"EFFECT OF STRAIN AND STIFFNESS ON MATRIX REMODELLING GENES"

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Thesis submitted for the Degree of Doctor of Philosophy
December 2006

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1

UMI Number: U592736

Dissertation Publishing

Acknowledgements

My warmest thanks go to my supervisor Dr. Mudera Vivek, for showing me the way into the wonderful world of science. I express my deepest gratitude to him for guiding and encouraging me during this study. With his vast knowledge, enthusiasm, and manner of scientific thinking, he has given me an admirable role-model as a researcher.

I extend my warm and sincere thanks to my second supervisor Professor Brown Robert. I have been privileged to benefit from his expertise. His personable support and guidance, along with his unique scientific knowledge have been of irreplaceable help.

I would like to thank especially some of my colleagues; Rebecca, Umber, Mariea, Burcak, Farhad, Mani, Partha and Alvena. It has been an honour working with such charismatic and intelligent people and I wish all the best.

I would also like to thank my family, both near and far, that have been by my side all along helping me out during all my frustrations. Without their encouragement and help I would have not coped. Special thanks to my parents, George Karamichos and Euagellia Karamichou, and my sister, Dr. Elina Karamichou, for everything they have given me, this thesis is for them.

Abstract

Cells embedded within tissues respond to mechanical, chemical and biological signals. However, the detail of how mechanical forces are transmitted to cells is poorly understood at present and represents a key missing link in Tissue Engineering. As cells attach to the fibrils in fibroblast-seeded 3D collagen scaffolds they generate contractile forces to levels, which depend on cell type, attachment, density, growth factors and matrix stiffness. The aim of this study was to use external applied strain to increase matrix stiffness in collagen constructs. Embedded resident cells (from three different sites) were then subjected to specific mechanical loading regimes in scaffolds of increasing stiffness and matrix remodelling genes quantified as markers of mechanoregulatory cellular response. Mechanical responses of cells were also quantified as contraction profiles over time.

Our findings indicated that collagen got stiffer with application of high strains and visco-elastic properties resulted in minimal transfer of applied loads as recorded by movement of indwelling markers. The mechanical and molecular responses of three different cell lineages: human dermal (HDF), neonatal foreskin fibroblasts (HNFF) and human bone marrow stem (hBMSC) cells seeded in constructs of increased stiffness was tested. Results indicated that in HNFFs contraction was predominantly attachment-dependent while in HDFs it was predominantly stiffness-dependent. hBMSCs showed differential response to serum levels. Molecular responses in progressively stiffer constructs investigated were MMP-2, MMP-3, MMP-9, TIMP-2,COL-1,COL-3 and IGF-1. Different cell types expressed specific variations in gene regulation. The effect of specific mechanical loading (slow and fast ramp)

regimes on regulation of matrix remodelling genes also showed a lineage dependent response.

The major impact of this project has been the identification of a strong co-relation between substrate stiffness, mechanical loading and regulation of key ECM turnover genes. This knowledge is crucial to successful tissue engineering outcomes. The differential lineage dependent response is a key finding and will have to be tailored depending on cell source and specific outcomes desired.

Contents   Page

5

List of Figures

10

Table of abbreviations

2-D:	2-Dimensional
3-D:	3-Dimensional
BSA:	Bovine Serum Albumin
CFM:	Culture Force Monitor
CIT:	Contraction Initiation Time
COL-1:	Collagen - 1
COL-3:	Collagen - 3
DMEM:	Dulbecco's Modification of Eagle's Medium
ECM:	Extracellular Matrix
FCS:	Fetal Calf Serum
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
HDF:	Human Dermal Fibroblast
HNFF:	Human Neonatal Foreskin Fibroblast
hBMSC:	Human Bone Marrow Stem Cells
IGF-1:	Insulin-like Growth Factor - 1
LPA:	Lysophosphatidic Acid
MEM:	Minimum Essential Medium
MMP-2:	Matrix Metalloproteinase - 2
MMP-3:	Matrix Metalloproteinase - 3
MMP-9:	Matrix Metalloproteinase - 9
PBS:	Phosphate buffered saline
PC:	Personal Computer
PCR:	Polymerase chain reaction
PDGF:	Physiological agonists platelet-derived growth factor
RNA:	RiboNucleic Acid
t-CFM:	Tensioning- Culture Force Monitor
TE:	Tissue Engineering
TIMP-2:	Tissue Inhibitors of Metalloproteinases

Chapter 1: INTRODUCTION

Tissue Engineering

Tissue engineering (TE) was defined firstly by Langer and Vacanti [1993] who stated it to be "*an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function*". [Langer et al., 1993]. MacArthur and Oreffo defined tissue engineering as "*understanding the principles of tissue growth, and applying this to produce functional replacement tissue for clinical use*" [MacArthur et al., 2005]. Recently Williams [2006] defined tissue engineering as "*the creation of new tissue for the therapeutic reconstruction of the human body, by the deliberate and controlled stimulation of selected target cells, through a systematic combination of molecular and mechanical signals*"

TE is a multi disciplinary field where biology, material science, modelling and engineering combine in order to replace or support damaged tissues and restore or improve their function. Any tissue or organ is subjected to a range of different mechanical forces as a result of body movement, contact with neighbouring tissues and due to direct cellular contraction. Cells embedded within those tissues/organs respond to mechanical/chemical/biological signals. The importance of external mechanical stimulation (applied loads) is now becoming appreciated and researchers have studied different loading regimes (some examples are: external agents such as thrombin, uniaxial forces and biaxial forces) applied to different scaffolds and none or a very wide range of effects (some examples are: actin cytoskeleton, cellular contraction and molecular gene regulation) have been shown [Harris et al., 1980; Kolodney et al., 1992; Brown et al., 1996; Mudera et al., 2000]. One of the most important decisions is the choice of the extra-cellular matrix (ECM), as ECM

material properties can completely alter the nature of external mechanical loads reaching resident cells. This can lead to regulation of gene expression, tissue differentiation and tissue architecture [Mudera et al., 2000; Cheema et al., 2005; Eastwood et al., 1998; Cullinane et al., 2003].

This study is focused on collagen Type I which is the most common occurring protein in the human body and the most distinctive property is its viscoelastic property. Because of this property collagen is used in TE as bio-artificial matrix, over the last decade [Eastwood et al., 1994; Tomasek et al., 1992; Brown et al., 1998; Mudera et al., 2000; Grinnell et al., 2002; Karamichos et al., 2006].

Collagen models

Collagen Type I provides tensile strength and stiffness to the tissues. When tissues are damaged their mechanical strength and integrity has to be recovered if full function is to be restored, and that is one of the main aims in TE.

Following an injury (such an accident) or surgery scar tissue will replace injured skin and underlying or damaged muscle. In these cases formation of scar tissue is inevitable, where on the other hand we have bone, epithelia, and gums where complete regeneration without scarring is possible. The amount of scarring is often unpredictable and may be determined by different factors such as: the size, depth, and location of the wound; as well as the age of the individual [Diegelmann et al., 2004].

The origin of scars is located at the cellular level, where as cells remodel and lay down new extracellular matrix (ECM), in this case collagen, contract the injured tissue resulting in a thick and dense fibrous connective tissue. Although, the scar

16

tissue, replaces (structurally) destroyed tissue, it cannot perform the functions of the missing tissue and the result is to loose tissue functional ability.

Normally scars become less noticeable with age, however, major injuries (such as burns) can cause loss of a large surface area of skin and may form hypertrophic scars (Figure 1). A scar is made up of 'connective tissue', ECM deposited in the skin by the fibroblasts to close the wound.
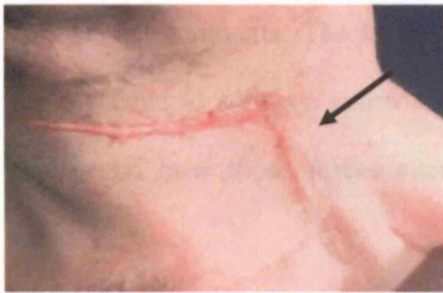
Figure 1. Hypertrophic scar formation with arrow pointing to the connective tissue formed to close the wound.|http://www.med.uottawa.ca/medweb/hetenyi/ayeni_figures.htm|

Host cells (at a scarred tissue) are mainly fibroblasts, and several studies have reported growth factors effects on normal skin fibroblasts contractility in collagen constructs [Clark et al., 1989; Montesano et al., 1988; Tingstrom et al., 1992; Gullberg et al., 1990] but not much has been reported on different cell types (including hypertropic scar fibroblasts) [Uppal et al., 2001]. Myofibroblasts are responsible for the generation of such contractile forces [Grinnell 1994, Tomasek et al., 2002, Serini et al., 1999]. Gabbiani et al. [1971] were first described myofibroblasts and since then, the role of myofibroblasts has been extensively studied [Desmouliere, 1995; Desmouliere and Gabbiani, 1996; Powell *et al.*, 1999; Moulin *et al.*, 2000; Hinz *et al.*, 2001; Van Beurden *et al.*, 2003]. Myofibroblasts cause the extracellular matrix to contract (Clark, 1996) and are involved in the regulation of proliferation and differentiation cell lineages such as: epithelial,

vascular, and neurogenic cells [Saunders and D'Amore, 1992; Yamagishi *et al.*,1993]. Myofibroblasts form stress fibers containing the major contractile elements actin and myosin II as reviewed by Tomasek et al. [2002]. The activation of myosin II that allows its cyclic attachment to actin filaments involves myosin light chain phosphorylation by the Ca21-calmodulin-dependent myosin light chain kinase (MLCK) [Tomasek 2006], as discussed later.

It would be necessary, therefore, to investigate how different cell lineages respond to such environments. The focus of this study was to quantify cellular responses, when seeded in collagen constructs, to external mechanical stimulation, in order to understand how those forces alter the organisation of new ECM and how cells respond.

Cell embedded scaffolds

Type I collagen is a biomaterial that has been used as 3-D Fibroblast populated construct to study the generated tensile forces in culture, where resident cells tend to reduce the dimensions (contraction) of the 3-D material [Tomasek et al., 2002; Grinnell, 1994]. When such constructs are attached to a force monitoring device such as the Culture Force Monitor (CFM) used here (Figure 2), the pattern of forces generated can be quantified and correlated with fibroblast motility/traction, contraction and fibril remodelling [Eastwood et al., 1994; Brown et al., 1998; Brown et al., 1996; Delvoye et al., 1991].
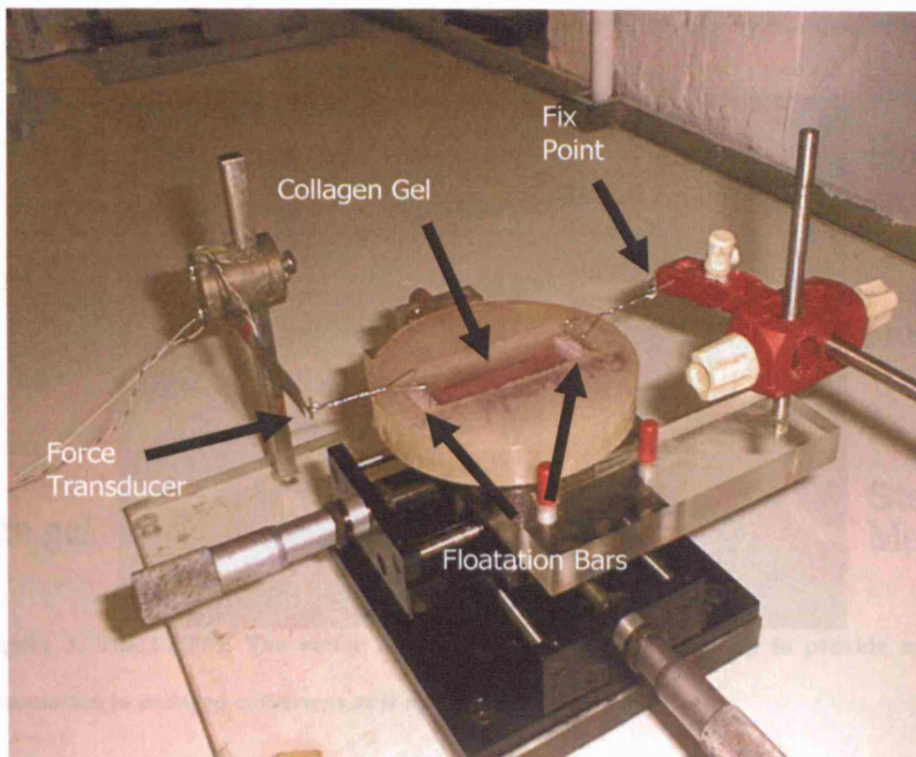
Figure 2. The CFM. Collagen construct attached between a fixed point and a force transducer which is then connected to a PC for real time contraction forces measurements.

Extension of such a monitoring device is the tensional-CFM (t-CFM) [Eastwood et al., 1998]. A motor attached to the stage of CFM provides the option of mechanical loads application to the collagen constructs (Figure 3). Unidirectional tensile loads in predetermined patterns applied throughout this study and will be described later. Mechanical forces transferred to the cells together with ECM surface biochemistry and soluble factor composition regulate tissue turnover and repair. In connective and contractile tissues cell control is often dominated by mechanical signals, cells also generate their own forces in response to different mechanical or chemical signals as part of normal tissue maintenance and renewal, hence the use of the t-CFM system.
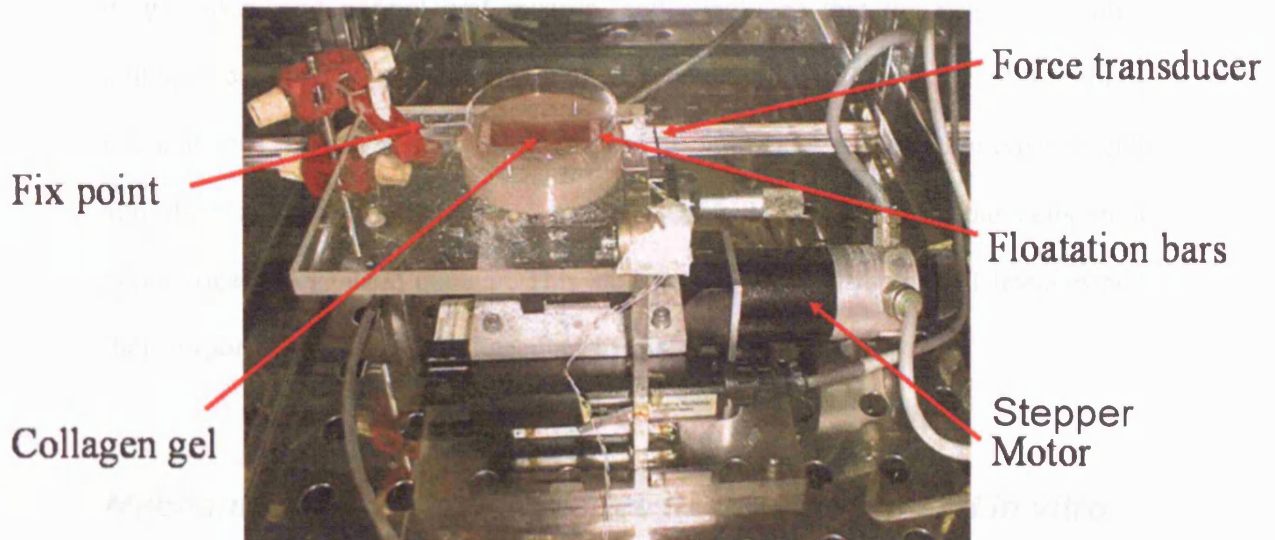
Figure 3. The t-CFM. The motor is been added to the CFM set-up to provide mechanical stimulation to collagen constructs as it moves the stage.

Such t-CFM systems have been used to predictably align cells and cellular reorientation in response to patterns of strain setup within the collagen gel construct [Eastwood et al., 1998; Tomasek et al., 1984; Delvoye et al., 1991]. Other studies have correlated changes in cell shape with cell attachment and force generation in similar constructs [Talas et al., 1997; Halliday et al., 1995] and alteration in fibronectin fibre assembly [Yamamoto et al., 2001]. Application of mechanical strains on collagen constructs means that collagen fibrils within the body of this material can be considered as a conduit by which mechanical information is transmitted both locally between cells and regionally across the construct. Interactions between collagen and cells are critical for the mechanical regulation of cell activity as well as for connective tissue homeostasis [Tomasek et al., 2002; Brown et al., 1998]. Investigators have reported that collagen contraction by resident cells reflects the mechanism of wound contraction. Tsai et al [1995] have analyzed

the contraction potency of fibroblasts that had been obtained from hypertrophic scar, normal skin, and normal oral mucosa, and concluded that the degree of initial collagen contraction was closely related to morphological changes of fibroblast resident cells. However, in vivo, tissues are assemblies of one or more types of cell and their associated extracellular matrix, which is produced by the cells in a geometrically organized manner. This study tested three different cell lineages and their responses (cellular and molecular) to stiff collagen matrices.

Mechanical forces in connective tissues in vivo and in vitro

Conventional mechanics deals with the strength and physical properties of any given material. Means of testing this is the load-deformation behaviour of the material. In the case of biomechanics the behaviour of the material that has been synthesized by the cells (i.e., extracellular matrix, ECM) is tested. The effects of mechanical strain on ECM/cell behaviour, has been reported in literature and discussed here. Mudera et al. [2000] showed significant up-regulation of matrix degrading genes following application of strain. Furthermore, there have been a number of studies describing different cell straining systems, as reviewed by Langelier et al [Langelier et al, 1999]. Those systems applied forces to cells when seeded on 2-D and/or 3-D environment. However, there are differences between the systems; for example Flexercell system [Banes et al.,1985] strain application was possible though force monitoring was not. At the other end of these systems, the tensional-Culture Force Monitor (t-CFM) [Eastwood et al., 1998] where tensile loading and force monitoring is possible. It is clearly important to assess each experiment and design the optimal strain values/regimes in order to characterize and quantify cellular responses.

Cell-matrix interactions

Collagen is found in almost every tissue and organ, particularly skin, cartilage, ligaments, bone. In fascia, ligaments and tendons, 80% of the dry weight is collagen where its hierarchical fibrillar organisation is key to overall gross tissue strength and stability. Fibroblasts synthesize and secrete the fibrils which are the principal source of tensile strength in tissues; define shape and form of tissues [Canty et al., 2005].

At present, there are 16 different types of Collagen [Ross et al., 1995] that are classified on the basis of chronology of discovery. Collagen gets its high tensile strength from its fibres. Collagen fibres appear as wavy structures of variable width and indeterminate length. They appear as a bundle of fine, thread-like subunits known as the collagen fibrils. Fibrils size varies between tissues; from 20nm in diameter for developing tissues to 200nm in dense regular connective tissue (for example tendons) or in tissues that are subject to considerable stress.

