same spread area as those cultured on 
much stiffer non-fibrous PA substrates, while a reduction in substrate thickness 
caused an increase in cell area in both collagen and PA substrates. The increase in 
cell area due to thickness was reduced as crosslinking density of collagen substrates 
was increased. Finite element simulations demonstrated that the effective modulus of 
a substrate increased when discrete fibres were included. The effective modulus was 
further increased as crosslinking density was increased and as substrate thickness 
was reduced. Interestingly, the effect of thickness on the effective modulus was 
lessened at higher crosslinking density, offering an explanation for the reduced effect 
of substrate thickness in highly crosslinked collagen. These results suggest that cell 
behaviour is regulated by the modulus of a substrate, as experienced at a local cell 
level, rather than bulk material properties. The results of this study may explain, in 
part, seemingly contradictory results as to the optimal stiffness for the promotion of 
osteogenic differentiation.
The final study of this thesis investigated the combined effects of fluid flow and substrate stiffness on osteocyte differentiation of MC3T3-E1 cells. Cells were cultured on collagen substrates previously shown to induce osteocyte differentiation (Study 1) and subjected to physiologically relevant levels of pulsatile fluid flow after 7 days of static culture. It was found that the application of flow at day 7 of culture followed by a further 7 days of static culture caused an increase in the percentage of cells undergoing osteocyte differentiation, as measured through morphological analysis and ALP activity.

Together these studies provide new evidence that osteocyte differentiation can be induced by culture of pre-osteoblast like cells on soft collagen substrates, provided substrate thickness and heterogeneity are also controlled, and that this differentiation may be initiated through the generation of isometric stress within the cell body. The importance of intercellular separation in osteocyte differentiation was also demonstrated for the first time, while the number of cells undergoing the differentiation process can be further enhanced through the application of fluid flow induced stress. Through the research studies of this PhD Thesis, fundamental information has been uncovered about the differentiation behaviour of osteogenic cells in response to extracellular mechanical cues, and these results can be used to inform future bone tissue engineering strategies.
Publications

The following publications have arisen from the work presented in this thesis:

Journal Article


Journal Articles in preparation

- Mullen CM, Haugh MG, Vaughan TJ, Billiar KL, McNamara LM “The effect of substrate thickness, stiffness and crosslinking density on osteogenic cell behaviour” In preparation for re-submission to Biophysical Journal
- Mullen CM, Haugh MG, McNamara LM “Osteocyte differentiation on soft collagen substrates is enhanced by pulsatile fluid flow” In preparation for submission to Journal of Biomechanics
Conference presentations

Peer reviewed international conferences

- Mullen CM, McNamara, LM “Regulation of Osteocyte Differentiation by Substrate Stiffness and Seeding Density”, Poster presentation at Tissue Engineering and Regenerative Medicine International Society EU meeting, Granada, Spain, June 2011.

Peer reviewed National Conferences

- Mullen CM, McNamara LM “Regulation of osteocyte differentiation by substrate stiffness and seeding density”, Podium presentation at Bioengineering in Ireland 17, Galway, January 2011.
- Mullen CM, McNamara LM “Effect of extracellular mechanical stimuli and intercellular separation on osteocyte differentiation” Podium presentation at Bioengineering in Ireland 18, Belfast, January 2012.
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Finally I would like to thank my parents, and sisters Eithne and Aisling for their confidence and support. I wouldn’t have managed this without you.
# Nomenclature

**Roman Letters**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>U</td>
<td>Velocity</td>
</tr>
<tr>
<td>h</td>
<td>Flow channel height</td>
</tr>
<tr>
<td>b</td>
<td>Flow channel breadth</td>
</tr>
<tr>
<td>Q</td>
<td>Flow rate</td>
</tr>
<tr>
<td>a</td>
<td>Contact radius of AFM tip</td>
</tr>
<tr>
<td>u,v</td>
<td>Displacement vectors</td>
</tr>
<tr>
<td>x,y,z</td>
<td>Deformed point co-ordinates</td>
</tr>
<tr>
<td>p,q</td>
<td>Reference points</td>
</tr>
<tr>
<td>F_{def}</td>
<td>Deformation gradient</td>
</tr>
<tr>
<td>V_0</td>
<td>Reference configuration</td>
</tr>
<tr>
<td>J</td>
<td>Determinant</td>
</tr>
<tr>
<td>L</td>
<td>Spatial velocity gradient</td>
</tr>
<tr>
<td>D</td>
<td>Rate of deformation tensor</td>
</tr>
<tr>
<td>C^{el}</td>
<td>4th order elastic moduli tensor</td>
</tr>
<tr>
<td>E</td>
<td>Young’s modulus</td>
</tr>
<tr>
<td>V</td>
<td>Undeformed volume</td>
</tr>
<tr>
<td>T</td>
<td>Temperature</td>
</tr>
<tr>
<td>N_i</td>
<td>Shape function at node i</td>
</tr>
<tr>
<td>B</td>
<td>Shape function matrix</td>
</tr>
<tr>
<td>D_{stiff}</td>
<td>Stiffness matrix</td>
</tr>
<tr>
<td>K</td>
<td>Element stiffness matrix</td>
</tr>
<tr>
<td>F</td>
<td>Element force vector</td>
</tr>
<tr>
<td>P</td>
<td>Force</td>
</tr>
<tr>
<td>R</td>
<td>Radius</td>
</tr>
<tr>
<td>k_{spring}</td>
<td>Spring constant</td>
</tr>
<tr>
<td>t</td>
<td>Thickness</td>
</tr>
<tr>
<td>d</td>
<td>Length</td>
</tr>
<tr>
<td>E_{eff}</td>
<td>Effective modulus</td>
</tr>
<tr>
<td>Re</td>
<td>Reynolds number</td>
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**Greek Letters**

- $\alpha$  
  AFM tip edge angle
- $\delta$  
  Contact depth/cantilever deflection
- $\varepsilon$  
  Logarithmic strain tensor
- $\rho$  
  Fluid density
- $\sigma$  
  Stress tensor
- $\nu$  
  Poisson’s ratio
- $\alpha_v$  
  Coefficient of expansion
- $\xi, \eta, \mu$  
  Reference geometry co-ordinates
- $\pi$  
  Potential energy
- $\delta$  
  Tip deflection
- $\gamma$  
  Piezo movement
- $\omega$  
  Deflection
- $\alpha$  
  Tip face angle
- $\tau$  
  Shear stress
- $\lambda$  
  Viscosity

**Acronyms**

- AFM  
  Atomic force microscopy
- ALP  
  Alkaline phosphatase
- ANOVA  
  Analysis of variance
- BMSC  
  Bone marrow stromal cell
- BMP  
  Bone morphogenic protein
- BMU  
  Bone multi-cellular unit
- CBFa1  
  Core-binding factor subunit alpha-1
- cDNA  
  Complementary DNA
- Col1A1  
  alpha 1, type 1 collagen
- DAPI  
  4',6-diamidino-2-phenylindole
- DMP-1  
  Dentin matrix protein 1
- DNA  
  Deoxyribonucleic acid
- ECM  
  Extracellular matrix
- EDAC  
  1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>ERK</td>
<td>Extracellular related kinase</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>EVOH</td>
<td>Ethylene vinyl alcohol</td>
</tr>
<tr>
<td>FA</td>
<td>Focal adhesion</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FE</td>
<td>Finite element</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>H₂O</td>
<td>Hydrogen dioxide (water)</td>
</tr>
<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>hFOB</td>
<td>Human foetal osteoblasts</td>
</tr>
<tr>
<td>IFF</td>
<td>Intermittent fluid flow</td>
</tr>
<tr>
<td>IL6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony stimulating factor</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OCN</td>
<td>Osteocalcin</td>
</tr>
<tr>
<td>OFF</td>
<td>Oscillatory fluid flow</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegrin</td>
</tr>
<tr>
<td>OPN</td>
<td>Osteopontin</td>
</tr>
<tr>
<td>OSF-2</td>
<td>Osteoblast specific factor 2</td>
</tr>
<tr>
<td>PA</td>
<td>Polyacrylamide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered solution</td>
</tr>
<tr>
<td>PCM</td>
<td>Pericellular matrix</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PFF</td>
<td>Pulsatile fluid flow</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PHEX</td>
<td>phosphate regulating endopeptidase homolog, X-linked</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>PIPES</td>
<td>Piperazine-N,N’-bis(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>pNPP</td>
<td>p-Nitrophenyl phosphate</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor kappa-B ligand</td>
</tr>
<tr>
<td>RhoA</td>
<td>Ras homolog gene family, member A</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-kinase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real time, Reverse transcription polymeric chain reaction</td>
</tr>
<tr>
<td>Runx2</td>
<td>Runt-related transcription factor 2</td>
</tr>
<tr>
<td>Sost</td>
<td>Sclerostin</td>
</tr>
<tr>
<td>SSF</td>
<td>Steady state fluid flow</td>
</tr>
<tr>
<td>TC</td>
<td>Tissue culture</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N’,N’-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine</td>
</tr>
<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>a-MEM</td>
<td>Alpha-Modified Eagles Medium</td>
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Chapter 1: Introduction

1.1 Bone Mechanobiology and Osteogenic Differentiation

Bone is an adaptive tissue capable of remodelling in response to the loads placed on it through daily physical activity. A commonly cited example is that of the dominant forearm in professional tennis players, which exhibits increased bone density and cross-sectional diameter, compared to the non-dominant arm (Buskirk et al., 1956, Krahl, 1994). The cells located within bone tissue, namely osteoblasts, osteocytes, osteoclasts, bone lining cells and mesenchymal stem cells (MSCs), are known to regulate bone mass and structure, through the bone remodelling process. This process is coordinated through a complex system of gene and protein expression (Rucci, 2008). Specifically, osteoblasts and osteocytes have been shown to be greatly affected by external mechanical stimuli and express factors that regulate the bone formation and bone resorption activities arising in bone tissue under altered loading. This has led many to cite the osteocyte in particular as a mechanosensor of bone tissue capable of initiating a tissue level response to mechanical stimuli (Bonewald, 2007).

Osteoblasts are bone forming cells derived from MSCs, and are also the precursor cells that ultimately differentiate into osteocytes. Osteoblasts have the ability to secrete and mineralise the bone tissue collagen matrix in their surroundings and so are fundamental in providing a strong, stiff tissue. As this mineralisation process is taking place, osteoblasts alter their shape from the cuboidal to one of a small rounded cell body with multiple long thin cell processes extending radially outwards (Cowin, 1989). The gene and enzyme expression profiles of the cell also undergo dramatic changes as they differentiate to become mature bone cells, known as
osteocytes (Cowin, 1989). This process of cell development from MSC to osteoblast and ultimately to osteocyte is termed osteogenic differentiation.

It has been widely shown that MSCs can be directed down the osteogenic pathway through mechanical stimulation, in particular through alterations in extra cellular matrix (ECM) mechanical properties, or control of substrate thickness (Leong et al., 2010), with cells exhibiting the spread morphology and gene and enzyme expression associated with mature osteoblasts. Interestingly, it has been shown that MSCs will differentiate into osteoblasts in 2D culture when grown on substrates of similar stiffness (~ 30 kPa ) to that of developing bone tissue (osteoid) (Engler et al., 2006). Similarly fluid flow induced stress has been shown to increase expression of osteogenic factors in cultured osteoblasts and osteoblast like cell lines (Hung et al., 1996, Donahue et al., 2003, Batra et al., 2005). However, these studies have focussed primarily on the differentiation of MSCs or osteoblast precursors to mature osteoblasts. It remains that the relationship between mechanical stimuli and osteocyte development, i.e. differentiation from osteoblasts to osteocytes, has not yet been established. In fact osteocyte differentiation has yet to be initiated in vitro in the absence of chemical growth factors (such as beta-glycerophosphate, ascorbic acid, dexamethasone).

Osteocytes are the most common cell contained in bone tissue and are thought to be vital for the regulation of bone formation and resorption (Bonewald, 2007, Cowin, 1989). They form junctions at the ends of their processes to generate a strain-sensing network extending throughout the tissue (Lanyon, 1993, Cowin, 1989), while expressing genes to control the behaviour of osteoblasts (Vezeridis et al., 2006), osteoclasts (Heino et al., 2002) and MSCs (Raheja et al., 2008, Birmingham et al., 2012). As such it is crucial for the effect of external mechanical forces on osteocyte
differentiation to be delineated if viable bone tissue engineering therapies and effective treatments for bone disease are to be developed.

One of the most dramatic changes associated with osteocyte differentiation is the morphological development from a spread, cuboidal cell, indicative of an osteoblast, to a small rounded cell expressing multiple long thin cell processes (Cowin, 1989). It is known that these processes facilitate intercellular communication (Civitelli, 2008), but it is not clear whether this is merely the end function of process formation or if processes are actively extended by the cell to form specific junctions with neighbouring cells. It is also plausible that the cell may alter its morphology in order to achieve a more desirable stress state (homeostasis) and that this is the method by which osteocyte differentiation due to ECM mechanics is initiated, but this has not yet been investigated.

1.2 Hypothesis formation

It is well known that extracellular mechanics have an important role to play in osteoblast development. However, as of yet the methods by which extra cellular mechanics dictate osteocyte differentiation are still not understood. The overall goal of this research is to investigate how osteocyte differentiation, a vital aspect of bone formation, is regulated by mechanical cues in the extracellular matrix. To address this, the following hypotheses have been formed, each of which will form the basis for the research described in Chapters 4 to 7.

Osteogenic differentiation of both MSC and ESCs has previously been shown to be affected by alterations in passive substrate stiffness (Engler et al., 2006, Khatiwala et al., 2006b). However, studies to date have focused primarily on osteoblast differentiation and the effect of extracellular mechanics on osteocyte differentiation.
is still poorly understood. Osteoblast differentiation of MSCs has also been shown to be influenced by initial cell seeding density (Kim et al., 2009), as a method of controlling intercellular separation, and this should be accounted for within in vitro experiments. Thus, a primary aim of this work is to test the hypothesis that “Osteocyte differentiation is initiated by substrate stiffness and intercellular separation”.

Dramatic changes in cell morphology accompany the gene and protein expression profile associated with osteocyte differentiation (Mikuni-Takagaki et al., 1995, Kato et al., 1997). However, the reasons for these initial changes in morphology and gene expression are still not known. Recently it has been shown that the morphology of MC3T3-E1 cells, as controlled by substrate patterning, can induce the release of factors associated with osteogenic differentiation (Ruirong et al., 2013). However, it is also known that the same cell line, as well as MSCs, will alter their morphology independently due to passive changes in substrate stiffness in conjunction with increased osteogenic gene expression (Mullen et al., 2013, Engler et al., 2006). Moreover, changes in morphology have previously been linked to intracellular tension in MSCs undergoing osteogenic or adipogenic differentiation (McBeath et al., 2004). This leads to the second primary objective of this PhD research is to test the hypothesis that “The cellular response to extracellular mechanics is mediated through intracellular stress, as the developing osteoblast alters its morphology in order to achieve a more appropriate stress state.”

Although numerous mechanobiology experiments have investigated the importance of substrate material parameters (e.g. substrate stiffness and thickness) for osteogenic differentiation of MSCs and osteoblasts, no single study has examined the effect of these factors in combination with one another. As mentioned previously, substrate
stiffness can have a profound effect on both osteoblast (Engler et al., 2006, Evans et al., 2009) and osteocyte differentiation (Mullen et al., 2013), while the thickness of both collagen and polyacrylamide substrates have also been shown to affect cellular behaviour (Leong et al., 2010, Lin et al., 2010). While substrate crosslinking is commonly used to alter substrate stiffness (Tan et al., 2008, Haugh et al., 2011) and is likely to additionally influence cell behaviour, the precise role of substrate crosslinking in dictating the extracellular mechanical environment of osteoblastic cells has never been investigated. Here it is hypothesised that “Substrate stiffness, thickness and heterogeneity all play a role in regulating cellular response.”

In vivo osteocytes are subjected to a wide range of mechanical stimuli, including changes in passive ECM stiffness and applied mechanical strain, as well as interstitial fluid flow through the lacunae, caused by compression at the tissue level (Cowin, 1989). In vitro studies have shown changes in biochemical signalling in response to various fluid flow stimulation regimes. Effects of this signal expression include the recruitment of MSCs through osteopontin (Raheja et al., 2008) and the inhibition of osteoclast differentiation (Tan et al., 2007). However, the lack of research into the combined effects of ECM stiffness and fluid flow induced shear stress means that the combined stimulation at the cellular level is not fully understood. Given that osteocytes are subject to both stimuli in vivo, it is imperative that their effects are examined in concert. For this reason, this thesis seeks to investigate the combined effect of the mechanical cues that exist in vivo, namely (1) substrate stiffness, representing extracellular mechanical signals imposed by developing bone tissue – osteoid, and (2) extracellular fluid flow, representing the flow of interstitial fluid through the lacunae, on the differentiation of osteocyte cells in vitro. In so doing the hypothesis that “Osteocyte differentiation is enhanced by
1.3 Objectives and Hypotheses

The global objective of this thesis is to add to the understanding of osteocyte mechanobiology through the delineation of the effect of mechanical stimuli on osteocyte differentiation. The primary specific objective of this research is to design a series of experiments to examine the effects of extracellular mechanics on the differentiation of MC3T3-E1 pre-osteoblast cells. The secondary specific objective is to determine whether the intracellular stress state could be a possible driver of osteocyte differentiation. The third specific objective is to derive an understanding of the influence of individual substrate parameters such as stiffness, thickness and crosslinking concentration on osteocyte differentiation. To address these objectives, four hypotheses have been defined, each of which will underpin the research of Chapters 4-7 of this thesis.

Hypothesis 1 - Osteocyte differentiation is initiated by substrate stiffness and intercellular separation.

Hypothesis 2 – The cellular response to extracellular mechanics is mediated through intracellular stress, as the developing osteoblast alters its morphology in order to achieve a more appropriate stress state.

Hypothesis 3 – Substrate stiffness, thickness and heterogeneity all play a role in regulating cellular response
Hypothesis 4 - Osteocyte differentiation is enhanced by fluid flow induced shear stress in addition to controlled substrate stiffness.

By testing each of these hypotheses, the research questions outlined above can be answered and the proposed research will deliver significant advances in the understanding of bone mechanobiology.

In vitro cell culture using the pre-osteoblast MC3T3-E1 cell line will be employed to examine the effects of substrate stiffness and intercellular separation on osteocyte differentiation (Hypothesis 1). Computational modelling will be employed to delineate the relationship between cell morphology and intracellular strain, and to determine whether the change in cell morphology caused by substrate stiffness could be due to the cell attempting to achieve a more desirable stress state (Hypotheses 2). A sloped collagen gelling technique will be used in conjunction with microscale material models to investigate the combined effects of substrate stiffness, thickness and crosslinking density on pre-osteoblast cell behaviour (Hypothesis 3). Finally, a parallel plate flow chamber will be used in conjunction with the optimal culture conditions found to best induce osteocyte differentiation to determine whether osteocyte differentiation can be further increased through the addition of fluid flow induced stress (Hypothesis 4). An improved understanding of the role of extra cellular mechanical cues for osteocyte differentiation may lead to the development of improved bone tissue engineering strategies, as well as the development of novel treatments for osteogenic disease.
1.4 Thesis structure

This thesis comprises the work completed for the duration of the candidates PhD studies. A review of current literature is presented in Chapter 2, providing a more in depth introduction to bone structure and osteogenic cell behaviour, a review of previous experimental studies on osteogenic mechanobiology and a summary of computational modelling of in-vitro cell culture. The theory behind finite element analysis and atomic force microscopy is discussed in Chapter 3. Chapter 4 examines the effect of substrate stiffness and intercellular separation on osteocyte differentiation in order to test Hypothesis 1. Hypothesis 2 is tested in Chapter 5, whereby finite element simulations of cellular behaviour on soft substrates are created. Chapter 6 tests uses a combination of experimental and computational approaches to examine the effects of various substrate parameters on cell behaviour (Hypothesis 3), while the effect of fluid flow induced shear stress in combination with optimal substrate stiffness and intercellular separation is investigated in Chapter 7 (Hypothesis 4). Finally Chapter 8 outlines the main findings of the thesis, places these in the context of the wider field of bone biology and tissue regeneration, and discusses recommendations for future research in the field.
Chapter 2: Literature Review

2.1 Bone

Bone is a strong, adaptable, cellular organ that provides structural support to the body while also fulfilling metabolic functions (Yaszemski et al., 1996). It also enables movement by providing attachment sites for skeletal muscle. The organ’s mechanical strength is attained primarily through the activities of osteoblast cells, which firstly synthesise a collagen matrix and subsequently express calcium phosphate to transform this surrounding acellular tissue, also known as the extracellular matrix (ECM), into a stiff, mineralised structure (Crockett et al., 2011). The ability of the tissue to remodel in response to external stimuli is achieved through the interplay between the various cell types present in the tissue, including mesenchymal stem cells (MSCs), osteoblasts, osteoclasts and osteocytes (Rucci, 2008). Any alteration in the behaviour of these cells can cause the bone to change its structure and therefore alter its mechanical strength and metabolic function.

2.2 Hierarchal structure of bone

Bone can be divided into two distinct types, namely cancellous and cortical. Cancellous bone, also known as trabecular bone, is found on the inside and the ends of long bones such as the femur, tibia, and fibula. It can be distinguished by its high porosity, giving it a mesh-like cross-sectional appearance, as can be seen in Figure 2.1. Cortical bone is a compact and stiffer than cancellous bone, and provides the skeleton with the bulk of the strength necessary to support the body (Augat and Schorlemmer, 2006). Bone marrow, which is located within and protected by cancellous and cortical bone, is a source of mesenchymal stem cells (MSCs), which have the potential to differentiate into many different cell phenotypes, including
adipocytes, osteoblasts and myoblasts (Gimble et al., 1996, Rickard et al., 1996, Ferrari et al., 1998).

**Figure 2.1:** Cortical and trabecular (cancellous) bone in sectioned upper humerus (Fawcett, 2014).

Cortical bone tissue is comprised of small cylindrical elements known as osteons, which are between 150 and 350 $\mu$m in diameter (van Oers et al., 2008). Running down the centre of these structures are small blood vessels known as Haversian canals. Trabecular bone is made up of non-vascular hemi-osteons, which are smaller than the osteons found in cortical bone. Both structures are themselves comprised of concentric lamellae, with those in cortical bone being more organised than those in trabecular bone (Rho et al., 1998). The lamellae themselves are comprised of collagen fibrils of approximately 500 nm thick, while these fibrils are made up of collagen molecules and hydroxyapatite (HA) crystals (Figure 2.2). It is the HA crystals that impart the mechanical stiffness to bone tissue (Augat and Schorlemmer,
2006, Guo, 2001), while the collagen in the structure regulates the ductility of the tissue (Burstein, 1975). Differences have been found in the relative HA/collagen content of the lamellae of cortical and cancellous bone and these differences, along with the differences in structure and organisation, are the reason behind the different mechanical properties between the two bone types (Guo, 2001). The hierarchical structure of bone is visualised in Figure 2.2 below.

**Figure 2.2:** Hierarchal structure of bone, a) Hydroxyapatite (HA) crystals and collagen fibrils, which form b) lamellar structures organised into c) osteons and d) hemiosteons, which comprise e) cortical and trabecular bone (Vaughan et al., 2012).

Within the lamellae are small cavities known as lacunae, each of which contains a single osteocyte, or bone cell. The osteocytes form connections with one another through small canals in the lamellae known as canaliculi. This structure is visualised in Figure 2.3 below.
2.3 Bone cells

Bone cells can be divided into four different types. Osteocytes are mature bone cells, each of which occupies its own individual lacunae. They are thought to be mechanoregulators of bone remodelling and adaptation (Bonewald, 2011a). Osteoblasts are pre-osteocytes and can be thought of as bone forming cells. They deposit and then mineralise a soft collagen matrix in their immediate vicinity. During this process they also undergo changes in their morphology and gene expression profile to become osteocytes. This process is known as osteocyte differentiation. Bone lining cells are inactive osteoblasts, which are located on the surface of the bone. Finally, osteoclasts are multinucleated, bone destroying cells that are extremely large compared to the other cells types present in the tissue. Their role is to resorb bone tissue in regions where remodelling is necessary. Also present in bone
(specifically in the bone marrow) are mesenchymal stem cells (MSCs), which have the potential to differentiate into a wide variety of cell phenotypes.

2.3.1 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are progenitor cells located in the bone marrow. They are characterised by a fibroblast-like morphology with a relatively large nucleus (Jiang, 2002) and are often identified by expression of phenotypic markers such as nestin, CD271 and α-smooth muscle actin (Renny et al., 2012, Bühring et al., 2007). Multiple investigations have determined a wide ranging gene expression profile of the cells during in vitro culture (Silva et al., 2003, Brendel et al., 2005). These cells are pluripotent and have the ability to differentiate into adipogenic, myogenic, neurogenic, chondrogenic, tenogenic or osteogenic cells (Minguell et al., 2001). Differentiation of MSCs along the osteogenic pathway is vital for normal bone biology during formation and bone remodelling. Among the genes involved in the differentiation of MSCs to osteoblasts are Runx2, Dlx5 and the bone morphogenic protein (BMP) family. Runx2 is required for the activation of the differentiation process as well as the maturation of osteoblasts (Hong et al., 2005). Dlx5 is vital for osteogenic development and reduced levels of the gene have been found in MSCs extracted from osteoporotic patients (Prall et al., 2013). BMPs, are members of the Transforming Growth Factor B superfamily of molecules and are heavily involved in osteoblast differentiation (Chen, 2004). Interestingly, it has been shown that BMP treatment can upregulate other osteogenic genes expressed by MSCs, such as RunX2 and osteocalcin (Prall et al., 2013).
2.3.2 Osteoblasts

Osteoblasts are bone forming cells differentiated from mesenchymal stem cells. They are involved in the creation of bone tissue through the excretion and mineralisation of a collagen matrix in their immediate vicinity (Cowin, 1989) and so are vital for the bone remodelling process. They are mononuclear cells, which exhibit a cuboidal morphology and contain a highly developed cytoskeleton, consisting of tensile actin filaments, compressive microtubule elements as well as intermediate filaments and focal adhesion sites, which allow attachment to their surroundings (Pavalko et al., 1998). Osteoblasts express a variety of genes and enzymes associated with the mineralisation of developing bone tissue, including alkaline phosphatase (ALP), osteocalcin and bone sialoprotein (BSP). ALP promotes mineralisation through binding to the mineralisation inhibitor pyrophosphate (Golub, 2007). Osteocalcin is the most abundantly found protein in bone, and is produced by osteoblasts during bone formation (Weinreb et al., 1990). It is also believed to be an inhibitor of excessive mineralisation (Wolf, 1996). BSP, also ubiquitous in bone tissue, is necessary for matrix mineralisation (Chen et al., 1994, Chen et al., 1992). Interestingly, BSP has also been detected in mineralised lesions in other tissues (Waltregny et al., 2000). Other genes involved in the regulation of osteoblast behaviour are Runx2, required for the maturation of osteoblasts (Komori, 1997) and osteoblast specific factor 2 (OSF2), which aids in cell adhesion and migration (Kruzynska-Frejtag et al., 2001, Takeshita and R Kikuno, 1993).

2.3.3 Osteoclasts

Osteoclasts are large multinucleated cells, which are directly responsible for bone resorption (Ash et al., 1980). Although relatively scarce in bone (comprising less than 1% of all bone cells), they are vital for the regulation of bone tissue and skeletal
remodelling. Osteoclasts are formed through the fusion of mononuclear osteoprogenitors, themselves members of the monocyte/macrophage family (Teitelbaum, 2000), through the binding of macrophage colony stimulating factor ((M-CSF) and RANKL to their respective receptors (Udagawa et al., 1990). They typically measure between 20 and 100 μm in diameter (Roodman, 1996) and contain between 5 and 15 nuclei (Blair, 1998). They express large amounts of acid producing proteins that are necessary to dissolve mineralised bone tissue (Ek-Rylander et al., 1991). Osteoclasts are known to interact with other osteogenic cells through gene and protein expression in order to regulate bone remodelling as appropriate. Among these proteins are osteoprotegrin (OPG), a RANKL inhibitor expressed by osteoblasts (Udagawa et al., 2000) and c-Fos, a proto-oncogene, the overexpression of which can impede osteoclast function and lead to the development of osteosarcomas (Sunters et al., 2004).