The collagen molecule (also called tropocollagen) measures about 300 nm in length and 1.5 nm thickness. It has a head and a tail which become aligned when forming a fibril. Head and tail are arranged in overlapping rows with a gap between the molecules within each row. The strength of the fibril is due to covalent bonds between collagen molecules of adjacent rows (Figure 3b).
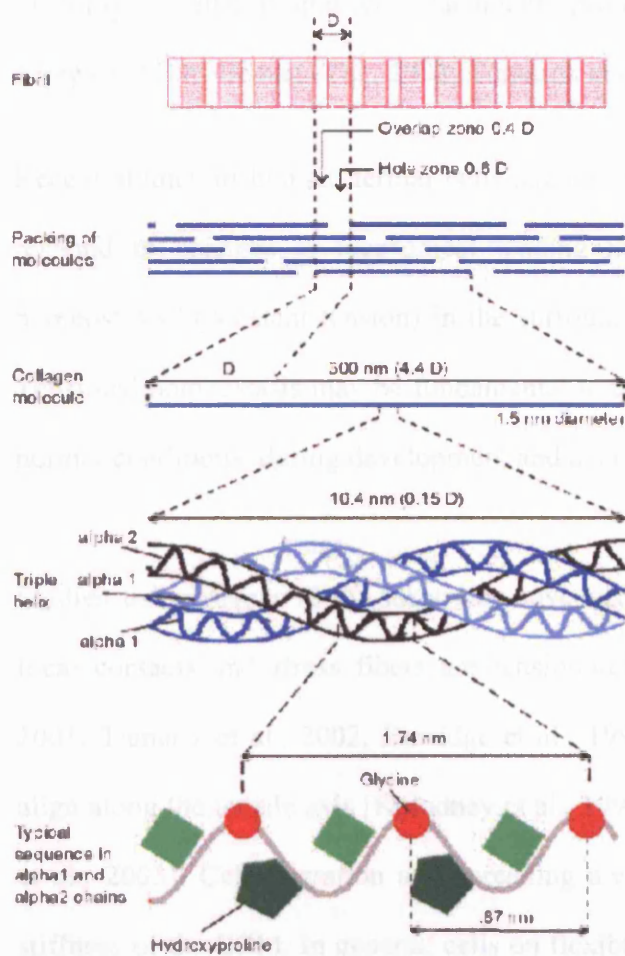
Figure 3b. Diagram shows the molecular character of a collagen fibril in increasing order of structure. [www.ccmbel.org/Chap5.html].

Furthermore, fibroblasts seeded in collagen ECM play a vital role in both immediate and long term responses to mechanical forces [Grinnell et al., 2002]. Mechanical stimuli on such constructs play a key role in regulating function (such as actin cytoskeletal network, integrins and signal transduction alteration) in a variety of cell types [Shyy et al., 2002; Sadoshima et al., 1997; Liu et al., 1999; Tummina et al., 1998]. Further, interactions between cells and surrounding collagen are critical for the mechanical regulation of cell activity and for connective tissue homeostasis [Eckes et al., 2004; Brown et al., 1998; Tomasek et al., 2002], as well as the balance

of functions like migration, attachment, proliferation, differentiation, and gene expression [Gentleman et al., 2003; Tomasek et al., 2002].

Recent studies in human dermal cells suggest that fibroblasts within 3-D matrices respond to changes in mechanical loading in a way that maintains "tensional homeostasis" (constant tension) in the surrounding matrix [Eastwood et al., 1994]. Tensional homeostasis may be fundamental to the regulation of tissue tension under normal conditions, during development and also in response to injury.

Studies using planar (2-D) substrates have demonstrated that the development of focal contacts and stress fibers are tension-dependent processes, [Riveline et al., 2001; Tamariz et al., 2002; Burridge et al., 1996] and that these structures tend to align along the tensile axis [Kolodney et al., 1992; Takakuda et al., 1996; Wakatsuki et al., 2003]. Cell migration and spreading are also influenced by the mechanical stiffness of the ECM. In general, cells on flexible substrates are more migratory and have smaller focal adhesions than those on more rigid substrates [Pelham et al., 1997]; cells also preferentially spread on more rigid substrates [Lo et al., 2000]. Although these studies using 2-D substrates have provided important insights into cell mechanical behavior, cells reside within 3-D extracellular matrices in vivo, and ECM geometry has been shown to effect both cell morphology, adhesion organization and mechanical behaviour [Bard et al., 1975; Tomasek et al., 1982] The mechanism of cell-mediated collagen contraction has been reported in literature [Eastwood et al., 1994; Mudera et al., 2000; Cheema et al., 2003] and involves interactions between cells and the surrounding matrix (ECM) via specific cell surface receptors [Klein et al.,1991]. In literature, examination of collagen contraction, by resident cells, at different time points showed that the rapid increase

in force generated (described by the authors as **traction phase**) corresponded with cell attachment and the extension of cell processes which are a prerequisite to any form of cell migration [Eastwood et al., 1996].

The rate and magnitude of contraction, though, is dependent on several factors such as cell density, collagen concentration and percentage of serum present (mainly due to LPA presence; discussed later) [Parizi et al., 2000]. Furthermore, the mechanical responses of fibroblast-seeded collagen constructs, has been reported in literature, by investigating different test parameters such as stretch rate and stretch amplitude [Wakatsuki et al., 2000]. It is important, therefore, to quantify contractile forces produced by the cells in response to different ECM environments and how these may alter the cytoskeleton structure and/or cellular orientation of the cells.

So far three model systems (Figure 4) with distinctly different mechanical properties have been developed and reported in literature: a) The floating collagen constructs model (Figure 4a) where fibroblasts spread and attach to collagen fibers. Tractional forces generated by the cells (contraction) lead to compaction of the collagen gel [Harris et al., 1981; Tomasek et al., 1992]. In this model, the collagen fibers are free to move in all directions; tension is therefore distributed isotropically, and the matrix remains mechanically relaxed [Grinnell 1994]. b) The stabilised collagen construct model (Figure 4b). Here the tractional forces result in reduction of matrix height; however because the collagen is attached to the underlying plastic substratum tension is distributed anisotropically along lines of stress [Mochitate et al., 1991; Tomasek et al., 1992]. Finally, c) The stabilised collagen model (Figure 4c). The collagen construct is released from the plastic substratum after 12h, leading to a

rapid contraction of the collagen [Mochitate et al., 1991; Tomasek et al., 1992] as a result of a contraction by the individual cells, involving an actin and myosin interaction similar to that which occurs in smooth muscle [Tomasek et al., 1992].
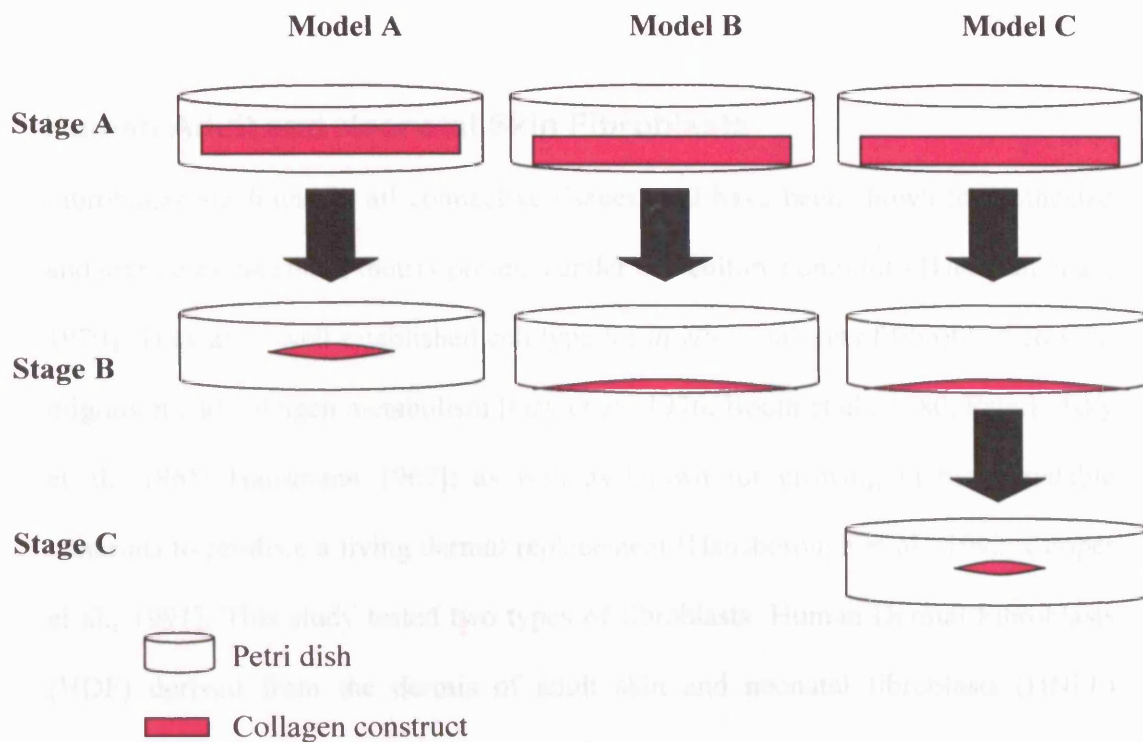
Figure 4. Three models of collagen constructs are shown. Model A: collagen is released from the plastic substratum at time=0h and contracted by resident cells; Model B: collagen is attached to the plastic substratum (Stage A) and remains attached during contraction (Stage B); Model C: collagen construct is attached to the plastic substratum (Stage A) and after 12h is released (Stage B), leading to a rapid contraction of the collagen (Stage C)

Different cell types respond differently to mechanics

Fibroblasts have been, so far, the most popular cell type seeded in collagen constructs. This study investigated how three different cell types respond to increased matrix stiffness and how may these be modulated by it.

Human Adult and Neonatal Skin Fibroblasts

Fibroblasts are found in all connective tissues, and have been shown to synthesize and secrete extracellular matrix proteins under cell culture conditions [Hedman et al., 1979]. They are a well established cell type for *in vitro* analysis of fibroblast growth, migration and collagen metabolism [Gay et al., 1976; Booth et al., 1980; Peterkofsky et al., 1965; Hausmann 1967]; as well as known for growing in biodegradable materials to produce a living dermal replacement [Hansborough et al., 1992; Cooper et al., 1991]. This study tested two types of fibroblasts: Human Dermal Fibroblasts (HDF) derived from the dermis of adult skin and neonatal fibroblasts (HNFF) derived from normal human neonatal foreskin.

Human embryonic and adult stem cells

Stem cell is a cell type with potential to repair damaged organs/tissues. From heart muscle tissue to bone and from cartilage to skin, stem cells have been associated with major input of repair of these organs [Korbing et al., 2003; Kelly et al., 2005; Grigoropoulos et al., 2006].

There are two main sources of stem cells: a) Human embryonic and b) adult stem cells (Figure 5) each with advantages and disadvantages.

27
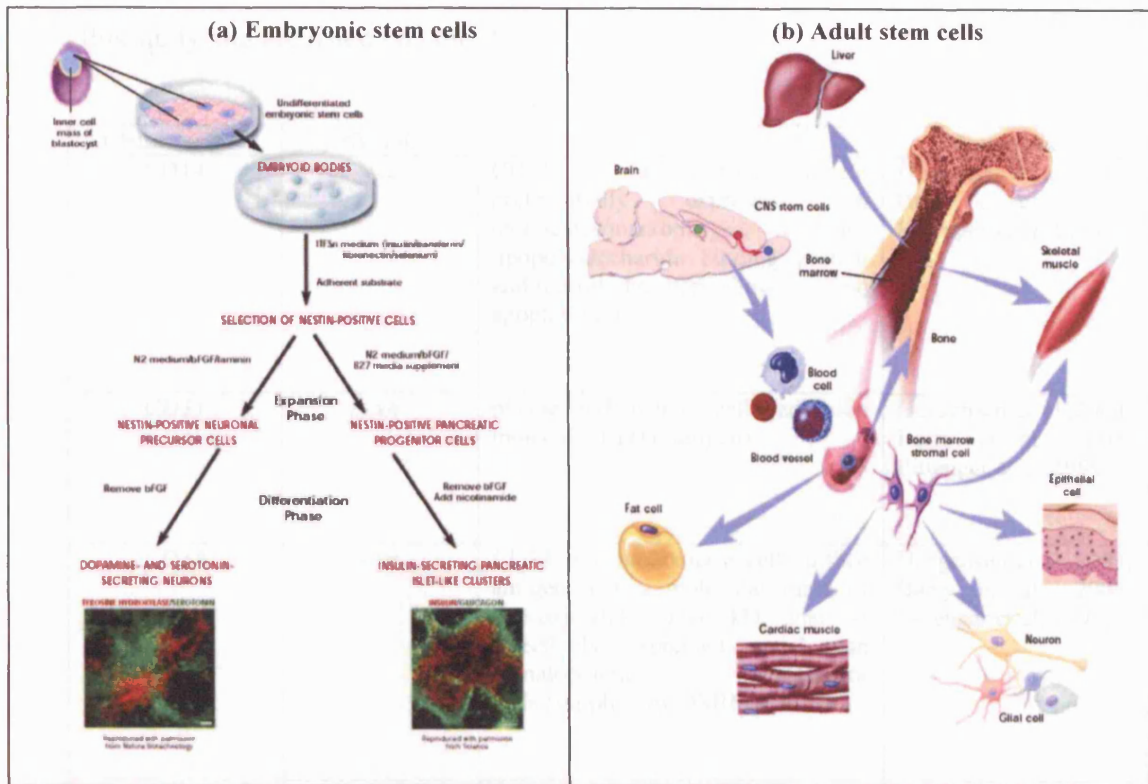
Figure 5.Embryonic stem cells (a) and Adult stem cells (b) differentiation potential is shown [The National Institutes of Health website: stemcells.nih.gov/info/basics/basics4.asp]

Adult Human Stem Cells have been isolated from a variety of tissues [Barry et al., 2004] where their differentiation potential is highly attractive. Many adult tissues contain stem cell population however the major source of adult mesenchymal stem cells is the bone marrow (hBMSCs). Researchers [Friedenstein et al., 1987; Kuznetsov et al., 1997] have shown that these hBMSCs are contributing to the regeneration of a variety of tissues, such as bone, cartilage, muscle, ligament, tendon, adipose and stroma. hBMSCs were first identified and isolated by Friedenstein [Friedenstein et al., 1966] from rat marrow. Despite the fact that hBMSCs are a very small fraction (0.001-0.01%) of the total population, isolation and expansion is possible in high efficiencies. hBMSC isolation and purified cultures are confirmed using specific surface marker proteins (according to the literature) [Jorgensen et

al.,2003; Barry et al., 2004; Pittenger et al.,1999]. Choices of markers were used in this study and are listed on table 1.

Characteristic	hBMSC		References
CD14	- ve	CD14 is a surface protein preferentially expressed on monocytes/macrophages. It binds lipopolysaccharide binding protein and recently has been shown to bind apoptotic cells.	[Jorgensen et al.,2003; Barry et al., 2004; Pittenger et al.,1999]
CD31	- ve	platelet/endothelial cell adhesion molecule (CD31 antigen)	[Jorgensen et al.,2003; Barry et al., 2004; Pittenger et al.,1999]
CD34	- ve	CD34 is a monomeric cell surface antigen with a molecular mass of approximately 110 kD that is selectively expressed on human hematopoietic progenitor cells.[supplied by OMIM]	[Jorgensen et al.,2003; Barry et al., 2004; Pittenger et al.,1999]
CD44	+ ve	CD44 The protein encoded by this gene is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration. It is a receptor for hyaluronic acid (HA) and can also interact with other ligands, such as osteopontin, collagens, and matrix metalloproteinases (MMPs).	[Jorgensen et al.,2003; Barry et al., 2004; Pittenger et al.,1999]
CD105	+ ve	CD105 is a homodimeric transmembrane glycoprotein highly expressed by endothelial cells	[Jorgensen et al.,2003; Barry et al., 2004; Pittenger et al.,1999]

Table 1. Surface marker proteins: CD14, CD31, CD34, CD44,and CD105, used for hBMSCs staining are shown together with a brief description and their expression (-ve or +ve) from hBMSCs.

Molecular genes

This study investigated the effect of increasing matrix stiffness on cell force generation as well as its effect on molecular mechanoresponsive genes. This is based on the findings that uniaxial pre-strain significantly alters the collagen stiffness.

Eastwood and co-authors [1998], using the t-CFM, has proved possible to identify mechano-responsive genes such as MMP-1, -2, and -3. Prajapati et al [2000] demonstrated strain dependence of HDFs in this 3-D system in terms of protease expression (MMP's and plasminogen activator). Mudera et al [2000] identified a sophisticated relationship between force vector and cell alignment operates to regulate gene expression of key matrix degrading enzymes. Increases in a range of proteins including tenascin and collagen levels in response to tension have been used. These studies suggest that cells respond to altered strain in their matrix in a number of ways, though correlation between cell response and the triggering aspect of mechanical loading is rarely investigated [Choquet et al, 1997].

In vivo, the early stage of wound healing in almost all tissues is the expression of a variety of extracellular proteolytic enzymes such as MMP-2 and MMP-9 investigated by Ritty and co-authors [2003] on tendon cells. These proteinases have several functions during healing and scar formation such as modulation and removal of compromised ECM.

Table 2 summarizes the genes used throughout this study as markers/indicators of matrix synthesis/degradation.

Gene Name	Gene Abbreviation	Characteristic	References
Matrix Metalloproteinase-2	MMP-2	This gene encodes an enzyme which degrades type IV collagen, the major structural component of basement membranes.	[Brinckerhoff et al., 2002]
Matrix Metalloproteinase-3	MMP-3	This gene encodes an enzyme which degrades fibronectin, laminin, collagens III, IV, IX, and X, and cartilage proteoglycans. The enzyme is thought to be involved in wound repair, progression of atherosclerosis, and tumor initiation.	[Brinckerhoff et al., 2002]
Matrix Metalloproteinase-9	MMP-9	The enzyme encoded by this gene degrades type IV and V collagens. Studies in rhesus monkeys suggest that the enzyme is involved in IL-8-induced mobilization of hematopoietic progenitor cells from bone marrow, and murine studies suggest a role in tumor-associated tissue remodeling.	[Brinckerhoff et al., 2002; Ritty et al., 2003]
Tissue Inhibitor of Metalloproteinase-2	TIMP-2	This gene is a member of the TIMP gene family. The proteins encoded by this gene family are natural inhibitors of the matrix metalloproteinases, a group of peptidases involved in degradation of the extracellular matrix	[Brinckerhoff et al., 2002]
Collagen-1	COL-1	Collagen facilitates successful adaptation of in vitro culture and enhances expression of cell-specific morphology and function. The high protein concentration results in a sturdier gel which provides maximal support to maintain the 3D environment	[Gautreau et al., 1999; Abir et al., 2001]
Collagen-3	COL-3	Type III collagen appears first in a wound and initiates the hemostatic process. In addition, 3-D matrix formulations (e.g., sponges) of recombinant human type III collagen demonstrate superior mechanical integrity, larger surface area, and higher hemostatic activity than bovine collagen type I in experimental models.	[Gelberman et al., 1980; Bailey et al., 1977]
Insulin Growth Factor-1	IGF-1	IGF-I is a complex gene that is regulated by multiple promoters and is capable of producing at least four different mature IGF-I precursor proteins (i.e. isoforms). Because of the variety of isoforms it remains unclear if all the forms of IGF-1 have similar effects.	[Hameed et al., 2004; Spangenburg, 2003]

Table 2. Gene markers used throughout this study are summarised here. MMP-2,-3,-9, TIMP-2, COL-1.-3, and IGF-1. A brief description and reference of each one of the genes are listed.

Thesis overview

The purpose of this study was to test how mechanical forces are transmitted through bio-artificial matrices using collagen as an example and to test the effect of mechanical stimuli on its physical properties as well as the responses by the resident cells. This study focused on an in vitro tissue model, i.e. fibroblast-populated collagen constructs. Native Type I collagen was used as the bio-artificial matrix. Collagen is the most common mammalian connective tissue protein, found in skin, cartilage, ligament and bone. Fibrillar Type I collagen is widely used to form bio-scaffolds for tissue engineering (TE) and repair applications [Brown et al., 2005; Ehrilch et al., 1990].

Hypothesis under test

"Stiffness of 3D collagen constructs can be controlled by applying external mechanical strain. The micro movement as perceived by the cells within these constructs is dependent on stiffness of constructs".

"Cells seeded in a collagen construct will up-regulate/down-regulate their pattern of force generation and expression of mechano-responsive genes (MMP-2, -3, -9, TIMP-2, COL-1, -3, and IGF-1) in response to changes in stiffness of the collagen construct".

"Up-regulation/down-regulation of cell responses (i.e. contraction forces and gene regulation), in increasingly stiff collagen constructs, will differentially be modulated between different cell lineages".

"Introduction of two different ramp loading regimes will up-regulate/down-regulate the expression of mechano-responsive genes (MMP-2, -3, -9, TIMP-2, COL-1, -3, and IGF-1) differentially, depending on cell lineages".

Aims and Objectives

The overall aim of this study was to progressively alter the properties (organisation and stiffness), of native Type 1 collagen constructs, and use the embedded resident cells (fibroblasts) as the load sensing system (i.e cyto-sensors) in order to quantify cellular (physical and molecular) responses.

The main objectives of this study are outlined below:

1) To test the ability to increase collagen matrix stiffness by applying increasing uniaxial pre-strains and optimising stiffness after visco-elastic relaxation of collagen independent of cell involvement.

2) To quantify micro movement as perceived by cells embedded within these constructs of increasing stiffness, using indwelling markers.

3) To quantify the mechanical contractile cellular responses of cells embedded in increasingly stiffer constructs in terms of contraction forces generated.

4) Test the effect of defined ramp loading regimes on regulation of relevant ECM genes (MMP-2, -3, -9, TIMP-2, COL-1, -3, and IGF-1) in cells embedded in progressively stiffer constructs.

5) Test lineage response (human dermal fibroblasts, neonatal foreskin fibroblasts and human bone marrow stem cells) of defined ramp loading regimes (molecular and contractile).

Chapter 2: Materials & Methods

Processing of Tissues

Fresh full thickness skin biopsy was obtained from the surgery theatres (Full ethical approval) and human dermal fibroblast cell culture was established using a routine explant technique [Burt et al., 1992]. Skin obtained was in a variety of tissue sizes (depending on operation/patient).
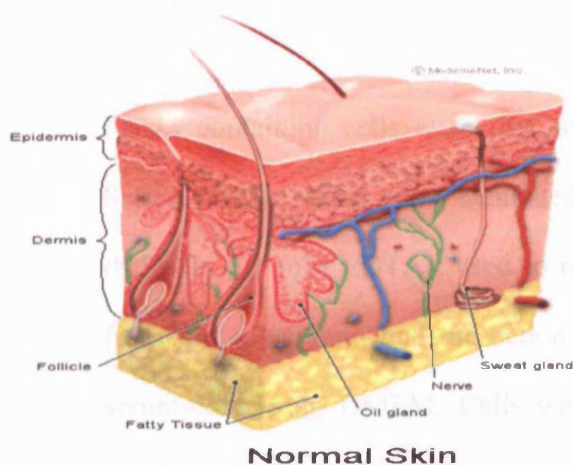
Figure 6. Layers of human skin consist of the epidermis follow by the dermis with a fatty layer beneath them [Medicinenet website: www.medicinenet.com/ melanoma/article.htm]

Skin comprise of three tissue layers (epidermis, dermis and underlying fat), as shown in figure 6. The layer of subcutaneous fat was scraped off the sample, using sterile carbon steel scalpel blade, and the remaining two layers were explanted. Small pieces of the skin (2mmx2mm) were cut, using scalpel blades, and skin pieces placed in flasks with the dermis side down. Explants were incubated at 37°C and 5% CO_2 for 1 hour, at which point explants were stuck down and dry and standard fibroblast growth medium DMEM was added [Invitrogen, Scotland, UK] supplemented with 10% foetal calf serum (FCS) [First Link Ltd, Birmingham, UK], 100u/ml Penicillin and 100ug/ml Streptomycin [Invitrogen, Scotland,UK] and L-Glutamine [2mM – SIGMA, Poole, UK].

Cell migration, from explants, was observed using microscopy and when cells had covered 70%-80% of the flask surface explants were removed gently using a plastic pipette, and washed off with sterile 0.1M Phosphate Buffered Saline (PBS).

Cell culture

Cells were passaged at 70-80% confluence and used for experiments between passages 3-10.

Flasks containing cells were first washed 2 times with 0.1M Phosphate Buffered Saline (PBS). Flasks were incubated for 5-10 minutes at 37^0C, containing trypsin (0.5% in 5.3 mM EDTA); used to release cells. Adherent cells were then released from the substratum and the action of trypsin was inhibited by addition of 15ml serum-containing DMEM. Cells were then centrifuged at 400g for 5 minutes to obtain cell pellet and re-plated at lower concentration (1:3), to allow cell expansion.

Human dermal fibroblasts (HDF's) used in this study were cultured in DMEM supplemented with 10% FCS and/or 20% FCS during experiments described later. HNFF's were only cultured in 10% FCS.

Human Neonatal Foreskin (HNFFs)

Human tissue explants and fibroblast cultures were established from freshly harvested neonatal foreskin tissue (full ethical approval) and foreskins derived cells (HNFF) were used for experiments in this study. HNFFs culture was identical to HDFs described above [HNFF cells were a gift from Dr. Jeffrey Teumer, Intercytex Ltd, USA].

Human Bone Marrow Stem Cells (hBMSCs)

Normal Human bone marrow aspirates (normally 3-5ml) were obtained from patients undergoing routine surgery for hip/knee replacement (full ethical approved). Marrow aspirates were withdrawn, using a sterile 21G needle inserted into the patient's bone (from hip/knee bones). Marrow was placed into tubes containing 3000 units of heparin and centrifuged at 900g for 10 minutes at room temperature to remove fat and debris. The supernatant was loaded onto a 20 ml ficoll (1.073g/ml density gradient; Amersham Pharmacia Biotech SE 75285). This was then centrifuged at 1160g for 30 minutes at room temperature and decelerated slowly (270sec. to standstill-in order to keep the interface intact). Following centrifugation the top layer including the interface (which contains the nucleated cell fraction with human Bone Marrow Stem Cells hBMSC's) was aspirated and placed into new sterile centrifuge tubes. PBS was then added, for washing, and centrifuged at 400g for 5 minutes. Finally, cells were cultured in DMEM (in T25 flasks) [Koller et al, 1998] supplemented with 10% and/or 20% FCS (reasons described later) and 100u/ml Penicillin and 100ug/ml Streptomycin.

The Culture force monitor (CFM) and the tensioning culture force monitor (t-CFM)

In this study the responses of fibroblasts have been investigated, when seeded in collagen constructs and subjected to external mechanical stimuli, using a specially fabricated device the tensioning-culture force monitor (t-CFM), Figure 7 [Eastwood et al.,1998].

A force monitoring system was first developed, by M.Eastwood [Eastwood et al., 1994] in which a fibroblast populated collagen construct (FPCL) was suspended between fixed points to give a rectangular, uniaxial force generation reading. A force transducer at one end and an anchoring point at the other provided the axis for measurement of overall contraction by the FPCL. This passive measuring instrument was termed the CFM (Culture Force Monitor) [Eastwood et al., 1994; Mudera et al., 2000].

This was then further developed, by Eastwood et al [1998] to a computer driven tensional loading device (t-CFM; Figure 7) capable of unidirectional controlled loading. t-CFM was used here to apply different predetermined programmed tensile loads (described later) to the collagen constructs, using PC-based software X150 (Parker Automation, City, USA). The analogue output was amplified, digitised and plotted using LabView software (National Instruments, v.6, Newbury, UK)
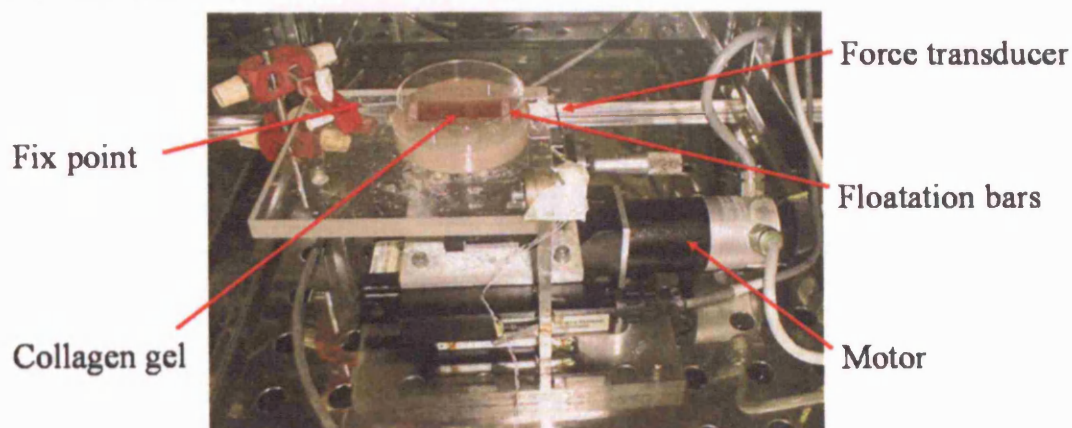
Figure 7. The t-CFM. Cell seeded collagen construct is placed between a fixed point and a force transducer. The motor shown was used to apply mechanical stimulus [Eastwood et al., 1998; Mudera et al., 2000].

Force transducer calibration

Measurement of contraction forces in this study was monitored using a force transducer, described before, mounted on a CuBe beam which was calibrated regularly, as follows.
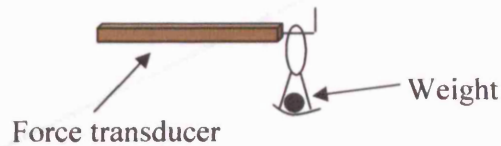
Figure 8. Weight and transducer, used, calibration position shown

The Cu-beryllium transducer beams were placed on the CFM stage with the flat surface facing parallel to the ground (Figure 8). Five weights plus a transducer beam free standing i.e 0g (0, 29.4, 49, 196, 294 and 490g) were placed on the hook at the tip of the transducer. Each mass gave a force reading and was recorded for 5 minutes for stable operation check. The calibration curve was plotted (Figure 9) as force (Dynes) against applied actual weight (grams).

Figure 9. Typical calibration curve is shown from one of the transducers used here. Force (Dynes) is plotted against weight used.

Linearity was important to ensure accurate force readings, using the transducer beams, and a value close to 1 was expected for the 'R^2' value (value indicating how close to linearity is the line plotted. 1 is perfect match to straight line). Transducers with 'R^2' < 0.95 were not used.

Preparation of collagen constructs

Collagen constructs were prepared as described previously [Eastwood et al., 1998]. 4ml of Type I rat tail collagen [First Link UK Ltd, Birmingham, UK] was added to 0.5ml of 10x Minimum Essential Medium Eagle [Invitrogen, Scotland, UK]. After drop-wise neutralization with 5M and 1M sodium hydroxide (until colour changed from yellow to light pink observed; pH from acid to neutral; 7.0), a suspension of 5 million cells (counted using a haemacytometer) in 0.5ml DMEM was added to the collagen mixture. Two plastic end floatation bars (Figure 10b) were pre coated with

neutralised collagen. The solution containing the cells and the collagen was poured into a 75 x 25 x 15mm well (with the floatation bars), and allowed to gel for 30 minutes at 37°C. One plastic spacer bar was positioned (before collagen incubation) at each of the short ends of the well to give 10mm spaces for mechanical extension and they were removed, after collagen had set (Figure 10a).

6(a)

6(b)
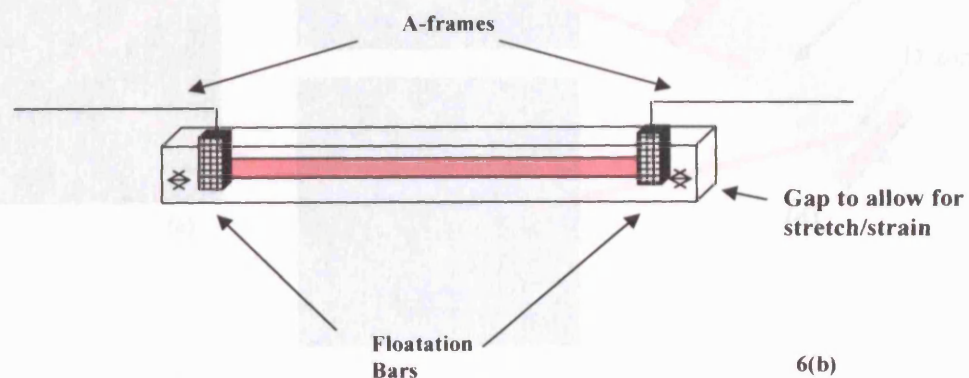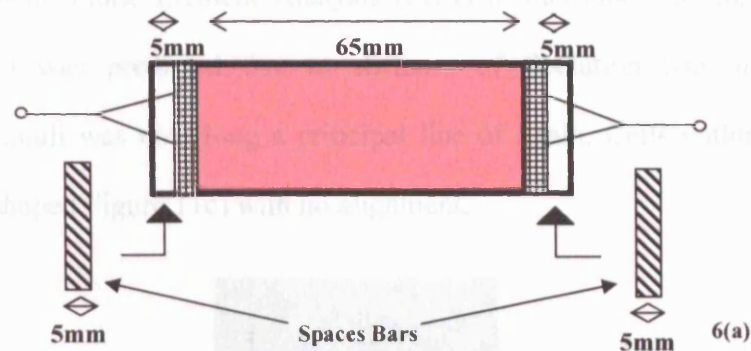
Figure 10. (a) Collagen gel shown with spaces bars added for 10mm space within the mold (5mm at each sort end) and (b) Collagen gel shown after spaces bars removed, with A-frames positions shown when hooked to the t CFM.

40

Constructs were hooked to the CFM/t-CFM (following 30 minutes incubation to allow constructs to set) via the A-frames attached to the fixed point at one end and the force transducer on the other (Figure 7). The force transducer was connected to the PC via an amplifier to provide force readings per second through LabView software, as described above.

Strain measurements within collagen constructs

Predictable cellular alignment (Figure 11b) to mechanical stimuli along the lines of principal strain, have been previously demonstrated [Eastwood et al., 1998], using the t-CFM. Using Finite Element Analysis (FEA) in this model a stress shielded zone (D-zone) was predicted due to stiffness of floatation bars used where mechanical stimuli was not along a principal line of strain. Cells within this zone were stellate shaped Figure 11c) with no alignment.
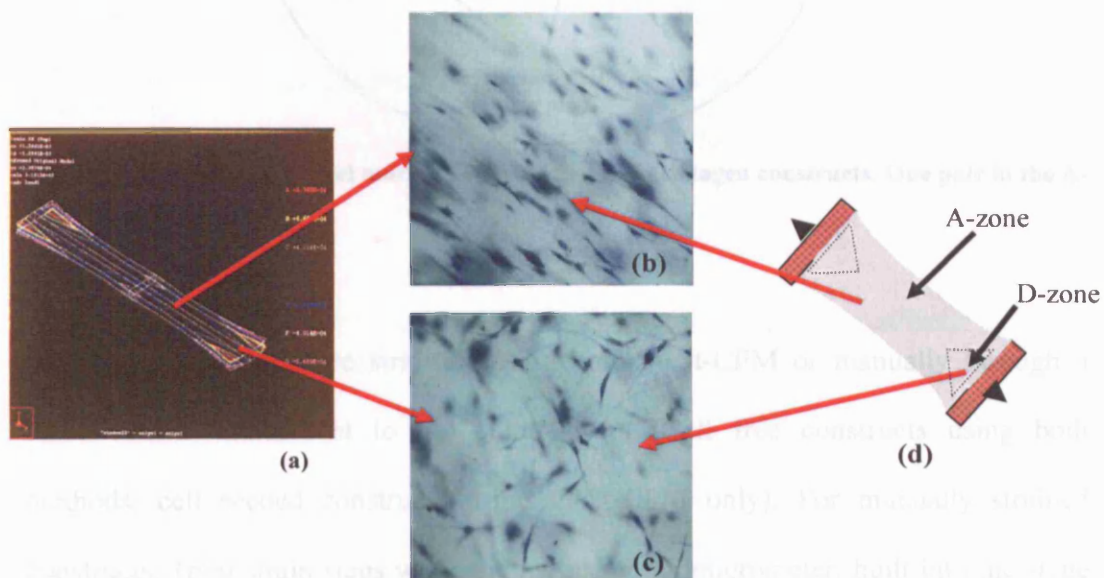
Figure 11. FEA analysis (a) predicted cell alignment (b) in the A-zone (d) of the collagen and cells with random orientation and stellate morphology in D-zone (c) [Eastwood et al., 1998]

41

For direct measurement of local strain within acellular collagen constructs, stainless steel markers (3x0.4mm) were cast into predetermined positions, (a) at the centre and (b) near the short floatation bar ends of each gel construct (Figure 12). One pair was parallel to the loading axis in the A – zone and one pair was perpendicular with the floatation bar in the D-zone [Karamichos et al., 2006]. The position and orientation of stainless steel markers were decided in order to mimic cell orientation/alignment (aligned parallel to the applied load axis in the A-zone and randomly orientated in the D-zone), as shown by Eastwood et al. [1998]
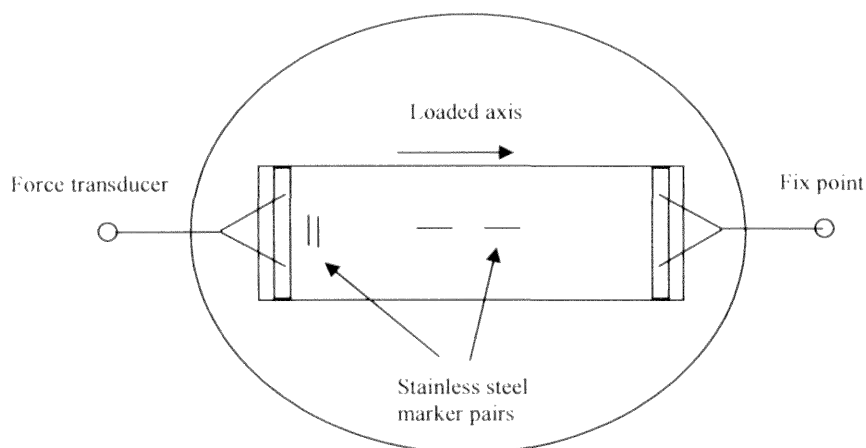
Figure 12. Four stainless steel markers embedded within collagen constructs. One pair in the A-zone and one in the D-zone.