![Diagram of osteoclast and osteoblast phenotypes](image)

**Figure 2.4:** Development of osteoclast and osteoblast phenotypes (Goldring, 2007). Osteoclasts are derived from hematopoietic precursors while osteoblasts differentiate from MSCs.
### 2.3.4 Osteocytes

Osteocytes are terminally differentiated osteoblasts, characterised by their small rounded cell body most of which is dominated by the nucleus (Cowin, 1989). Long thin cell processes, sometimes known as dendrites, extend from the cell body in an unorganised branched pattern (Bonewald, 2011a). These structures may number between 40 and 60 per cell (Sugawara et al., 2005) and are primarily composed of actin (Tanaka Kamioka et al., 1998). TEM imaging has revealed small protrusions that form integrin based attachments between the osteocyte cell process and the wall of the canaliculae in which they reside (shown in figure 2.5 and 2.6) (McNamara et al., 2009). The cells are surrounded by a stiff mineralised matrix and are immobile when engulfed in this matrix. Intercellular communication is facilitated by the formation of gap junctions and functional synctitia at the ends of the cell processes (Civitelli, 2008). The presence of this interconnected network, and the ubiquitous distribution of osteocytes throughout bone tissue has led many researchers to postulate that these cells function as mechanosensors, which regulate the remodelling of bone tissue (Burra et al., 2010, Aarden et al., 1994). There is some debate over the gene expression profile of developing osteoblasts and osteocytes (Hadjiargyrou et al., 2001, Franz-Odendaal et al., 2006, Schulze et al., 1999). Dentin matrix protein 1 (DMP1) and E11 are expressed as osteoblasts are developing into osteocytes, as shown in Figure 2.7. Both proteins are necessary for cell process formation and are found in greater quantities in the vicinity of the cell processes (Rios, 2005, Schulze et al., 1999). DMP1 has also been shown to increase mineralisation in MSCs cultured in-vitro (Albazzaz and Karthikeyan Narayanan, 2009). Sclerostin is a late stage osteocyte specific gene with expression of the gene
only occurring after cell processes have formed (Winkler et al., 2003). It works to regulate bone formation by interrupting the Wnt signalling pathway (Poole et al., 2005, Li et al., 2005). Research into the behaviour of osteocytes has so far been hindered by virtue their location deep within the mineralised tissue, making primary cell extraction difficult. Further to this, they have been shown to significantly alter their behaviour when cultured in vitro, demonstrating a reduction in cell process number as well as changes in gene expression (Yang, 2009).

**Figure 2.5:** TEM image of osteocyte in lacunae fixed with acrolein method (McNamara et al., 2009). Osteocyte processes can be observed within the canaliculi.
**Figure 2.6:** Image showing protrusions connecting osteocyte processes to canaliculi walls (McNamara et al., 2009)

**Figure 2.7:** Osteoblast to osteocyte development (Bonewald, 2011b) (Bonewald, 2011b). Osteoblasts, derived from MSCs develop into osteocytes.
2.3.5 Osteogenic cell lines

The use of primary osteoblasts and osteocytes for in vitro experiments is limited by the difficulty in extraction from their in vivo environment, although various isolation techniques have been used successfully, such as cell migration (Jones and Boyde, 1977) or chemical digestion (Peck et al., 1964, Nijweide et al., 2003). As well as this the use of primary osteocytes is made more difficult by their inability to proliferate owing to their terminally differentiated nature (Cowin, 1989). This has led to the creation of several immortalised cell lines, which share many of the characteristics of osteoblasts or osteocytes. Among the commonly used osteoblastic cell lines are MC3T3-E1, MG-63 and SaOS-2, each of which have their own advantages and disadvantages.

SaOS-2s are a human osteosarcoma derived cell line that express a wide variety of osteolastic markers, including ALP and osteocalcin expression as well as matrix mineralisation (Rodan et al., 1987). However, the ALP expression of the cells has been shown to vary greatly according to both culture time (Saldaña et al., 2011) and passage number (Hausser and Brenner, 2005), while cell proliferation is also much higher than that of primary osteoblasts (Pautke et al., 2004). MG-63 cells are also a human derived osteosarcoma cell line and have been shown to exhibit a range of osteoblast qualities including osteocalcin and ALP expression when cultured in the presence of 1,25-(OH)_{2}D_{3} (Lajeunesse et al., 1990). However they are subject to the same high proliferation rates as the SaOS-2 cell line (Pautke et al., 2004) while expressing very low levels of ALP (Saldaña et al., 2011) in the absence of 1,25-(OH)_{2}D_{3} and are considered to be arrested in the pre-osteoblast state (Czekanska, 2012). MC3T3-E1s are a murine derived pre-osteoblast cell line, which have been shown to form mineralised nodules when cultured in vitro (Sudo et al., 1983). They
exhibit a similar ALP expression profile to primary osteoblasts (Quarles et al., 1992) and have been cited as an appropriate osteoblast model in bone formation and remodelling studies in particular (Czekanska, 2012).

The MLO-Y4 cell line is a murine derived osteocyte cell line, exhibiting a dendritic morphology similar to that of primary osteocytes, as well as high levels of E11 and low levels osteoblast specific factor 2 (OSF2) and ALP (Kato et al., 1997, Bonewald, 1999, Zhang, 2006). They exhibit lower levels of DMP1 (Stern, 2012) and sclerostin (Papanicolaou et al., 2009) than primary osteocytes, but remain the most commonly studied osteocyte cell line due to their overall similarity with primary osteocytes. MLO-A5 is a recently derived osteocyte cell line cited as being a more immature model of osteocyte behaviour than the MLO-Y4 which has been shown to spontaneously mineralise in culture (Kato et al., 2001).

2.4 In vitro studies of osteocyte biology

2.4.1 Osteogenic cell mechanobiology

Osteogenic cells have been shown to alter their morphology and gene expression profile due to external mechanical stimuli. It is these changes in cell behaviour that demonstrate the differentiation of the cells along the osteocyte lineage. Further to this, behaviours such as adhesion, migration and proliferation have also been shown to be affected by the application of external mechanical stimuli. This interpretation of mechanical stimuli to influence cell behaviour is facilitated by a variety of mechanosensory machinery. The various cytoskeletal components of the cell are vital in this regard. Studies have shown that reorganisation of actin, vinculin and tubulin occurs when osteoblasts are subjected to mechanical stimulus (Li et al., 2007, Liu et al., 2010). It has also been shown that cellular response to mechanical stimuli
is reduced when cytoskeletal components are removed (McGarry et al., 2005b, Pavalko et al., 1998, Meazzini et al., 1998), with cytoskeletal suppressors such as cytocalasin D or Nocadazole being utilised to knock out the relevant cellular machinery. Further to this it has been suggested that cytoskeletal reorganisation is itself a driver of osteogenic differentiation (Higuchi et al., 2009).

The conversion of external mechanical forces into cellular signalling is thought to involve the release of calcium ions from strain controlled ion channels. Indeed it is known that calcium signalling plays a role in driving a variety of cellular functions (Berridge and Bootman, 2000), while the release of calcium and PGE2 has been induced by a variety of applied stimuli (Chen et al., 2003, Ajubi et al., 1999, Lu et al., 2012). In particular the effect of fluid flow induced shear stress on calcium expression in osteoblasts has been studied in great detail (Hung et al., 1996, Donahue et al., 2003).

Integrin attachment proteins such as $\alpha_v\beta_3$, $\alpha_v\beta_5$ and the $\beta_1$ subunit, have been shown to be necessary for the transmission of extra cellular forces into intracellular responses (Lee et al., 2010). $\beta_1$ has been shown to be involved in mechanotransduction in vitro (Litzenberger et al., 2010), while $\beta_1$ knockout mice have been shown to exhibit reduced bone mass as well as irregular canaliculi formation (Zimmerman et al., 2000). $\alpha_v\beta_3$ has been shown to be heavily involved in cell adhesion through its interaction with osteopontin, vitronectin, fibrinogen and other similar adhesive proteins (Ross et al., 1993, Wozniak et al., 2000).

Primary cilia are microtubule based structures that extend into the extracellular space and have been shown to be involved in regulating cellular response to fluid flow induced shear stress (Anderson et al., 2008, Hoey et al., 2012, Malone et al., 2007).
They are present in a variety of cell types, including MSCs, endothelial cells and osteocytes, and are limited in number to a single cilium per cell (Satir et al., 2010). They consist of nine microtubule doublets organised in a radial pattern and are believed to convert mechanical signals into cellular response through the presence of ion channels around the cilium base (Satir et al., 2010). It has been shown that the removal of primary cilia from MC3T3-E1 cells results in reduced levels of PGE₂ and osteopontin in response to fluid flow, while their removal from MLO-Y4 cells similarly inhibits OPG/RANKL and Cox2 expression in response to flow (Malone et al., 2007).

2.4.2 Effect of ECM mechanical properties on osteogenic cell development

Osteogenic cells are strongly affected by mechanical stimuli such as substrate stiffness, as demonstrated by Khatiwala et al through the culture of MC3T3-E1 osteoblasts on type 1 collagen coated polyacrylamide gels (Khatiwala et al., 2006a). The stiffness of the substrates was adjusted by changing the percentage of bis-acrylamide in the underlying PA gel, similar to an earlier study by Pelham and Wang (Pelham and Wang, 1997). An increase in stiffness from 12 to 39 kPa was found to increase the migration speed of the MC3T3-E1 cells and to increase the number of focal adhesions present between the cell and substrate. Von Kossa staining was also carried out and revealed that the stiffest substrate (in this case 39 kPa), resulted in the largest percentage mineralised substrate area, indicating the highest level of osteogenic differentiation.

The differentiation of MSCs into various lineages based on substrate stiffness was investigated by Engler et al (Engler et al., 2006). Cells were cultured on substrates of three different stiffnesses; 1 kPa, 11 kPa, and 34 kPa respectively, using identical media in each case. Cells cultured on the softest substrates displayed a branched
morphology, those on the intermediate stiffness displayed a spindle like morphology, while those on the stiffest substrate displayed a spread morphology similar to that of osteoblasts. The differentiation profiles were confirmed by cells on the soft, intermediate and stiff substrates showing an upregulation (between 4 and 6 fold) in neurogenic (β2tub), myogenic (MyoD, Desmin) and osteogenic (osteocalcin, Cbfα1) specific markers respectively. One of the most interesting aspects of this study was that the MSCs differentiated into brain cells when cultured on stiffnesses similar to that of brain tissue (Engler et al., 2006, Flanagan, 2002), those cultured on stiffnesses similar to muscle tissue differentiated into muscle cells, while those cultured on stiffnesses similar to unmineralised osteoid differentiated into osteoblasts (Engler et al., 2006, García and Reyes, 2005).

Evans et al performed similar experiments using embryonic stem cells (Evans et al., 2009) culturing cells on type 1 collagen coated polydimethylsiloxane (PDMS) substrates. It was found that mineralisation and relative expression of osteopontin and Runx2 increased as substrate stiffness was increased from 41 kPa to 2.7 MPa. It is interesting to note these stiffness levels are up to orders of magnitude higher than those reported to induce osteogenic differentiation in MSCs and osteoblastic cell lines. It is also in apparent disagreement with work by Kong et al., which showed that the osteoblast differentiation of MC3T3-E1 cells increased as substrate stiffness was reduced from 110 kPa to 20 kPa (Kong et al., 2005). Subtle differences in substrate chemistry could be responsible for the disagreement in results present in the literature. This is perhaps best illustrated in the work of Rowlands et al (Rowlands et al., 2008). The osteogenic differentiation of MSCs on type 1 collagen, as examined through expression of Runx2, increased with substrate stiffness from 0.7 kPa to 80 kPa. However, when cultured on type 4 collagen, fibronectin or
Laminin 1, expression peaked on substrates of 25 kPa. This indicates that if the effect of stiffness on osteogenic differentiation is to be fully understood, other substrate parameters such as surface chemistry must either remain constant, or be investigated themselves as regulators of differentiation.

The effects of ECM stiffness have also been shown to affect osteoblast behaviour in three dimensional culture. Haugh et al showed cell proliferation into crosslinked collagen scaffolds to be increased on softer scaffolds using either 1-ethyl-3-3-dimethyl aminopropyl carbodiimide (EDAC) or dehydrothermal crosslinking methods (Haugh et al., 2011). An increase in crosslinking density has also been shown to increase osteogenic differentiation as examined through OCN and OPN expression (Chen et al, 2006), although scaffold stiffness was not measured in this case. Osteoblast differentiation of MC3T3-E1 cells, as examined by ALP and calcium phosphate expression has also been shown to be highest on stiffer PEG scaffolds (225 kPa compared to 12 kPa) (Chatterjee et al., 2010). This is in contrast to the results of Keogh et al, who showed higher levels of OPN and OCN on softer scaffolds (0.48 kPa, compared to 1.17 and 1.37 kPa) (Keogh et al, 2010). Interestingly, these values are well below those found to encourage osteocyte differentiation in 2D (between 25 and 40 kPa).

2.4.3 Experimental approaches to alter the ECM stiffness

One of the most common methods of altering ECM stiffness is by coating cytotoxic polymers of tuneable stiffness with cell adhesive ligands such as collagen (Engler et al., 2006, Evans et al., 2009), laminin (Rowlands et al., 2008) and fibronectin (Rowlands et al., 2008, Altmann et al., 2011). The ease with which the stiffness of polymers such as polyacrylamide (Engler et al., 2006) and PDMS (Evans et al., 2009) can be altered makes them ideal materials for mechanotransduction
experiments when coated appropriately. Both biological and non-biological materials can also be crosslinked by a variety of methods, including chemical dehydrothermal crosslinking and exposure to ultraviolet light (Haugh et al., 2011, Keogh et al., 2010b, Tan et al., 2008, Bian et al., 2013).

A method of applying direct strain to cells in culture, consisting of a four point bending device capable of stretching/relaxing cell seeded (with rat BMSCs in this case) was developed by Qi (Qi et al., 2008), shown in Figure 2.8 below. However, the use of non-linearly elastic substrates (which comprise the vast majority of biological substrates) allows devices such as this to also allows for the control of the passive stiffness of the substrate. As shown in Figure 2.9, the relationship between stress and strain in a range of commonly used substrates can change dramatically according to the level of strain applied. In this way the application of a strain to these substrates prior to cell culture can alter the stiffness as experienced by the cells without changing the surface chemistry or ligand density.

**Figure 2.8:** Four point bending apparatus for substrate loading, adapted from Qi (Qi et al., 2008). Bending load applied to the substrate induces tension in the substrate.
Figure 2.9: Stress-strain relationships for a range of biological substrates. Data taken from (Lee et al., 2013) (Polyacrylamide), (Ge et al., 2012) (Gelatin) and (Kinugasa, 2012) (Collagen).

The effect of substrate stiffness on cell behaviour can also be altered by changing the thickness of the substrate. MSCs cultured on collagen coated PA gels of 0.5 mm, have been shown to exhibit the same spread morphology as those cultured on collagen substrates of 34 kPa, whereas those cultured on thicker substrates (2 mm) of identical composition show similar results to those cultured on 1 kPa collagen gels (Amnon Buxboim and Discher, 2010). The effect of substrate thickness as a modulator of cell mechanotransduction has been further investigated by others (Maloney et al., 2008, Rudnicki et al., 2013), while it has also been shown to affect cells on a larger length scale (Leong et al., 2010). The work of Leong et al is of particular interest as it demonstrates the use of gel thickness as a driver of osteogenic
differentiation over 14 days of culture rather than just of cell spreading over a relatively short time scale. The effect of reduced substrate thickness can be explained through the induction of displacements in the substrate by the cell, which can extend through to be resisted by the underlying coverslip (usually glass or plastic), and so the method allows for investigation into the role of substrate stiffness in cell behaviour without any alterations in substrate composition. The difficulty with the method lies in the manufacture of very thin gels in a reproducible manner. Microbeads have been widely used to accurately control substrate thickness (Amnon Buxboim and Discher, 2010, Lin et al., 2010). However, the beads themselves are many orders of magnitude stiffer than typical biological substrates and as such can influence the stiffness experienced by the cells in the same way. Recently wedge shaped gels have been used to vary substrate thickness along its length (Merkel et al., 2007, Rudnicki et al., 2013), showing that cell area, stiffness and traction all increased as substrates became thinner. However, this method is limited in that cell population markers such as gene expression cannot be analysed.

2.4.4 Effect of fluid flow on osteogenic cell development

Osteocytes in vivo are subjected to fluid flow through the lacunae, which applies stress to the cells. Much research has been conducted into understanding the effect of fluid flow on bone cell behaviour. Various flow regimes, including steady state (SSF), intermittent (IFF), oscillatory (OFF) and pulsatile (PFF) fluid flow, have all been simulated in order to examine their effect on osteogenic cells in vitro. As well as this changes in both the frequency (where applicable) and flow rate can have a substantial effect on cellular response. Fluid flow studies necessitate the design of appropriate bioreactor systems where the shear stress can be controlled through both the initial design and the applied fluid flow rate. A commonly used design is the
parallel plate bioreactor, shown in Figure 2.10, where fluid is passed through a thin channel over a cell monolayer (Hochmuth and N. Mohandas, 1973). Flow is kept laminar provided the Reynolds number, calculated from equation 2.1, is kept below 1000 (Panton, 2013).

\[ Re = \frac{U h \rho}{\mu} \]  \hspace{1cm} (2.1)

where \( U \) is the fluid velocity, \( h \) the channel height and \( \rho \) and \( \mu \) the density and viscosity of the fluid respectively. Due to the height of the monolayer being many times less (usually 1-2 orders of magnitude) than that of the flow channel, the shear stress at the channel wall is taken as equal to that which is exacted on the cells, and can be calculated from equation 2.2, where \( Q \) is the volumetric flow rate and \( b \) is the channel breadth.

\[ \tau = \frac{6Q\mu}{bh^2} \]  \hspace{1cm} (2.2)
**Figure 2.10:** Syringe pump controlled parallel plate flow chamber system. Flow is controlled through Alladdin pump system (World Precision Intruments).
Figure 2.1: Spinning flask bioreactor and Rotating wall bioreactor. Adapted from (Martin et al., 2004). Bioreactors generate force through motion or simulation of microgravity.
Osteoblasts have been shown to alter their morphology, ALP expression and proliferation in response to 30 minutes of steady state fluid flow (Kapur et al., 2003), while after an hour of flow at 20 dynes/cm² substantial cytoskeletal reorganisation occurs (Liu et al., 2010), with cells elongating in the direction of flow. Further to this, a variety of flow patterns, including SSF, OFF and IFF have been shown to provoke an immediate calcium expression response in osteogenic cells (Hung et al., 1996, Donahue et al., 2003, Batra et al., 2005).

While it has been established that SFF fluid flow plays a role in osteoblast maturation, it has also been demonstrated that process formation in the osteocyte like MLO-Y4 cell line is upregulated in response to OFF compared to static controls, whereas no statistical upregulation was observed when cells were subjected to SFF (Ponik et al., 2007), indicating that OFF may be a more potent driver of osteocyte differentiation. The same study found that Cox2 and osteopontin activity were
equally upregulated in response to both SFF and OFF. However, it has also been shown that human foetal osteoblasts (hFOBs) are more sensitive to steady state or pulsatile flow than OFF (Jacobs, 1998).

MSCs exposed to OFF have been shown to respond with an increase in proliferation, mediated through the upregulation of calcium and extracellular signal regulated kinases (ERKs) (Riddle et al., 2006). OFF has also been shown to lead to highly increased levels of osteogenic factor expression (65% increase in osteopontin and 44% increase in osteocalcin) in MSCs when compared to static controls (Li et al., 2004). However, the cells experienced no change in collagen type 1, \( \alpha_1 \) (Col1A1) activity and a decrease in ALP activity, both indicators of osteoblast maturation, indicating that although MSCs may be directed towards osteogenesis by OFF, other influences are needed to ensure cells progress along the osteogenic differentiation pathway.

It is generally accepted that osteocytes in vivo are subjected to a combination of steady state and pulsatile flow (Jacobs, 1998). It has been shown that pulsatile fluid flow (PFF) induces an increase in ALP activity in osteoblasts. Interestingly, pulse frequency had no effect on ALP expression, while mineralisation was found to be unaffected by fluid flow, indicating that pulsatile flow alone is not sufficient to induce osteocyte differentiation. Other studies involving pulsatile flow have involved the co-culture of osteocytes and osteoblasts, and these have observed that PGE\(_2\) expression was upregulated in response to PFF (Vezeridis et al., 2006, Klein-Nulend et al., 1995). The release of PGE\(_2\) by osteocytes initiates a signalling cascade in both osteoblasts and osteoclasts regulating bone resorption and deposition (Kawaguchi, 1995).
The use of bioreactor systems in 3 dimensional culture has also been investigated with spinner flask, rotating wall and flow perfusion bioreactors being the most widely used. A spinner flask bioreactor is characterised by a rotating arm or stirrer located in the cell media (see Figure 2.11) (Sikavitsas et al., 2002). They have been shown to induce increased ALP, osteocalcin, Col1A1 and Runx2 in human MSCs (Wang et al., 2009b, Stiehler et al., 2009). A rotating wall bioreactor (see Figure 2.11) operates on a similar principle with two concentric walls moving relative to one another to induce a microgravity environment in the scaffold (Schwarz et al., 1992). Increases in expression of Col1A1 and osteocalcin have also been reported in MC3T3-E1 cells exposed to shear stress through the use of a rotating wall bioreactor (Kasper, 2007), while ALP activity and mineralisation have been induced in hMSCs through the use of similar devices (Zhang et al., 2009). In both cases care must be taken to ensure scaffolds are kept separate from moving reactor parts. Both spinning flask and rotating wall bioreactors must also include a mechanism for the exchange of media to prevent the build-up of metabolic waste and ensure cell viability through the provision of nutrients. It has also been claimed that both designs fail to achieve the necessary perfusion of nutrients into the centre of the scaffold (Yeatts and Fisher, 2011). Flow perfusion bioreactors (see Figure 2.12) involve the propulsion of fluid through the scaffold, usually by means of a mechanical pump and so achieve greater infiltration into the scaffold centre and provide for easier replenishment of growth media, through the use of media reservoirs. Intermittent SFF applied through a perfusion bioreactor has been shown to increase osteocalcin expression in primary osteoblasts (Goldstein, 2004). Interestingly, this study found that flow magnitude, which was varied between 0.36 and 2.7 dynes/cm² had no effect on osteocalcin expression.
The effect of scaffold properties on cellular response to fluid flow must also be scrutinised. Partap et al subjected MC3T3 cells seeded in collagen-GAG scaffolds to steady state and intermittent fluid flow and found that Col1A1, osteopontin and ALP were increased when rest periods were included in the flow pattern (Partap S, 2009). Perfusion bioreactors have also been shown to induce osteogenesis in cells cultured on ethylene vinyl alcohol (EVOH), polycaprolactone (Gomes et al., 2003), titanium fibre (Holtorf et al., 2005) as well as calcium phosphate scaffolds (Janssen et al., 2006). Comparing these studies would indicate that fluid flow induced stress may be a driver of osteogenic differentiation independent of scaffold structure. However, given that scaffold mechanical properties have been shown to affect osteoblast differentiation without fluid flow induced shear stress, the effect of the stimuli in combination cannot be ignored. As of yet effects of mechanical stiffness and fluid flow in concert on osteoblast differentiation have not been investigated.

It must be noted that the majority of work thus far has investigated osteoblast differentiation and the effect of fluid flow on osteocyte development is very poorly understood. A dialysis bioreactor was shown to regulate the differentiation of MC3T3-E1 cells into osteocyte like cells, expressing specific gene markers such as DMP1 and E11 as well as trace expression of Sost (Krishnan et al., 2010). The fact that this upregulation of genes could only be achieved with the addition of osteogenic growth factors over an extended time (10 months) could be seen as a limitation as some have questioned the viability of osteogenic growth factors for use in future tissue engineering applications (Rowlands et al., 2008). However, this study does point to an exciting potential for the use of fluid flow induced stress in the promotion of osteocyte differentiation.
2.5 Computational Modelling of Biological cells

2.5.1 FE modelling of in-vitro experiments

Recent advances in finite element methods have led to the method being applied to derive an understanding of biological phenomena, including cell mechanics. As discussed in section 2.2.2, cells are not homogenous but are in reality composed of numerous smaller structures. Including all cellular geometry in finite element models is computationally expensive. As well as this, many cell sub-structures have a negligible effect on overall cellular mechanics.

Early modelling techniques combined approximations in morphology with homogenous elastic material properties to examine the strains generated in osteocytes in vivo (McCreadie, 1997). More recently linear elastic cell models have been further developed to examine inhomogeneities in modulus throughout the cell body (Charras and Horton, 2002), the influence of structural inhomogeneities on cell response to fluid flow (Ferko et al., 2007) and the effect of the nucleus on the cell adherence (McGarry et al., 2005c). More recently, an elastic cell material model was used in conjunction with realistic cell geometries to examine the response of endothelial cells to fluid flow (Dailey et al., 2009).

However, it must be noted that cells do not behave as linear elastic materials, but rather as highly complex (Weiss, 1962), time-dependant (Pravincumar et al., 2012, Canetta, 2005, Mills, 2004, Darling et al., 2008), non-linear (Kang et al., 2008, Mills, 2004) structures, which are also capable of altering their mechanical properties as part of the differentiation process (Bongiorno et al., 2013). Therefore viscoelasticity has been introduced by Karcher et al, who reported a superior prediction of cellular response lag through the use of a Maxwell continuum model.
over that of a Kelvin-Voigt equivalent (Karcher et al., 2003). Further to this, an 
upper-convected Maxwell model, used to describe large scale viscoelastic 
deformations was used to describe the recovery of chondrocytes subject to 
micropipette aspiration (Trickey et al., 2006). Viscoelastic models have also been 
used in combination with micropipette aspiration to determine the viscoelastic 
properties of valve interstitial cells (Zhao et al., 2009). Milner et al, also used a 
Maxwell model with instantaneous and equilibrium moduli of 6.5 kPa and 4.3 kPa 
respectively to examine an osteoblast on a membrane undergoing cyclic strain 
(Milner et al., 2012).

The effect of the nucleus on the compressive behaviour of endothelial cells was 
investigated by Caille et al, using an axisymmetric Mooney-Rivlin model (Caille et 
al., 2002). Meanwhile, the deformation of osteoblasts under fluid flow (Vaughan et 
al., 2013) has been investigated using a Neo-Hookean material model, a method also 
used by Bidhendi et al, who claimed it necessary for the study of large scale cellular 
deformation such as those experienced during micropipette aspiration (Jafari 
Bidhendi and Korhonen, 2012). However, recent work by Reynolds et al has shown 
that an active material model is necessary for accurate predictions of cellular 
behaviour under such high levels of strain (Reynolds et al., 2014). Although it is 
accepted that the use of a continuum model is sufficient in many cases, the inclusion 
of individual cellular components such as the cell nucleus, membrane or 
cytoskeleton is necessary where such structures are of interest to the final solution 
(Zhu et al., 2000). The cell nucleus is the most common individual structure to be 
included, with the nucleus stiffness usually being cited as between 4 and 10 times as 
stiff as the remainder of the cell body (McGarry et al., 2005c, McGarry and 
McHugh, 2008, Vaughan et al., 2013). A further complication was addressed by
Vaziri et al, who found that the individual components of the nucleus, the nuclear envelope and nucleoplasm, resulted in the distribution of forces throughout the nucleus due to the bending and stretching behaviour of the nuclear envelope (Vaziri, 2006).