Collagen constructs were strained, using both the t-CFM or manually through a screw-vernier attachment to the culture bars (Cell free constructs using both methods, cell seeded constructs using the t-CFM only). For manually strained constructs, 1mm strain steps were applied using the micrometer, built into the stage (maximum displacement of 12mm) with 1minute periods between each strain point. A fibre optic microscope (Moritex Scopeman 504) was used to monitor changes in the position and angles of the stainless steel markers, as shown in figure 13. Digital images were captured at the end of each strain step and stored (Macintosh G4 PC)

for image analysis and measurement using OpenLab software (Improvision, Birmingham, UK).

Strain direction
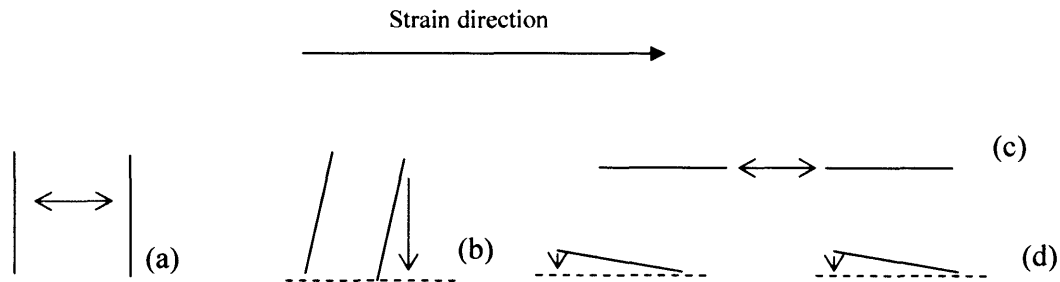
Figure 13. Red arrows indicate (a) Distance measured in D-zone, (b) Angle measured in D-zone, (c) Distance measured in A-zone and (d) Angle measured in A-zone

These marker displacements were used to calculate the local magnitude and vector of forces within the two construct zones. Distances indicated by the red arrows were measured and averaged for each construct. For the x-plane markers (Figure 13a and 13c) the distance between the two markers was measured. For the y-plane markers (Figure 13b and 13d) the distances for each individual marker was measured and averaged to give a value for the specific construct.

Effects of FCS starvation on cell attachment and effect of cell seeding on viscoelastic properties of collagen

External strains (applied as described above) of 5%, 10% and 15% were applied using the t-CFM, each over 5 seconds (Figure 14 – Red arrow). Both cell-seeded (1million cells/ml density) and acellular collagen constructs were tested. Direct cell contribution through force generation, spreading, contraction was minimised by complete removal of Fetal Calf Serum (FCS) to prevent attachment/contraction (described below). After applied load, constructs were held at the constant final displacement for one hour (to allow for strain relaxation) which was the point used

for stiffness calculations (Figure 14 – blue arrow). In other words, final strain

relaxation of the collagen constructs was measured relative to the initial applied

displacement to determine material stiffness (Total applied strain – Strain relaxation

= Stiffness accumulated in collagen).
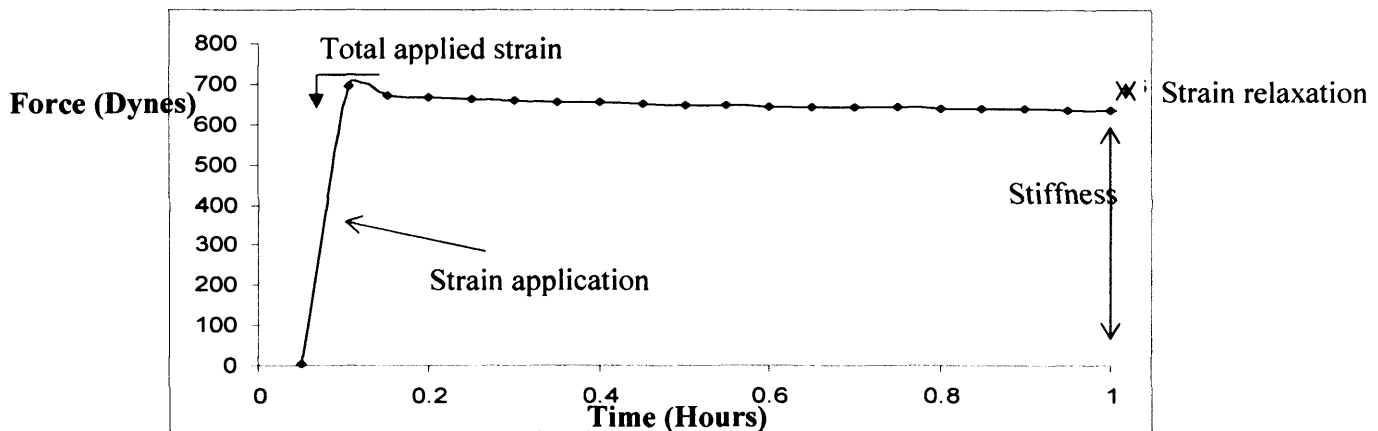
Figure 14. External uniaxial strain applied to collagen (red arrow) and value recorded 1h post strain (Blue arrow)

Again the differences in collagen mechanical properties between the A and Delta

zone under defined loads represented a key part of the collagen material properties

investigation.

Fetal Calf Serum Starvation

Cell seeded collagen constructs were prepared without FCS in order to minimize

cell-matrix attachment (attachment = force generation) [Eastwood et al., 1998;

Mudera et al., 2000]. Experimental cell seeded constructs were FCS starved for 60

minutes and then each one stained with live/dead stain (Live/Dead

Viability/Cytotoxicity Kit, Molecular Probes, Paisley,UK) for cell viability

(examined using fluorescence microscopy) and also fixed using 10% Formal saline

for routine microscopy (whole constructs visualised using an inverted microscope) to

visualise for lack of cell attachment using morphology as an output. Further, mechanical output was used, i.e these constructs produced no force for 60 minutes of FCS starvation.

Mechanical loading application on collagen constructs

X-ware software X150 [Parker Automation, City, USA] was used to control the motor attached to the CFM stage. In this part of the study pre-strain was introduced to the collagen constructs in order to vary the stiffness of the collagen. HDF's, HNFF's and hBMSC's cell types were all included in the pre strain experiments. The first part of this study investigated the cellular responses to increasing matrix stiffness, in terms of force generated under externally applied mechanical pre-strains (i.e mechanical loads applied at time 0).

Motor calibration

Before any mechanical stimulation could be performed on the constructs, t-CFM machines were calibrated for linear operation within the pre-strain range used for our experiments (0%, 5% and 10%).

All the motors used in this study, had a step angle of $1.8°$ which translates to 200 full steps per revolution. Each revolution of the motor related to a certain distance by which the stage moved, as explained below. The default motor set up was 4000 steps (which is one revolution) which permitted accurate calculation of the relationship between motor and distance travelled (i.e strain applied). For example:

$$\frac{Dis\tan ce(D)}{Time(T)} = \frac{No.Steps}{Seconds} = \frac{10.000}{4.000} = 2.5revs/\sec. \quad (1)$$

Using a screw pitch of 0.5mm displacement (2), we could calculate the distance (mm) travelled:

From (1) and (2) we can equate:

10.000steps = 2.5revs/sec = 1.27mm displacement   (3)

Hence, in order to apply a 10% strain to constructs of 65mm (length) an applied displacement of 6.5mm was needed (10% of 65mm), using the following motor motion:

From (3) 10.000 steps = 1.27mm, i.e 6.5mm = 51.160 steps   (4)

The software controlling the motor was X150 [Parker Automation, Dorset, UK]. Speed and distance travelled was selected and calculated as above as distance (D), related to the strain which was needed (Table 3). In this study the predetermined strains were: 0%, 5%, 10% and 15%. Therefore:

Strain	Distance (mm)	Motor Step No.
0%	0	0
5%	3.25	25558
10%	6.5	51160
15%	9.75	76760

Table 3. Strain (%) used for CFM/t-CFM collagen constructs translated to distance (mm) travelled by the motor.

Model development

Three different speeds (V), were initially investigated, 0.1, 0.3 and 0.5revs/sec (Figure 16) in order to make sure we operate on linear motor regions. Results showed (Figure 16) linearity for all speeds at all strains (0%, 5%, 10%, and 15%) and hence the faster speed (0.5) was adopted throughout this study.
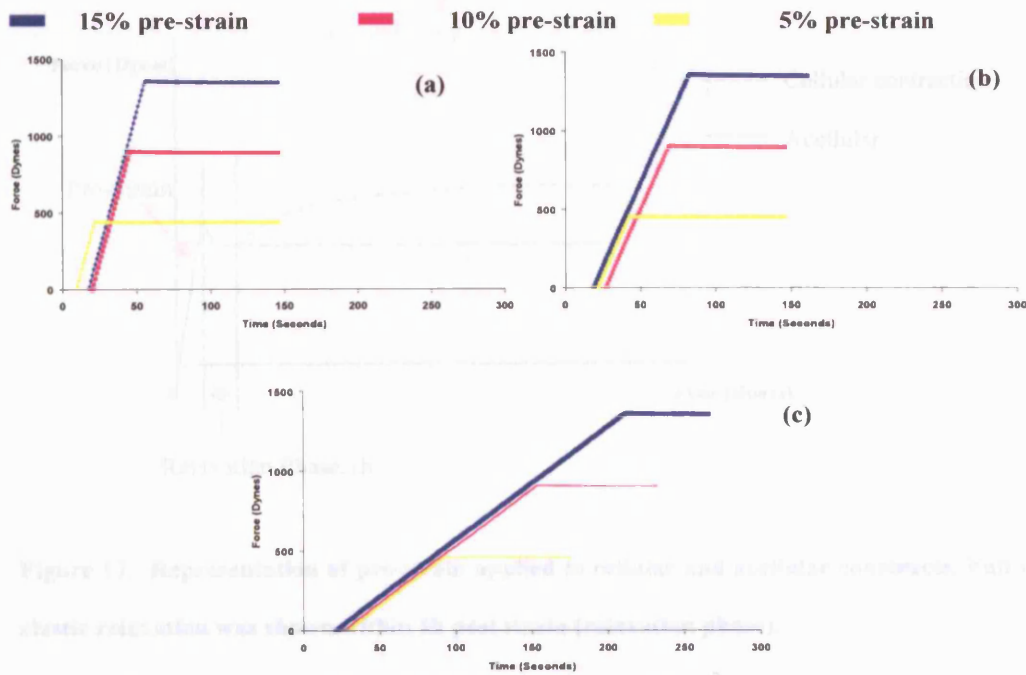
Figure 16. Three different speeds were tested for 0%, 5% and 10% pre-strain: a) 0.5revs/sec., b) 0.3revs/sec., and c) 0.1revs/sec.

Importantly, 15% pre-strain was later excluded from this study. Initial results showed no force generation by the 3 different cell types (described above: HDF, hBMSC and HNFF) when cell-seeded collagen constructs were subjected to 15% pre-strain. As described later, one aspect of this study was to investigate forces generated by cells when mechanically stimulated, therefore 0 (dynes) force generated following 15% pre-strain led to exclusion, from this study, of the 15% regime.

Mechanical stimulus to collagen constructs

Constructs (cellular and acellular) were pre set and placed on the t-CFM and a pre strain of 0%, 5% and 10% was applied (Figure 17; Red arrow). Constructs were held at the constant final displacement for 24 hours (as described earlier) and contraction force monitored. Figure 17 shows response examples from cellular and acellular constructs (dotted and continues line respectively).
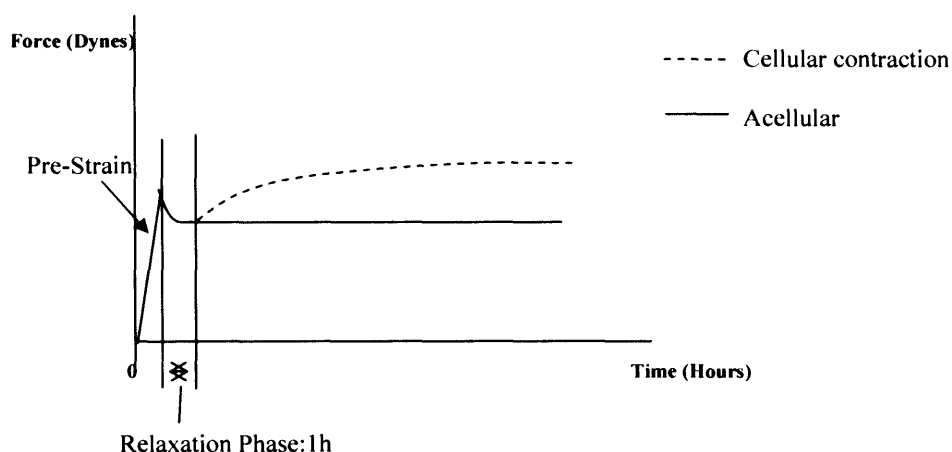
Figure 17. Representation of pre-strain applied to cellular and acellular constructs. Full visco-elastic relaxation was shown within 1h post strain (relaxation phase).

As presented later on, full visco elastic relaxation was shown at 1h independent of cell presence and/or pre-strain levels.

FCS starvation model

Pre strain experiments were repeated using HNFF's and HDF's by FCS starving the embedded cells for 1h. More specifically, cells were embedded in collagen with serum free medium and allowed to set (as described before). At time 0h constructs were floated in serum-free DMEM and pre-strain was applied (0%, 5% and 10%). Due to viscoelastic properties of the collagen, relaxation will always follow strain. It was determined (Results section) that the optimal viscoelastic relaxation time for collagen constructs adaptation to pre-strain was 1hour irrespectively of the amount of pre-strain. Constructs were held under tension and at 1h post-strain (optimal relaxation time) FCS was added to the DMEM to give a final concentration of 10% (red arrow, Figure 18), using a 10ml syringe to deliver. Force generated data was recorded for 24h and then experiments stopped.
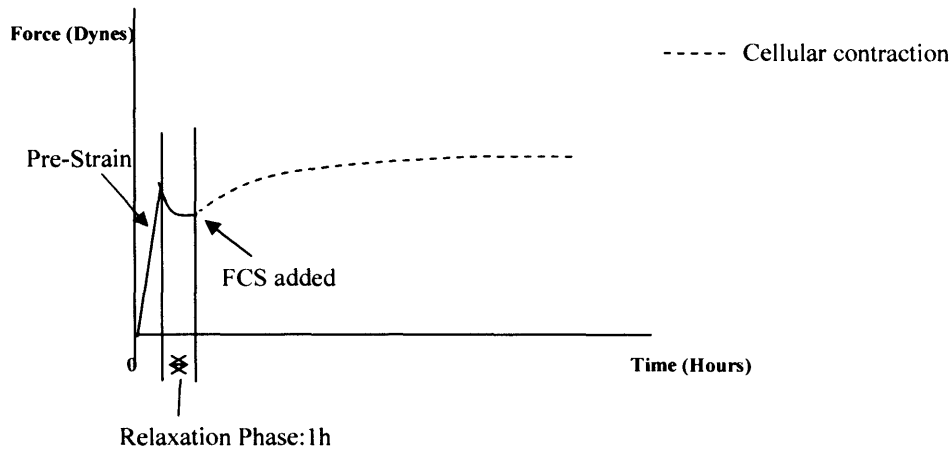
Figure 18. Representation of pre-strain applied to cells seeded collagen constructs without FCS. 10% FCS addition indicated by black arrow.

Pre-strain loading

After all the programming details were determined, the t-CFM motor was programmed. Pre-strain of 0%, 5% and 10% was applied with the velocity (V=0.5revs/sec., as described above) remaining constant throughout. Constructs were kept at the final displacement (post-strain) for 24h during which force generated by the cells recorded.

Ramp mechanical loading

Specific programmes were written for ramp mechanical loading. Previous studies have shown specific genes regulation due to ramp loading [Cheema et al., 2005]. In this study we used this knowledge to combine two different ramp loading regimes (described below) with the constructs subjected to 0% and 5% pre-strain. We allowed 12h post-strain, before 10% strain was applied over 1h or 12h.