McElfresh et al have used a variation on the Canham-Helfrich approach, which describes the bending behaviour of fluid filled membranes (Canham, 1970, Helfrich, 1973) to simulate the bending behaviour of the cell membrane (itself a composite layered structure) and how this affects the force generated in the cell when subjected to AFM indentation (McElfresh, 2002, Rudd, 2001). A difficulty occurs in validating such models as the mechanical properties of individual cellular components such as the cell membrane and nucleus are only beginning to be understood (Ovalle-García et al., 2011, Caille et al., 2002). Interestingly, Karcher et al have demonstrated the negligible effect of the cell membrane on overall cell response (Karcher et al., 2003), although this may be as an artefact of the Hertzian model used in corroborating AFM experiments.

A further complication is the introduction of tensile and compressive cytoskeletal elements into the cell body. A cell model developed by McGarry et al. including a separate nucleus, membrane and cytoplasm as well as compressive microtubule and tensile actin elements (shown in figure 2.13), was used to examine cellular response to substrate strain and fluid flow. Such an approach was also used by Jean (Jean and Christopher S. Chen, 2005) and has been expanded by Bursa to include a larger number (120) of discrete elements, more akin to a cell during in-vitro culture (Bursa and Fuis, 2010). While there is no doubt that cytoskeletal elements greatly affect cell stiffness (Wang et al., 1993) and response to mechanical stimulation (Heidemann and Stefanie Kaech, 1999), the internal cytoskeleton of osteogenic cells is far more
developed than other eukaryotic cells. This makes the inclusion of a realistic discrete cytoskeleton computationally impossible, while also improving the accuracy of a continuum approximation.

![Finite element model of adherent cell including; nucleus, cell body, cell membrane; actin filaments and microtubules. Model is split for display purposes (McGarry et al., 2005a).](image)

**Figure 2.13:** Finite element model of adherent cell including; nucleus, cell body, cell membrane; actin filaments and microtubules. Model is split for display purposes (McGarry et al., 2005a).

Perhaps one of the most interesting recent developments in the field is the introduction of an active material model (Ronan et al., 2012). Stress fibre formation was initially introduced in response to an exponentially decaying signal and maintained through the tension present in the system. Strain was then introduced to the system based on the stress fibre formation through a Hill-like equation. This material model has been used to investigate cell spreading, compression (Ronan et al., 2012), cyclic strain application (Dowling et al., 2013), shear force application (Dowling et al., 2012) and AFM compression (Weafer et al., 2013), with results showing improved adherence to experimental results compared to previous passive hyperelastic material models.
2.5.2 Simulating cell-ECM interaction

The interaction of the cell with its ECM also poses challenges for the construction of computational models. The first challenge when modelling cell-ECM interactions is the inclusion of focal adhesions. They are themselves complex structures, consisting of numerous adhesion proteins and integrin receptors connected to actin stress fibres as shown in Figure 2.14. However, in most cases their size is orders of magnitude smaller than the cell as a whole, enabling them to be modelled as discrete attachment points or regions. In the model shown in Figure 2.14, the connection points of the microtubule and microfilament elements acted as focal adhesions (McGarry et al., 2005a). This was suitable in that specific model, as the aim of the study was to observe cytoskeletal re-organisation. However, in a model where the cytoskeleton or focal adhesions are not the area of interest, focal adhesions can be modelled as nodal attachments between the cell and it surroundings along the majority, or all, of the cell-substrate interface. Charras et al., assumed continuous adherence of the cell along the entire interface in order to predict cellular stress distribution in response to cyclic substrate strain (Charras and Horton, 2002). The same method was later used by Deguchi et al. to investigate the effects of cell material properties such as stiffness and prestress on cell height (Deguchi et al., 2009).
**Figure 2.14:** Schematic of focal adhesion complex composed of actin stress fibre, focal adhesion proteins and ligands present in the ECM.

The generation of node sets to act as discrete focal adhesion sites has been used (Mack et al., 2004) to examine force distribution across focal adhesion sites, with nodes on the cell-substrate interface outside these sets left to translocate horizontally. A similar, though not identical method was employed by Milner et al, to examine osteoblast response to cyclic substrate strain, where discrete stress fibres in the cell were attached as pin constraints to allow for only rotational motion at the point of attachment (Milner et al., 2012). The inclusion of specific focal adhesion in endothelial cell models subjected to shear stress caused increases in stress of up to 100 fold in the immediate vicinity of the adhesion sites (Ferko et al., 2007). While these results demonstrate that focal adhesion sites have a key role to play in the distribution of intracellular force, it must be noted that, as is the case with other subcellular components, their inclusion in finite element models is dependent on the region of interest of the model.
A Xu-Needleman cohesive zone has been used to model the cell-substrate interface behaviour during cyclic deformation of the substrate, with zone material properties such as characteristic length and the strength of bonds chosen based on previous literature (McGarry et al., 2005c). This model was further developed in subsequent work by the authors to demonstrate that the adhesion strength between cell and substrate depends on both adhesion strength and cell stiffness. (McGarry and McHugh, 2008). This same study showed that similar total adhesive strength was reported when discrete focal adhesion sites were included; however it must be assumed that the presence of discrete attachment sites leads to stress concentrations in their immediate vicinity.

In order to fully understand how the various organelles and structures on the cell-ECM interface come together to affect cell behaviour, all influential cellular organelles and structures should be included. However, this is made computationally impossible by the size differences present throughout the different intercellular components. As is the case when modelling the cell itself, the level of detail included must be tailored around the particular area of interest of the simulation.

2.6 Summary

Chapter 2 has presented a detailed overview of bone; its structure, composition and function, with particular attention paid to the behaviour of osteogenic cells. To summarise, bone is a highly adaptive tissue, capable of altering its structure in response to the load placed upon it. These loads are perceived by osteocytes, the most abundant cell type in bone, while the response of the tissue as a whole is governed by the interplay between all cell types present. As such an understanding of the differentiation of these cells is of vital importance if viable bone tissue engineering techniques are to be developed for the replacement of tissue lost through
disease or trauma. The studies referenced in this chapter demonstrate the effects that mechanical stimuli, both passive differences in ECM stiffness and externally applied fluid flow, have on osteogenic differentiation. However, it is also shown that the effects of mechanical stimuli of osteocyte differentiation is very poorly understood at present.

To address this, chapter 4 of this work investigates in detail the effects of both substrate stiffness and intercellular separation distance on osteocyte differentiation. Chapter 5 uses finite element analysis to examine the intercellular stress generated as osteoblasts undergo the morphological changes associated with osteocyte differentiation. Chapter 6 uses a combination of experimental and computational techniques to examine the role of the substrate in MC3T3-E1 behaviour in greater detail, and thus further develops the understanding of osteocyte differentiation gained from the results of Chapter 4. Finally, Chapter 7 investigates the combined effects of substrate stiffness and fluid flow induced stress on osteocyte differentiation, by subjecting MC3T3-E1 cells to physiologically relevant fluid flow when cultured on substrates previously shown to induce early osteocyte differentiation in MC3T3-E1s.
Chapter 3: Theory

3.1 Computational modelling

Computational modelling techniques have been developed to investigate the mechanics of materials and structures too complex to otherwise be understood. Biological cells and biomaterials are complex in terms of both material properties and structure, and as such several computational approaches have been utilised to provide an enhanced understanding of their mechanical behaviour, which is often challenging to characterise using experimental or analytical approaches. In this work, computational methods are used to investigate the effects of ECM stiffness on the mechanical stimulation experienced by osteogenic cells (Chapter 5), in particular investigating the role of cell morphology and attachment to the substrate. Computational methods are also applied to investigate the mechanical behaviour of biomaterial substrates themselves (Chapter 6), in particular investigating the role of fibres and cross-linking in the determination of local substrate mechanics. This section outlines the theory behind the computational methods applied in Chapters 5 and 6 of this thesis.

3.1.1 Continuum mechanics

The finite element (FE) analyses in this thesis are based on “Large deformation kinematics”, which describes the deformation of a reference configuration into a current or deformed configuration. As shown in Figure 3.1, the location of a point in the reference and deformed configurations are given by the vectors $\mathbf{x}$ and $\mathbf{y}$ respectively. Thus the displacement vector, $\mathbf{u}$, is given by Equation 3.1 and the velocity vector, $\mathbf{v}$, by Equation 3.2.
\[ \mathbf{u}(x, t) = \mathbf{y}(x, t) - \mathbf{x} \]  
(3.1)

\[ \mathbf{v}(x, t) = \frac{d\mathbf{u}}{dt} \]  
(3.2)

The deformation occurring between the reference and deformed configurations is calculated by relating the distance between two points (p and q in Figure 3.1) in the two configurations according to Equation 3.3.

\[ \mathbf{F}_{def} = \frac{d\mathbf{y}}{d\mathbf{x}} \]  
(3.3)

Where \( \mathbf{F}_{def} \) is the deformation gradient and \( d\mathbf{x} \) and \( d\mathbf{y} \) are the distance between the points in the reference and deformed configuration respectively.

*Figure 3.1* Finite deformation kinematics showing the displacement and deformation of a reference configuration, \( V_0 \) (Fagan, 1992)
It follows that the total volume change from referenced to deformed configuration is given by $J$, the determinant of the deformation gradient.

$$J = \det(F_{def})$$  \hspace{1cm} (3.4)

The spatial velocity gradient, $L$, can also be derived from the deformation gradient according to Equation 3.5.

$$L = dF_{def} \cdot F_{def}^{-1} = \frac{dv}{dy}$$  \hspace{1cm} (3.5)

Following on from this, the symmetric rate of deformation tensor, $D$, can be derived from the spatial velocity gradient through equation 3.6.

$$D = \text{sym}(L) = \frac{1}{2}(L + L^T)$$  \hspace{1cm} (3.6)

$D$ can then be integrated with respect to time to give the logarithmic strain tensor, $\varepsilon$, the most commonly used measure of finite strain.

$$\varepsilon(t) = \int_{0}^{t} D dt$$  \hspace{1cm} (3.7)

A linear elastic material model is used to describe a material whose stress strain relationship does not depend on the rate of deformation. In a linear elastic model the relationship between stress and strain is described by the generalised Hooke’s law, shown in Equation 3.8.

$$\sigma = C^{el} \varepsilon$$  \hspace{1cm} (3.8)

Where $\sigma$ is the stress tensor, $\varepsilon$ is the strain present in the material and $C^{el}$ is the fourth order tensor of elastic moduli. In a linear elastic material $C^{el}$ is not affected by
\( \varepsilon \), the strain tensor. However, \( \sigma \), the Cauchy stress is affected by material
deformation as it is defined as the force per unit area placed on the material in the
deformed configuration. The three dimensional Cauchy stress tensor is shown in
Equation 3.9.

\[
\sigma_{ij} = \begin{bmatrix}
\sigma_{11} & \sigma_{12} & \sigma_{13} \\
\sigma_{21} & \sigma_{22} & \sigma_{23} \\
\sigma_{31} & \sigma_{32} & \sigma_{33}
\end{bmatrix}
\] (3.9)

This tensor is symmetric (\( \sigma_{ij} = \sigma_{ji} \)), which is also true of the strain tensor, shown in
Equation 3.10.

\[
\varepsilon_{ij} = \begin{bmatrix}
\varepsilon_{11} & \varepsilon_{12} & \varepsilon_{13} \\
\varepsilon_{21} & \varepsilon_{22} & \varepsilon_{23} \\
\varepsilon_{31} & \varepsilon_{32} & \varepsilon_{33}
\end{bmatrix}
\] (3.10)

The symmetry present in these tensors allows Hooke’s law to be written in matrix
form as follows in Equation 3.11.

\[
\begin{bmatrix}
\sigma_{11} \\
\sigma_{22} \\
\sigma_{33} \\
\sigma_{23} \\
\sigma_{31} \\
\sigma_{12}
\end{bmatrix} =
\begin{bmatrix}
C_{11} & C_{12} & C_{13} & C_{14} & C_{15} & C_{16} \\
C_{21} & C_{22} & C_{23} & C_{24} & C_{25} & C_{26} \\
C_{31} & C_{32} & C_{33} & C_{34} & C_{35} & C_{36} \\
C_{41} & C_{42} & C_{43} & C_{44} & C_{45} & C_{46} \\
C_{51} & C_{52} & C_{53} & C_{54} & C_{55} & C_{56} \\
C_{61} & C_{62} & C_{63} & C_{64} & C_{65} & C_{66}
\end{bmatrix}
\begin{bmatrix}
\varepsilon_{11} \\
\varepsilon_{22} \\
\varepsilon_{33} \\
\varepsilon_{23} \\
\varepsilon_{31} \\
\varepsilon_{12}
\end{bmatrix}
\] (3.11)

where (\( \sigma_{ij} = \sigma_{ji} \)), (\( \varepsilon_{ij} = \varepsilon_{ji} \)), etc. For isotropic materials, such as is assumed in Chapter
5 of this Thesis, \( C^{el} \) can be defined in terms of 2 material variables, \( E \), the Young’s
modulus, and \( v \), the Poisson’s ratio, as shown in Equation 3.12. It should be noted
that the orthotropic behaviour of these models is captured through the application of
an orthotropic thermal contraction, rather than the material behaviour, the effect of which is to set \( \sigma_{22} \) and \( \sigma_{33} \) to zero in Equation 3.11.

\[
\mathbf{C}^{el} = \frac{E}{(1 + \nu)(1 - 2\nu)} \begin{bmatrix}
1 - \nu & \nu & \nu \\
\nu & 1 - \nu & \nu \\
\nu & \nu & 1 - \nu \\
0 & 0 & 0 \\
0 & 0 & 1 - 2\nu \\
0 & 0 & 0 \\
\end{bmatrix}
\]

The methods utilised in Chapter 6 include 2 dimensional plane stress analysis, for which \( \sigma_3 \) goes to zero. This generates the equivalent Hooke’s law relationship given in Equation 3.14.

\[
\begin{bmatrix}
\sigma_{11} \\
\sigma_{22} \\
\sigma_{21}
\end{bmatrix} = \frac{E}{(1 - \nu^2)} \begin{bmatrix}
1 & \nu & 0 \\
\nu & 1 & 0 \\
0 & 0 & 1 - \nu
\end{bmatrix} \begin{bmatrix}
\varepsilon_{11} \\
\varepsilon_{22} \\
\varepsilon_{21}
\end{bmatrix}
\]

3.1.2 Cell contraction

Cells cultured in vitro have been shown to contract their substrate through actin-myosin contraction of their cytoskeleton (Peterson et al., 2004, Wang et al., 2007, Guolla et al., 2012), shown in Figure 3.2. The resistance of a substrate to cell contraction generates isometric stress within the cell (Goeckeler and Wysolmerski, 1995, Bodmer et al., 1997). Chapter 5 of this Thesis hypothesises that this intracellular stress is the method by which osteoblasts interpret the stiffness of their surrounding ECM and that they change their morphology and stiffness in order to experience a more desirable level of cell tension. Thermal contraction properties were assigned in order to simulate this cell contraction, while the intracellular tension generated due to the resistance of the substrate was indicated by the stress generated in the direction of contraction.
The thermal expansion coefficient of a material is defined as the percentage change in volume relative to a change in temperature, also shown in Equation 3.14.

\[
\alpha_v = \frac{1}{V} \frac{\delta V}{\delta T}
\]  

(3.14)

where \(\alpha_v\) is the volumetric coefficient of thermal expansion, \(V\) is the undeformed volume and \(T\) is temperature.

In an unrestricted volume element, as shown in Figure 3.4, the applied thermal load causes a change in volume of the element, without the generation of stress. However, a mechanical restriction of the element, such as an applied boundary condition as also shown in Figure 3.4, leads to the generation of stress due to the applied restriction. This principle was used in Chapter 5 of this Thesis to simulate the stress generated in a cell body when cell contraction occurs against the resistance of a substrate.

A cylindrical co-ordinate system was assigned to the cell as shown in Figure 3.3. Anisotropic thermal expansion properties were then assigned to the cell body allowing the thermal expansion properties of the cell to act in the direction of principal fibre alignment (denoted by \(R\) in the models shown in Figure 3.3). A
negative thermal load was applied to the cell body, in order to generate cell contraction, while the intracellular tension was taken as the stress generated in this direction ($\sigma_{RR}$).

Figure 3.3 Cylindrical co-ordinates system relating to the direction of principal fibre alignment in spread and dendritic cell morphologies.

3.1.3 Finite element method
The ABAQUS software used in Chapters 5 and 6 of this thesis is based on the finite element method. This method involves discretisation of the structure to be analysed into individual node sharing elements, where the strain distribution at each node is calculated through polynomial algebraic functions. This results in the creation of a mesh of connected nodes representing the structure to be analysed. The density of this mesh can be altered to achieve more accurate and more relevant results as appropriate.
The element is defined as the area or volume enclosed by a series of connecting nodes (as shown in Figure 3.4). In this thesis, one dimensional truss elements are used to model collagen fibres in Chapter 6, while two dimensional plane stress elements are used to model the non-fibrous portion of the same collagen gels. Meanwhile, three dimensional quadratic tetrahedral and hexahedral stress elements were used to model the cell and substrates examined in Chapter 5. Diagrams of each of these elements are shown in Figure 3.4. Langrangian interpolation is used to define deformation within the element according to set values of deformation at the element nodes through the use of shape functions.

The geometry and displacement at node i is defined by $N_i$, the shape function at that node, as shown in Equations 3.15 and 3.16. Shape functions must satisfy the criterion that $N_i = 1$, at i and $N_i = 0$, at all other nodes. As such they are defined in terms of n variables for n dimensions. A table of the shape functions used in this thesis are given in Appendix 1.
Figure 3.4 Elements used as part of this thesis include 2 node truss element, 8 node quad element, 10 node tet element and 20 node hex element.

\[
\begin{bmatrix}
    x \\
    y \\
    z
\end{bmatrix} =
\begin{bmatrix}
    x_1 & \cdots & x_i \\
    y_1 & \cdots & y_i \\
    z_1 & \cdots & z_i
\end{bmatrix}
\begin{bmatrix}
    \tilde{N}_1 \\
    \vdots \\
    \tilde{N}_i
\end{bmatrix}
\] (3.15)

\[
\begin{bmatrix}
    u_x \\
    u_y \\
    u_z
\end{bmatrix} =
\begin{bmatrix}
    u_{x1} & \cdots & u_{xi} \\
    u_{y1} & \cdots & u_{yi} \\
    u_{z1} & \cdots & u_{zi}
\end{bmatrix}
\begin{bmatrix}
    \tilde{N}_1 \\
    \vdots \\
    \tilde{N}_i
\end{bmatrix}
\] (3.16)

From Section 3.1.1, the strain, \( \varepsilon \), is equal to the derivative of the displacement with respect to the direction. This is given in matrix form for a three dimensional element in Equation 3.17.
\[
\begin{bmatrix}
N_{x1} & 0 & 0 & \ldots & N_{xi} & 0 & 0 \\
0 & N_{yi} & 0 & \ldots & 0 & N_{yi} & 0 \\
0 & 0 & N_{zi} & \ldots & 0 & 0 & N_{zi} \\
N_{x1} & N_{yi} & 0 & \ldots & N_{xi} & N_{yi} & 0 \\
0 & N_{yi} & N_{zi} & \ldots & 0 & N_{yi} & N_{zi} \\
N_{x1} & 0 & N_{zi} & \ldots & N_{xi} & 0 & N_{zi}
\end{bmatrix}
\begin{bmatrix}
u_{x1} \\
u_{y1} \\
u_{z1} \\
u_{xi} \\
u_{yi} \\
u_{zi}
\end{bmatrix} = Bu
\] (3.17)

Where \( N_{xi}, \ N_{yi} \) and \( N_{zi} \) are the derivatives of the shape function \( N_i \) with respect to \( x, y \) and \( z \), calculated as shown in Equations 3.18 to 3.20.

\[
\frac{\partial N_i}{\partial x} = \frac{\partial N_i}{\partial \xi} \frac{\partial \xi}{\partial x} + \frac{\partial N_i}{\partial \eta} \frac{\partial \eta}{\partial x} + \frac{\partial N_i}{\partial \mu} \frac{\partial \mu}{\partial x}
\] (3.18)

\[
\frac{\partial N_i}{\partial y} = \frac{\partial N_i}{\partial \xi} \frac{\partial \xi}{\partial y} + \frac{\partial N_i}{\partial \eta} \frac{\partial \eta}{\partial y} + \frac{\partial N_i}{\partial \mu} \frac{\partial \mu}{\partial y}
\] (3.19)

\[
\frac{\partial N_i}{\partial z} = \frac{\partial N_i}{\partial \xi} \frac{\partial \xi}{\partial z} + \frac{\partial N_i}{\partial \eta} \frac{\partial \eta}{\partial z} + \frac{\partial N_i}{\partial \mu} \frac{\partial \mu}{\partial z}
\] (3.20)

Where \( \xi, \eta \) and \( \mu \) are the co-ordinates of the reference element. From Section 3.1.1, the stress generated in the element is given by Equation 3.21.

\[
\sigma = D\varepsilon = DBu
\] (3.21)

Where \( D \) is the stiffness matrix relating the element stress to element strain. Minimum potential energy is used to predict element behaviour. The potential energy of a system is equal to the strain energy of the system less the work done by the system, each of which are defined in Equation 3.22.

\[
\pi = \Lambda - W = \frac{1}{2} \int_V \sigma^T \varepsilon \ dV - \int_V u^T F \ dV
\] (3.22)
Introducing shape function transformation to Equation 3.22 generates Equation 3.23. This is rearranged to form Equation 3.24, where the element stiffness matrix, $K$, and element force vector, $F$ are defined according to Equations 3.25 and 3.26.

$$
\pi = \frac{1}{2} \int_V (DBu)^T Bu \, dV - \int_V (Nu)^T F \, dV \pi = \frac{1}{2} u^T Ku - u^T F \quad (3.23)
$$

$$
\pi = \frac{1}{2} u^T Ku - u^T F \quad (3.24)
$$

$$
K = \int_V B^T DB \, dV \quad (3.25)
$$

$$
F = \int_V N^T F \, dV \quad (3.26)
$$

Thus Equation 3.27 is derived from the principle of minimum potential energy, and re-written in Equation 3.28.

$$
\frac{\partial \pi}{\partial u} = 0 = Ku - F \quad (3.27)
$$

$$
Ku = F \quad (3.28)
$$

The individual stiffness matrices of each element are then combined computationally to provide information on the behaviour of a discretised structure. The automation of this process and the solving of the relevant equations allow for complicated geometries as well as force and displacement states to be analysed and is a key tool of engineering design and analysis.

### 3.2 Atomic force microscopy

Atomic force microscopy (AFM) is a widely used method of characterising the mechanical properties of soft biological substrates, tissues and cells (Pietuch and
Janshoff, 2013, Evans et al., 2009, Huang et al., 2013, Engler et al., 2006). The self-contained system consists of a laser emitting diode, a photosensitive receptor diode (photodiode), a system of piezo motors and a specially designed tip. The tip is made up of a tip holder, known as a chip, and a flexible cantilever with one reflective side (see Figure 3.5 (B)), which comes to a sharp, highly machined tip. Tips may be conical, spherical, parabolic or pyramidal (as shown in Figure 3.5 (A)), in shape. While the tip radius usually ranges between 5 nm and 30 nm, specially designed tips of up to 750 nm are commercially available. Tipless cantilevers are also available, while extremely large spherical tips have been used to measure the global mechanical properties of entire cells (Weafer et al., 2012).

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

**Figure 3.5 A** Pyramidal AFM tip on cantilever (NanoWorld, 2014), **B** Tip holder and cantilever (Nanoscience-instruments, 2014)

The operation of the system is begun by centering the laser on the cantilever. The reflection of the laser from the cantilever is then directed to the photodiode through
the use of an adjustable mirror, as shown in Figure 3.6. As the Z-piezo, the piezo which controls movement in the vertical direction, is lowered, the tip comes into contact with the surface to be measured. This causes the cantilever to bend which moves the position of the laser on the photodiode. The relationship between the respective positions of the laser on the photodiode and the Z-piezo is used to determine the elastic properties of the material being tested.

Figure 3.6 AFM system set up. Deflection of the cantilever is measured by the position of the laser on the photodiode.

The most common method of relating these properties to the material properties is the Hertz contact model, shown in Equation 3.32, originally developed for shallow contact between two spheres, as shown in Figure 3.7.
\[ P = \frac{4}{(3\sqrt{\delta^2})} \left( \frac{E_1E_2}{E_2(1 - v_1^2) + E_1(1 - v_2^2)} \right) \sqrt{\frac{R_1R_2}{R_1 + R_2}} \] (3.32)

**Figure 3.7** Contact between two spheres, demonstrating contact area defined by \( a \) and \( \delta \).

Where \( P \) is the force generated, \( \delta \) is the deflection of the sphere (or the reduction in radius at the point of contact), \( E_1, E_2 \) and \( v_1, v_2 \) are the Young’s moduli and Poisson’s ratios of the spheres 1 and 2 and \( R_1 \) and \( R_2 \) are the radii of spheres 1 and 2. This model assumes frictionless, non-adhesive contact between two linearly elastic spheres. It is also assumed that \( \delta \), the deflection of the spheres, is small relative to their radii.

Several derivations have been developed for describing the contact between different geometries, including specific formulae for contact between spherical, conical,
parabolic and pyramidal tips and a flat surface. A pyramidal tip indenting flat surface (substrate) was used in measurements conducted in Chapter 4 of this thesis, while in Chapter 5 the cells measured were large enough relative to the AFM tip to be treated as a flat surface. In this model, the tip is considered rigid, a valid assumption due to both the bending of the cantilever and the relative stiffness of the tip, thus removing the material properties of the tip from the equation. Meanwhile, the radius of one sphere becomes infinitely large (flat), while the contact radius, $a$, is approximated according the pyramid angle as shown in Equation 3.33. This generates the equation for the force induced by a pyramidal tip on a flat surface as shown in equation 3.34

$$a = \frac{\tan \alpha}{\sqrt{2}} \delta$$  \hspace{1cm} (3.33)

$$F = \frac{E}{(1 - v^2)} \frac{\tan \alpha}{\sqrt{2}} \delta^2$$  \hspace{1cm} (3.34)

where $\alpha$ is the face angle of the pyramid. The deflection in this case is obtained through the difference between the distance travelled by the Z piezo and the deflection of the cantilever, as shown in Figure 3.8.

![Figure 3.8](image)

**Figure 3.8** Relationship between Z-piezo movement ($h$) and cantilever deflection ($\delta$). $\omega$ is calculated through Equation 3.35.
\[
\omega = \frac{P}{k_{spring}}
\]  

(3.35)

Where \( k_{spring} \) is the spring constant of the cantilever, determined by finding its resonance frequency. The atomic force microscopy approach is used in this thesis to assess the mechanical properties of specific substrates and cells, and is described in Chapters 4 and 5. These experiments were conducted using a silicon-nitride pyramidal tip, with a face angle of 35˚ and tip radius of 5 nm (cell measurements) or 10 nm (material measurements).

3.3 Summary

The theory outlined Sections 3.1.1 and 3.1.3 in this chapter forms the basis of computational simulations of cell and substrate behaviour reported in Chapters 5 and 6 respectively. Similarly, the theory described in Section 3.1.2 is employed in Chapter 5 to simulate the active cell contraction which occurs in in-vitro experiments. Finally the theory outlined in Section 3.2 was used to experimentally investigate the mechanical properties of both the substrates used in Chapter 4 and the cells and substrates investigated in Chapter 5.
Chapter 4: Osteocyte differentiation is regulated by extracellular matrix stiffness and intercellular separation.