Cellular contraction responses following mechanical loading

All cell types used here (HDF, HNFF, and hBMSC) reached a maximum contraction force within 12h. Pre-strain (0% and 5%) was applied at t=0h and constructs were kept under tension for 12h. At 12h two different Ramp loading regimes were applied (for programs see above). A 10% total strain was applied using two ramp loads; a) Over 1h and b) over 12h. Figure 19 shows an example of the two ramp loads applied for 0% pre-strained constructs.
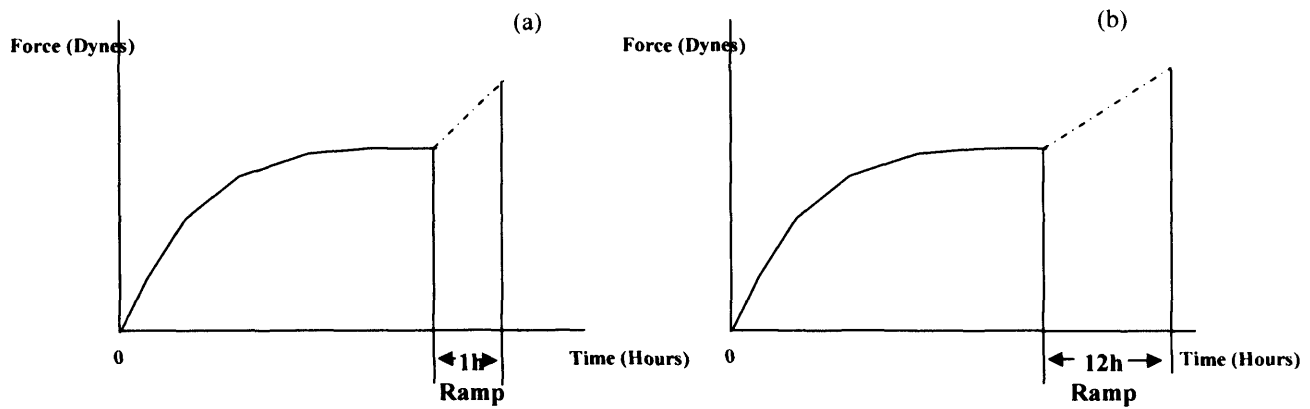
Figure 19 Ramp loading regimes applied after 12h culture on the CFM (a) 1h duration and (b) 12h duration

Figure 20 shows an example of the two ramp loads applied for 5% pre-strained constructs.
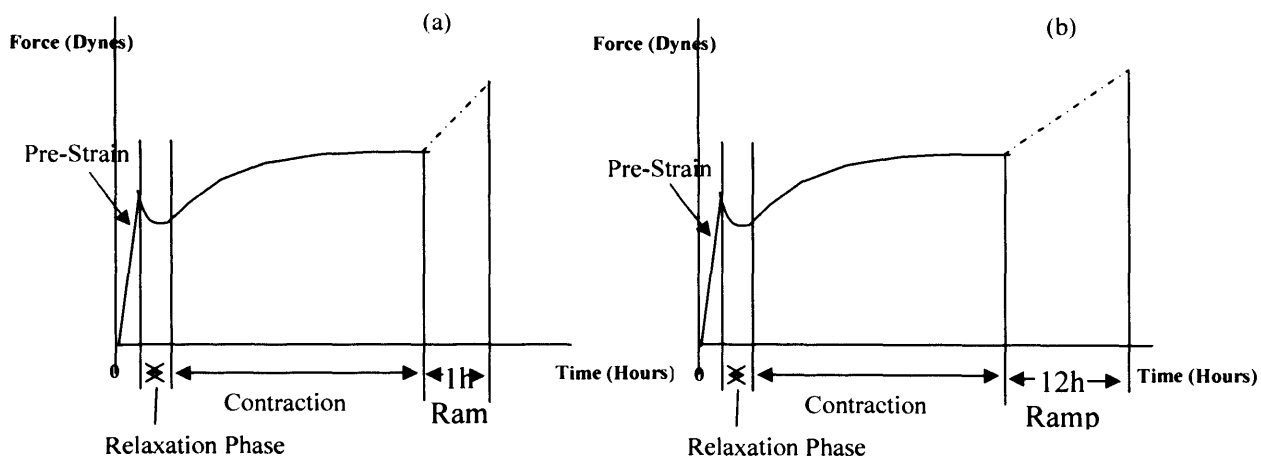
Figure 20 Ramp loading regimes applied after 12h culture on the CFM, on pre-strain (5%-Red arrow) constructs (a) 1h duration and (b) 12h duration

These experiments investigated the effect of different ramp loading regimes on expression of molecular outputs using specific genes responsible for matrix regulation. All the experiments were stopped at 24h and processed for molecular outputs (described later).

Statistical analysis

Statistical analysis of data (minimum of n=3 for all the experiments) were performed using the non-parametric, ANOVA (Analysis of Variance) testing (Kruskal-Wallis), using GraphPad Prism (Orism, Graphpad software, San Diego). t-test was also used, where needed. ANOVA and t-test are closely related and the only difference is that ANOVA can test the differences between the means of two or more groups. t-test was used here only to compare the means of two groups.

52

Histology: H&E

Constructs were processed for light microscopy and wax embedded. Sections were cut (5μm) on a microtome and dried onto glass slides. Sections were de-waxed in xylene, rehydrated and stained with Haematoxylin and Eosin (H&E; both VWR, Lutterworth, Leicestershire, UK).

Sections were then dehydrated in a graded series of alcohols, and mounted with DePX (VWR, Lutterworth, Leicestershire, UK) and mounted with coverslip. Sections were viewed (nuclei in blue, cytoplasm pale pink and collagen pink) using a light microscope (Olympus BH-2, Olympus, Japan).

Immunohistochemistry

Immunohistochemical staining of hBMSC was performed. hBMSC cultured on glass coverslips were fixed in ice cold acetone for 5 minutes and then washed twice in PBS for 5 minutes. They were then incubated in PBS containing 1%BSA and 1% Tween 20 for 30 minutes at room temperature.

The following primary monoclonal mouse anti human Ig G antibodies were diluted with PBS containing 1%Tween and 1%BSA, CD105 diluted 1:10 (R&D Systems, Abingdon, Oxon, UK), CD44 diluted 1:25 (BD Bioscience, Oxford, UK), CD45 diluted 1:25(BD Bioscience, Oxford, UK), CD31 diluted 1:40 (DAKO, Ely, Cambridgeshire, UK), and CD34 diluted 1:25(Serotech, Oxford, UK)

A total volume of 120μl of the diluted primary antibody was placed onto parafilm "stuck" onto the bench. The coverslips with the fixed cells were then placed cell side

down onto the primary antibody droplet. These were then covered with a box to prevent drying out and left at room temperature for one hour.

This was followed by 3x 5 minute PBS washes and then incubation in the secondary antibody for one hour at room temperature. The secondary antibody was a goat antimouse IgG, Alexiaflour, 594nm, (Invitrogen Ltd, Paisley, UK) diluted 1 in 250 with PBS. The cells were then washed three times in PBS for five minutes. The coverslips were then gently blotted and mounted in Permaflour (Beckman Coulter UK Ltd, Bucks, UK) and viewed under a Olympus BH2-RFC fluorescent microscope, (Olympus BH-2, Olympus, Japan).

RNA extraction

The total cellular RNA was isolated from all the cell populated collagen lattices, using Qiagen Rneasy kits (Qiagen, UK). In addition to this kit a QIA shredder kit was used in order to break up the collagen before total RNA isolation occurred.

All gels were flash frozen in liquid nitrogen, following the end of CFM/t-CFM experiments, and stored at -80° C to prevent RNA degradation. When gels were processed, 1ml of 'RLT buffer' was added to each one of them. RLT buffer contains guanidine isothiocyanate which denatures RNAses. Samples were kept at room temperature for 15-20 minutes and then passed through the membrane in the spin column provided with the QIA shredder kit. This step was added in order to break up collagen completely. From that stage onwards the standard Qiagen Rneasy kit protocol was used (standard manufacturer protocol).

1ml of 70% ethanol was added to each sample and mixed well, by pipetting. 650ul of this mixture was added to the spin column and centrifuged for 15 seconds (7,000g).

This process was repeated until the whole sample has passed through the spin column. A silica-gel membrane in the spin column kept the RNA attached to the membrane. The flow through was discarded. 700ul of 'RW1 buffer' (to wash away any contaminants) was added to the spin column and again centrifuged for 15 seconds (7,000g).

500ul of 'RPE buffer' (to wash away any contaminants) was then added and centrifuged for 15 second (7,000g). 500ul of 'RPE buffer' was added again, this time centrifuged for 2 minutes (7,000g), helping washing away any remaining contaminants. Finally 50ul of RNase-free water was added to the membrane and centrifuged for 1minute (7,000g). The resulted elute was collected, which contained the total RNA. Readings were taken at 260nm (absorption of nucleic acids) and 280 nm (absorption of proteins present in the sample) using Genespec 1 (Naka instruments, Japan) spectrophotometer. Typical ratios 260/280nm, obtained from samples was 1.6-2.0, indicating good quality of RNA, i.e. correct size (above 200 bases).

c-DNA synthesis

The total RNA was reverse transcribed into cDNA using Expand reverse transcriptase (Roche Diagnostics, East Sussex, UK). The RT reaction generates a single-stranded DNA molecule, which is complementary to the RNA (cDNA). Reaction for cDNA synthesis contained 2μgm/10μl of RNA (for every sample), 0.4μl dNTPs (deoxynucleoside triphosphates-5mM), 5μl First Strand Buffer, 3μl MgCl$_2$ (25mM), 0.5μl OligoDT primer, 2μl MMLV (200U/μl) reverse transcriptase enzyme, 0.25μl RNAse inhibitor and 28.85μl of water to give a total of 50μl reaction mix. This 50μl

mix was mixed and a drop of mineral oil was added on top before running through

the RT cycle, using a PCR thermocycler (Hybaid OMN-E). RT reaction included the

following sequence: 25 ° C for 10 minutes, 42 ° C for 60 minutes, and 94 ° C for 5

minutes.

Samples were then stored at -80° C. Transcribed cDNA was used later as a template

in the Polymerase Chain Reaction (PCR), described below.

PCR amplification

PCR was performed to amplify specific region of the cDNA from each sample. The

cDNA is amplified exponentially via cycles of denaturation, annealing and

extension.

Relative Quantitative PCR was performed using Applied Biosystems 7300 Real-

Time PCR System (California, USA), with the TaqMan Universal PCR Master Mix

(Applied Biosystems) which contained AmpliTaq Gold® DNA polymerase for

better yield and AmpErase® UNG to prevent carry over contamination. 10μl of the

cDNA was used per reaction and TaqMan® Gene Expression Assays were provided

(Applied Biosystems) and used for gene expression. Assays use universal cycling

conditions, eliminating the need to optimize conditions individually. For each gene

expression 50μl of Master Mix, 5μl of Gene Expression Assay and 35μl of Rnase

free water used in conjuction with the cDNA; a total of 100μl per sample per gene

assay, from which 25μl pipette into the 96 well plates. Triplicates were performed

for each sample and each gene assay.

Applied Biosystems 7300 Real-Time PCR System combines thermal cycling, fluorescence-dye technology, and application-specific software enabling detection of the PCR products cycle-by-cycle accumulation in a single-tube reaction (of 25µl).

Results (see Results) were expressed relative to housekeeping gene GADPH which was found not to be significantly altered by the loading regimes. In order to relatively quantify genes upregulation / downregulation we compared the Threshold Cycle (Ct) values of the samples of interest with a control or calibrator such as a non-treated sample. The Ct reflects the cycle number at which the fluorescence generated within a reaction crosses the threshold. The Ct assigned to a particular sample thus reflects the point during the reaction at which a sufficient number of amplicons have accumulated, within that well/sample, to be at a statistically significant point above the baseline.

The C_t values of both the calibrator and the samples of interest are normalized to the appropriate endogenous housekeeping gene, in this case GADPH.

Primers used in this study are listed in table 4 Sequences are as provided by Applied Biosystems (confidential).

Genes tested	
MMP-2	COL-3
MMP-3	TIMP-2
MMP-9	IGF-1
COL-1	

Table 4. Genes tested here are listed

57

Chapter 3: Cell force generation is dependent on collagen substrate stiffness and serum concentration

An elastic material deforms under stress and upon removal of the load, tends to return to its original shape. A viscous material though, is permanently deformed and does not return to its original shape after removal of the load. Fibrillar collagen protein, in terms of its material properties, lies between these two types of behaviour (elastic and viscous), hence it is visco-elastic.

The main objectives of the first part of this study were: a) To test stress – relaxation of collagen constructs following external mechanical forces, i.e to quantify visco-elastic properties of collagen construct and adaptation to external strain and stiffness changes b) Test the effects of external uniaxial loading (pre-strain) on collagen fibril orientation, c) Test the effect of FCS starvation on fibroblast attachment and contraction.

Collagen stress-relaxation

Initially the stress relaxation of the collagen constructs was tested. Collagen constructs, whether cell-seeded (1million/ml) or cell-free, were mechanically loaded (pre-strain) in the t-CFM. As described in Methods, the pre-strain levels applied to the constructs, using the t-CFM, were 0%, 5% and 10% (Figure 17). Following pre-strain application, constructs were maintained under tension for 24h, to quantify the time of viscoelastic relaxation.

The time taken for complete relaxation was determined here and found to be 1h (n=4 for each pre-strain level), independently to the amount of pre-strain (average of 1.1±0.2h for cellular and 1.07±0.3h for acellular constructs). The force reached at

that time point (1h) was termed here Stress-Relaxation (SR). **SR** is defined here as the *amount of force* (in dynes) ''lost'' following pre-strain.

Figure 21 shows representative example of 5, 10 and 15% pre-strain (n=4 each) applied to collagen construct and the relaxation over 1h. As shown in Figure 10a, b and c, optimal viscoelastic relaxation time was 1hour irrespective of the amount of pre-strain (5%, 10% and 15% respectively).
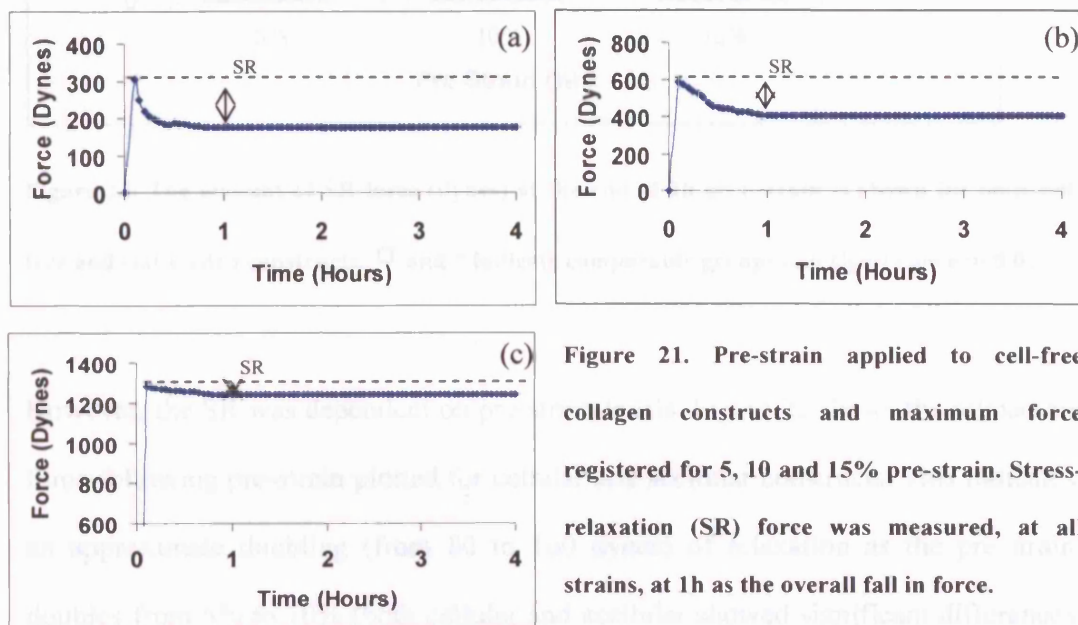
Figure 21. Pre-strain applied to cell-free collagen constructs and maximum force registered for 5, 10 and 15% pre-strain. Stress-relaxation (SR) force was measured, at all strains, at 1h as the overall fall in force.

Collagen constructs cell free and/or cell seeded were fully viscoelastic relaxed, within 1h, following pre-strain. This 1h was independent of the pre-strain regimes applied here.
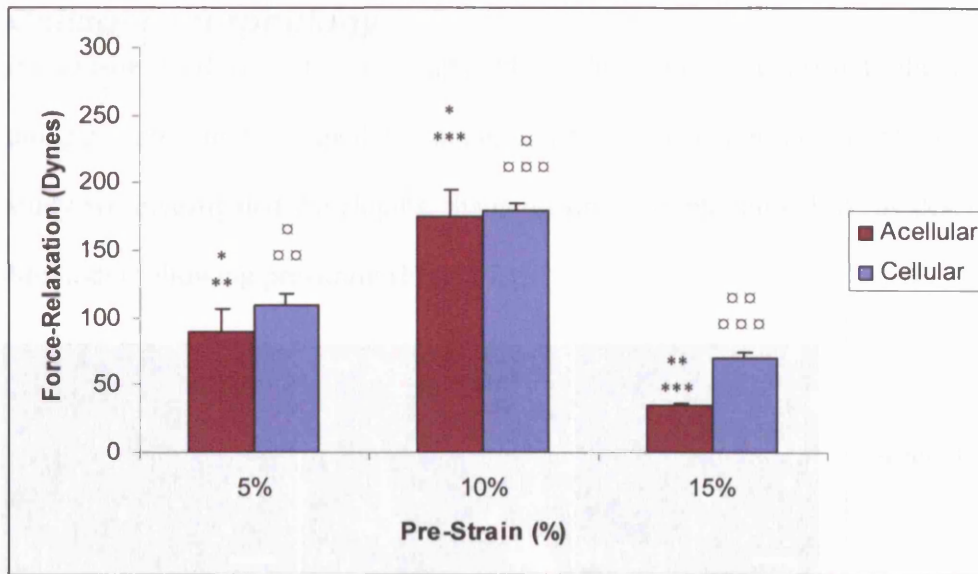
Figure 22. The amount of SR force (dynes) at the end of 1h post-strain is shown for both cell free and cell-seeded constructs. □ and * indicate comparable groups and significance p<0.05.

However, the SR was dependent on pre-strain levels. Figure 22 shows the relaxation force following pre-strain plotted for cellular and acellular constructs. This indicates an approximate doubling (from 80 to 160 dynes) of relaxation as the pre-strain doubles from 5% to 10% (both cellular and acellular showed significant differences between 5%-10% pre-strain; p=0.001 and p=0.04 respectively). When pre-strain was increased further, both cellular and acellular constructs showed significant (both p<0.01) reduction on the relaxation force. At that pre-strain level (i.e 15%) there was a significant lower relaxation force for acellular constructs (30%; p=0.004) compared to cellular. Therefore, *the amount of relaxation (SR) was directly dependent on pre-strain levels.*

Collagen morphology

As tensile load (pre-strain) is applied to the collagen construct, the construct elongates and fluid is expelled leading to fibril compaction and orientation. In this study we investigated developing/changing fibril orientation (SEM; as described in Methods) following pre-strain (Figure 23).

Force direction
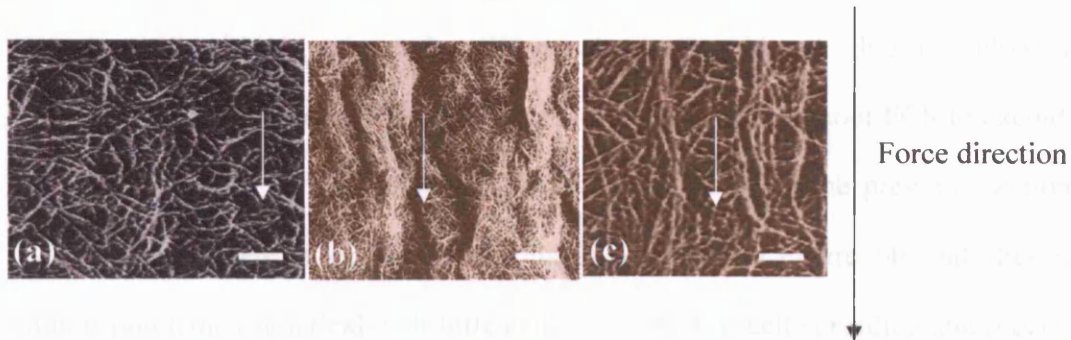
Figure 23. Electron Microscopy images of acellular collagen gels before and after different external mechanical loading (Bar = 5um). (a) Free floating, (b) 7% pre-strain, (c) 20% pre-strain [Karamichos et al., 2006]

Strain measurements were correlated with ultra structural pictures of constructs (pre-strain applied and constructs kept under tension for 1h before being fixed in 1.5% glutaraldehyde) under 0%-25% applied strain (n=3; Figure 23). Figure 23a shows a non pre-strained construct where collagen fibrils are randomly oriented. 23b and 23c shows collagen constructs subjected to 7% and 20% strain respectively, showing collagen fibrils aligned (visual observation) in the direction of the applied strain direction. All the strain levels used in this study showed similar results.

These results showed that there was a progressive reorganization and alignment of collagen fibrils on application of increasing amounts of strain (with no cellular involvement).

Distinction between cellular contraction and collagen matrix

Cell-seeded in collagen constructs have been used as in vitro models and have shown cellular ability to generate a measurable force over the experimental period (24h) [Eastwood et al., 1994; Mudera et al., 2000]. In this study, we tested the hypothesis that the presence of cells (at 1 million/ml concentration normally used in experiments) within increasingly stiff constructs, would not alter the physical properties of the construct. Constructs (n=3) were prepared without FCS to exclude cell-matrix attachment and so force generation, as shown in the previous section. Cells did not adhere or spread in the collagen as shown in Figure 24c and after 60 minutes remained spherical with little cell-cell contact or cell spreading and process elongation (Figure 24c; Arrow). However, when FCS was added (after 60 minutes), cells started to elongate and take a stellate morphology (Figure 24d and 24e; Arrows) as well as generate significant forces (above 10 dynes; Figure 24b).Cells with FCS present (n=3) from t=0h started to generate significant forces (above 10 dynes; Figure 24a), within 20 minutes, as well as attach and spread exhibiting normal morphology of fibroblasts as they attach to this substrate (Figure 24g and 24h) [Karamichos et al., 2006].
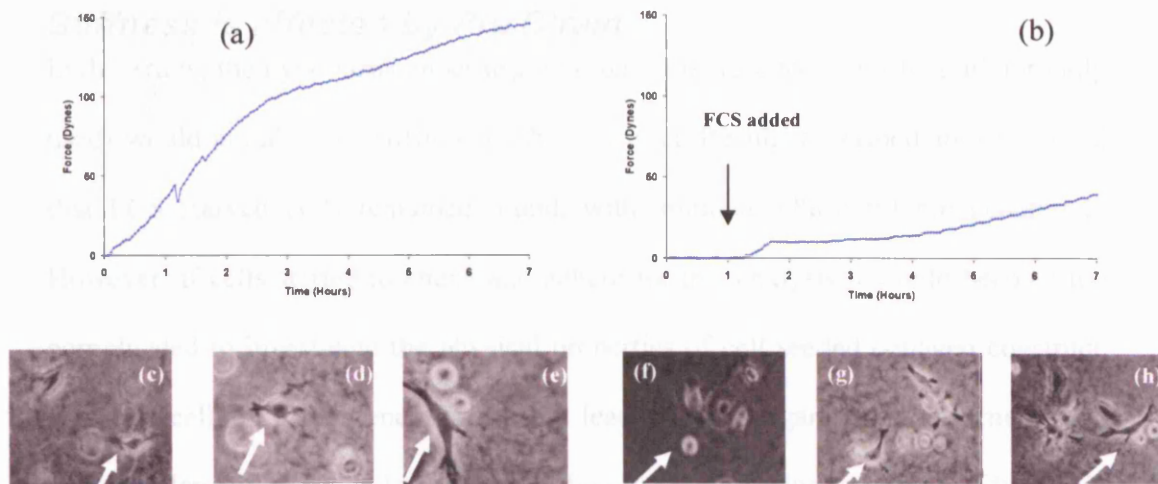
Figure 24. (a) Contraction profile over 7h hours is shown for a) cell seeded construct with FCS presence from t=0h and b) cell seeded construct following 1h FCS starvation. Note: Delay in CIT in (b) where co-related to cell attachment at 0.5h, 2.5h and 24h. With FCS presence at t=0h: (c) shows cell attachment as early as t=20min with progressive increase in cell spreading over time (d and e). With FCS presence at t=1h: (f) shows a delay in cell attachment and spreading due to FCS starvation which correlates with 0 dynes force generation. (g) and (h) shows progressive increase on cell spreading with time, co-related with increased force generation.

This experiment showed that cells remain round (in morphology) for 1h FCS starvation and upon re-introduction of FCS are capable of attaching, spreading and generating significant forces to the collagen construct [Karamichos et al., 2006]. *Therefore, FCS starvation was used to distinguish between cell contributions from collagen matrix physical properties (i.e stiffness, fibril alignment).*

Stiffness is affected by Pre Strain

In this study, the hypothesis under test was that, cell presence (1 million/ml normally used) would not alter the stiffness of the construct. Results described above showed that FCS starved cells remained round, with minimal adhesion/force generation. However, if cells started to attach and adhere for this analysis it would become too complicated to investigate the physical properties of cell seeded collagen construct. Onset of cellular force generation would lead to fibril organization/alignment (and collagen deposition in the long term) leading to an increasing in overall stiffness (in this case within 24h) of the construct [Karamichos et al., 2006], and so FCS starvation was used as mechanism to temporarily block this cell-mediated change of material properties in order to assess its relative influence of simple cell presence (unattached) versus cell force generated [Karamichos et al., 2006]. Cell-free and cell-seeded (FCS starved) collagen constructs were compared.

0, 5%, 10% and 15% pre-strain was applied to both cell free and cell seeded collagen constructs (FCS starved; both n=4). Stiffness was calculated at the end of full viscoelastic relaxation determined earlier (i.e 1h post pre-strain) for all the constructs (cellular and acellular) for all pre-strain regimes. Stiffness for 2.5% was calculated (approximation) from values obtained from higher strains. Figure 25a shows stiffness plotted against applied strain in mm.
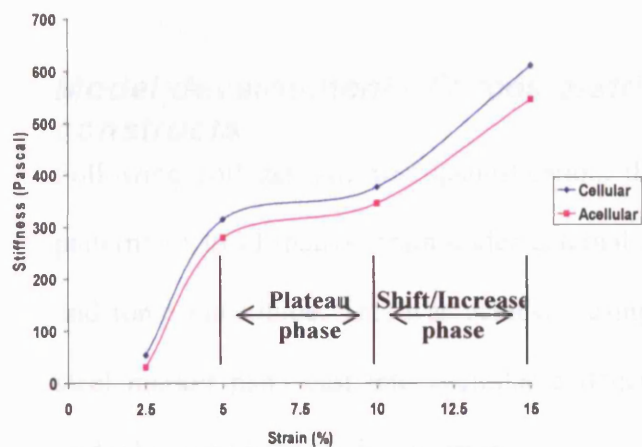
Figure 25. Stress-Strain curves are shown for both cell free and cell-seeded constructs. Matrix stiffness is plotted against pre-strain applied (%).

There was no significant difference in stiffness (Figure 25) between cellular and acellular constructs at any pre-strain level. Collagen construct stiffness was increased with pre-strain in a non linear manner by 70Pa for 2.5% and rose up to 310Pa at 5% ($p<0.05$), 380Pa at 10% ($p<0.05$), and 630Pa ($p<0.05$), for 15% pre-strain. There are two main phases: (a) a plateau region (Figure 25) between 5% and 10% pre-strain (b) a shift in the rate of changes stiffness (Figure 25) after the 5% and 10% plateau. *This data suggests a non linear response of the collagen matrix to external strain.*

Pre-strain leads to increased collagen stiffness independent of cellular presence, i.e. cells at 1mil/ml did not alter material properties of collagen when tested for increased stiffness.

Model development - Forces distribution in acellular collagen constructs

Following collagen stiffness quantification, this part of the study investigated the patterns of local matrix strain under external uniaxial load in terms of both vector and force magnitude. This was achieved using micro displacement of the stainless steel marker pairs cast into acellular collagen constructs, to quantify local strain under known applied uniaxial strains.

As described in Materials (Figure 11), cells in the collagen align parallel to the lines of principle strain, over 24h on the CFM, within the A-zone of the construct and are randomly oriented within the D zone due to stiffness of the floatation bars [Eastwood et al., 1994]. In order to simulate these shifts in principle strain, stainless steel markers were positioned (Figure 26) in two axes (a) parallel, in the A-zone, and (b) perpendicular, in the D-zone, to the applied load.
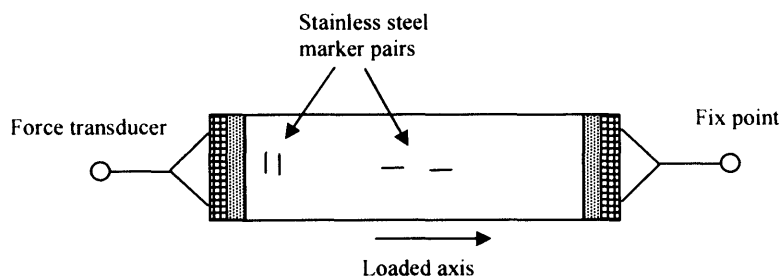
Figure 26. Stainless steel embedded in collagen lattices are shown, as well as the axis of the applied load.

Initial distance between each marker pair, was measured immediately after set up (prior to loading). The measured distances that were used to quantify and analyse local collagen matrix strain are shown in Figure 27.
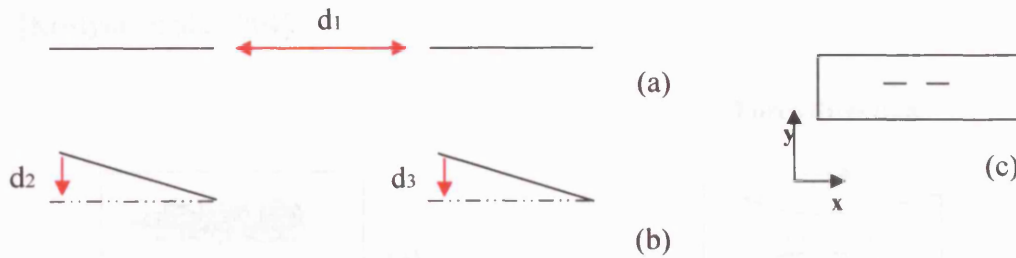
Figure 27. Stainless steel markers positioned in the A zone. Arrows indicate measurements taken on the axis parallel and perpendicular to the loaded axis. (a) and (b) respectively. (c) Shows the position of the markers within the collagen construct.
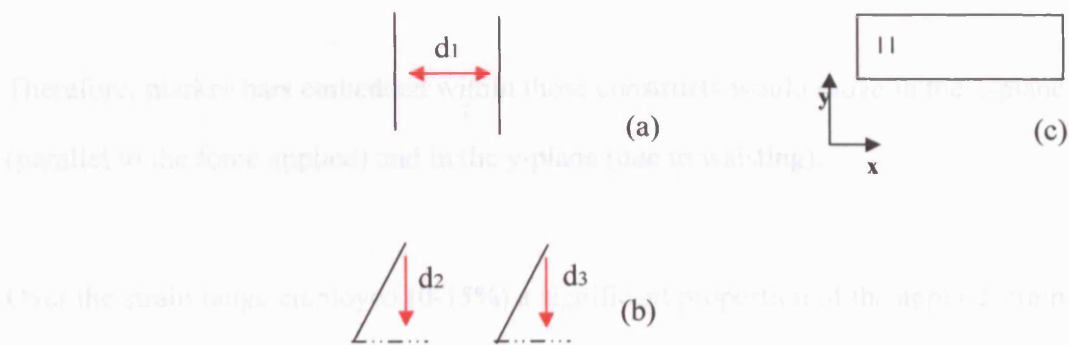
Figure 28. Stainless steel markers positioned in the D zone. Arrows indicate measurements taken on the axis parallel and perpendicular to the loaded axis applied (a) and (b) respectively. (c) Shows the position of the markers within the collagen construct.

Marker movement was measured in two planes (x and y), as described in Methods. On the x-plane d_1 was measured and on the y-plane d_2 and d_3. z plane could not be quantified and hence was not included in this study.

When pre-strain was applied, collagen constructs showed a waist effect as shown in figure 29b. Literature has shown [Eastwood et al., 1994; Mudera et al., 2000] that cells will have identical effect (waist) on collagen shape and structure when they contract the matrix. Similar alignment is produced by externally applied strain [Kostyuk et al., 2004].
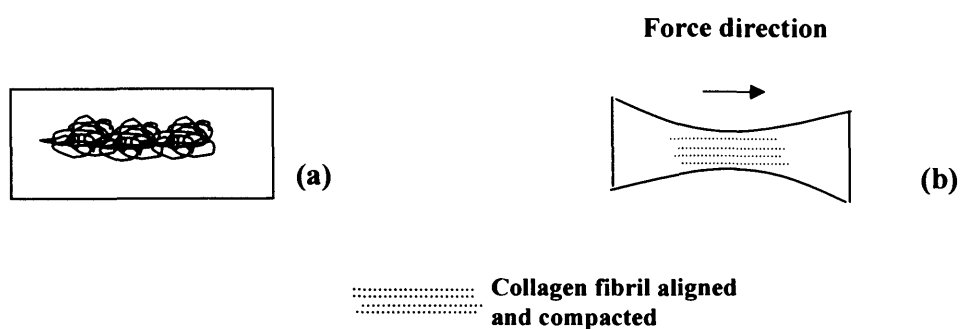
Figure 29. (a) Initial collagen shape and fibril mesh (prior to pre-strain) is shown and (b) Waist effect and fibril alignment and compaction are shown following pre-strain.

Therefore, marker bars embedded within those constructs would move in the x-plane (parallel to the force applied) and in the y-plane (due to waisting).

Over the strain range employed (0-15%) a significant proportion of the applied strain was lost in elastic deformation of the compliant construct (approximately 50%). Figures 30 and 31 show the strain transmitted through the collagen constructs, and registered as detectable marker bar displacement, plotted against the applied strain (%). In other words, an applied displacement of 3.25mm will be 5% strain on the x-axis, calculated according to original construct length which was 65mm. Following this we measured the local displacement between the end points of each marker bar at each point in applied strain range and plotted on the y-axis.

Therefore, a measured displacement of 0.5mm between the marker bars following an applied strain of 1mm represents a 50% marker response, plotted on the y-axis.
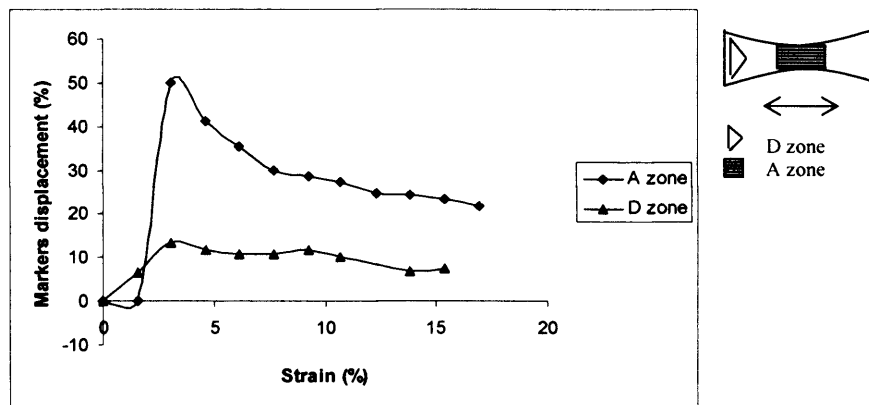
Figure 30. Marker displacement (parallel to the long axis of the construct, as shown by arrow) for applied strains 0 to 15% plotted for both A and D zone of the constructs.

Figure 30 shows the displacement measured parallel to the applied load. The maximum displacement (50%) transfer rose up to 3% applied strain, at which point half of the applied strain was transferred to the markers. Beyond 3% applied strain there was a steady decrease (down to 28%) in the proportion of the displacement detected by the markers with increasing applied strain. Over this range the proportion of applied strain which was detected as displacement of the markers dropped from 50%, at 3% strain, to only 22% by the end of the series at 17% applied strain. This suggests that after 3% applied strain the material properties of the construct in the A-zone became progressively stiffer, since marker displacement was reduced (i.e inherent fibrilar movement is limited with increasing external strain and consequently increasing stiffness). The 3% point therefore represents a key change in overall material behaviour.

The D-zone behaved in a similar manner to the A-zone with a maximum observed displacement at 3% applied strain. However, in this case the maximum detected displacement was only a quarter (12%) of that seen in the A-zone, indicating that the displacement magnitude adjacent to the construct attachment bars was much lower than in the main bulk of the construct, consistent with the idea that the collagen construct of the D-zone is strain-shielded by the attachment bar [Eastwood et al., 1998] and that cells experience forces in different directions, in the D-zone. In contrast, in the A-zone strain is highly aligned-parallel with the loading axis.
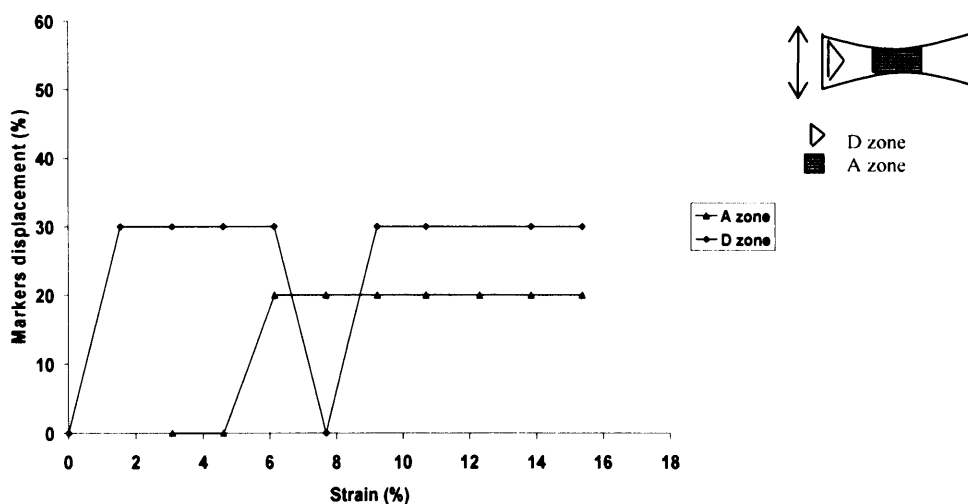
Figure 31. Marker displacements (across the long axis of the construct, as shown by arrow) for strains 0 to 15% plotted for both A and D zone of the constructs.

The comparable analysis of responses in the plane perpendicular to the applied load is shown in Figure 31. With an applied strain of 6%, in the A-zone there was a marker displacement of 20%. Surprisingly further increase in applied strain, up to 15%, produced constant detectable displacement (20%) of the markers for each ramp strain step. Indeed, in this perpendicular plane it was the D-zone markers, which produced the greater actual displacement (in this case the response was characteristically cyclical in nature). There was an early response at 1.5% applied

70

strain of 0.45% actual marker displacement (30% of applied strain). However, there was a constant 30% displacement for 4 consecutive strain steps, upto an applied strain of 7.7%, where the markers suddenly flipped back to the zero displacement position (ie. starting position). With further increase in applied strain to 9.2% the markers responded with a flip back to the displacement position (30% of displacement) where again a constant displacement was shown for increasing strains up to 15%. This movement then represented a double displacement between 0% and 30% of the applied strain. Again these results suggested that even in terms of force vectors in the D-zone, resident cells would have experienced equal and opposite direction forces at different stages during the strain process [Karamichos et al., 2006]. It is important to note at this point that position of the markers on the z plane was not controllable.