4.1 Introduction

Osteocytes make up over 90% of cells in mature bone (Boukhechba et al., 2009). They play a vital role in skeletal health by acting as mechanosensors that monitor the mechanical environment within bone tissue and control signalling to osteoblasts and osteoclasts to remodel the tissue so that bone strength is maintained throughout life (Klein-Nulend et al., 1995, Lanyon, 1993, Jee, 2001). Osteocytes are formed when osteoblasts undergo a dramatic phenotypic transition as they become embedded within newly deposited bone matrix. During this transition their morphology is altered from cuboidal to the dendritic shape associated with osteocytes, which is defined by a rounded cell body, a large nucleus, and long cell processes that extend from the cell body. These processes contact neighbouring cells both within and on the surface of the bone (Marotti et al., 1996, Palazzini et al., 1998, Jee, 2001), and thereby form functional syncitia by establishing gap junctional communication at these contact points (Donahue, 1998, Palazzini et al., 1998). The gene expression pattern of the cells also undergoes a dramatic change. Expression of the osteoblast marker enzyme alkaline phosphatase (ALP) is greatly reduced (Jee, 2001, Nakano et al., 2004), along with a reduction in Col1A1, Bone morphogenetic protein 2 (BMP-2) and osteoblast specific factor 2 (OSF-2, also known as periostin) expression (Igarashi et al., 2002, Santos et al., 2011, Wilde et al., 2003). Expression of osteocalcin (Weinreb et al., 1990, Boivin et al., 1990) and E11 (Zhang, 2006) is increased, while osteocyte specific markers such as PHEX (Westbroek et al., 2002),
Sclerostin (Sost) (Poole et al., 2005) and dentin matrix protein 1 (DMP-1) (Feng, 2003, Rios, 2005) are either induced de-novo or dramatically upregulated.

Col1A1 and OSF-2 are early stage osteocyte markers which are downregulated as osteoblasts begin to develop into osteocytes in vitro (Kato et al., 1997, Kato et al., 2001). DMP-1 is upregulated as the cells begin to extend processes and mineralise their surrounding matrix, while Sost is a late stage osteocyte marker necessary for regulation of mineralisation (Atkins et al., 2009). The highly selective expression of molecules such as Col1A1 and OSF-2 in osteoblasts, and Sost and DMP-1 in osteocytes has led to their use as phenotypic markers in many studies (Kato et al., 2001, Gu et al., 2006, Gooi et al., 2010, Kramer et al., 2010, Krishnan et al., 2010). However, there is still very little understanding of the cues that control the phenotypic shift from osteoblasts to osteocytes.

Among the possible stimuli for the phenotypic shift from osteoblast to osteocyte are changes in the stiffness of the cell’s extracellular matrix (ECM). ECM stiffness has been shown to strongly regulate a variety of cell behaviours such as migration, proliferation, and differentiation in both osteogenic and non-osteogenic cells (Pelham and Wang, 1997, Khatiwala et al., 2006b, Hsiong et al., 2008). It has also been shown that MSC differentiation along different phenotypic lineages (i.e. adipogenic, myogenic, osteogenic) is dependent on substrate stiffness (Engler et al., 2006). In particular MSCs were shown to differentiate into osteoblast-like cells when cultured on collagen coated polyacrylamide substrates, with stiffnesses in the range of 25-40 kPa, whereas cells take on the characteristics of neurons or myoblasts when cultured on substrates with stiffness values similar to those of brain (≈1 kPa) and muscle tissue (≈11 kPa) respectively.
The initial cell seeding density of in vitro experiments has long been used as a method of controlling cell separation and nutrient supply (Mauck et al., 2003, Bitar et al., 2007). The separation distance of the cells, as controlled in this manner, has also been shown to play a role in regulating osteoblast proliferation and matrix mineralisation in three-dimensional constructs in-vitro (Holy et al., 2000, Zhou et al., 2006), while the seeding density of human bone marrow stromal cells is an important factor for the development of cell matrix constructs for bone tissue engineering (Lode et al., 2008). Osteogenic differentiation of MSCs in two dimensional culture, as examined by ALP and BMP-2 expression, has also been shown to be increased when cells are cultured at a low seeding density (3 X 10^4 cells/cm^2) (Kim et al., 2009). To date however, research into the individual effects of both ECM stiffness and cell separation on osteogenic differentiation has focussed on osteoblast differentiation rather than the osteoblast-osteocyte transition. Osteocyte differentiation is necessary for mature bone tissue formation and as such an understanding of this stage of the differentiation pathway is crucial for the development of future tissue engineering strategies for bone.

In this study we test the hypothesis that osteocyte differentiation is regulated by both ECM stiffness and intercellular separation. To address this we employ in vitro culture of MC3T3-E1 cells to compare cellular differentiation on substrates of different chemical composition and stiffness as well as on substrates of identical chemical composition but different stiffness. The effect of intercellular separation is investigated through the culture of cells at varied initial seeding densities on each of the chosen substrates. Osteocyte differentiation is then examined by quantifying cellular morphology, ALP activity and matrix mineralisation, as well as expression of the osteogenic genes Col1A1, OSF-2, DMP-1 and Sost.
4.2 Materials and Methods

Experiments were designed to test whether MC3T3-E1 (a pre-osteoblast cell line) cells would undergo phenotypic changes associated with osteoblast-osteocyte transition in vivo, under specific experimental conditions that investigated the role of ECM composition, ECM stiffness and cell seeding density. Morphological changes in MC3T3-E1 cells were assessed, paying particular attention to cell process formation. ALP activity was quantified using a colorimetric assay, mineralisation was assessed using an alizarin red/cetylpyridinium chloride assay while expression of Col1A1, OSF-2, DMP-1 and Sost genes were quantified by RT-PCR.

4.2.1 Experimental Design: Substrate stiffness and seeding density experiments

Preliminary studies investigated the effect of a range of different substrates and seeding densities on MC3T3-E1 morphology. The results of these studies were used to select the experimental conditions outlined here. MC3T3-E1 cells were plated at seeding densities of $10^3$ or $10^4$ cells/cm$^2$ and cultured on: (a) NaOH neutralised collagen (Col), (b) NaOH neutralised collagen crosslinked with EDAC at 20 μM/mg collagen (ColEDAC1), (c) NaOH neutralised collagen crosslinked with EDAC at 100 μM/mg collagen (ColEDAC2), (d) Acetic acid neutralised collagen (ColAA), (e) Thin matrigel (MatThin), (d) Thick matrigel (MatThick) and (f) Uncoated tissue culture plastic (TC plastic). Cells were allowed to grow on all substrates for 1, 4, 9, 14 or 21 days respectively. Triplicate wells were analysed for each condition to quantify cell morphology, ALP expression, ECM mineralisation and gene expression. MLO-Y4 cells were cultured for 4 days on acetic acid neutralised collagen (ColAA) as a positive control (Kato et al., 1997).
4.2.2 Cell culture

Two bone cell lines were used in this study. MC3T3-E1 cells are a murine derived osteoblast cell line which can express high amounts of ALP, Col1A1 and OSF-2 (Hurley et al., 1993, Oshima et al., 2002). The cells are capable of differentiating into osteocytes and mineralising their surrounding matrix (Sudo et al., 1983). They are considered to be a good model of primary osteoblasts (Quarles et al., 1992). For these studies MC3T3-E1 cells were maintained in Alpha Modified Eagle’s Medium (α-MEM) supplemented with 10% foetal bovine serum (FBS), 100 U/mL penicillin streptomycin and 100 ug/mL L-glutamine (all Sigma Aldrich) prior to all experiments.

MLO-Y4 cells are a murine derived cell line which share numerous characteristics with primary osteocytes such as high production of osteocalcin and E11 and low expression of alkaline phosphatase and OSF-2, as well as the extension of numerous dendritic processes per cell (Kato et al., 1997, Bonewald, 1999). MLO-Y4 cells were maintained on type 1 collagen at 0.15 mg/mL in acetic acid and cultured in Dulbecco’s MEM supplemented with 5% foetal bovine serum, 5% foetal calf serum (FCS) (Sigma Aldrich), 100 U/mL penicillin streptomycin and 100 ug/mL L-glutamine as recommended by Kato et al (Kato et al., 1997).

4.2.3 Preparation of ECM substrates

Type 1 rat tail collagen (Life Technologies) was neutralised with NaOH (Sigma Aldrich) at 18.4 µM /g collagen, and diluted with 10% Phosphate Buffered Saline (PBS) (all Sigma Aldrich) and 68% distilled H₂O. The mixture was then pipetted in 150 µL volumes onto 13 mm diameter coverslips (Sarstedt) and incubated for 30 minutes at 37°C, before being double rinsed with sterile PBS. This resulted in the
formation of a soft, thick, gel like coating on the coverslips (Col). To produce substrates of different mechanical stiffness but identical ligand density, substrates were crosslinked with 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDAC) (Sigma Aldrich) by incubating at 20 µM/mg collagen (ColEDAC1) or 100 µM/mg collagen (ColEDAC2) EDAC for 3.5 hours at room temperature as described previously (Haugh et al., 2011). Substrates were then rinsed with PBS and incubated in fresh PBS for 3 hours at room temperature to remove any remaining EDAC before being washed twice with sterile distilled H₂O.

Type 1 rat tail collagen was also neutralized with acetic acid (Sigma Aldrich) at 2.34 µM /mg collagen and incubated for 1 hour at room temperature to create a thin collagen coating (ColAA) of identical ligand density to the NaOH neutralized collagen substrates described above. This mixture was pipetted onto 13 mm diameter coverslips in 150 µL volumes and allowed to incubate at room temperature for 60 minutes. Excess liquid was then removed and substrates were double rinsed with sterile PBS prior to cell plating.

Matrigel (Sigma Aldrich) was diluted 1:3 in α-MEM and plated on 13 mm coverslips in volumes of 200 µL. The coverslips were then incubated for either 30 minutes at 37°C or 1 minute at room temperature to create thick (>1 mm - MatThick) and thin (< 50 µm - MatThin) substrates. Again substrates were washed twice with sterile PBS prior to cell plating.

4.2.4 Substrate stiffness measurement by Atomic Force Microscopy (AFM)

Substrate stiffness measurements were conducted using an Agilent 5500 Atomic Force Microscope (AFM). A pyrex-nitride pyramidal tip of face angle 30° was used. Force-distance curves were obtained from each of the following substrates; Col,
ColEDAC1, ColEDAC2, ColAA and TC. These curves were then used to calculate the material stiffness according to equations 3.34 and 3.35.

The spring constant, \( k_{\text{spring}} \), was calculated by finding the resonant frequency of the cantilever during testing (52 N/m), while the Poisson’s ratio was assumed as 0.2, in keeping with previous measurements of collagen gels (Bloom et al., 2008, Raub et al., 2010, Knapp et al., 1997). Using this equation substrate stiffness was measured five times at three different locations on each substrate and the values averaged to generate a stiffness value for each measured substrate. A single tip was used for all measurements obtained.

4.2.5 Morphological analysis of cell phenotype

Cultures were fixed using 4% paraformaldehyde (Fluka) in piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) buffer (Sigma Aldrich) after 1, 4, 9 or 14 days of culture. Cells were permeabilised with Triton-X100 (Sigma Aldrich), diluted to 0.05% in PBS, and then incubated in phalloidin-TRITC according to the manufacturer’s protocol (Life technologies) to stain the actin cytoskeleton. Cells were then counterstained with DAPI dilactate (Sigma Aldrich) and rinsed with PBS prior to being mounted in DPX mounting media (Sigma Aldrich) for imaging.

Images were taken using an Olympus IX50 inverted fluorescence microscope at different locations on the coverslips at 10X magnification, giving a 0.62 cm\(^2\) image area. In total 15 images were taken at each time-point, for each condition (5 on each replicate coverslip). Cell processes were defined as cellular features located at the cell membrane which had a cross sectional diameter of no more than 1 \( \mu \text{m} \) and extended for a distance of at least 5 \( \mu \text{m} \). Using this classification cells exhibiting cell processes were classified as Dendritic and cells without cell processes were classed as Spread. The term Transitional cells was used to describe cells exhibiting features of both spread and dendritic cells. These cells were
identified by their large spread area and a single cell process. Examples of Spread and Dendritic and Transitional morphologies are shown in Figure 4.1.

![Figure 4.1: Examples of Spread, Dendritic and Transitional morphologies used to perform morphological analysis of the cells on each substrate. All cells were stained with rhodamine-phalloidin. A cell process was defined as an actin structure with cross section of less than 1 μm, extending for a minimum length of 5 μm.](image)

4.2.6 Quantification of Cell number

Cell number was quantified through a Hoechst 33258 assay kit according to the manufacturer’s protocol (Abcam). Hoechst fluorescently labels double-stranded DNA which can then be detected using a plate reader (Perkin Elmer 2030).
4.2.7 Quantification of ALP activity

ALP activity was analysed on Col, ColEDAC1, ColEDAC2, and TC plastic. Col and ColEDAC1 were chosen as they had the highest percentage of dendritic cells by morphological analysis. ColEDAC2 was chosen as it is chemically identical to ColEDAC1. This allowed for the effect of substrate stiffness to be examined while minimising the chemical differences between the substrates. TC plastic was used as a control substrate. Intracellular ALP activity was measured using a colorimetric ALP assay (Sigma Aldrich) as described previously (Birmingham et al., 2012), at 1, 4, 9 and 14 days for all conditions. Briefly, cells were lysed by freezing at -80°C and thawing at room temperature before being mechanically scraped from the substrate. The ALP assay uses p-nitrophenyl phosphate (pNPP) which changes its emission wavelength when dephosphorylated by ALP. The change in emission wavelength was measured at 540 nm on a plate reader (Perkin Elmer 2030). Results were then normalized to cell number as determined through the Hoescht assay outlined above. Cell culture media from the same substrates (Col, ColEDAC1, ColEDAC2, and TC plastic) was also analysed to quantify extracellular ALP production. Media was changed 24 hours before cell culture media was harvested and analysed using the p-nitrophenyl phosphate assay described above.

These experiments sought to analyse changes in ALP production over time as an indicator of osteoblast to osteocyte transition. It is known that immature MC3T3-E1 cells initially produce low levels of ALP, but as these cells differentiate into mature osteoblasts they upregulate ALP production while as they differentiate into osteocytes they then decrease ALP production (Mikuni-Takagaki et al., 1995).
4.2.8 Mineralisation

Mineralisation of the extracellular matrix by MC3T3-E1 cells was analysed after 4, 9 and 14 days using an alizarin red/cetylpyridinium chloride assay (Sigma Aldrich), as described previously (Birmingham et al., 2012, Brennan et al., 2012, Stanford et al., 1995), on specific substrates (Col, ColEDAC1, ColEDAC2, TC plastic). Media was removed and cells were rinsed twice in PBS before being incubated with a 2% alizarin red solution (Sigma Aldrich) for 20 minutes on an orbital rocker. The solution was removed and the substrates were washed three times in deionised H₂O to remove any unbound alizarin red. Cells were then incubated with 10% cetylpyridinium chloride solution (Sigma Aldrich) on an orbital rocker for 20 minutes at room temperature to disintegrate bound alizarin red. The absorbance of these samples at 562 nm was measured using a plate reader (Perkin Elmer 2030) to determine calcium deposition.

4.2.9 Gene expression

Expression of osteoblast specific (Col1A1, OSF-2) and osteocyte specific (DMP1, Sost) gene’s was analysed by RT-PCR on specific substrates (Col, ColEDAC1, ColEDAC2) after 14 and 21 days of culture. These substrates were chosen based on the results of the morphological studies, as cells cultured on Col and ColEDAC1 at 10³ cells/cm² had the highest levels of dendrite formation indicative of osteocyte differentiation, whereas cells cultured on ColEDAC2 and/or plated at 10⁴ cells/cm² were predominantly spread indicative of osteoblast differentiation.

Cells were lysed using 1 mL TRI reagent before phase separation by the introduction of 200 µL chloroform. RNA was precipitated using 70% ethanol and then washed using an ENZA RNA isolation kit according to the manufacturer’s protocol (Omega Bio-tek) and dissolved in 30 µL of RNAse free water (Qiagen). The quality of the
RNA was measured using a Nanodrop spectrometer (Thermo-scientific) before being converted to cDNA using a cDNA synthesis kit (Omega Biosciences) and Gene Amp 9700 A Thermal cycler (Applied Biosystems).

RT-PCR was performed on a Step-One plus analyser (Applied Biosystems) using Taqman probes (Applied Biosystems) for Col1A1 (Mm00801666_g1) and OSF-2 (Mm00450111_m1) on the cells harvested after 14 days of culture and DMP-1 (Mm00803833_g1) and Sost (Mm04208528_m1) on cells harvested after 21 days of culture. RT-PCR data was analysed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), with GAPDH (Mm03302249_g1) as a housekeeping gene and all reactions were conducted in biological and technical triplicates.

4.2.10 Statistical Analysis

Cell morphologies were compared to a positive (MLO-Y4 cells cultured on ColAA) and a negative (MC3T3-E1 cells cultured on TC plastic) control. Statistical significance of the effects of substrate stiffness and seeding density on cell morphology, ALP activity, mineralisation and gene expression was determined using a one way ANOVA. Pierce’s criterion was used to determine statistical outliers in ALP and mineralisation data.

4.3. Results

4.3.1 AFM mechanical properties

TC plastic had a stiffness of 80.1 ± 4.8 MPa, while silicone, tested as a control of known stiffness (1MPa), was measured as 1.18 ± 0.2 MPa. Col was the softest substrate with a stiffness of 9.7 ± 0.3 Pa. As expected substrate stiffness increased as a function of the concentration of crosslinking agent. ColEDAC1 and ColEDAC2 had stiffnesses of 286 ±
22.1 Pa and 957 ± 12.0 Pa respectively. ColAA had a similar stiffness to ColEDAC2 at 921 ± 9.6 Pa. These results are summarised in the graph in Figure 4.2. Matrigel is liquid at room temperature and so the stiffness of these two substrate could not be measured.

**Figure 4.2:** Substrate stiffness measurements by Atomic Force microscopy. Error bars indicate standard deviation. Graph is on a logarithmic scale due to the large variation in the stiffnesses of the respective substrates.

### 4.3.2 Morphological analysis of cell phenotype

After 9 days, MC3T3-E1 cells cultured on TC plastic at $10^4$ cells/cm$^2$ (control condition) exhibited a predominantly spread morphology with 66.7% of cells classed as spread while only 13.8% exhibited cell processes (shown in Figure 4.3). Similarly MC3T3-E1 cells grown on stiff collagen based substrates (ColEDAC2) at the higher initial seeding density ($10^4$ cells/cm$^2$) had a predominantly spread morphology with 53.7% of cells being classed as spread and 26.8% of extending cell processes. Cells cultured at lower seeding density on TC plastic or stiff collagen showed similar morphologies with 54.4% (ColEDAC2) and 46.7%
(TC plastic) being classed as spread and 23.8% (ColEDAC2) and 14.1% (TC plastic) extending cell processes. Cells cultured at \(10^4\) cells/cm\(^2\) on the softest two collagen substrates (Col and ColEDAC1) showed higher percentages of cells with processes at 35.8% and 29.0% respectively. However a higher portion of the cells exhibited a spread morphology in both cases (49.6% and 45.7%). Less of the cells cultured at \(10^3\) cells/cm\(^2\) on the Col and ColEDAC1 substrates exhibited a spread morphology (31.0% and 20.0% respectively) while 31.0% (Col) and 41.5% (ColEDAC1) produced cell processes. The remainder of cells cultured at these conditions were classed as transitional. After 14 days 70.1% of MC3T3-E1 cells cultured on the TC plastic control were classed as spread with 24.5% extending cell processes, as shown in Figure 4.4. Cells cultured on the stiff (ColEDAC2) or intermediate (ColEDAC1) stiffness collagen based substrates at high initial seeding density showed a similar morphological pattern with approximately 56% of cells classed as spread and 31% exhibiting cell processes at both culture conditions. A lower percentage of spread cells (46.0%) was observed on the softest substrate at the higher seeding density of \(10^4\) cells/cm\(^2\), while 36.8% of cells on these substrates extended cell processes. Cells cultured at the lower seeding density of \(10^3\) cells/cm\(^2\) on ColEDAC2 or TC plastic also predominantly exhibited a spread morphology with over 63% of cells being classed as spread and less than 28% extending cell processes under both culture conditions. In contrast, cells cultured on the softest (Col) or intermediate stiffness (ColEDAC1) substrates at \(10^3\) cells/cm\(^2\) were predominantly osteocyte like with over 50% extending cell processes under both culture conditions, while less than 37% of the cells cultured under these conditions were classed as spread. 47% of the MLO-Y4 control cells were classed as spread while 48.6% extended cell processes. Sample images of MC3T3-E1 cells on Col and TC plastic are shown in Figure 4.5. MLO-Y4 cells were cultured for 4 days on ColAA as a positive control with 50.3 ± 2.7 % of these cells classed as dendritic, see Figure 4.4.
Figure 4.3: Percentage of cells displaying the dendritic morphology typical of osteocytes after 9 days of culture on each substrate at an initial seeding density of either $10^3$ cells/cm$^2$ or $10^4$ cells/cm$^2$. $\text{a}$ indicates statistical difference with negative control (MC3T3’s on TC plastic at $10^4$ cells/cm$^2$, $p < 0.01$). $\text{b}$ indicates statistical difference with positive control (MLO-Y4’s on ColAA at $10^4$ cells/cm$^2$, $p < 0.01$).
Figure 4.4: Percentage of cells displaying the dendritic morphology typical of osteocytes after 14 days of culture on each substrate at an initial seeding density of either $10^3$ cells/cm$^2$ or $10^4$ cells/cm$^2$. a indicates statistical difference with negative control (MC3T3’s on TC plastic at $10^4$ cells/cm$^2$, p < 0.01). b indicates statistical difference with positive control (MLO-Y4’s on ColAA at $10^4$cells/cm$^2$, p < 0.01).
Figure 4.5: Sample morphologies from A) TC plastic at $10^4$ cells/cm$^2$ for 14 days, B) Col at $10^3$ cells/cm$^2$ for 14 days. White arrows in B) indicate cell processes.

4.3.3 ALP activity of cells

Both intracellular and extracellular ALP activity increased continuously in MC3T3-E1 cells cultured on TC plastic and ColEDAC2 for the duration of culture for both seeding densities, as shown in Figures 4.6 and 4.7. All cells cultured at the higher seeding density of $10^4$ cells/cm$^2$ on all substrates also showed an increase in ALP activity for the duration of culture. Similarly ALP expression increased significantly up to 9 days of culture in MC3T3-E1 cells cultured at a low seeding density ($10^3$ cells/cm$^2$) on the two softest substrates (Col and ColEDAC1). Expression in cells on these two substrates at the lower seeding density of $10^3$ cells/cm$^2$ was then downregulated after 14 days of culture (although this change was not statistically significant).
Figure 4.6: Extracellular ALP activity over time of MC3T3’s on: Col, ColEDAC1, ColEDAC2, TC plastic, measured from media extracted from wells upon harvest.

Error bars indicate standard deviation of repeat wells. a indicates statistical difference from previous timepoint of same condition (p < 0.01). b indicates statistical difference from control (MC3T3’s on tissue culture plastic at $10^4$ cells/cm$^2$) and same timepoint (p < 0.01).
Figure 4.7: Intracellular ALP activity over time of MC3T3’s on: Col, ColEDAC1, ColEDAC2, TC plastic. Error bars indicate standard deviation of repeat wells. * indicates statistical outlier. a indicates statistical difference from previous timepoint of same condition (p < 0.01). b indicates statistical difference from control (MC3T3’s on tissue culture plastic at $10^4$ cells/cm$^2$) and same timepoint (p < 0.01).

4.3.4 Mineralisation

No increase in mineral production over time by MC3T3-E1 cells on tissue culture plastic at either $10^3$ or $10^4$ cells/cm$^2$ was observed, see Figure 4.8. Similarly, on the stiffer collagen substrate (ColEDAC2) with a seeding density of $10^3$ cells/cm$^2$, no significant change in mineralisation over time was observed. However a significant increase was observed from day 4 to day 9 on this substrate with a seeding density of $10^4$ cells/cm$^2$ (see Figure 4.8). The highest levels of mineralisation were observed on Col and ColEDAC1 when cells were
cultured at $10^3$ cells/cm$^2$. An increase in mineralisation over time was also observed in these culture conditions. An increase in mineralisation over time was also observed on ColEDAC1 with a seeding density of $10^4$ cells/cm$^2$. However, levels of mineralisation under this condition were significantly lower than in either Col or ColEDAC1 with a seeding density of $10^3$ cells/cm$^2$ (see Figure 4.8).

Figure 4.8: Mineralisation of ECM over time caused by MC3T3’s cultured on: Col, ColEDAC1, ColEDAC2, TC. Error bars indicate standard deviation of repeat wells. 

* indicates outlier from statistical data. a indicates statistical difference from previous timepoint (p < 0.05). b indicates statistical difference from control (MC3T3’s on tissue culture plastic at $10^4$ cells/cm$^2$) at same timepoint (p < 0.05).

4.3.5 Gene Expression

MC3T3-E1 cells cultured on TC plastic for 24 hours were chosen as the control from which to measure the relative expression of all genes examined. Col1A1 expression was lowest in cells cultured on ColEDAC1 at the lower seeding density. Low levels of Col1A1 expression were also observed in cells cultured on Col and ColEDAC2 at the higher seeding density of $10^4$ cells/cm$^2$ (see Figure 4.9). OSF-2 was lowest in cells cultured on ColEDAC1 at $10^3$
cells/cm², as shown in Figure 4.10, with a low level of expression also observed in cells cultured on the softest substrate (Col) at $10^3$ cells/cm². DMP-1 expression after 21 days of culture was highest in cells cultured Col at the $10^3$ cells/cm² (see Figure 4.11), while Sost was detected in trace amounts in cells cultured on ColEDAC1 at both seeding densities, but not in cells cultured on any other substrates.

**Figure 4.9:** Col1A1 expression in MC3T3’s cultured on: Col, ColEDAC1, ColEDAC2 for 14 days. a indicates statistical difference from Col at the same seeding density. b indicates statistical difference from ColEDAC1 at the same seeding density. c indicates statistical difference from $10^3$ cells/cm² seeding density on the same substrate (p < 0.05).
Figure 4.10: OSF-2 expression in MC3T3’s cultured on: Col, ColEDAC1, ColEDAC2 for 14 days. **a** indicates statistical difference from Col at the same seeding density. **b** indicates statistical difference from ColEDAC1 at the same seeding density. **c** indicates statistical difference from $10^3$ cells/cm$^2$ seeding density on the same substrate ($p < 0.05$).
Figure 4.11: DMP-1 expression in MC3T3’s cultured on: Col, ColEDAC1, ColEDAC2 for 21 days. \( \text{a} \) indicates statistical difference from Col at the same seeding density. \( \text{b} \) indicates statistical difference from ColEDAC1 at the same seeding density. \( \text{c} \) indicates statistical difference from \( 10^3 \) cells/cm\(^2\) seeding density on the same substrate (p < 0.05).

4.4 Discussion

The results of this study show that MC3T3-E1 cells progress along the osteogenic lineage to become osteocyte-like cells when cultured on soft collagen substrates at low seeding density (\( 10^3 \) cells/cm\(^2\)), as indicated by phenotypic changes associated with early osteocyte differentiation (dendritic morphology, reduction in ALP expression, ECM mineralisation). Furthermore, downregulation of the osteoblast specific genes Col1A1 and OSF-2 as well as the upregulation of the osteocyte specific genes DMP1, Sost can be induced in MC3T3-E1 cells through culture on
collagen based substrates of approximately 286 Pa at an initial seeding density of $10^3$ cells/cm$^2$. In contrast, cells cultured on stiffer collagen based substrates, or at a high initial cell seeding density proliferated and displayed the spread morphology, continually high ALP expression, low levels of ECM mineralisation, and high levels of Col1A1 and OSF-2 expression associated with the osteoblast phenotype. As a whole these findings show that the mechanical and compositional properties of the ECM, as well as the necessity for the cells to establish a communication network, contribute greatly to osteocyte differentiation.