It is concluded, then, that application of increasing external mechanical strain on 3D collagen constructs cells perceive different forces in both magnitude and vector in different zones and areas of the collagen matrix, particularly at the edges and loading/anchoring region.

Discussion

The influence of the viscoelastic relaxation (amount of force for stress relaxation-SR) has not been carefully examined in biologic soft tissues. Other studies have reported preconditioning protocols, but without comment on rest periods during the test [Huang et al., 1993; Wakatsuki et al., 2000; Seliktar et al., 2000]. The first part of this study was to test stress–relaxation of the collagen constructs following a uniaxial fast-ramp load. More importantly we investigated both cellular and acellular

constructs in terms of the time taken for them to viscoelastic relax following 0-15% pre-strain levels. *The optimum time for full visco-elastic relaxation was 1h for all the constructs, independent of the presence of cells or the amount of pre-strain.*

One of the main drawbacks with the use of collagen as a TE construct is its characteristic randomly oriented fibril network and its poor tensile strength. There is a need, then, to devise some means to align and strengthen collagen fibrils and so to increase strength. To address these issues the next part of the study was to analyze the effects of pre-strain on alignment (i.e. collagen fibril orientation) and on collagen stiffness.

Alignment of collagen matrix without cell contribution, using the pre-strain model, was shown in this study. Normally cells embedded within a collagen fibril mesh reorganise the mesh by generating force onto those fibrils over time. Use of externally applied pre-strain gives the opportunity to take over this task from the resident cells which will have implications in terms of eliciting predictable molecular responses.

Results showed alignment of the fibrils (Figure 23) at a pre-strain as low as 5% together with an increase in stiffness. In terms of stiffness, cellular and acellular constructs showed a non linear increase, with no significant difference between the two (Figure 25).

Cell-ECM interactions

Studies have not yet been able to identify any effect of the presence of cell bodies on mechanical properties of constructs under strain when the cell-matrix interactions (i.e integrin attachment and cytoskeleton) are minimal. This study tested the effect of the simple presence of cells on collagen matrix stiffness, minimising cell-collagen attachment by FCS removal.

Serum dependence and cell shape changes and attachment is well known in the literature; Grinnell and co workers [2002] have reported differences on cell morphology when treated with growth factors and other agents, eg. LPA or PDGF or basal medium containing only ovine serum albumin, where fibroblasts showed stellate morphology following 4 h in PDGF medium, whereas cells in LPA medium tended to become bipolar. Further studies have emphasized the bipolar morphology of cells [Elsdale et al., 1972; Tomasek et al., 1984], which might be a response to the LPA in serum however in this study this has not been investigated.

Following addition of FCS (within 20min.) cells started to attach and spread exhibiting normal morphology of fibroblasts as they attach to this substrate and also contract the collagen matrix. These findings agree with other studies where contractile force increased significantly while cells are spreading into the collagen matrices in the presence of serum [Wakatsuki et al., 2003] compared to cells lacking serum, where no substantial matrix remodelling and/or production of contractile force was shown [Wakatsuki et al., 2003]. Since the strain-dependent increase in material stiffness was the same in the presence and absence of cells (i.e independent of cell-matrix attachment) this study concluded that the contribution of the cell cytoplasm was minimal in terms of cytoskeleton assembly. At least until all attach

and assemble a strong cytoskeleton, then, this implies that the mechanics are dominated by scaffold material properties. This is important in cell-scaffold design in tissue engineering, though it might be cell density dependent beyond the range tested here [Karamichos et al. 2006].

Novel method for measurement of forces transmitted through collagen constructs

Little is known of how cells perceive applied external strain, though Wakatsuki et al [2003] have determined the basic parameters linking contraction and cytoskeletal organization. Visco-elastic tensile test systems recently developed have shown that strains applied to cell seeded collagen constructs vary between different sites in the construct. In this case greater strains occur in the centre region of constructs. However this did not take account of the ECM material properties immediately surrounding cells. Here we have generated data using stainless steel markers and showed that force distribution is non-linear and differs in magnitude between the different areas of the same construct.

The main core of constructs (A-zone) was subjected to higher forces in the parallel axes (parallel to forces applied) compared to the D-zone. However, forces in the D-zone perpendicular to the applied load, displayed a non-linear response to increasing strain; interestingly in this instance the D-zone registered a higher magnitude of displacement than the A-zone.

Diagrammatic analysis of stresses in the D zone of constructs suggested that strains set up as the construct edges elongate and are extended to take up the longer curved dimension as the centre of the gel (A-zone) narrows to a 'waist'. Formation of the

construct waist means that the greatest strain will occur in these construct edges. In turn stress will be transferred, as suggested by the arrows (Figure 32a-c), to the end piece anchoring bar. However, since each of the two edges contributes equally to these stresses, with opposite vectors, this analysis suggests that failure is most likely at the centre of the gel-anchor interface. Direct observation of the gel (Figure 32d) indicated that this was indeed the site where gel-anchor bar failure occurred.

The most likely explanation of the marker movement in the y-plane, D zone is a stress relaxation from a central failure point (Figure 32b) moving out towards the edge and growing in magnitude (Figure 32c), allowing the permanent relaxation of the edge which is seen after stress recovery. Direct experimental (Figure 32d) observation identified that the construct failed first in the central area of the edge, consistent with the predictions in Figure 32b and 32c. Early stages of this failure point, with potential contribution of relaxation of the edge, would explain the flips in strain identified in the y plane: D zone. Since markers in D zone were positioned very close to the attachment point any micro fracture would result in force transmission and so displacement of the markers. Not surprisingly markers far from this focus, in the A zone did not show such movement in the y plane. Equally, a combination of localised gel edge failure and focal failure at the anchor bar are likely to explain the discontinuous strain transfer detected by bars in the x plane.
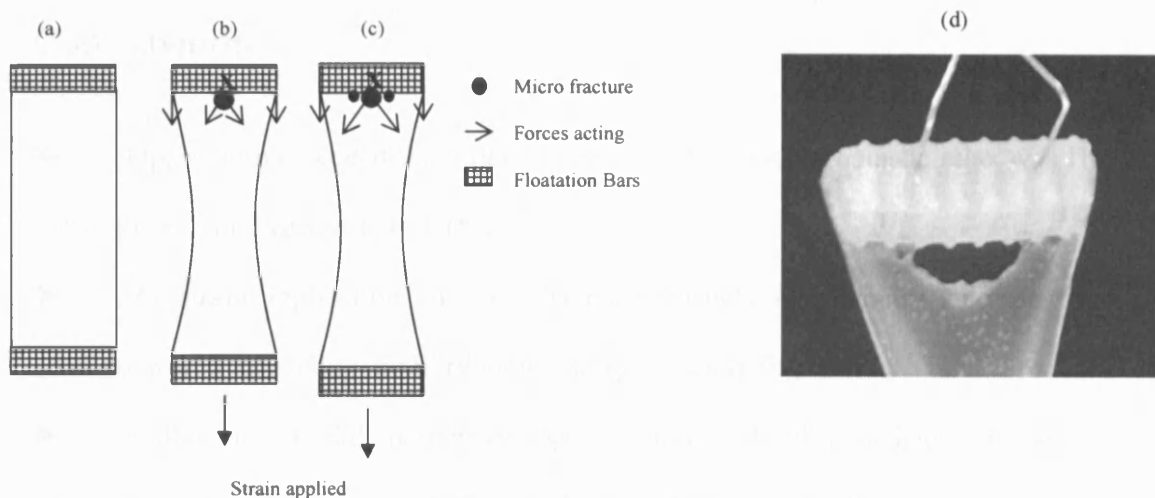
Figure 32. Diagramatic representation of force distribution as strain is increasing from a) 0% to b) 10% and c) 20%. Arrows indicates stress transfer to the gel-anchor interface which increases as strain increases. d) Shows direct observation of the gel indicating failure of the gel construct at the gel-anchor interface [Karamichos et al., 2006].

The results obtained from this experiment give insight into the baseline properties of the collagen constructs. Beyond tissue engineering, the importance of understanding the mechanism of construct 'failing' (ie breaking) at the attachment point will lead to clinical answers. A striking example is the bone-ligament-bone complex, extensively studied by Woo and co workers [1999]. Woo et al have developed a tensile loading system in order to be able to investigate both structural and mechanical properties of the bone-ligament-bone complex [Woo et al., 1983]. During normal activity, ligaments are easily elongated to maintain normal movement and allow the joint to move easily and smoothly. At higher externally applied loads, such as during exercise, the stiffness of the ligament increases to restrict any excessive motion in the joint [Woo et al., 1999]. However, if the applied load exceeds the maximum limits of the ligament, the risk of ligament damage increases. Consequently point of failing will be at the attachment point with bone.

Conclusions

▶   Optimal time needed for collagen constructs to fully viscoelastic relax was 1h for all pre-strain levels tested (0-15%).

▶   Pre-strain application on 3D collagen constructs significantly increases its stiffness whilst increasing the fibril network organization/alignment.

▶   Collagen material properties (ie stiffness and fibril orientation) were independent of cell presence upto the density tested here (1mil/ml).

▶   FCS starvation was used successfully to exclude cellular attachment and contraction of the collagen matrix

▶   Cells perceive different forces in both magnitude and vector in different zones and areas of the collagen matrix, particularly at the edges and loading/anchoring region.

Chapter 4: HDF cellular and molecular responses to increasing collagen matrix stiffness

HDF's contraction forces regulated by mechanical stimulation

Stiffness and fibril orientation (Chapter 3) of a collagen matrix were shown to be affected by the application of external mechanical pre-strain [Karamichos et al., 2006]. Stiffness was predictably increased by increasing the levels of pre-strain and collagen fibrils were orientated and aligned parallel to the principal strain (i.e applied load) [Karamichos et al., 2006]. In this study we used Collagen Type I as a scaffold, which cells have the ability to contract and align by generating forces on its fibrous structure [Eastwood et al., 1996; Eastwood et al., 1998]. Hypothesis under test was that cells seeded in a collagen construct will alter their pattern of force generation and expression of mechano-responsive genes (those related to matrix turnover) in response to changes in stiffness of the collagen fibril construct.

Human Dermal Fibroblasts (HDFs) were embedded in collagen matrices that were subjected to 3 different pre-strain regimes: 0%, 5% and 10%, which produce a stiffness range up to 380Pa (310Pa at 5% and 380Pa at 10% pre-strain; Figure 25). Figure 33 shows the contraction profile over 24h of HDF's, for the 3 different stiffness levels.
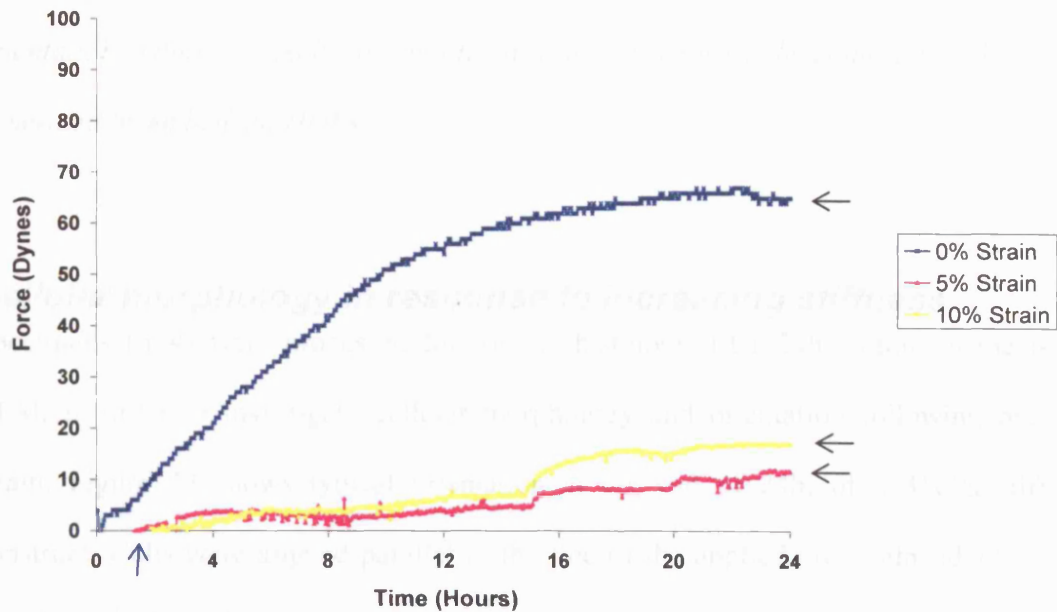
Figure 33. Contraction force profiles generated by HDF seeded constructs, with 10% FCS, following 0%, 5% and 10% pre-strain (n=3 each). Black arrows show final force reached by seeded cells and blue arrow shows Contraction Initiation Time levels.

The maximum mean contraction force (70 Dynes) was recorded when cells were embedded within non-strained constructs, i.e 0% pre-strain. As pre-strain increased, contraction force decreased (70% decrease to 23dynes for 5% and 65% to 12dynes for 10% pre-strain). This was significant only between 0% and 5%, 0% and 10% (p=0.05). Contraction forces between the 5% and 10% pre-strain (310Pa and 380Pa) were not significantly different. These results indicate that increasingly stiffer collagen matrices result in lower measurable force generation by the HDFs seeded within. The next part of this study was to examine cellular morphology and specific genes expression, in response to pre-strain and define specific cellular responses.

Increasing collagen stiffness (0%, 5%, and 10% pre-strain) and higher fibril orientation within it results in significantly lower measurable contraction forces generated by embedded HDFs.

Cellular morphology in response to increasing stiffness

Specimens (n=4) were processed for routine histology after 24h culture in the t-CFM, in order to investigate cellular morphology and orientation following pre-strain. Figure 34 shows typical orientation at the end of 24h, of a 310Pa stiff construct. Cells were aligned parallel to the line of the applied pre-strain. Identical morphological results were observed for all three pre-strain regimes (0%, 5% and 10%).

Long axis of collagen

x 20
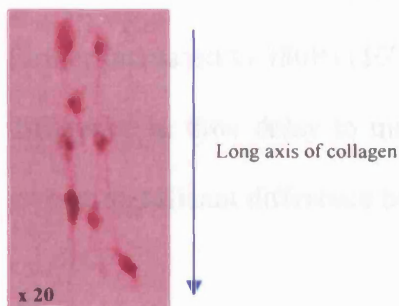
Figure 34. HDF seeded collagen constructs showing orientation (Blue arrows) parallel to the line of principle strain.

Results here can be extrapolated and co-related with Chapter 3 where movement of markers embedded within collagen were quantified, in response to pre-strain. Cells align parallel to the lines of the applied pre-strain (Figure 34) [Eastwood et al., 1998] Results in Chapter 3 showed alignment of collagen matrix with increased stiffness at a 'gross level'.

Cell alignment was independent of the amount of the pre-strain (i.e stiffness) since uniform alignment at all pre-strain levels was observed.

The Contraction delay effect

Figure 33 showed HDF responses to increased collagen stiffness. Increasing stiffness also altered the time point where the cells started to quantitatively generate contraction forces (in above a nominal 5 dynes threshold). This time delay was termed here as Contraction Initiation Time (**CIT**) and is plotted on the y-axis of Figure 35 against the pre-strain levels (0%, 5% and 10%).

Critically there was a significant ($p<0.05$) time delay when cells started to quantitatively contract the stiffer collagen constructs. In constructs subjected to 0% pre-strain there was measurable force generation in less than 20 minutes, but when stiffness was increased to (310Pa) by 5% pre strain there was a significant time delay of almost 7 hours in measurable contraction ($p<0.05$). When the stiffness was further increased to 380Pa (10% pre-strain), there was again a statistically significant difference in time delay to measurable contraction (5 hours; $p<0.05$) though there was no significant difference between 5% and 10% pre-strained constructs.

Results here indicated that increase in collagen matrix stiffness also delayed initiation of cellular contraction apparently to a maximal level at 5% pre-strain (hence no further increase in CIT).
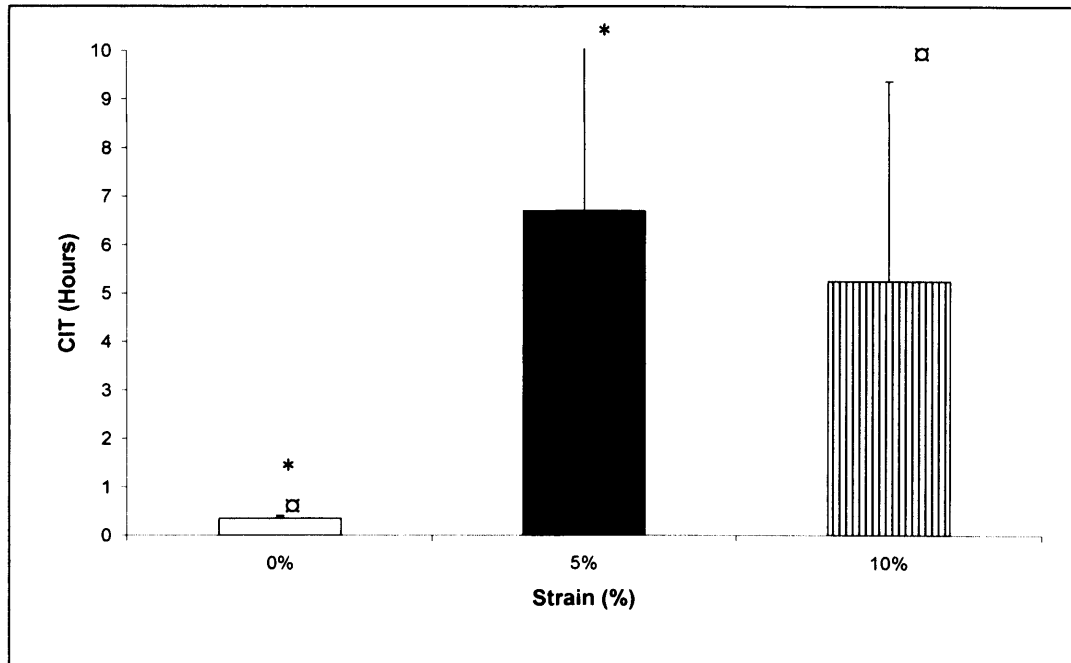
Figure 35. CIT is shown for HDF seeded constructs at the three different stiffness regimens (n=3 each). There was a significant difference in CIT between 0%-5% (*; p<0.05) and 0%-10% (◻; p<0.05). No significant difference in CIT was noted between 5%-10% pre-strain.

In summary pre-strain applied to cell-seeded collagen constructs will alter the fibril organisation of the collagen, by aligning cells and collagen fibrils along the lines of the applied pre-strain (Chapter 3) [Karamichos et al., 2006]. It also delayed cellular force generation and the onset of contraction. In terms of cellular morphology, cells were round with minimal attachment to the collagen during the period of no force generation (i.e CIT). Cells were round in morphology during the period of CIT, however, were able to spread and generate forces later, indicating that were unable to contract fast, a stiffer matrix.