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 respectively (Sudo et al., 1983, Kato et al., 1997, Quarles et al., 1992, Bonewald, 1999). MLO-Y4 cells exhibit low levels of ALP activity, which does not change over time (Kato et al., 1997). Other osteocyte-like cell lines, such as MLO-A5 and MLO-C2, were deemed to be inappropriate given their excessively high levels of ALP activity (Kato et al., 2001). Primary osteoblasts/osteocytes were not used because of difficulties in cell isolation and characterisation (Hentunen, 2010) and the necessity for large cell numbers to satisfy the numerous experimental conditions and time points in this study. An added limitation in the use of the MLO-Y4 cell line is in the lack of osteocyte specific genes expressed by the cells, particularly in two dimensional studies (Papanicolaou et al., 2009). This is reflected in the use of this cell line as a positive control for morphological studies only.

The current studies were also conducted using only three different substrate stiffness’s (for substrates of identical ligand density) and two different seeding
densities ($10^3$ or $10^4$ cells/cm$^2$). However preliminary studies were conducted to select these final experimental conditions. Investigation of cell morphology and ALP expression in these initial experiments showed that higher seeding densities produced similar results to the $10^4$ cells/cm$^2$ seeding density, while substrates stiffer than the CoLEDAC2 substrate described in Section 4.2.3 produced similar results to this substrate. Cells cultured at initial seeding densities of less than $10^3$ cells/cm$^2$ failed to show signs of proliferation after 4 days of culture. Further to this the $10^3$ cells/cm$^2$ density used in this study is similar to low seeding densities used in previous research involving the MC3T3-E1 cell line (Byskov et al., 2012, Arnsdorf, 2009). Another possible limitation exists in the use of AFM for substrate stiffness measurements. The effect of tip shape has been shown to be of great importance in accurately converting AFM deflection data to substrate stiffness (Bhushan et al., 2009). However the accuracy of the AFM methods used was confirmed in this work by testing a silicone sample of known stiffness of 1 MPa (shown in Figure 4.2), and therefore the use of the Hertzian equation described in Section 4.2.4 was considered an accurate method of stiffness measurement.

It should be noted that these cells did not express significant levels of Sost, which is known to be a late stage osteocyte marker, involved in the regulation of bone formation. However, Sost expression has only previously been induced in vitro in the MC3T3-E1 cell line through the addition of osteogenic growth factors (Mattinzoli, 2012, Uchihashi et al., 2013). Indeed it has been shown in vivo that Sost is not expressed until after mineralisation has begun (Poole et al., 2005). Furthermore, increased DMP-1 expression has not previously been reported without the addition of osteogenic growth factors (Krishnan et al., 2010, Mattinzoli, 2012). The results presented here show a significant increase in DMP-1 expression and
trace amounts of Sost when cells are cultured on soft collagen based substrates (286 Pa) at low initial seeding density ($10^3$ cells/cm$^2$). This highlights the importance of substrate mechanical properties even in the absence of commonly used osteogenic factors.

Osteocyte differentiation has been induced in MC3T3-E1 cells through treatment with fibroblast growth factor 2 (FGF-2) (Gupta et al., 2010), or by culture of the cells on collagen based substrates in the presence of the osteogenic factors (Uchihashi et al., 2013). In the current study, similar results are presented based solely on the substrate stiffness and intercellular separation of the cells. Previous studies have also shown substrate stiffness to affect osteogenic cell behaviour. Cell migration, focal adhesion formation as well as calcium deposition by MC3T3-E1 cells have been shown to depend on substrate stiffness (Khatiwala et al., 2006a). Furthermore, mineralisation by embryonic stem cells has been shown to increase on stiffer substrates (Evans et al., 2009), while perhaps most interestingly, culture of pluripotent mesenchymal cells on a relatively rigid ECM substrate (40 kPa) favored osteoblastic differentiation, whereas culture on a more flexible substrate (1 kPa) led to a neural differentiation pathway (Engler et al., 2006). However, previous studies have only examined pre-osteoblast to osteoblast, or stem cell to osteoblast, development and no study has ever monitored osteocyte differentiation as a function of ECM stiffness. In the current study the importance of ECM stiffness for regulating pre-osteoblast to early osteocyte transition is shown for the first time. While osteoblast differentiation is enhanced on stiffer substrates (20-40 kPa (Engler et al., 2006, Kong et al., 2005)), the current studies suggest that osteocyte differentiation is in fact governed by softer substrates (<300 Pa). In vivo osteocytes are formed when certain osteoblasts become embedded in newly formed osteoid,
which is a soft, non-mineralised collagen matrix (Franz-Odendaal et al., 2006, Dallas and Bonewald, 2010). The process initiates when mature osteoblasts secrete a collagen matrix while simultaneously undergoing the morphological changes associated with osteocyte differentiation (Doty, 1981). The cells begin to downregulate certain osteoblast specific genes, such as Col1A1 and OSF-2, while upregulation occurs in genes involved in controlling osteocyte function, such as DMP-1 (Yang, 2004, Rios, 2005) and Sost (Yang, 2009, Winkler et al., 2003). It is interesting to note from the results of this study that the cells which displayed the traits of osteocyte differentiation were cultured on soft collagen based substrates. It is therefore proposed that this substrate provides a similar extracellular mechanical environment to osteoid (Engler et al., 2006), which is necessary for osteoblast to osteocyte transition.

Cell seeding density has previously been shown to affect osteoblast differentiation. ALP activity in rat bone marrow stromal cells after 4 days of culture was found to be higher when cells were seeded at a lower initial seeding density of $3 \times 10^4$ per cm$^2$, compared to a higher density of $14.9 \times 10^4$ per cm$^2$ (Kim et al., 2009), which indicated the importance of seeding density for differentiation of BMSCs into osteoblasts. However these cells were cultured on TC plastic and seeding densities were an order of magnitude larger than those used in this study. Bone formation on implanted HA scaffolds, plated with goat bone marrow cells, was shown to be increased where cells were plated at higher seeding densities up to $47.8 \times 10^6$ cells/cm$^3$ in osteogenic media for 7 days prior to implantation (Wilson et al., 2002). MG-63 osteosarcoma cells cultured at a low initial seeding densities in 3D collagen scaffolds showed significantly higher rates of ALP expression than those at higher seeding densities by 2 days (Bitar et al., 2007). Although the difference in cell
phenotype prevents direct comparison with this study, the results presented here show similar changes in ALP expression over time due to initial cell seeding density. It has already been widely hypothesised that the cells extend processes to facilitate intercellular communication (Donahue, 1998, Palazzini et al., 1998). It is intriguing to speculate that the combined effect of substrate stiffness and cell seeding density observed here is driven by the need for osteocytes to establish a communication network. The osteocyte network is akin to that of neurites and it has previously been reported that neurite formation is increased on substrates in the range of 250-500 Pa (Georges, 2006, Estell, 2012). Therefore, it is proposed that the formation of dendrites is driven by ECM stiffness and intercellular separation across a range of phenotypes. It is possible that cells on stiffer substrates find it easier to proliferate allowing the formation of gap junctions between their cell bodies without the need to establish cell processes. Indeed previous studies have shown the presence of gap junctions both in the MC3T3 cell line (Yamaguchi et al., 1994) as well as between MC3T3s and MLO-Y4s (Yellowley et al., 2000), when cells are cultured on tissue culture plastic at high seeding densities. However cells on softer substrates might be prevented from proliferating sufficiently due to the mechanics of the substrate, and after a time might resort to extending processes to establish a communication network with neighbouring cells. Indeed it is important to note that, although osteocyte-like cells were initially formed on soft collagen substrates at a low initial cell seeding density, these cells no longer produced dendrites if sufficient proliferation subsequently occurred to enable close contact between cell bodies. Furthermore, dendrite formation in MC3T3-E1 cells was not ubiquitous across the substrates; while a large proportion of cells were dendritic at the low seeding density on soft substrates, in certain regions cells became quite confluent and maintained an
osteoblast-like phenotype. This likely arose where cells were initially sufficiently close (due to seeding variability) so that they could spread and establish communication junctions without the necessity to form dendrites. Nonetheless an overall increase in dendrite formation was observed in cells cultured on softer substrates at lower initial seeding densities. It is expected that if cell seeding and separation could be precisely controlled then the observed effects would in fact be more pronounced.

While the mechanisms underlying specific phenotypic changes associated with the osteoblast-osteocyte transition remain to be identified, several candidate molecules are known. For example, ECM dependent changes in cell shape and gene expression in many cell types are mediated by integrins and other adhesion receptors that associate intracellularly with both the actin cytoskeleton and with multiple phosphorylation based intracellular signaling pathways (Wang et al., 2009a). In particular, small GTPases including Rac, Rho and CDC42 have specific functions related to cellular spreading, migration and the extension of cellular processes (Huveneers and Danen, 2009), crucial elements in establishing the osteocyte’s characteristic form and its capacity to interact with matrix and with neighbouring cells (Tanaka Kamioka et al., 1998, Kamioka et al., 2007, McNamara et al., 2009). Loss of ALP activity, as observed in this study, involves not only a change in gene expression but also loss or inactivation of pre-existing enzyme in osteoblasts. Such losses could occur through shedding of membrane-derived vesicles or by enzymatic cleavage of the enzyme itself (Dean et al., 1996, Xie, 1995). Finally, diffusible signals may also play important roles in the osteoblast-osteocyte transition; it has recently been demonstrated that sustained stimulation of osteoblastic cells with the oncostatin-M, a member of the interleukin 6 (IL6) family, could induce a broad
range of gene expression changes consistent with osteocyte formation in cultured osteoblasts (Brounais et al., 2008).

These experiments represent the first investigation into the effects of both substrate stiffness and cell seeding density on osteoblast-osteocyte transition and shed light on the external cues necessary for osteocyte differentiation. For the first time, pre-osteoblast MC3T3 cells have been induced to undergo early osteocyte differentiation without the addition of growth factors or application of mechanical loading. An understanding of these effects is vital in order to inform future tissue engineering strategies.

4.5 Conclusion

The results of this study show that substrate stiffness and intercellular separation are vitally important in osteogenic differentiation. It is shown that MC3T3-E1 cells can be induced to differentiate into early osteocytes without the addition of osteogenic growth factors or the application of direct mechanical loading when cultured on soft collagen based substrates provided intercellular separation necessitates process formation. Furthermore, for the cells to differentiate further along the osteogenic pathway, an optimal stiffness of approximately 286 Pa must be used. It is proposed that this simulates the in-vivo environment of the cells, where osteoblasts develop on osteoid, a soft collagen based matrix, and subsequently differentiate when they become embedded within this matrix. Knowing how the mechanical environment affects osteocyte development is a vital step in the regeneration of viable bone tissue for implantation.
Chapter 5: Cell Morphology and Focal Adhesion Location Alters Internal Cell Strain

5.1 Introduction

Extra cellular mechanical cues, such as differences in passive substrate stiffness, externally applied mechanical strain and fluid flow induced shear stress, have been shown to affect many aspects of cell behaviour, including migration, proliferation and differentiation (Pelham and Wang, 1997, Saez et al., 2007, Bian et al., 2013, Kapur et al., 2003, Guo et al., 2012). The internal machinery of the cell, including tensile (actin) and compressive (microtubule) elements as well as focal adhesion attachment complexes are known to play a role in the translation of extracellular forces and consequently alter fundamental cell behaviours, such as viability and migration, through the generation of intracellular tension (Galbraith and Sheetz, 1998). Osteogenic cells have a highly developed cytoskeleton and it is known that their differentiation is regulated in part through mechanical forces imposed by their surrounding environment (Khatiwala et al., 2006a, Kapur et al., 2003).

One of the most dramatic examples of osteogenic differentiation is the change in morphology from cuboidal osteoblasts to osteocytes, which display numerous, long, thin cell processes extending from a small, rounded cell body (Dallas and Bonewald, 2010). Various studies have demonstrated the effect of extracellular mechanics on osteogenic differentiation. In particular it has been shown that osteoblastic differentiation of both mesenchymal and embryonic stem cells (MSC and ESC) is controlled by passive differences in extra cellular matrix (ECM) stiffness (Engler et al., 2006, Evans et al., 2009), while Chapter 4 of this Thesis investigated the specific effect of substrate stiffness on the later stage of osteogenic differentiation, namely
osteoblast to osteocyte development (Mullen et al., 2013). The results of Chapter 4 demonstrated that MC3T3-E1 cells will adopt the dendritic morphology of an osteocyte when cultured on soft collagen based substrates. Changes in cell morphology have recently been shown to affect both intracellular tension (McBeath et al., 2004) and osteoblast differentiation (Ruirong et al., 2013). It is intriguing to speculate that the dramatic change in morphology observed in Chapter 4 of this thesis could result in a change in intracellular tension.

Cell stiffness has been shown to be influenced by cell morphology, specifically by cell height (Vichare et al., 2012), and substrate stiffness (Solon et al., 2007). The stiffness of various cell types has been measured using AFM (Kuznetsova et al., 2007, Sato et al., 2004, Sen et al., 2005), and it has recently been shown that cell stiffness can indicate osteoblast differentiation of MSCs (Bongiorno et al., 2013). Therefore, differences in the stiffness of the cells themselves, arising on the various substrates examined in Chapter 4, could also play a role in the control of the differentiation process, but this yet to be examined.

Focal adhesions (FAs) are multicomponent protein complexes which adhere cells to their ECM (Berginski et al., 2011), and facilitate the transfer of external force through the stress fibres of the cell (Ridley and Hall, 1992, Nobes and Hall, 1995). They are known to be specifically involved the osteogenic differentiation of MSCs (Titushkin and Cho, 2011) and are thought to be of particular importance in osteoblast differentiation on collagen based substrates (Mathieu, 2012). Focal adhesion formation is also known to be affected by changes in substrate stiffness (Khatiwala et al., 2006a). However, as of yet, little is known about the interplay between focal adhesion formation, cell morphology and intracellular tension and their effect on osteogenic differentiation. The effects of these parameters on
osteocyte differentiation must be uncovered if a greater understanding of osteocyte mechanobiology is to be achieved.

Finite element modelling techniques have been widely used to investigate the effect of various stimuli, such as fluid flow (McGarry et al., 2005a, Vaughan et al., 2013), externally applied strain (Stops, 2008, Stern and Van Dyke, 2012) or strain applied directly to individual cells (Rudd, 2001, Mijailovich et al., 2002), on the intracellular loading state. Typically these models are built using a variety of passive material descriptions, which may be linearly elastic (McCreadie, 1997, Charras and Horton, 2002), hyperelastic (Caille et al., 2002) or viscoelastic (Karcher et al., 2003, Trickey et al., 2006) in nature. Recently an active material model has been used to examine the effects of extracellular mechanics on stress fibre formation (Weafer et al., 2013, Ronan et al., 2012) as well as the force generated by individual focal adhesions in MSCs (Ronan et al., 2013), thus highlighting the importance of the inclusion of realistic focal adhesion locations in such models. Other studies have demonstrated the effects of cell morphology (Dailey et al., 2009) and osteocyte process formation (Verbruggen, 2012) on the internal force generation of the cell. Together these studies demonstrate that realistic cell morphologies are also vital in the creation of finite element cell models.

In this study we used a combination of experimental and finite element techniques to test the hypothesis that a link exists between internal cell stiffness, intracellular strain, focal adhesion formation and morphological alterations of the cells occurring as a result of changes in substrate stiffness. MC3T3-E1 pre-osteoblast cells were cultured at low initial seeding density ($10^3$ cells/cm$^2$) on soft collagen based substrates, previously shown to induce early osteocyte differentiation (Mullen et al., 2013). Cell morphologies, stiffnesses and the location of focal adhesion complexes
were quantified and used to create finite element models of cell contraction (through applied thermal contraction material properties) against passively resistant substrates. Focal adhesion location and cell morphology and stiffness were varied according to experimental results.

5.2 Methods

5.2.1 Experimental Methods

5.2.1.1 Collagen substrate preparation
Type 1 rat tail collagen (Life Technologies) was neutralised with NaOH (Sigma Aldrich) at 18.4 μM /g collagen, and diluted with 10% Phosphate Buffered Saline (PBS) (all Sigma Aldrich) and 68% distilled H₂O. The mixture was then pipetted in 150 μL volumes onto 13 mm diameter coverslips (Sarstedt) and incubated for 30 minutes at 37°C, before being rinsed with sterile PBS. This resulted in the formation of a soft, thick, gel like coating on the coverslips (Col). To produce substrates of different mechanical stiffness but identical ligand density, substrates were crosslinked with 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDAC) (Sigma Aldrich) by incubating at 20 μM/mg collagen (ColEDAC1) or 100 μM/mg collagen (ColEDAC2) EDAC for 3.5 hours at room temperature as described previously (Haugh et al., 2011, Mullen et al., 2013). Substrates were then rinsed with PBS and incubated in fresh PBS for 3 hours at room temperature to remove any remaining EDAC before being washed twice with sterile distilled H₂O. Uncoated tissue culture plastic was used as a control.

5.2.1.2 Cell culture
MC3T3-E1 cells are a murine derived osteoblast cell line, which display the spread morphology associated with the osteoblast phenotype (Sudo et al., 1983, Quarles et
al., 1992). The cells are capable of differentiating into osteocytes and mineralising their surrounding matrix (Sudo et al., 1983) and are considered to be a good model of primary osteoblasts (Quarles et al., 1992). As such they are widely used in the study of osteoblast biology (Maeda et al., 2001, Ducy et al., 1997). For these studies MC3T3-E1 cells were maintained in Alpha Modified Eagle’s Medium (α-MEM) supplemented with 10% foetal bovine serum, 100 U/mL penicillin streptomycin and 100 ug/mL L-glutamine (all Sigma Aldrich) prior to all experiments. Cells were cultured at an initial seeding density of $10^3$ cells/cm$^2$. These culture conditions have been previously shown to allow for a change in cell morphology from the spread cuboidal shape of osteoblasts to the osteocyte morphology, characterised by numerous long cell processes extending from a small rounded cell body (Mullen et al., 2013).

5.2.1.3 Stiffness measurements

Cell and substrate stiffness measurements were taken after 4 days of culture, using a JPK Cellhesion 200 atomic force microscope (JPK Instruments, Berlin). The Young’s modulus of each substrate was measured in the vicinity (within 20 mm) of the cell. Following this, the Young’s moduli of five spread and five dendritic cells were measured on each substrate. All cells were measured at both the proximal (directly over the cell nucleus) and distal (within 3 μm if the cell edge) regions of the cell cytoplasm. A silicon-nitride pyramidal tip with tip radius of 5 nm was used for all measurements, with the Young’s modulus of both the cells and substrates being related to the force generated by the AFM system through Equations 3.34 and 3.35.

To ensure the cell measurement is not influenced by the stiffness of the underlying material, tip indentation should less than 10% of the total cell depth (Oliver and Pharr, 1992). To verify that this was the case for these experiments, the height of
each cell was measured by approaching the surface both at the point of interest and directly adjacent to the cell and recording the absolute height values. Force-distance curves were then only analysed up to a maximum of 10% indentation.

5.2.1.4 Cell staining for focal adhesions

Cultures were fixed after 7 days of culture using 4% paraformaldehyde (Fluka) in piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES) buffer (Sigma Aldrich). Cells were permeabilised with Triton-X100 (Sigma Aldrich), diluted to 0.05% in PBS, before being treated with primary mouse anti-vinculin (V9131, Sigma-Aldrich) and secondary goat anti-mouse (Alexa fluor 488, Life Technologies). Cells were then counterstained with tetramethylrhodamine (TRITC) labelled rhodamine-phalloidin (Life Technologies) to identify the actin cytoskeleton and mounted in 4',6-diamidino-2-phenylindole (DAPI) (Vector Labs) containing hard set mounting media for imaging.

5.2.1.5 Morphological analysis of cell phenotype

Images were taken using a Zeiss LSM 510 Axiovert Inverted Confocal Microscope at different locations on the coverslips at 10X magnification. Cell processes were defined as cellular features located at the cell membrane which had a cross sectional diameter of no more than 1 μm and extended for a distance of at least 5 μm. Cells with cell processes were classified as “dendritic” while cells without any cell processes were classified as “spread”. Example morphologies are shown in Figure 5.1. The number of processes on each dendritic cell, the longest and shortest axes of each spread cell as well as the cell body diameter and length of each process on each dendritic cell were measured. All parameters were measured for a minimum of 10
cells on each substrate and the average values were calculated for each parameter on each substrate.

**Figure 5.1:** Cell morphological examples. Short and long axes in Spread cell morphology and cell body diameter in Dendritic cell morphology are labelled. White arrows indicate cell processes.

5.2.1.6 Focal adhesion location

Focal adhesions were imaged using a Zeiss LSM 510 Axiovert Inverted Confocal Microscope. Cells of each morphology were broken into regions and the number of focal adhesions in each region was quantified for each cell morphology, with a focal adhesion defined as an area of stain of over 1 $\mu m^2$ in area. The cellular regions, as shown in Figure 5.2, were as follows: (1) **Nucleus** – the region of the cell directly under the cell nucleus, (2) **Distal end** – the cell border perpendicular to the direction of principle actin alignment (spread cells only), or the end of the cell process (dendritic cells only), (3) **Edge** – the cell border parallel to the direction of principle actin alignment (spread cells only), or the cell border excluding the cell process (dendritic cells only), (4) **Process** – the cell process excluding the most distal 1 $\mu m$ (dendritic cells only), (5) **Cell body** – the remainder of the cell body. This was repeated for at least 10 cells of each morphology on each of the three substrates.
Figure 5.2: Focal adhesion location on each cell morphology. Cellular regions labelled; Nucleus, Edge, Distal end and process are labelled. White arrows indicate focal adhesion complexes as identified through vinculin staining.

5.2.1.7 Statistical analysis

A one way ANOVA was performed on substrate stiffness measurements and on cell stiffness measurements to compare cells to one another. A paired t-test was conducted to compare the stiffness of the proximal and distal regions of the cell, while a one way ANOVA was used to determine statistical difference between average cell measurements, as described in section 5.2.1.5, on each substrate.
5.2.2 Methods - Computational

5.3.2.1 Model development

Two cell models, representing spread or dendritic cell morphologies, were created using ABAQUS software according to the average measured parameters described in Table 5.1. The substrates had an outer edge 50 μm from the most distal point of the cell, with a depth of 10 μm below the cell bottom. Preliminary studies showed that edge effects were minimised using these dimensions (see Appendix 2). Rounded edges were included wherever feasible to minimise the effect of stress concentrations due to geometry. For improved computational efficiency, models were reduced to sixth or eighth geometries where symmetry allowed.

<table>
<thead>
<tr>
<th></th>
<th>Spread</th>
<th>Dendritic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nucleus</strong></td>
<td>18.3 ± 2</td>
<td>18.3 ± 1.1</td>
</tr>
<tr>
<td><strong>Long axis</strong></td>
<td>82 ± 15.8</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>Short axis</strong></td>
<td>45.2 ± 13.6</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>Cell diameter</strong></td>
<td>n/a</td>
<td>20.8 ± 2.1</td>
</tr>
<tr>
<td><strong>No. Processes</strong></td>
<td>n/a</td>
<td>3.7 ± 1.4</td>
</tr>
<tr>
<td><strong>Process length</strong></td>
<td>n/a</td>
<td>31.6 ± 15.1</td>
</tr>
<tr>
<td><strong>Middle/end ratio</strong></td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

**Table 5.1:** Cell measurements for Spread and Dendritic cell morphologies. All units of length are in μm
The highly developed cytoskeleton of osteogenic cells made the discrete modelling of cytoskeletal components computationally prohibitive. Instead the cytoskeleton and cytoplasm were modelled as a linearly elastic continuum, with elastic moduli of both cells and substrates assigned based on cell stiffness measurement outlined in section 5.2.1.3 and shown in Table 5.2.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Soft</th>
<th>Intermediate</th>
<th>Stiff</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spread cell</td>
<td>600 Pa</td>
<td>1800 Pa</td>
<td>10 kPa</td>
</tr>
<tr>
<td>Dendritic cell</td>
<td>7 kPa</td>
<td>13 kPa</td>
<td>18 kPa</td>
</tr>
<tr>
<td></td>
<td>8 kPa</td>
<td>12 kPa</td>
<td>24 kPa</td>
</tr>
</tbody>
</table>

**Table 5.2:** Young’s moduli of substrates after 4 days of culture, as well as Young’s moduli of spread and dendritic cells on each substrate at the same timepoint, as measured through AFM procedure described in Section 5.2.1.3. *, ^ and ˦ indicate statistical difference between values.

The cells were attached to the substrate by three different methods. The first method involved the application of tie constraints along the entire cell substrate interface. In the second method one individual focal adhesion site, measuring 1 μm² in area was assigned per model. A high density mesh was used at this site and at the corresponding substrate site to attach the cell to the substrate. For the final method numerous focal adhesion complexes were assigned to the cell, according to the experimental results described below (Table 5.3). Quadratic tetrahedral C3D10R elements were used for all models.
<table>
<thead>
<tr>
<th>Cell type</th>
<th>Nucleus</th>
<th>Distal End</th>
<th>Edge</th>
<th>Body</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spread</td>
<td>4.1 ± 1.4</td>
<td>12.2 ± 6.8</td>
<td>7.3 ± 6.1</td>
<td>17.4 ± 15.1</td>
<td>n/a</td>
</tr>
<tr>
<td>Dendritic</td>
<td>4.4 ± 2.7</td>
<td>3.5 ± 1.2</td>
<td>1.8 ± 1.3</td>
<td>2.5 ± 1.6</td>
<td>6.0 ± 3.4</td>
</tr>
</tbody>
</table>

Table 5.3: Focal adhesion location per cell region. Cell regions are described in section 5.2.1.6 and shown in Figure 5.2.

5.3.2.2 Boundary conditions and loading

In all cases, boundary conditions were applied to the cell, nucleus and substrate to account for symmetry. Further boundary conditions were applied to prevent radial movement of the distal surface of the substrate and axial movement of the bottom surface of the substrate, so as to simulate an infinitely stiff well plate/petri dish (relative to the stiffness of the substrate), as shown in Figure 5.3. An isotropic thermal contraction was applied to the cell body in the rr direction shown in Figure 5.3. A thermal expansion coefficient of 0.05 K⁻¹ was used in conjunction with a negative thermal load of 1 K, similar to previous techniques use to simulate active cell contraction (Stops, 2008). These values were selected so as to cause a 5% decrease in the radius of the cell body when unrestrained. The stress generated in the cell body due to the resistance of the substrate against the applied contraction was measured and compared across the different cell models.
Table 5.4: Spread cell geometry, showing axial and radial boundary conditions (black arrows). Symmetry boundary conditions (not shown) prevented angular displacement of the model.

5.3 Results

5.3.1 Cell geometry

As shown in Table 5.1, spread cells were found to have a larger area with an average long axis of 82 µm and an average short axis of 45 µm, compared to dendritic cells, which had an average diameter of 22 µm, excluding processes. Dendritic cells were also found to have an average of 3.7 processes per cell with an average process length of 31.6 µm (see Table 5.1). The nucleus diameter of the cells ranged from 17.8 to 19.1 µm but no statistical difference in nucleus size existed between the three morphologies. No statistical difference due to substrate stiffness existed in the parameters of the various cell morphologies. However, it should be noted that the total focal adhesion density (across all cell types) did vary according to substrate stiffness, as shown in Table 5.4.
### 5.3.2 Focal adhesion location

It was observed that spread cells contained far more focal adhesion complexes than dendritic cells (see Table 5.3), with an average of 41 compared to 18. The majority of focal adhesions on spread cells were located in the centre of the cell, whereas on dendritic cells more adhesion sites were observed in the cell processes than anywhere else on the cell. It was also observed that a single focal adhesion was located on the distal end of all cell processes. No statistical difference due to substrate stiffness existed in focal adhesion location in the various cell morphologies.

### 5.3.3 Cell stiffness

The Young’s moduli of the substrates were measured as 0.6, 1.8 and 10 kPa respectively, as shown in Table 5.2, with all substrates being statistically different to one another. It was found that the stiffness of both spread and dendritic cells increased as the substrate stiffness was increased. On substrates of 0.6 kPa, spread and dendritic cells measured 7 kPa and 6 kPa respectively, while on substrates of 1.8 kPa, this increased to 13 kPa and 12 kPa respectively. As the substrate was further increased to 10 kPa, the stiffness of the cells increased to 18 kPA (spread) and 24

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Soft</th>
<th>Intermediate</th>
<th>Stiff</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA per cell</td>
<td>$29.9 \pm 5.0^*$</td>
<td>$35.1 \pm 6.4^\text{c}$</td>
<td>$48.2 \pm 7.3^\text{***}$</td>
</tr>
</tbody>
</table>

**Table 5.5:** Focal adhesion location per cell on each substrate. * and ^ indicate statistical difference in focal adhesion density between different substrates. ± indicates standard deviation.
kPa (dendritic) respectively. The stiffness of both cell morphologies measured on the 10 kPa substrate were found to be significantly higher than that measured on the 0.6 kPa substrate, but not that measured on the 1.8 kPa substrate. No statistical difference was observed between the proximal and distal stiffness measurements from either cell type or between the stiffnesses of different cell morphologies on the same substrates. Sample force distance curves are included in Appendix 3 of this thesis.

5.3.4 Intracellular stress (computational)

On all substrates, it was observed that higher levels of stress were generated in both morphologies when cells were attached to the substrate through the entire cell-substrate interface, while the lowest stress levels were generated when cells were attached through a single focal adhesion site per model. For example, as shown in Figure 5.4, the average stress generated in a spread cell on a substrate of stiffness 600 Pa was computed to be 190 Pa when the cell was attached through the entire cell substrate interface. This reduced to 110 Pa when the cell was attached through realistic focal adhesion locations and decreased further to 100 Pa when the cell was attached through a single adhesion site. It was also observed that the substrate stiffness had less effect on the intracellular stress for the dendritic cell morphologies compared to spread cells. This can be seen in Figure 5.4, where the average stress in the dendritic cell increases from 160 Pa on the softest (600 Pa) substrate to 370 Pa on the stiffest (10 kPa) substrate, while the average stress in the spread cell morphology increases from 110 Pa to 470 Pa over the same two substrates. Figure 5.5 represents the percentage of cell volume experiencing each stress band and further demonstrates that substrate stiffness has less influence over the stress experienced by dendritic cells. It was also observed that the highest levels of
intracellular stress are generated in the immediate vicinity of the focal adhesion attachment complexes. Moreover a higher level of stress is generated in the process of the dendritic cell morphology compared to the remainder of the cell body, as shown in Figures 5.6 and 5.7.

**Figure 5.4:** Average internal cell stress in the direction of principal fibre alignment.

Average stress was calculated by normalising element stress to element volume.
Figure 5.5: Percentage of (A) Spread cell morphology and (B) Dendritic cell morphology finite element models under various stress level when attached to substrate through realistic focal adhesion locations.
Figure 5.6: Stress generated in the radial direction in spread cell morphology, when cell is attached to substrate through experimentally based discrete locations. The location of two focal adhesions is indicated on the 1800 Pa model to demonstrate the stress concentration in the region surrounding the adhesion site.
Figure 5.7: Stress generated in the radial direction in dendritic cell morphology, when cell is attached to substrate through experimentally based discrete locations.

The location of two focal adhesions is indicated on the 1800 Pa model to demonstrate the stress concentration in the region surrounding the adhesion site.
5.4 Discussion

These results show that cell stiffness, morphology, focal adhesion density and location, as well as substrate stiffness all have a role to play in dictating intracellular stress. It is interesting that the method of cell attachment (focal adhesion complexes) strongly influences the intracellular stress generated through cell contraction, with a larger adhesion area resulting in higher internal cell stress. This finding highlights the importance of the inclusion of realistic focal adhesion locations in cell-substrate models. When realistic focal adhesion complexes are included it is shown that substrate stiffness has a greater effect on the stress profile of the spread cell morphology than that of the dendritic morphology. Most interestingly, it is notable that the dendritic morphology experiences a higher average stress than the spread morphology on the two softest substrates. These results suggest that when cultured on softer substrates, MC3T3s might alter their morphology and stiffness in order to achieve a more desirable stress state.

A possible limitation in this work is the use of a linearly elastic continuum to model complex, time dependant, hyperelastic structures. Other studies have used more complex modelling techniques to include non-linear elasticity (Mijailovich et al., 2002, Ohayon J, 2004) as well as discrete (McGarry et al., 2005a) or active cytoskeletal components (Ronan et al., 2012). However, the relatively simplistic material model used in the current study still allows for the comparison between the stress profiles of cells of different morphologies on substrates of different stiffnesses. Further to this, as the cytoskeleton becomes more developed (due to most of the cell body of osteogenic cells being composed of actin), the dissimilarity between a continuum model and the inclusion of a discrete cytoskeleton decreases. This is the case for the MC3T3 cells studied here, which have a highly developed cytoskeleton.
Meanwhile, the consequences of directionality in the transfer of force through the actin framework are also accounted for by the inclusion of an orthotropic contraction within the models in the direction of principal actin fibre alignment. This allowed for the effects of cell morphology, substrate stiffness and focal adhesion location on intracellular stress to be investigated without excessive computational expense.

Cell morphology is a widely used indicator of the differentiation stage of osteogenic cells (Palumbo et al., 1990, Yamanouchi et al., 1997), and MC3T3-E1 cells have previously been shown to alter their morphology in response to the same changes in the extracellular mechanical environment as induced here (Mullen et al., 2013). The dimensions of the dendritic cells measured in this study were similar to those reported for osteocytes in vivo (Mullender et al., 1996), as was the average cell process length (Sugawara et al., 2005). However, the number of processes present was fewer than either osteocytes in vivo (Sugawara et al., 2005) or isolated primary osteocytes cultured on two dimensional substrates (Tanaka Kamioka et al., 1998). Nucleus shape and dimensions have also been shown to vary when cells are subjected to mechanical force (Nathan et al., 2011), while control of nuclear shape has been shown to induce osteocalcin expression in isolated pre-osteoblasts (Thomas et al., 2002). Interestingly, the results of this study show no statistical change in nuclear dimensions due to differences in substrate stiffness, indicating that another method of cell mechanosensation must be driving the change in cell morphology previously observed (Mullen et al., 2013).

Focal adhesion complexes are known to play a key role in force transfer and cell mechanosensation (Ponik and Pavalko, 2004, Lim et al., 2007, Boutahar et al., 2004), while the density of attachment complexes in MC3T3-E1 cells has been shown to be affected by changes in substrate stiffness (Khatiwala et al., 2006a).
Meanwhile finite element simulations have shown that cell generated force depends strongly on focal adhesion area (Ronan et al., 2013). Despite this, a common method of force transfer between a cell and its underlying substrate used in finite element simulations is the generation of tie constraints across the entire cell substrate interface (Charras and Horton, 2002, Deguchi et al., 2009), while other studies have used single, arbitrarily assigned attachment sites (De Santis, 2011, Milner et al., 2012). The results presented here show that these approximations greatly alter both the magnitude and distribution of stress throughout the cell body, meaning that in order to accurately model cell substrate interaction, realistic focal adhesion densities and locations must be included.

Cell tension has been previously been shown to affect the osteogenic differentiation of MSCs (McBeath et al., 2004). Briefly, cell tension was reduced through Rho kinase (ROCK) inhibition of myosin activation by culture in Y-27632 containing media. It was found that this reduction in cell tension induced adipogenic rather than osteogenic differentiation, as measured by expression of liposome lipase (adipogenic) as well as ALP and CBFα1 (osteogenic). However, the interdependence between cell morphology and tension cannot be ignored as it has been shown that the disruption of the cytoskeleton through ROCK inhibition will prevent the formation of cell processes in MC3T3-E1 cells cultured on collagen substrates (Gellynck, 2013). Cell morphology has also been shown to affect cell generated tension, with bovine pulmonary smooth muscle cells and 3T3 mouse fibroblasts both shown to induce higher forces on micropost substrates when allowed to expand to a larger cell area (Tan et al., 2003), a phenomenon shown to require the active formation of stress fibres within the cell (McGarry et al., 2009).
It is intriguing to speculate that the cell adopts a dendritic morphology in order to achieve a more suitable stress state and that this stress state is a crucial aspect of osteocyte differentiation. Indeed, homeostasis of the intracellular tension has been widely cited as a cellular response to loading in various cell types. Studies have shown that fibroblasts subjected to external loading alter their stress fibre formation in order to maintain their internal stiffness (Mizutani et al., 2004, Brown et al., 1998), while the concept of tissue level homeostasis in bone has been widely accepted (Cowin, 2005, Rodan, 1997, Bonewald, 2006, Klein-Nulend et al., 2013). This study sheds more light on the interplay between cell morphology and tension. It is hypothesised that the cell changes its morphology in order to achieve a more suitable stress state and allow osteocyte differentiation to occur. However, this change in morphology is itself driven by cytoskeletal reorganisation (Vogel and Sheetz, 2006, Wozniak and Chen, 2009), with the cytoskeleton itself being another method of controlling intracellular tension (Parsons et al., 2010, McBeath et al., 2004). With this in mind the effects of intracellular tension and cell morphology cannot be discussed as independent drivers of cell differentiation.

It is clear that cell morphology and tension are key drivers of cell differentiation, the mechanisms behind the process are still poorly understood (McBeath et al., 2004, Gellynck, 2013). While the relationship between substrate stiffness and the formation of stress fibres and focal adhesion complexes has been demonstrated computationally by Ronan et al (Ronan et al., 2013). However, the chemical factors involved in these changes in cell behaviour are as of yet poorly understood. Many enzymes and proteins are involved in the cellular differentiation process, and some have already been identified as also being affected by cell tension. In particular RhoA, a known regulator of cytoskeletal mechanics (Sit, 2011, Peck et al., 2002),
has been shown to be affected by cell morphology in MSCs and in so doing affect the morphology and differentiation pathway of the cells (McBeath et al., 2004). As well as this, RhoA, along with another member of the GTPase family Rac1, have been shown to specifically affect process formation in neurites (Tashiro and Yuste, 2004). It is possible that similar methods are used by osteogenic and neurogenic cells to achieve and maintain cellular processes and that the continued activity of these molecules is regulated through intracellular tension (McBeath et al., 2004, Bongiorno et al., 2013). This study provides a novel insight into the interplay between cell morphology and tension, specifically in relation to the development of cell processes during osteocyte differentiation.

5.5 Conclusion

The results presented here show that intracellular tension is affected by substrate stiffness and that this effect is mediated through focal adhesion complexes, the location and density of which influence the effect of substrate stiffness. We also show that both the levels and distribution profile of intracellular stress directly affected by cell morphology and stiffness. Specifically we observe that the stress profile of cells which adopt a dendritic morphology, similar to that of an osteocyte are less affected by changes in substrate stiffness. It is therefore hypothesised that by changing their morphology, these cells can obtain a more desirable stress state and that this is an essential cue in the differentiation process.
Chapter 6: The Effects of Substrate Stiffness, Thickness and Crosslinking Density on Osteogenic Cell Behaviour

6.1 Introduction

Osteogenic cells possess a highly developed cytoskeleton (Tanaka Kamioka et al., 1998, Bonewald, 2011a), and have been long regarded as efficacious mechanosensors (Kapur et al., 2003, You et al., 2008). There has been widespread investigation into the effect of various mechanical forces, including substrate stiffness (Engler et al., 2006, Mullen et al., 2013), fluid flow induced shear stress (Ponik et al., 2007) and applied substrate strain (Weyts et al., 2003) on osteogenic cell behaviour. While a general consensus is evolving that an understanding of mechanobiology is necessary for the treatment of disease originating at the cellular level (Papachroni et al., 2009, Bonewald, 2011a, Santos et al., 1999), the exact nature of the methods by which cells interact with their environment must be delineated if the mechanobiology of osteogenic cells is to be better understood. Specifically the effects of bulk material stiffness, substrate thickness and heterogeneity within the substrate have yet to be separated to investigate both their individual and combined effects on cell behaviour.

One of the most common methods of investigating mechanobiology is the culture of cells on substrates of controllable stiffness and it has been shown that a change in substrate stiffness can affect osteoblast behaviour, including proliferation, migration and differentiation (Keogh et al., 2010b, Tsai et al., 2012, Evans et al., 2009). There are various approaches for altering the stiffness of substrate materials for in vitro cell culture applications. Polyacrylamide is widely used in mechanobiology studies due to the relative ease and reliability with which its stiffness can be altered through the
percentage of acrylamide and bis-acrylamide used in the polymerisation process (Wang and Pelham Jr, 1998, Tse and Engler, 2001), while a range of other polymers including polydimethylsiloxane (Evans et al., 2009), polyethylene glycol (Khatiwala et al., 2006b) and polymethyl methacrylate (Dalby et al., 2007) have also been used, all of which can have their stiffness controlled through the polymerisation process. Collagen, the primary component of the matrix on which bone cells develop, can be modified using a variety of crosslinking methods including chemical crosslinkers such as glutaraldehyde and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) and exposure to ultraviolet light to achieve specific bulk substrate stiffness (Tierney et al., 2009, Chau et al., 2005). However, this change in material properties is not homogenous, as the formation of crosslinks is dependent on the position and density of fibres in the collagen. In order for the effect of crosslinking on cell behaviour to be analysed, particular attention must be paid to the heterogeneity present in the substrate material.

Recently, substrate thickness has been used as a method of varying the stiffness as experienced by the cell (Leong et al., 2010, Sen et al., 2009). On thin substrates (< 5 μm) cell-induced contractile forces can propagate through the entirety of the substrate allowing the underlying material, often glass coverslips or tissue culture plastic, to govern cell behaviour. However, the effect of substrate thickness is also governed by the structure of the substrate in question. Specifically it has been demonstrated that cell induced forces travel only a few microns through linear, homogenous substrates such as polyacrylamide (Maloney et al., 2008), whereby the average focal adhesion site area in 3T3 fibroblasts was shown to decrease as substrate thickness was increased up until a total substrate thickness of 5 μm, above which no further decrease in area was observed. This is in marked contrast to the
behaviour of cells on fibrous substrates, which have been shown to be influenced by structures which are hundreds of microns away (Leong et al., 2010, Feng et al., 2013), as demonstrated through the underlying coverslip influencing cell morphology on fibrous substrates of up to 130 \( \mu \text{m} \). This difference in the effect of substrate thickness has been attributed to the fibrous nature of biological substrates, which has been proposed to occur as the cell induced force propagates through individual fibres to interact with the underlying coverslip (Rudnicki et al., 2013).

It is well known that the fibrosity of biological tissues greatly alter the forces experienced at the cellular level from those induced at the tissue level (Han et al., 2013, Upton et al., 2008). The specific effect of fibres on the mechanical properties of fibrous gels has also been investigated through computational approaches. A microscale discrete fibre representative element has been linked to a Galerkin macroscale model to study the effects of fibrosity on bulk collagen gel properties (Stylianopoulos and Barocas, 2007), demonstrating the non-uniform deformation of a fibrous gel. This method has been further developed to investigate both fibrous wound behaviour (Chandran, 2007) and the response of cells to gel fibrosity (Rudnicki et al., 2013). However, this approach prevents the fibres from extending between elements at the macroscopic level. In the relatively thin (70 \( \mu \text{m} \)) substrates used in cell mechanobiology experiments (Sen et al., 2009) collagen fibres may extend through the entire gel thickness. Further to this the effect of varying crosslinking density, a common method of altering substrate stiffness (Mullen et al., 2013, Tierney et al., 2009, Bian et al., 2013, Erickson et al., 2009), on force transfer through fibrous gels has yet to be investigated. The range of methods used to alter substrate stiffness experienced by the cell has led to apparent contradictions in results. For example osteoblast differentiation has been shown to occur on substrates
between 20 and 40 kPa (Engler et al., 2006, Khatiwala et al., 2006b), or stiffer substrates of up to 80 kPa. Meanwhile the results of Chapter 4 of this Thesis have shown that osteoblasts differentiation occurs on type 1 collagen substrates of 1 kPa, while softer substrates of 300 Pa induce osteoblast differentiation followed by early osteocyte differentiation. The precise stiffness experienced by the cell, here termed $E_{\text{eff}}$, the effective modulus, can only be found through investigating the combined effects of material stiffness, substrate thickness and substrate heterogeneity on cell behaviour.

In this Chapter we compared the cell spread area (which has shown to be an indicator of osteogenic differentiation potential of MSCs (Song et al., 2011)) of MC3T3-E1 cells, due to the combined effects of substrate stiffness and thickness. MC3T3-E1 cells were cultured on flat and wedge shaped non-fibrous polyacrylamide and fibrous collagen gels, while cell spread area was used to determine the effects substrate stiffness, structure and thickness on cell behaviour. Finite element simulations using discrete tensile collagen fibres in a soft linearly elastic gel were used to investigate the transfer of force through collagen gels of different crosslinking density over a range of gel thicknesses. We hypothesise that the effective stiffness experienced by the cell is influenced by gel Young’s modulus, thickness and crosslinking density and that attention must be paid to all these factors when investigating mechanobiology in vitro.
6.2 Methods

6.2.1 Experimental methods

6.2.1.1 Substrate manufacture

Flat and sloped collagen gels were prepared as described previously (Rudnicki et al., 2013). Briefly, rat tail type 1 collagen (Sigma-Aldrich) was neutralised using 10 mM NaOH, and diluted to 4 mg/mL with dH₂O. Glass slides were activated by sonicating in 1% 3-aminopropyltrimethoxy silane (Sigma-Aldrich) before being incubated in 0.5% glutaraldehyde (Sigma-Aldrich) overnight. The mixture was pipetted onto activated glass coverslips (Electron Microscopy Sciences) while the top coverslip was treated with Sigmacote (Sigma-Aldrich) to allow the gel to set in the required shape (flat or wedge) without attaching to the upper coverslip. Flat gels were formed at approximately 70 μm thick, while sloped gels ranged from 0 to 150 μm over a lateral distance of 50 mm.

Polyacrylamide gels were formed by varying the concentrations of acrylamide and bis-acrylamide (BioRad) to alter the gel stiffness as described previously (Tse and Engler, 2001), before being polymerised with 0.15% TEMED. Gels were formed between treated glass coverslips as described above. SulfoSANPAH (Fischer Scientific), a heterobifunctional crosslinker was diluted to 1 mg/mL in HEPES buffer and used to bind acetic acid diluted collagen (4 mg/mL) to the gel surface to allow for cell attachment.

6.2.1.2 Cell culture

MC3T3-E1 cells, an immortalised pre-osteoblast cell line were maintained in Alpha Modified Eagle’s Medium (α-MEM) containing 100 ug/mL L-glutamine and supplemented with 10% foetal bovine serum and 100 U/mL Antibacterial-
Antimytotic (all Life Technologies). Cells were fixed with 4% paraformaldehyde following 24 hours culture at 37 °C and 5% CO₂. Membrane permeabilisation was conducted with 0.1% Triton in Phosphate Buffered Saline (PBS) (both Sigma-Aldrich) and cells were stained with Fluorescein isothiocyanate (FITC) labelled rhodamine-phalloidin (actin cytoskeleton) and Hoescht (nuclei) (both BD Biosciences). Cells were imaged using a Leica DM2700 inverted microscope at 10X magnification.

6.2.2 Finite element methods

The material under study is a type 1 collagen gel whose microstructure, a fibrous arrangement of interconnected collagen fibres is shown in Figure 1. The behaviour of these gels may be varied by chemical crosslinking, allowing a greater number of adjacent fibres to form zero length molecular bonds between one another, thus affecting the materials mechanical properties (Haugh et al., 2011). To understand the role of microstructural crosslinking density on mechanical response of these gels, a micromechanical model was developed using ABAQUS finite element software, representing the fibrous and non-fibrous phases of this material discretely with a finite element framework. This micromechanical approach was used to predict the effective properties of random collagen fibre distributions, having a range of crosslinking densities to study their effect on macroscopic material behaviour.
Figure 6.1: A) Ion conductance microscopy (ICM) image of collagen gel fibres (Azonano, 2013), and B) sample finite element representation of fibres within a gel.

6.2.2.1 Model generation

The fibres within collagen gels exhibit no preferential orientation and can therefore be considered to be randomly oriented. They measure approximately 200 nm (McDaniel et al., 2007) in diameter and account for approximately 20% of the material volume fraction (Saidi et al., 1995, Driessen et al., 2003). A numerical algorithm was developed to create representative distributions of these fibrous gels in two-dimensions, whereby each fibre was assigned a centre point, C, that was randomly chosen within a domain measuring 450 by 450 µm. The length of each collagen fibre was chosen randomly between the limits 10 and 500 µm, while the orientation of each fibre was also chosen to be a random angle \( \theta \), where \( -\pi \geq \theta > \pi \). A resulting collagen fibre distribution generated using this procedure is shown in Figure 6.1 (B) and Figure 6.2. The fibre end points were then generated by extrapolating half the length in both the direction of the angle \( \theta \) and it’s opposite angle \( \theta - 180 \). Fibres were truncated so as to fall within the confines of the gel as
appropriate. Models were generated for a range of crosslinking densities with crosslinked and non-crosslinked fibres being introduced to the model separately. For each model, the relevant crosslinked fibres were imported directly to the ABAQUS sketch facility as a single part using a python script. This created a series of fibres where each intersecting point was assigned a unique node, while a single element joined corresponding nodes to one another. Non-crosslinked fibres were introduced as discrete nodes and elements to the relevant ABAQUS input file, with a single element spanning the entire length of each fibre. Thus non crosslinked fibres were prevented from directly interacting with one another, although indirect interaction between non-crosslinked fibres could still occur through the gel.

**Figure 6.2:** Boundary conditions placed on micromechanics model. A uniaxial tensile test was simulated on the structure through allowing horizontal displacement of one side and applying a 1% strain displacement to the top edge.
6.2.2.2 Finite Element Model

Two dimensional plane stress finite element models were created to simulate a 50 μm wide section of a soft gel containing relatively stiff collagen fibres. Fibres were set as linear, truss elements (T2D2) with a circular cross-section of radius 100 nm which were embedded into the plane stress element (CPS8R) containing gel region. A Young’s modulus of 10 Pa was assigned to the gel based on previous atomic force microscopy measurements, reported in Chapter 4. A Young’s modulus of 2 MPa was assigned to the fibres in order to achieve a range of equivalent moduli on the same scale as the range present in the polyacrylamide substrates investigated.

A uniform normal displacement boundary condition was applied to the top edge of the micromechanical model that resulted in a 1% normal strain, while the bottom edge and one side edge were kept horizontal and vertical respectively (see Figure 6.2 for boundary conditions). A surface based homogenisation scheme was used to determine the effective properties of the micromechanical models. This involved computing the average stresses from surface tractions acting on the edges of the model due to an applied surface strain. The effective normal stress, \( \bar{\sigma}_{xx} \), may be determined using the following relation,

\[
\bar{\sigma}_{xx} = \int_{0}^{d} F \, d
\]

where the integral is the resultant forces acting on the edge due to the normal reaction force vector \( F \), \( d \) is the length of the model along the top edge and \( t \) is the plane stress thickness. The effective modulus, \( E_{\text{eff}} \), was defined as the relationship between the stress and the predefined strain (1%). The procedure was repeated for a range of crosslinking densities. The effect of thickness on the equivalent tensile modulus was investigated by reducing the vertical dimension of the gel, and truncating the fibres contained within as appropriate.
6.3 Results

6.3.1 Experimental results

On polyacrylamide (PA) gels of 0.6 or 1.2 kPa, cells adopt an encapsulated morphology and converge into groups as shown in Figure 6.3 (A). The average cell spread area on these substrates is 400 \( \mu m^2 \). Above this stiffness cells separate from one another and begin to elongate, increasing their area to between 700 and 800 \( \mu m^2 \) on substrates of between 2.4 kPa and 9.6 kPa. The cells do not appear to group together on these substrates as demonstrated by the dispersed nature of the cells in Figure 6.3 (B). On stiffer substrates (above 9.6 kPa), cells increase their area to an average of between 1200 and 1600 \( \mu m^2 \) and adopt the spread morphology associated with osteoblasts, see Figure 6.3 (C).
**Figure 6.3:** Representative images of MC3T3-E1 cells on polyacrylamide gels of: A) 600 Pa, B) 9.6 kPa and C) 38.4 kPa and Collagen gels crosslinked with: D) 0mM EDAC, E) 50 mM EDAC and F) 150 mM EDAC. White arrows on A) indicate multiple cells grouped together
Cell area on crosslinked collagen substrates increased as the crosslinking density used was increased. As shown in Figure 6.5, the lowest average spread area is approximately 800 $\mu$m$^2$, which occurred on the non-crosslinked (shown in Figure 6.3 (D)) and 20 mM crosslinked gel. The cells typically display an elongated morphology, similar to that observed on the 9.6 kPa PA gel, shown in Figure 6.3 (B). On gels with a higher density of crosslinking (50 to 150 mM/mg collagen), the cells adopt the spread morphology as observed on 38.4 kPa PA gels, shown in Figure 6.3 (F), while the average spread cell area increases to between 1200 mm$^2$ and 1600 mm$^2$. 

Figure 6.4: Area of MC3T3-E1 cells on Polyacrylamide gels for 24 hours. a indicates statistically higher than 0.6 kPa to 4.8 kPa gels. b indicates statistically higher than 0.6 kPa to 9.6 kPa gels. c indicates statistically higher than 0.6 kPa to 19.2 kPa gels. $P > 0.05$. 
Figure 6.5: Area of MC3T3-E1 cells on crosslinked rat tail collagen for 24 hours. * indicates statistically higher than 0mM and 20 mM EDAC per mg collagen concentration. P > 0.05.

Cell spread area on collagen gels was also shown to increase as the gel thickness was decreased, and this effect was more pronounced on gels with a lower crosslinking density. As shown in Figure 6.6, a large rise in cell area, from 400 $\mu$m$^2$ to just over 1500 $\mu$m$^2$ was observed as the thickness of a non-crosslinked collagen gel was decreased from 150 mm to 5 $\mu$m. On more densely crosslinked gels (50 to 150 mM), the thickness of the structure had less effect on the spread cell area. This is shown in Figure 6.6, where the average cell spread area on a 150 mM crosslinked gel increases from 1400 $\mu$m$^2$ to 1700 $\mu$m$^2$. 
6.3.2 Finite element results

The inclusion of discrete fibres to the finite element model increased the effective modulus from 10 Pa (non-fibrous gel) to approximately 25 kPa. The relative stress borne by the gel and fibres can be seen in Figure 6.7 (A). It is also shown in Figure 6.7 (B) that the transverse fibres are involved in load transfer through adjoining crosslinks. This results in an increase in effective modulus from 25 kPa to 90 kPa observed as the percentage of crosslinked fibres in increased from a structure with no crosslinks to one where all intersecting fibres were connected to one another. The linking of fibres within the structure enables load transfer through multiple connected fibres throughout the entire gel depth. This can be seen by comparing the two plots contained in Figure 6.7. In the non crosslinked model (Figure 6.7A) fibres that do not span the depth of the gel bear considerably smaller loads. Meanwhile in the crosslinked model (Figure 6.7B), fibres in the transverse direction bear loads comparable to that borne on fibre extending axially through the entire gel depth.

Figure 6.6: Area of MC3T3-E1 cells on sloped crosslinked collagen substrates.

Average cell area at relevant thickness for each gel is shown.
Figure 6.7: Micromechanical simulation showing the relative stress states in collagen gels with (A) non-crosslinked fibres and (B) crosslinked fibres. The load borne by transverse fibres in (B) is much greater than that borne by transverse fibres in (A) indicating the transfer of force through fibre crosslinks.

The change in equivalent modulus was also dependent on the thickness of the gel in question. As shown in Figure 6.8, as the thickness of a gel with no crosslinks between the fibres reduced from 150 μm to 10 μm, the effective modulus of the structure increased from 25 kPa to 55 kPa. Reducing the thickness of a gel containing a high percentage of linked fibres has very little effect on the equivalent modulus of the structure. This is best observed in Figure 6.8, where the effective modulus of a gel with 100% crosslinked fibres was found to be 85 kPa at 150 μm and increased to just 90 kPa when the gel depth was reduced to 10 μm. It was also observed that a larger number of fibres span the entire depth of the gel as the total gel depth is reduced. For example, it can be seen in Figure 6.9 that 12 fibres span the depth of the 10 μm gel, whereas only one fibre spans the depth of the 150 μm gel.
**Figure 6.8:** Equivalent tensile stiffness of crosslinked collagen gels of different thicknesses as calculated using ABAQUS software as percentage of crosslinked fibres was altered from 0 to 100%. Equivalent tensile stiffness was calculated through the relationship between the reaction force per μm along the top edge of the gel to the strain applied to the structure (1% in all cases).
Figure 6.9: Demonstration of the effect of substrate thickness on the force distribution within collagen fibres. Stress distribution plots of (A) 10 μm thick gel and (B) 150 μm thick gel. An increased number of fibres extend through the entire gel depth in the 10 μm gel shown in (A) compared to the 150 μm gel shown in (B).
6.4 Discussion

Cell spread area has been shown to be an indicator of the differentiation stage of MSCs, with osteoblast differentiation found to correlate with increased cell area (Song et al., 2011, Kilian et al., 2010). The results of this study demonstrate that cells on non-fibrous PA gels exhibited a larger spread cell area on stiffer gels. It was also shown that cells cultured on relatively soft collagen substrates exhibited the same large spread area as observed on stiff (>38 kPa) PA gels. The finite element simulations in this study predict that the equivalent modulus of a fibre reinforced soft gel is significantly greater than that of a non-reinforced gel. It was also seen that, as the concentration of crosslinking agent was increased, the cell spread area also increased (observed in experimental results). This is proposed to be due to an increase in the effective modulus (as predicted by the FE simulations). Gel thickness also had an effect on both cell spread area and effective modulus, as thinner substrates resulted in an increase in cell spread area and effective modulus as measured through FE simulations.

A possible limitation of this study is the use of the MC3T3-E1 cell line as a model of primary osteoblasts. However, these cells have been shown to be a suitable model for several aspects of osteoblast behaviour, including ALP expression and matrix mineralisation (Quarles et al., 1992, Sudo et al., 1983), while they have also been used to investigate cell spread area in response to substrate stiffness (Khatiwala et al., 2006a). Another possible limitation is that cell differentiation was not directly measured through long term experiments, but inferred from the very early indicator of spread area (Song et al., 2011, Cheng et al., 2013). This was necessary as osteoblasts change the mechanical properties of their substrate over time (Buxton et al., 2008), by means of matrix mineralisation, and as such examination of the exact
stimuli experienced by the cell is not possible once the cells have begun to differentiate. Further to this, cell area immediately after plating has been shown to be a good predictor of osteogenic differentiation of MSCs after 7 and 21 days culture (Song et al., 2011).

The effects of substrate stiffness and thickness on cell differentiation have both been studied in detail. However, the interplay between the various factors involved has yet to be elucidated. The results presented in this study, provide a new insight into the combined effects of substrate stiffness, fibrosity and thickness on bone cell behaviour. Moreover this study provides a possible explanation for previous contradictions concerning the differentiation of osteogenic cells in response to extracellular matrix stiffness, which have either shown osteoblast differentiation to occur on soft substrates between 20 and 40 kPa (Engler et al., 2006, Khatiwala et al., 2006b), or stiffer substrates of up to 80 kPa (Rowlands et al., 2008). Gel thickness has been shown to affect cell behaviour to different extents depending on the nature of the gel material, with cells on fibrous materials being influenced by gel thicknesses of up to over 130 μm (Leong et al., 2010), while cells on non-fibrous materials are influenced only by structures within 10 μm of the cell (Sen et al., 2009, Maloney et al., 2008). Substrate crosslinking meanwhile has been shown to affect many aspects of cellular behaviour (Haugh et al., 2011). The experimental results presented here suggest that the change in substrate stiffness caused by collagen crosslinking is sufficient to explain the change in area experienced by MC3T3-E1 cells when cultured on crosslinked collagen substrates. Furthermore this is the first time that the effects of substrate stiffness, thickness and crosslinking density have been studied in tandem.
To understand the mechanisms behind this behaviour we must examine the finite element micromechanics simulations. These simulations predict that for fibrous collagen substrates, force was predominantly transferred through the fibres rather than through the gel, with fibres which span the entire depth of the gel transferring the majority of the load. This provides an explanation for the increase in cell spread area on thinner collagen gels. It was also shown that there was a very slight increase in effective modulus as the gels became thinner when all of the fibres in the gel were joined through crosslinks. This corresponds to the reduced effect of gel thickness on cell spread area on highly crosslinked collagen gels. The high level of crosslinking between the fibres allows force to be transferred through multiple fibres through the entire gel depth, thus increasing the effective modulus and therefore the cell spread area.

6.5 Conclusion

The results presented here demonstrate that commonly utilised methods of stiffness measurement cannot accurately interpret the stiffness as experienced by the cell, and that comparison of cell behaviour on different substrates is not possible without knowledge of the substrates thickness and micromechanical structure. This is the first time the combined effects of substrate stiffness, thickness and crosslinking density on cell behaviour have been investigated and this provides an enhanced understanding of the forces actually experienced by osteogenic cells in 2D in-vitro experiments. The findings in this study can improve the current understanding of osteogenic mechanobiology and pave the way for a more successful transfer of in-vitro experimental findings to tissue regeneration strategies.
Chapter 7: Osteocyte Differentiation on Soft Collagen Substrates is Increased by Pulsatile Flow

7.1 Introduction

Osteocytes, derived from bone forming osteoblasts, are terminally differentiated bone cells which comprise over 90% of the cells in mature bone (Boukhechba et al., 2009). The differentiation of osteoblast to osteocytes is characterised by dramatic changes in morphology and gene expression. The spread, cuboidal osteoblast adopts a small rounded cell body while extending multiple long thin cell processes (Cowin, 1989), which contact neighbouring cells to form gap junctional communications (Donahue, 1998). Among the genes involved in process formation are dentin matrix protein 1 (DMP-1) and E11 (Rios, 2005, Schulze et al., 1999, Zhang, 2006), while the prostaglandin PGE$_2$, also involved in bone formation and resorption (Jee et al., 1985, Nefussi and Baron, 1985), has been shown to be vital in gap junction formation (Cheng et al., 2001). This morphological change is accompanied by a decrease in alkaline phosphatase (ALP) expression as the cells mineralise their surrounding extracellular matrix (ECM) (Jee, 2001).

Osteoblast and early osteocyte differentiation has been shown to be initiated by passive control of substrate stiffness in in-vitro experiments, with MSCs shown to induce osteoblast differentiation, through an increase substrate mineralisation, when cultured on stiff (25-40 kPa) collagen substrates (Khatiwala et al., 2006a, Engler et al., 2006). More recently it has been demonstrated that MC3T3-E1 cells adopt an osteocyte morphology and increase expression of DMP-1, a phenotypic marker of osteocytes, when cultured on soft (300 Pa) collagen substrates (Mullen et al., 2013). These studies, in conjunction with developing bone tissue (osteoid) itself being a
collagen based matrix, have led many to hypothesise that osteogenic differentiation is more likely to take place on substrates that mimic the in vivo environment (Engler et al., 2006, Mullen et al., 2013).

In vivo osteocytes, embedded within the mineralised collagen matrix, are surrounded by a fluid-saturated pericellular matrix and under mechanical loading the flow induced in this fluid applies shear stress and deformation to cell membranes as well as drag on the PCM (You et al., 2001, Han et al., 2004). This strain-derived fluid flow is a secondary means of manifestation of the strain applied under everyday loading (Cowin et al., 1995), and has been cited as a key regulator of osteocyte activity (Burger et al., 2003). The role of fluid flow for in vitro differentiation of osteoblasts has been widely investigated, and it has been demonstrated that a variety of flow profiles induce osteoblast differentiation (Riddle et al., 2006, Jacobs, 1998). In particular exposure of MSCs to oscillatory fluid flow of 20 dynes/cm² has been shown to induce an increase activity of the extracellular signal regulated kinases (ERKs) (Riddle et al., 2006), which are vital for osteoblast differentiation (Liu, 2009, Ge et al., 2007), as well as the osteogenic proteins osteocalcin and osteopontin (Li et al., 2004). Moreover, steady state fluid flow of 20 dynes/cm² applied for between 30 and 60 minutes has been shown to induce both an increase in ALP activity (Kapur et al., 2003) and cell elongation and reorientation (Liu et al., 2010) in primary osteoblasts. The differentiation state of the cells is of vital importance in predicting cellular response to fluid flow. Foetal osteoblasts have been shown to be more sensitive to steady state or pulsatile flow than oscillatory flow of identical peak shear stress through measurement of their calcium expression (Jacobs, 1998), while it has been shown that the osteocyte like MLO-Y4 cell line demonstrated increased cell process formation, a key feature of osteocyte differentiation, when exposed to
oscillatory fluid flow but not under steady state flow (Ponik et al., 2007). Thus the role of fluid flow for osteocyte differentiation is likely very important, but is not yet well understood.

While separately, both fluid flow (Riddle et al., 2006, Kapur et al., 2003, Li et al., 2004) and substrate stiffness (Engler et al., 2006, Evans et al., 2009, Mullen et al., 2013) have been shown to promote osteoblast and early osteocyte differentiation, late stage osteocyte differentiation has yet to be induced in vitro. In vivo, bone cells are subjected to both distinct ECM stiffness and interstitial fluid flow, but to date no in vitro study has sought to investigate the combined role of these stimuli in osteocyte differentiation. Interestingly, substrate chemistry has been shown to alter the response of osteoblasts to steady state fluid flow (Li et al., 2013). Thus it is intriguing to hypothesise that passive substrate mechanics could also alter the cellular response to fluid flow.

In this study, the hypothesis that osteocyte differentiation is regulated by a combination of ECM stiffness and fluid flow induced stress was addressed. The pre-osteoblast MC3T3-E1 cell line, were cultured at low density on collagen based substrates of stiffness’s previously shown to induce early osteocyte differentiation (Mullen et al., 2013). After 7 days of static culture, the cells were subjected to pulsatile flow for 1 hour per day over 1 or 7 days. Cell process formation, proliferation and the expression of ALP and PGE$_2$ were quantified as a measure of osteocyte differentiation and mechanoresponsiveness. Flow is pulsatile in nature and the calculated shear stress (10 dynes/cm$^2$) was within the expected physiological range imposed on osteocytes in vivo (8-30 dyn/cm$^2$) (Weinbaum et al., 1994).
7.2 Methods

7.2.1 Substrate manufacture
Soft collagen based substrates were used in this work, based on the authors previous studies (described in Chapter 4) that established that these substrates were suitable to induce early osteocyte differentiation of MC3T3-E1 pre-osteoblasts (Mullen et al., 2013). Briefly, rat tail type 1 collagen (BD biosciences) was neutralised at 4 mg/mL using 1N NaOH (Sigma Aldrich), before being crosslinked with 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC)/ N-hydroxysuccinimide (Sigma Aldrich) at 20 mg/mL collagen. This forms zero length molecular crosslinks without bonding to the collagen itself, thus avoiding any cytotoxicity (Powell and Boyce, 2006, Park et al., 2002) and results in a soft gel, previously measured as having a Young’s modulus of 286 Pa (Mullen et al., 2013).

7.2.2 Cell culture
Pre-osteoblast MC3T3-E1 cells were cultured in α-MEM supplemented with 10% foetal bone serum (FBS), 1% penicillin-streptomycin and 0.5% L-Glutamine (all Sigma-Aldrich). Cells were seeded at an initial density of 10^3 cells/cm^2, which was previously shown to induce early osteocyte differentiation (Mullen et al., 2013). MC3T3-E1 cells are considered a good model of osteoblast behaviour. They have been shown to express similar levels of alkaline phosphatase to primary osteoblasts and have been cited as a particularly suitable model for osteoblast differentiation studies (Quarles et al., 1992). They have also been shown to be capable of osteocyte differentiation (Sudo et al., 1983), although normally the use of osteogenic growth factors such as β-glycerophosphate and ascorbic acid is required (Quarles et al., 1992, Krishnan et al., 2010).
7.2.3 Fluid flow

Cells were divided into 5 culture conditions labelled; Static7, Static14, Flow7, Flow14 and Flow7-14. The Static7 and Static14 groups were not subjected to flow and were fixed/harvested (as detailed below) after 7 and 14 days of static culture respectively. Cells in groups Flow7 and Flow14 were subjected to flow for 1 hour on day 7 of culture and fixed/harvested on day 8 and day 14 respectively, while cells in Flow7-14 were subjected to flow for 1 hour daily from day 7 to day 13 of culture before being fixed/harvested on day 14. For all flow groups, a pulsatile fluid flow of 0.18 mL/min was applied to the cells through a parallel plate flow chamber, using a computer controlled syringe pump (World Precision Instruments). The solid aluminium flow chamber was designed in house and manufactured by Riteway Engineering, Galway. The flow channel had a height of 200 µm, breadth of 18 mm and a total length of 142 mm. Part drawings are included in Appendix 3. A truncated sine wave pattern was applied to induce pulsatile flow over the cells, with a peak shear stress of 10 dynes/cm², within the predicted level of shear stress experienced by osteocytes in vivo (Weinbaum et al., 1994).

7.2.4 Morphological staining

Cells were fixed with 4% paraformaldehyde (Sigma–Aldrich), permeabilised with 0.025% Triton (Sigma-Aldrich) in phosphate buffered saline (PBS) and stained using Tetramethylrhodamine (TRITC) labelled rhodamine-phalloidin (Sigma-Aldrich). Cells were then placed in 4',6-diamidino-2-phenylindole (DAPI) containing mounting media (Vector laboratories) before being imaged using an Olympus IX50 inverted fluorescent microscope and examined for the presence of osteocyte-like cell processes as previously described in Chapter 4 (Mullen et al., 2013). Briefly, cell processes were identified as features composed of actin, with a cross sectional
diameter of less than 1 μm, extending for a distance of at least 5 μm. Cell alignment was measured by quantifying the percentage of cells with a long axis within 30° of the direction of flow. Cell process alignment was measured in the same way, with processes occurring within 30° of the direction of flow being classed as aligned.

7.2.5 Biochemical assays
Cultures were lysed using the freeze-thaw method (Johnson, 1994). Briefly, cultures were incubated under static conditions for 2 hours after flow and rinsed twice with PBS, before being immersed with deionized water. The entire well plate was frozen and thawed 3 times to disrupt the cell membrane. A Hoescht DNA assay (Abcam) was used to determine the total DNA content per mL of cell lysate. Cells were placed in fresh media for 2 hours prior to extracellular enzyme expression in the supernatant being analysed (ALP and PGE₂). ALP activity was quantified in both the lysate (intracellular) and the extracted supernatant using a p-nitrophenol assay (Abcam). PGE₂ activity was quantified using an enzyme immunoassay (Cayman Chemical), while all results were normalised to DNA content of the lysate.

7.2.6 Statistical analysis
A one way ANOVA was used to determine statistical differences in cell process formation, ALP activity and PGE₂ activity as well as total DNA content per culture condition. A p-value of 0.05 was used in all cases. Morphological analysis was conducted in biological triplicate, while 10 images (approx. 150 cells per image) were analysed per replicate. All biological assays were conducted in biological and technical triplicate. Pierce’s criterion was used to remove outliers from technical replicates.
7.3 Results

7.3.1 PGE\textsubscript{2} activity

As shown in Figure 7.1, PGE\textsubscript{2} expression was significantly higher in cells exposed to flow at day 7 (Flow7), than in all other groups. No statistical difference in PGE\textsubscript{2} activity occurred between cells cultured under static conditions for 7 or 14 days (Groups Static7 and Static14). Meanwhile a statistically lower level of PGE\textsubscript{2} activity was observed in cells of group Flow14 (Cells exposed to flow at day 7 and cultured under static conditions for a further 7 days) than in cells cultured under static conditions (Static7 and Static14). Finally activity of the enzyme in cells exposed to one hours of flow each day over 7 days (Flow7-14) was not significantly different than cells cultured under static conditions (Static7 and Static14).

Figure 7.1: PGE\textsubscript{2} activity of MC3T3-E1 cells on EDAC crosslinked collagen subjected to pulsatile flow of 10 dynes/cm\textsuperscript{2} under various flow patterns. a indicates statistically lower than Flow7 group (flow at day 7 with cells incubated in media for 2 hours after application of flow). b indicates statistically higher than Flow14 group (flow at day 7 only and reading taken at day 14). p<0.05.
7.3.2 Cell proliferation

Cell number (as measured by DNA content), was found to increase as culture time was increased from 7 to 14 days, as shown through the 3 fold increase from Static7 to Static14 in Figure 7.2. An increase in cell number was observed in all groups cultured for 14 days compared to group Static7. Application of fluid flow followed by continuous static culture (group Flow14 in Figure 7.2) induced a significant increase in cell number compared to all other groups, while fluid flow over 7 days induced an increase in cell number compared to Static7 (cells under static conditions for 7 days) but not compared to Static14 (cells under static conditions for 14 days).

**Figure 7.2:** DNA content of MC3T3-E1 cells on EDAC crosslinked collagen subjected to pulsatile flow of 10 dynes/cm² under various flow patterns. a indicates statistically higher reading than group Static7 (cells cultured under static conditions for 7 days), b indicates statistically lower reading than group Flow14 (Flow applied on day 7, followed by a further 7 days of static culture). p<0.05.
7.3.3 Morphological analysis

Representative images of the morphology of cells for each group are shown in Figure 7.3. A higher percentage of MC3T3-E1s exhibiting cell processes were observed after 14 days of static culture compared to 7 days of static culture, although this increase was not statistically significant. Application of fluid flow on day 7 caused an initial significant decrease in the percentage of cells exhibiting processes compared to static controls, see Figure 7.3. Repeated exposure to flow from day 7 to day 13 caused a further significant decrease in the percentage of cells exhibiting processes. The highest percentage of MC3T3-E1s containing cell processes was in Flow14 (one period of pulsatile fluid flow at day 7, followed by 7 days of static culture), at 56%. This was the only group that showed a significant increase in the percentage of cells exhibiting processes compared to 7 days of static culture (Static7), as shown in Figure 7.4. No change in cell alignment, or cell process alignment was observed, as shown in Figure 7.5.

Figure 7.3 Sample morphologies of MC3T3-E1 cells on EDAC crosslinked collagen subjected to pulsatile flow of 10 dynes/cm² under various flow patterns. White arrows show sample cell processes.
Figure 7.4 Morphology of MC3T3-E1 cells on EDAC crosslinked collagen subjected to pulsatile flow of 10 dynes/cm² under various flow patterns. a indicates statistically higher than group Static7 (cells cultured under static conditions for 7 days), b indicates statistically lower than group Static14 (cells cultured under static conditions for 14 days). P<0.05.

Figure 7.5 Percentage of MC3T3-E1 cells on EDAC crosslinked collagen aligned within 30° of the direction of flow. No statistical difference was reported. P<0.05.
7.3.4 ALP activity

Intracellular ALP activity was significantly reduced from day 7 to day 14 of static culture, as shown through the decrease from Static7 to Static14 in Figure 7.6. Cells subjected to flow on day 7 of culture (Flow7) did not experience any change in ALP activity compared to cells cultured under static conditions for 7 days (Static7). A significant reduction was also observed when cells were subjected to flow at day 7 and cultured under static conditions for a further 7 days (Flow14 in Figure 7.5) and when cells were subjected to flow for 1 hour each day over 7 days (Flow7to14 in Figure 7.6) compared to cells cultured under static conditions for 7 days (Static7).

Extracellular ALP activity was shown to also decrease significantly from day 7 to day 14 of static culture (Static7 to Static14 in figure 7.7). Similarly to the intracellular ALP activity, cells subjected to flow on day 7 of culture (Flow7) did not experience any change in extracellular ALP activity compared to cells cultured under static conditions for 7 days (Static7). The application of flow followed by static culture for a further 7 days (Flow14) led to a further reduction in ALP activity, with a significantly lower level of expression observed than in cells in static culture for 14 days (see groups Static14 and Flow14 in Figure 7.7).
**Figure 7.6:** Intracellular ALP activity of MC3T3-E1 cells on EDAC crosslinked collagen subjected to pulsatile flow of 10 dynes/cm² under various flow patterns. \( \textit{a} \) indicates statistically lower reading than group Static7 (Static culture for 7 days), \( \textit{b} \) indicates statistically lower reading than group Flow7 (Flow on day 7 and reading taken on day 7) \( P<0.05 \).
Figure 7.7: Extracellular ALP activity of MC3T3-E1 cells on EDAC crosslinked collagen subjected to pulsatile flow of 10 dynes/cm² under various flow patterns. a indicates statistically lower activity than Static7 group (Static culture for 7 days), b indicates statistically lower reading than Static14 (Static culture for 14 days), c indicates statistically lower reading than Flow7 group (Flow on day 7 and reading on day 7) P<0.05.
7.4 Discussion

The results of this study indicate that MC3T3-E1 cells, cultured on collagen substrates that mimic developing osteoid (approx. 30 kPa), differentiate towards the osteocyte lineage and that a greater percentage of cells adopt the osteocyte morphology and ALP expression profile through the addition of a single bout of pulsatile fluid flow. The static conditions used here, have previously been shown to induce early osteocyte differentiation in MC3T3-E1 cells through examination of morphological changes, ALP activity, mineralisation and gene expression profile (Chapter 4), and the results of the static cultures presented here further support this. The increase in PGE$_2$ when cells were subjected to flow indicates the response of the cells to the flow stimulus. While PGE$_2$ activity did not increase in cells subjected to flow for 1 hour per day over 7 days (Flow7-14), previous studies have not examined the effects of long term fluid flow on PGE$_2$ activity, and it has been proven that short term increases in PGE$_2$ activity are not indicative of long term response to fluid flow in primary osteoblasts (Nauman et al., 2001). Furthermore, when subjected to flow at day 7 of culture and cultured under static conditions for a further 7 days, the cells experienced a further decrease in ALP activity, both intracellular and extracellular, while the percentage of cells which exhibit cell processes also increased. These results indicate that a single bout of pulsatile fluid flow encouraged the differentiation of a greater proportion of the cell population along the osteocyte pathway. It was also found that application of fluid flow over consecutive days did not increase levels of osteocyte differentiation as evidenced from the lower percentage of cells exhibiting cell processes under these conditions. These results demonstrate that an appropriate fluid flow pattern is vital when seeking to encourage osteocyte differentiation of MC3T3-E1 cells.
A possible limitation of this study is in the use of the MC3T3-E1 cell line. However, these cells have been shown to be an excellent model for osteoblast behaviour (Quarles et al., 1992), exhibiting ALP expression and matrix mineralisation (Sudo et al., 1983) and have previously been used in many studies to investigate osteoblast behaviour (Maeda et al., 2001, Ducy et al., 1997). In particular they have been cited as an appropriate cell model for the study of osteoblast differentiation (Franceschi et al., 1994). Meanwhile, the use of primary osteoblasts for in vitro cell culture experiments is made difficult by complex procedures for cell extraction and isolation (Jonsson, 1999, Bakker, 2012). A further possible limitation is the investigation of a single fluid flow pattern. Pulsatile fluid flow was chosen due to this pattern being cited as being characteristic of in vivo conditions (Jacobs, 1998), while the calculated levels of shear stress induced at the cellular level were within the range commonly cited as being physiologically relevant (Weinbaum et al., 1994). The lack of gene expression analysis conducted in this study could also be seen as a limitation. Osteocytes are characterised not just by their morphology and low ALP activity, but also by their expression of specific genes such as DMP-1, E11 and sclerostin (Bonewald, 2011a). However, the static culture conditions used in this experiment have been shown in Chapter 4 to induce the downregulation of the osteoblast specific genes Col1A1 and OSF-2 as well as an upregulation in the osteocyte specific gene DMP-1. The results presented here indicate that a greater percentage of the total cell population are stimulated to undergo osteocyte differentiation when exposed to pulsatile fluid flow than previously shown through static culture conditions.

Alkaline phosphatase production by primary osteoblasts has previously been shown to be reduced compared to base levels in response to pulsatile fluid flow of 5
dynes/cm², with both an immediate and sustained downregulation in activity being recorded (Hillsley and Frangos, 1997). However, an increase in ALP activity was observed when primary osteoblasts were subjected to a higher rate (20 dynes/cm²) of steady state fluid flow (Kapur et al., 2003). Previous studies have also shown that osteoblast response to flow is inextricably linked to the flow pattern used, with human foetal osteoblasts shown to be more sensitive (as measured through calcium expression) to pulsatile or steady state flow than oscillatory flow (Jacobs, 1998). Logically, the flow pattern used should be an approximation of in vivo conditions. Although there is some debate over the exact nature of in vivo flow in osteocyte lacunae, it is generally accepted that a combination of steady state and pulsatile flow is involved (Jacobs, 1998). The results of this study are in agreement with those of Hillsley and Frangos (that pulsatile flow reduces ALP activity), and indicate that the response of osteoblasts or osteoblast cell lines to fluid flow depends greatly on the flow pattern used.

It has previously been shown that the culture of MC3T3-E1 cells under the static conditions used here (cells cultured at 10³ cells/cm² on type 1 collagen crosslinked with EDAC/NHS at 20 mM/mg collagen) result in early osteocyte differentiation (Mullen et al., 2013), and this initial early osteocyte differentiation allows for comparison between the work presented here and previous studies, which examined the effects of fluid flow on osteocytes and osteocyte like cell lines. It has been shown that the osteocyte like cell line MLO-Y4, which exhibit the long thin cell processes associated with the osteocyte phenotype, experienced an increase in the percentage of cells exhibiting more than 3 cell processes when exposed to oscillatory flow of 8 dynes/cm², but that under steady state flow of the same magnitude the number of processes is reduced compared to static controls. (Ponik et al., 2007). This same
study exposed the pre-osteoblast MC3T3-E1 cell line to identical flow regimes and found an increase in stress fibre formation in response to steady state, but not oscillatory flow. However, it must be noted that no process formation was induced in MC3T3-E1 cells in response to either flow pattern (Ponik et al., 2007). This highlights how the response of osteogenic cells to fluid flow induced shear stress differs according to their differentiation stage. It is interesting to note that the response of osteogenic cells to other mechanical stimuli, such as substrate stiffness and ligand density is also dependant on the differentiation stage of the cells, with uncommitted MSCs being less affected by mechanical stimuli (Hsiong et al., 2008). It is shown here that a single bout of pulsatile fluid flow induces differentiation of a larger population of MC3T3 cells compared to static controls, as indicated by the further increase in process containing cells and the decrease in ALP activity when cells are subjected to pulsatile flow on day 7. These results indicate that fluid flow may play an additional important role in inducing differentiation of osteoblasts to osteocytes by day 14 of culture. This is the first study to investigate the effects of the two stimuli together and an understanding of the combined effects of ECM mechanics and fluid flow induced shear stress, which are both experienced to in vivo, is vital if the effect of extracellular mechanics on osteocyte differentiation is to be understood.

It was also observed that cells cultured under these conditions were not heterogeneously located throughout the culture area, as is common in in vitro experiments (Lindström et al., 2009). Although intracellular tension of individual cells was hypothesised to be a driver of osteocyte differentiation, the intracellular tension of cells in confluent culture is not known. It is possible that the application of pulsatile fluid flow could induce a similar change in intracellular tension in adjacent
cells to that observed in Chapter 5 of this thesis, and that this change in tension could
induce some of those cells in confluent regions of the culture area to differentiate
towards the osteocyte phenotype. Indeed applied fluid flow has previously been
shown to induce normal and shear cellular stress in both experimental and
computational studies (Vaughan et al., 2013, Song et al., 2012).

ALP expression by osteoblasts promotes matrix mineralisation though the hydrolysis
of inorganic pyrophosphate (Hessle et al., 2002, Rezende, 1994), an anion which
suppresses the formation of hydroxyapatite crystals (Meyer, 1984). It is known that
erly osteocyte development can be induced on the soft collagen based substrates
used in this study (as demonstrated in Chapter 4 of this thesis). However, this is the
first time that fluid flow induced stress has been used in conjunction with the control
of substrate stiffness to induce osteocyte differentiation of MC3T3-E1 cells, and the
results presented here have important implications for future osteocyte
mechanobiology studies.

7.5 Conclusion
The response of osteogenic cells to fluid flow depends greatly on the differentiation
stage of the cells in question. MC3T3-E1 cells have been shown to differentiate into
early stage osteocyte after 21 days of static culture on identical substrates to those
used in this study (Mullen et al., 2013). After 7 days of culture 37% of cells are
shown to exhibit cell processes associated with osteocytes, indicating partial
differentiation towards the osteocyte phenotype. After 14 days, this percentage is
increased to 48%, while a decrease in ALP activity is also observed. It was found
that the application of fluid flow, of a similar rate and pattern to that experienced in
vivo, at day 7 of culture induced a further increase in osteocyte differentiation as
demonstrated by cell process formation in 56% of cells and decreased overall ALP
activity. From this it is proposed that a combination of a soft collagen based substrate and physiologically relevant fluid flow is needed for osteocyte differentiation of a large proportion of cells to occur in vitro.
Chapter 8: Discussion and Conclusions

8.1 Introduction

This chapter summarises the main findings of the thesis, drawing together the insight obtained from the different experimental and computational techniques performed into the effects of extracellular mechanics on osteocyte differentiation. The relationship between the individual chapters of this thesis and previous highly relevant publications is demonstrated in the flow chart in Figure 8.1, and discussed in more detail in Section 8.3. Key findings are discussed together with other relevant studies. Finally recommendations are made for future work to be conducted in the fields of osteocyte mechanobiology and bone tissue engineering, given the outcomes of this PhD research.

Figure 8.1: Summary of relationship between research Chapters, with key studies used in hypotheses formation included. References: 1 (Maloney et al., 2008), 2 (Leong et al., 2010), 3 (Engler et al., 2006), 4 (Evans et al., 2009), 5 (Kapur et al., 2003), 6 (Vezeridis et al., 2006), 7 (Bongiorno et al., 2013)
8.2 Main findings of the thesis

The research studies of this thesis have focused on delineating the effect of extracellular mechanics on osteocyte differentiation. Experiments were conducted to investigate the effects of passive differences in ECM stiffness, the distance between cells during culture and the effect of fluid flow induced shear stress in conjunction with substrates which mimic the in vivo environment. A combination of experimental and computational approaches was used to investigate in more detail the effects of extracellular mechanics on intracellular tension as well as to uncover the nature of the extracellular mechanics as experienced by the cell. These studies are discussed below in the context of the hypotheses that underpin the research of this thesis.

**Hypothesis 1:** Osteocyte differentiation of an osteoblast-like cell line is initiated by substrate stiffness and intercellular separation.

The first study of this thesis, described in Chapter 4, investigated the effects of substrate stiffness and intercellular separation on the osteocyte differentiation of MC3T3-E1 pre-osteoblast cells. It was found that early osteocyte differentiation occurs on collagen based substrates of 300 Pa, provided intercellular separation is large enough to necessitate cell process formation. The results of this study provide essential information on the mechanobiology of osteocytes and confirm the hypothesis that osteocyte differentiation is initiated by substrate stiffness.

**Hypothesis 2:** The cellular response to extracellular mechanics is mediated through intracellular stress, as the developing osteoblast alters its morphology in order to achieve a more appropriate stress state.
The second study, presented in Chapter 5, of this thesis investigated the effect of substrate stiffness on the internal stress profile of the cell. Realistic cell morphologies, stiffnesses and focal adhesion locations were used to create finite element models of the experiments performed in the previous study (Chapter 4). It was found that cell morphology greatly influenced the stress generated within the cell body, particularly for a spread cell geometry. Interestingly, it was seen that the stress generated in the dendritic cell morphology is less affected by changes in substrate stiffness. The results of this chapter support the hypothesis that the cellular response to extracellular mechanics is mediated through intracellular stress, and moreover, that the developing osteoblast alters its morphology in order to achieve a more desirable stress state.

**Hypothesis 3: Substrate stiffness, thickness and heterogeneity all play a role in regulating cellular response.**

The third study of this thesis, presented in Chapter 6, applied a combination of novel experimental and computational techniques to uncover how the individual aspects of substrate mechanics, specifically bulk material stiffness, substrate thickness and heterogeneity, are experienced by the cell. The experimental studies demonstrated that cells differentiate similarly, as indicated by cell spread area, on a soft fibrous substrate as on a much stiffer non-fibrous substrate. The computational investigations revealed that this was likely a result of the transfer of load being facilitated through the fibres in the substrate, which effectively altered the local mechanical stimuli experienced by the cells on fibrous substrates. It was also demonstrated that reduced substrate thickness increased the stiffness experienced by the cell due to an increase in the load transfer through the fibres. Interestingly, this thickness effect was less prominent on crosslinked substrates, as load transfer was
facilitated through multiple fibres. Together these results confirm the hypothesis that osteogenic mechanobiology is influenced by the nature of substrate mechanics regulation.

*Hypothesis 4: Osteocyte differentiation is enhanced by fluid flow induced shear stress in addition to controlled substrate stiffness.*

The final study presented in Chapter 7, investigated used a parallel plate flow chamber to apply physiologically relevant fluid flow to MC3T3-E1 cells cultured on the substrates which in the first study (Chapter 4) were shown to promote osteocyte differentiation. It was demonstrated that a greater percentage of cells undergo osteocyte differentiation in cultures that were first subjected to one period of fluid flow after 7 days of static culture and allowed to recover for a further 7 days, compared to either cells cultured under static conditions or cells subjected to multiple bouts of flow on consecutive days, as indicated cell morphology and ALP activity. These results support the hypothesis that osteocyte differentiation is enhanced by fluid flow induced shear stress in addition to controlled substrate stiffness.

### 8.3 Insight gained into osteocyte mechanobiology

The effects of extracellular mechanics on osteoblast differentiation have been well documented, with MSCs, ESCs and MC3T3s all shown to differentiate into mature osteoblasts when cultured on collagen based substrates with stiffnesses in the region of 25 kPa to 40 kPa (Engler et al., 2006, Evans et al., 2009, Khatiwala et al., 2006b), which is similar to the stiffness of osteoid, the tissue in which osteoblasts reside (Engler et al., 2006). Moreover, has been shown that MSCs can be induced to
differentiate towards the neurogenic, myogenic or osteogenic phenotype when cultured on substrates mimicking the stiffness of brain, muscle and bone tissue respectively (Engler et al., 2006). Until now however, the effects of extracellular mechanics on osteocyte differentiation have not been examined in the same detail. The results presented in this Thesis provide a novel insight into the effects of extracellular mechanics on osteocyte differentiation. This is the first time that osteocyte differentiation, as evidenced by cell morphology, ALP activity and specific gene expression profile, has been induced in the MC3T3-E1 cell line without the addition of osteogenic growth factors. In apparent contrast to earlier investigations into osteoblast differentiation, these studies show osteocyte differentiation to occur on collagen based substrates of approximately 300 Pa. At first glance this is in strong disagreement both with earlier research, and the accepted hypothesis that cell differentiation to a certain phenotype occurs when cells are cultured on a substrate with similar mechanical properties to the tissue in which those cells reside in vivo. However, the results discussed in Chapter 6, describe how the local mechanical cues experienced by the cell can differ dramatically from the stiffness of the substrate as measured through conventional methods. In particular the resistance of a collagen substrate to cell induced tension is substantially different to that shown to a compressive AFM tip during substrate stiffness measurement. This is due to both the cell interacting directly with fibres through ligand-focal adhesion interactions, as well as the increased transfer of force through the substrate by the collagen fibres. This may further explain the low substrate stiffness found to induce osteocyte differentiation in the studies presented in this thesis.

In addition to this, intercellular separation was found to play a key role in inducing osteocyte differentiation, with a high degree of separation, controlled through a low
initial seeding density, leading to increased osteocyte differentiation. Low seeding density has previously been shown to induce osteoblast differentiation in MSCs (Kim et al., 2009), while in vivo studies have also highlighted the importance of initial seeding density in bone tissue formation in scaffolds (Holy et al., 2000).

Osteocyte cell processes, a widely used indicator of the osteocyte morphology (Dallas and Bonewald, 2010, Tanaka Kamioka et al., 1998), are known to be involved in intercellular communication (Marotti et al., 1996, Jee, 2001). It may be that the separation distance between these cells, which initially prevented cell-cell contact, forced the formation of cell processes in order to facilitate this intercellular communication. Furthermore, cell process formation is a key aspect of osteocyte differentiation and it is possible that if cellular communication can take place without these features, as is the case at higher initial cells seeding densities where direct cell-cell contact is established quickly, process formation will not occur.

The results presented in Chapter 5 show for the first time that cell process formation also greatly affects the intracellular stress generated in the developing osteocyte. Specifically it was shown that the stress generated in cells which exhibit the small rounded cell body and long thin processes associated with the osteocyte phenotype (Bonewald, 2011a), are less sensitive to changes in substrate stiffness. This is an intriguing result when examined in combination with with the main findings of Chapter 4, which showed that osteocyte differentiation, and specifically cell process formation, is regulated by substrate stiffness and intercellular separation. From this it is hypothesised that the change in the intracellular stress generated on soft substrates is a key driver of osteocyte differentiation, i.e. that process formation occurs in an effort to achieve a homeostatic stress state, as cells exhibiting a dendritic morphology are less affected by differences in substrate stiffness. Indeed, it has
previously been shown that intracellular tension, as controlled through ROCK inhibition plays a key role in osteoblast differentiation of MSCs (McBeath et al., 2004). The results presented in this thesis demonstrate that cell morphology, intracellular stress and extracellular mechanics are inextricably linked and together regulate the process of osteocyte differentiation.

Fluid flow induced shear stress has been shown to be a potent stimulus for the initiation of osteoblast differentiation (Kapur et al., 2003, Vezeridis et al., 2006), and it is known that osteocytes in vivo are subjected to the flow of interstitial fluid through their lacunae (Cowin, 2002). However, the combined effect of fluid flow and substrate mechanics has not previously been investigated. The results presented in Chapter 7 of this thesis demonstrate that the application of pulsatile fluid flow within the expected physiological range (Weinbaum et al., 1994), can induce a greater percentage of MC3T3-E1 cells to commence osteocyte differentiation. Specifically, levels of osteocyte differentiation were increased compared to static controls when cells were cultured for 7 days under static conditions, subjected to a single bout of fluid flow, before being cultured for another 7 days under static culture conditions. Several studies have demonstrated a difference in internal cell stress in cells subjected to fluid flow (Dailey et al., 2009, Charras and Horton, 2002) and it is intriguing to speculate that a change in intracellular stress could lead to an increase in the percentage of cells undergoing osteocyte differentiation when exposed to fluid flow.

When analysed together, the individual studies reported on in this thesis provide a novel insight into the effects of extracellular mechanics on osteocyte differentiation, as outlined in Figure 8.2. Optimal substrate stiffness and external fluid flow have the ability to regulate intracellular stress, which is closely related to cell morphology.
Cell morphology, as shown in Chapter 4, is also dependant on intercellular separation, with process formation more likely to occur when required for intercellular communication. Further to this, cell process formation (Dallas and Bonewald, 2010) and intracellular tension (McBeath et al., 2004) are both required for osteogenic differentiation to occur. An additional requirement is the presence of a fibrous collagen matrix. In vivo, ECM mineralisation is mediated through the addition of HA crystals to the collagen fibrils in the ECM (Boskey, 1996, Anderson, 1995), and is an essential aspect of both osteocyte differentiation and bone tissue formation (Cowin, 1989). The fibrous nature of this collagen matrix also alters the mechanical cues experienced at the cellular level as discussed in Chapter 6.
Figure 8.2: Graphical representation of osteocyte mechanobiology as discussed in section 8.3. Fibrous collagen, controlled substrate stiffness, applied fluid flow and intercellular separation all combine to influence cell morphology and intracellular tension, which in turn drive osteocyte differentiation.
8.4 Implications for the fields of bone mechanobiology and bone tissue engineering

These results of this Thesis have important implications for the fields of bone mechanobiology and bone tissue engineering. For the first time, early osteocyte differentiation from MC3T3-E1 pre-osteoblasts, as described in Chapter 4, has been induced without the addition of osteogenic growth factors such as β-glycerophosphate or ascorbic acid. Previous studies have concentrated on the control of ligand density (Harbers and Healy, 2005, García and Reyes, 2005, Lee et al., 2004) or substrate stiffness (Engler et al., 2006, Evans et al., 2009) individually. By comparing these studies to the results of this Thesis it is demonstrated that the effects of these two parameters on osteogenic differentiation cannot be separated and particularly, that while osteoblast differentiation may be initiated through the control of substrate stiffness alone, a collagen matrix is necessary for the mineralisation of the surrounding ECM, a vital part of osteocyte differentiation. Further to this the effects of intercellular separation has been shown for the first time to be vital for osteocyte differentiation. This key aspect of osteogenic cell development has been largely ignored in two dimensional studies (Engler et al., 2006, Khatiwala et al., 2006b, Harris et al., 1994). These effects must be considered in the design of future mechanobiology studies and tissue regeneration approaches.

Until now osteogenic differentiation studies have relied on conventional stiffness measurement techniques such as AFM at the nanoscale (Engler et al., 2006, Witkowska-Zimny, 2013) or tensile testing of the bulk material properties (Khatiwala et al., 2006a, Rowlands et al., 2008). The results presented in Chapter 6 of this thesis demonstrate that the results generated through these techniques may not reflect the mechanical stimulation experienced at the cellular level. This is of
particular significance as fibrous materials, in particular collagen-GAG (Tierney et al., 2009, Duffy et al., 2011, Keogh et al., 2010a), and collagen-HA scaffolds (Al-Munajjed et al., 2009), as well as HA-polymer composites (Ma et al., 2001, Lao et al., 2011, Kim et al., 2005) and have been widely used in bone tissue engineering strategies thus far. The results of this Thesis show that the effect of substrate thickness and heterogeneity must be taken into account when extrapolating the effects of mechanical forces on cell behaviour.

8.5 Future work

These studies provide a novel understanding of mechanobiology in osteocytes. Based on the findings of this thesis, the following recommendations are made for future research:

8.5.1 The effects of substrate stiffness and intercellular separation on the osteogenic differentiation of MSCs

Osteocyte differentiation of the MC3T3-E1 cell line, as outlined above, could lead to the development of novel bone tissue engineering strategies for the replacement of tissue lost through disease or trauma. However, before clinical applications can be investigated, the effects of substrate stiffness on human MSC must be investigated. Previous studies have demonstrated that the differentiation of MSCs is possible through control of substrate stiffness. However, the full differentiation process from MSC to osteocyte has never been induced through control of extracellular mechanics. Chapter 6 of this thesis demonstrated that the stiffness experienced by the MC3T3-E1 cells in the study described in Chapter 4, was likely to be similar to that experienced by MSCs shown to undergo osteoblast differentiation in previous
studies (Engler et al., 2006). Instead the experimental differences between the studies lie in the dense collagen substrates and controlled intercellular separation used in this thesis. The culture of MSCs on soft collagen gels at low initial seeding density might eventually lead to osteocyte differentiation in these cells.

8.5.2 Osteocyte mechanobiology in three dimensional scaffolds

While progress has been made into the effects of extracellular mechanics on osteocyte differentiation in two dimensional cultures, cellular differentiation in three dimensional scaffolds must be delineated if clinical applications are to become a reality. The behaviour of various cell types has been shown to vary between two and three dimensional culture experiments (Ribeiro, 2012), while mechanical properties have been shown to vary dramatically due to an increase in dimensions. However, the understanding of osteocyte mechanobiology presented here still has the potential to inform future three dimensional approaches for bone tissue engineering.

8.5.3 Effect of cell morphology on cell generated traction

The findings of Chapter 5 of this thesis highlight the link between substrate stiffness, cell morphology and intracellular stress, through the application of a thermal contraction to the cell body. The effect of this contraction was to generate stress in the cell based on the resistance of the substrate to cellular deformation. The generation of this stress is directly related to the force generated through actin-myosin interaction within the cell body, often measured directly through traction force microscopy (Dembo and Wang, 1999, Huang et al., 1998, Munevar, 2001). Cell traction force microscopy examines cell generated force through the movement of fluorescent microbeads embedded within a substrate. It has been shown that the force generated by MSCs is affected by ECM stiffness (Gershklak et al., 2013, Murat and Jason, 2012) and that the force generated by osteoblasts is related to focal
adhesion formation (Tan et al., 2014). Meanwhile, cell spread area and scaffold stiffness have been shown to regulate cell traction forces and in turn drive osteogenic differentiation of MSCs (Khetan et al., 2013). The results presented in this work indicate that cell morphology, specifically cell process formation in developing osteocytes, could have just as important a role in the control of cell traction, but this cannot be confirmed without experimental observation. For this reason an extensive study into the relationship between cell morphology and cell induced traction could generate a novel insight into osteogenic mechanobiology.

8.5.4 Can combined fluid flow and ECM stiffness induce mature osteocyte differentiation?

It has been shown in this thesis that MC3T3-E1 cells will undergo early osteocyte differentiation when cultured on soft collagen substrates at low initial cell seeding density. Further to this, it has also been shown that the application of pulsatile fluid flow during this culture can induce a greater percentage of cells to differentiate towards the osteocyte phenotype. However, as of yet, the differentiation of MC3T3-E1 cells to mature osteocytes has not been achieved. While the application of fluid flow to pre-osteoblasts cultured on biological scaffolds has already been investigated, with studies demonstrating an increase in osteopontin and PGE₂ expression (Partap S, 2009, Batra et al., 2005), a true understanding of the effects of fluid flow and ECM stiffness on osteogenic differentiation cannot be obtained without the realisation of mature osteocyte differentiation in vitro. A greater understanding of the mechanical cues behind osteocyte differentiation is essential if viable bone tissue engineering strategies are to be designed.
8.5 Conclusion

In conclusion, this Thesis has presented experimental and computational studies conducted throughout the course of the authors PhD studies in the field of osteocyte mechanobiology. A comprehensive literature review of the current state of research in the area was presented, along with a detailed rationale behind the hypothesis formation for each of the studies completed. The findings of each study were described and the implications for the field as a result of the findings were discussed in each chapter.

Together these studies provided novel information on the effects of extracellular mechanics on osteocyte differentiation. The specific effects of substrate stiffness and intercellular separation on osteocyte differentiation were uncovered and osteocyte differentiation of MC3T3-E1 cells was induced without the addition of osteogenic growth factors for the first time. Computational modelling techniques were used to demonstrate the effects of substrate stiffness, cell morphology and stiffness as well as focal adhesion location on intracellular stress, and the stress generated was proposed as a possible mechanism by which osteocyte differentiation occurs. Meanwhile, an in depth investigation into the effects of substrate parameters on cell behaviour was conducted, demonstrating that substrate thickness, stiffness and heterogeneity all affect cell behaviour. Finally, the effects of fluid flow induced shear stress in conjunction with controlled substrate stiffness on osteocyte differentiation were investigated. Together these studies have provided a greater insight into the effects of extracellular mechanics on osteocyte differentiation, and could provide vital information for the development of future bone tissue engineering strategies.
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Appendix 1

The following is a list of the transformed co-ordinates and shape functions of element types used throughout thesis

1. Linear truss element

![Diagram of 2-node linear truss element]

<table>
<thead>
<tr>
<th>Node</th>
<th>( \xi )</th>
<th>( \eta )</th>
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</table>

Transformed co-ordinates

Shape functions

\[
N_1 = \frac{1}{2}(1-\xi) \\
N_2 = \frac{1}{2}(1+\xi)
\]

2. Quadratic quadrilateral element

![Diagram of 8-node quadratic quadrilateral element]

<table>
<thead>
<tr>
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<th>( \eta )</th>
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</tbody>
</table>

Transformed co-ordinates

Shape functions

\[
N_1 = -\frac{1}{4}(1-\xi)(1+\eta)\xi\eta \\
N_2 = \frac{1}{4}(1+\xi)(1+\eta)\xi\eta \\
N_3 = -\frac{1}{4}(1+\xi)(1-\eta)\xi\eta \\
N_4 = \frac{1}{4}(1-\xi)(1-\eta)\xi\eta \\
N_5 = \frac{1}{2}(1-\xi^2)(1+\eta)\eta \\
N_6 = \frac{1}{2}(1+\xi^2)(1-\eta)\eta \\
N_7 = -\frac{1}{2}(1-\xi^2)(1+\eta)\eta \\
N_8 = -\frac{1}{2}(1+\xi^2)(1-\eta)\eta
\]
3. Quadratic tetrahedral element

Shape functions

\[ N_1 = \zeta_1(2\zeta_1 - 1) \]
\[ N_2 = \zeta_2(2\zeta_2 - 1) \]
\[ N_3 = \zeta_3(2\zeta_3 - 1) \]
\[ N_4 = \zeta_4(2\zeta_4 - 1) \]
\[ N_5 = 4\zeta_1\zeta_2 \]
\[ N_6 = 4\zeta_2\zeta_3 \]
\[ N_7 = 4\zeta_3\zeta_4 \]
\[ N_8 = 4\zeta_4\zeta_1 \]
\[ N_9 = 4\zeta_1\zeta_3 \]
\[ N_{10} = 4\zeta_2\zeta_4 \]

Transformed co-ordinates

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<th>( \zeta_4 )</th>
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<td>( \frac{1}{2} )</td>
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<tr>
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<td>( \frac{1}{2} )</td>
<td>( \frac{1}{2} )</td>
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</table>
4. Quadratic hexahedral element

Shape functions

\[ N_1 = \frac{1}{8} (\xi + 1)(\eta + 1)(\zeta + 1)(\xi + \eta + \zeta + 2) \]
\[ N_2 = \frac{1}{8} (1 + \xi)(1 + \eta)(1 + \zeta)(\xi + \eta + \zeta - 2) \]
\[ N_3 = \frac{1}{8} (1 - \xi)(1 - \eta)(1 - \zeta)(\xi - \eta - \zeta - 2) \]
\[ N_4 = \frac{1}{8} (1 - \xi)(1 + \eta)(1 + \zeta)(\xi - \eta - \zeta - 2) \]
\[ N_5 = \frac{1}{8} (1 - \zeta)(1 - \eta)(1 - \xi)(\eta - \xi - \zeta - 2) \]
\[ N_6 = \frac{1}{8} (1 - \xi)(1 - \eta)(1 + \zeta)(\xi - \eta - \zeta + 2) \]
\[ N_7 = \frac{1}{8} (1 + \xi)(1 + \eta)(1 + \zeta)(\xi + \eta + \zeta - 2) \]
\[ N_8 = \frac{1}{8} (1 + \xi)(1 + \eta)(1 + \zeta)(\xi + \eta + \zeta - 2) \]
\[ N_9 = \frac{1}{4} (1 - \xi^2)(1 - \eta)(1 - \zeta) \]
\[ N_{10} = \frac{1}{4} (1 - \eta^2)(1 + \xi)(1 - \zeta) \]
\[ N_{11} = \frac{1}{4} (1 - \zeta^2)(1 + \eta)(1 - \xi) \]
\[ N_{12} = \frac{1}{4} (1 - \xi^2)(1 - \eta)(1 - \zeta) \]
\[ N_{13} = \frac{1}{4} (1 - \zeta^2)(1 + \eta)(1 - \xi) \]
\[ N_{14} = \frac{1}{4} (1 - \xi^2)(1 + \eta)(1 + \zeta) \]
\[ N_{15} = \frac{1}{4} (1 - \zeta^2)(1 + \eta)(1 + \xi) \]
\[ N_{16} = \frac{1}{4} (1 - \xi^2)(1 + \eta)(1 + \zeta) \]
\[ N_{17} = \frac{1}{4} (1 - \zeta^2)(1 - \eta)(1 - \xi) \]
\[ N_{18} = \frac{1}{4} (1 - \zeta^2)(1 - \eta)(1 - \xi) \]
\[ N_{19} = \frac{1}{4} (1 - \zeta^2)(1 + \eta)(1 + \xi) \]
\[ N_{20} = \frac{1}{4} (1 - \zeta^2)(1 + \eta)(1 - \xi) \]

Transformed co-ordinates

<table>
<thead>
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<th>Node</th>
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<th>$\zeta$</th>
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</table>
Appendix 2

The parameter study outlined here was used to determine the appropriate size of the substrates used in Chapter 5 of this thesis. The radius and depth of the substrate was varied according to Table A.2.1 below, where \( d \) is the substrate depth and \( r \) is the distance from the most distal part of the cell to the substrate edge, as shown in Figure A.2.1. A spread cell morphology was modelled on the stiffest (10 kPa) substrate to be used in the study. The remaining parameters were identical to those described in Chapter 5. Briefly, the Young’s modulus of the cell body and nucleus was set as 18 kPa while the Poisson’s ratio of the cell body, nucleus and substrate was set as 0.45. A thermal coefficient of 0.05 was assigned to the cell body and an orthotropic thermal load of -1 K was assigned in the direction of principal fibre alignment. The stress in the cell body in the direction of principal actin alignment was compared across the various models.

<table>
<thead>
<tr>
<th>Model</th>
<th>( d ) (( \mu m ))</th>
<th>( r ) (( \mu m ))</th>
<th>Average cell stress (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2 ( \mu m )</td>
<td>2 ( \mu m )</td>
<td>842 Pa</td>
</tr>
<tr>
<td>B</td>
<td>5 ( \mu m )</td>
<td>20 ( \mu m )</td>
<td>876 Pa</td>
</tr>
<tr>
<td>C</td>
<td>10 ( \mu m )</td>
<td>50 ( \mu m )</td>
<td>943 Pa</td>
</tr>
<tr>
<td>D</td>
<td>25 ( \mu m )</td>
<td>100 ( \mu m )</td>
<td>955 Pa</td>
</tr>
<tr>
<td>E</td>
<td>50 ( \mu m )</td>
<td>200 ( \mu m )</td>
<td>930 Pa</td>
</tr>
</tbody>
</table>

Table A.2.1: Size of substrate and average stress in the cell body of each model investigated
**Figure A.2.1:** Model of spread cell morphology on substrate showing depth (d) and distance between cell and substrate edge (r). Example shown in model C, with \( d = 10 \mu m \) and \( r = 50 \mu m \).

**Figure A.2.2:** Percentage of cell body at experiencing a given stress.

As shown in Figure A.2.2, models C, D and E experienced very similar levels of stress to one another. Of these three models, model C is the most computationally efficient. For this reason model C (substrate depth of 10 \( \mu m \) and cell to substrate edge distance of 50 \( \mu m \)) was chosen for the simulations conducted in Chapter 5 of this thesis.
Appendix 3

Figure A-3.1: Sample force-distance curve for an MC3T3-E1 cell displaying a spread morphology on the stiffest (10 kPa) substrate.

Figure A-3.1: Sample force-distance curve for an MC3T3-E1 cell displaying a dendritic morphology on the stiffest (10 kPa) substrate.