CIT was increased with collagen stiffness i.e CIT was directly dependent on collagen stiffness up to 5% pre-strain, though there was no significant increase in CIT above 5% pre-strain.

Cellular contraction regulated by FCS starvation

As shown in previous chapters, cells in collagen constructs remained viable even with persistent round shape morphology, when FCS starved for 1h, but generated little or no force, due to restricted substrate attachment. Subsequent addition of FCS, at t=1h, after cell seeding led to the onset of cell spreading/elongation and force generation within 20 minutes (Chapter 3- Figure 24). Here we tested the hypothesis that cellular responses to pre-strain may be altered by FCS pre-starvation, i.e excluding cellular contribution to force generation as the collagen viscoelastic relaxes and cells are unable to attach and generate their own forces during this process, i.e cells attach only after the stiffness levels are at equilibrium. Cells in 0% FCS were pre-strained as before and allowed 1 hour (pre determined) to allow for visco elastic relaxation of collagen, before addition of FCS to a final concentration of 10%. Force output was then measured for a further 23h at each level (0,5 and 10%) of pre-strain constructs.

Figure 36 shows that total force generated (at 24h) decreased dramatically for stiffer matrices from 84 dynes (0% pre strain) to 8.9 dynes at 10% pre strain (p=0.05), as seen previously with no serum starvation. However in this case maximal effect of reduced force generation was clearly produced at the highest matrix stiffness (10% pre-strain), indicating that the force generated was directly dependent on matrix stiffness, over the full range of stiffness.
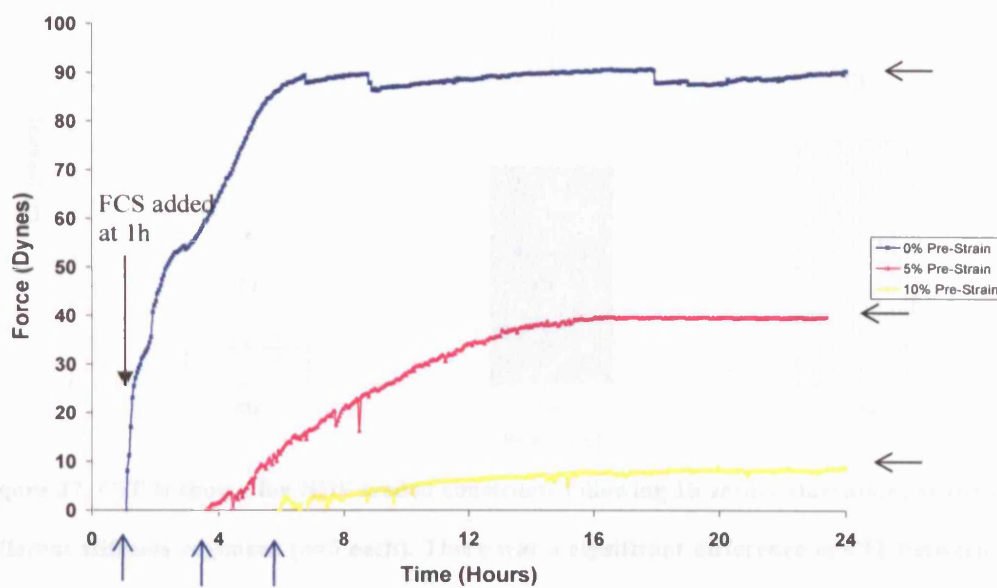
Figure 36. Contraction force profiles generated by HDF seeded constructs following 1h FCS starvation for 0%, 5% and 10% pre-strain (n=3 each). Black arrows show final force reached by seeded cells and blue arrows show CIT levels.

FCS starvation led to similar results in terms of contraction forces (i.e decreased force with increased pre-strain; from 84 to 40 and 9dynes for 0,5, and 10% pre-strain respectively), with a significant CIT (Figure 37; $p<0.05$).

CIT was stiffness dependent and not due to FCS starvation/presence. Figure 37 shows CIT for constructs with 1h FCS starvation. Similar significance was shown; between 0% - 5% (p<0.05; 5 times) and 0% - 10% (p<0.05; 6 times) pre strained constructs.
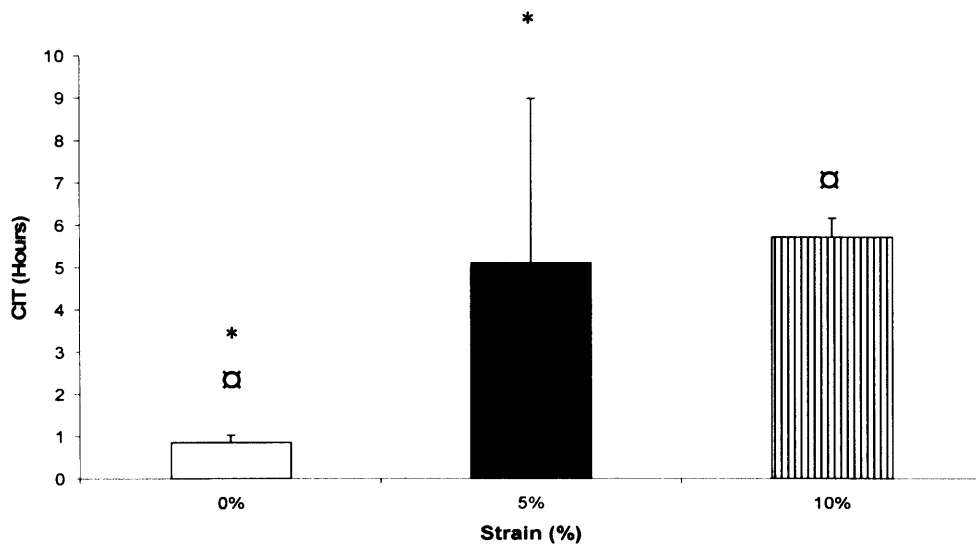
Figure 37. CIT is shown for HDF seeded constructs following 1h serum starvation, at the three different stiffness regimens (n=3 each). There was a significant difference in CIT between 0%-5% (*; p<0.05) and 0%-10% (□; p<0.05). No significant difference in CIT was noted between 5%-10% pre-strain.

Comparison of CIT responses with and without FCS starvation showed that FCS starvation only altered CIT significantly in the case of the least stiff constructs (0% pre-strain: Figure 38: p=0.05).
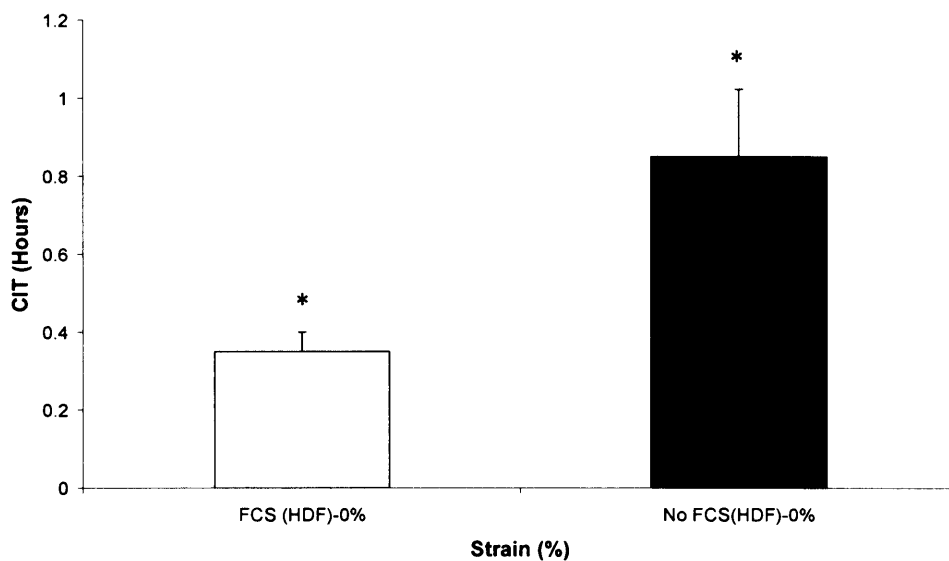
Figure 38. CIT is shown for HDF seeded constructs (n=3 each) under 0% pre-strain, with and without FCS starvation (*; p<0.05).

These results represent clearly the role of serum in attachment and initiation of contraction in increasingly stiffer collagen constructs. Crucially these results suggested that FCS starvation will be significant only at less stiff constructs (0% pre-strain) when control of cellular attachment and contraction is desired.

Fast and slow contraction phase

Many cell types (including HDF's) generate a characteristic contraction force pattern [Eastwood et al., 1994; Mudera et al., 2000], often with a force 'plateau' after 4-10h of culture. Initial force generation (0-8h) is thought to be due to traction as cells attach to and spread through the collagen constructs [Tomasek et al., 2002]. Hence it is an attachment and motion dominated process. Understanding of this traction phase is important since it both facilitates and perhaps predicts the level force generation (at the end of contraction phase) and matrix remodelling [Tomasek et al., 2002].

In this study tension homeostasis was reached within 10-12h of culture. We therefore investigated the initial rate of contraction (traction phase; as described in Methods) between 0h and 4h [Karamichos et al., 2006].
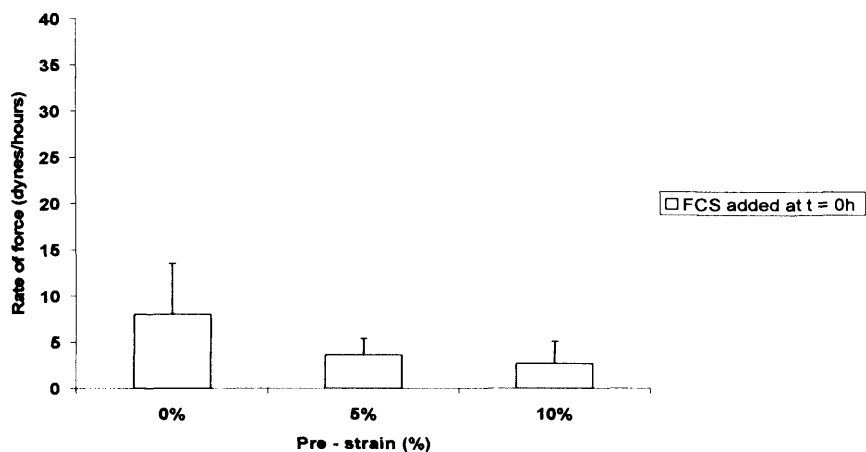
Figure 39. Rate of force generation is shown (0-8h) for HDFs seeded within increasingly stiffer collagen constructs with FCS at t=0h (n=3 each). No significant differences were observed.
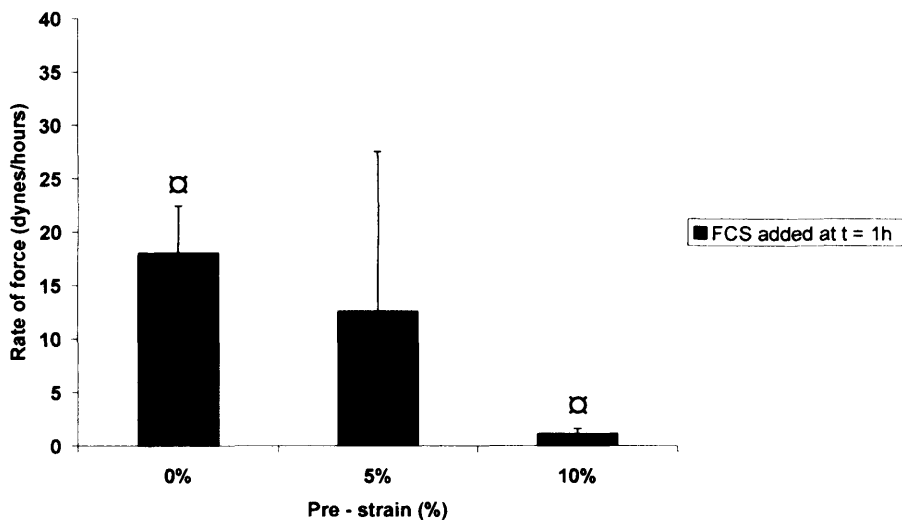
Figure 40. Rate of force generation is shown (0-8h) for HDFs seeded within increasingly stiffer collagen constructs with FCS added at t=1h (n=3 each). Significant differences were observed between 0% and 10% pre-strain for FCS starved constructs (α; $p<0.05$).

Non-strained, FCS starved HDF constructs showed a significant (Figure 40; p<0.05)

17 times faster (in terms of rate between 0-8h) contraction than constructs with the

highest stiffness (i.e 10% pre-strain). In contrast when FCS was present at t=0h no

significant difference was shown in the contraction rate between the three levels of

constructs stiffness (Figure 39), tested here. However, at 0% pre-strain, constructs

with FCS presence were significantly different compared to the serum starved ones,

by two fold (p=0.05). These indicated that despite cells requiring more time to attach

to collagen (CIT), they were able to rapidly attach/contract the collagen upon

reintroduction of FCS [Karamichos et al., 2006].

Reintroduction of FCS at 1h, mediates a rapid force generation, particularly in low

stiffness constructs. In addition this attachment-traction phase of force generation

was most important in the least stiff constructs, with or without serum starvation.

Collagen constructs seeded with HDF's and 20% FCS supplement

As described in Methods, cell seeded collagen constructs were pre-strained with 20%

FCS present, in order to investigate the effect of serum increase on forces generated

by seeded HDFs. This part of the study investigated the effect of increased FCS

(20% FCS) on force generated by the seeded cells, in this case HDF's.

Figure 41 shows the mean (n=4) contraction forces generated following 0%, 5% and

10% pre-strain. Peak force was massively increased at 0% pre-strain, when 20% FCS

was used (305 dynes compared to 70 with 10% FCS). Furthermore, peak force

(Black arrows) was significantly different for 0% and 5% (305 and 32 dynes

respectively; p=0.05) as well as 0% and 10% (305 and 24 dynes respectively;

p=0.05). No significant difference was shown between 5% and 10% pre-strain. The significance shown here is exactly the same as constructs with 10% FCS and/or even with 1h FCS starvation. *In conclusion the increase in FCS concentration had no significant effect on peak force generation in stiffer matrices (310 and 380Pa), but it was significantly increased in less stiff matrices.*
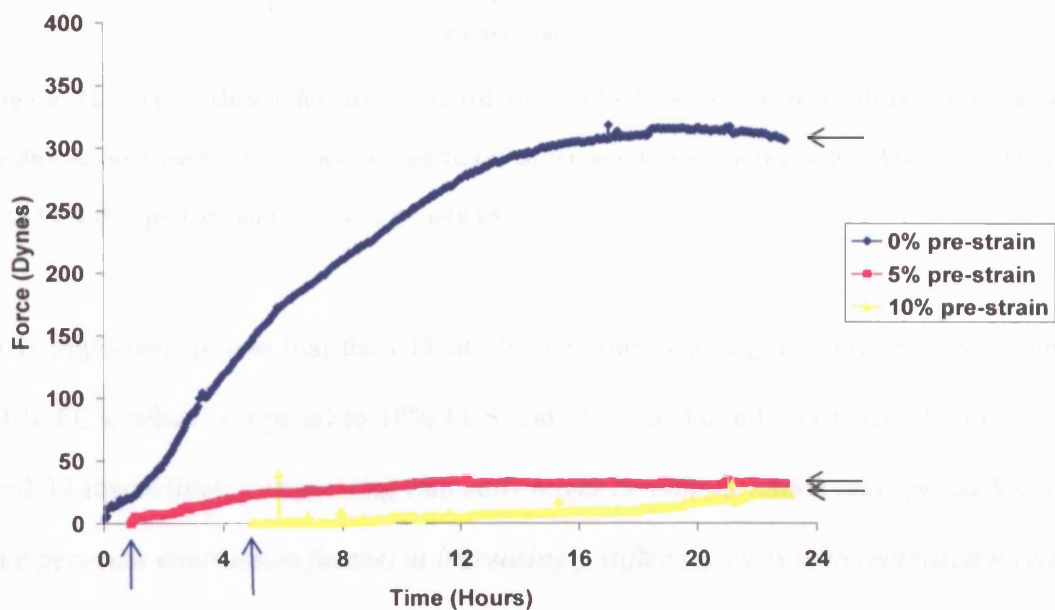
Figure 41. Contraction force profiles generated by HDF seeded collagen constructs, with 20% FCS, following 0%, 5% and 10% pre-strain (n=3 each). Black arrows show final force reached by seeded cells and blue arrows show CIT levels.

In terms of CIT (Figure 42), there was significant delay between all the pre-strain regimes i.e 0%-5%, 0%-10% and 5%-10% (all three; p=0.05). HDFs with 20% FCS supplement are the first in this study to show significant attachment delay (CIT) between the two stiff constructs (310 and 380Pa), when compared with constructs supplemented with 10% FCS. *These results indicated that FCS levels increase (20%) have a significant effect on cellular responses to stiffer matrices.*
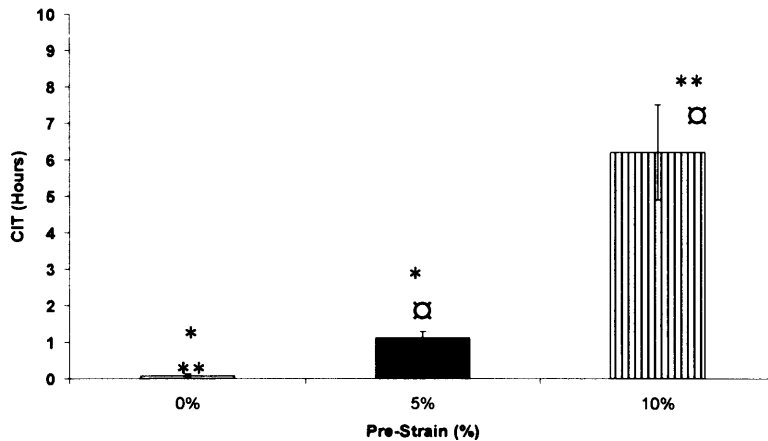
Figure 42. CIT is shown for HDFs seeded with 20% FCS, at the three different stiffness regimens (n=3 each). There was a significant difference in CIT between 0%-5% (*; p<0.05), 0%-10% (**; p<0.05) and 0%-10% (□; p<0.05).

It is important to note that the CIT at 5% pre-strain was significantly reduced with 20% FCS, when compared to 10% FCS and 1h FCS starved constructs (Figures 35 and 37 respectively), *suggesting that cells might be able to attach and spread faster (i.e generate contraction forces) at increasingly stiffer matrices with increased levels of FCS acting as stimuli.*

Gene expression of HDF seeded progressively stiffer matrices

From the previous section it was demonstrated that stiffness of the collagen gel increased as pre-strain was increased (Chapter 3). It was also shown that collagen fibrils are aligned parallel to the lines of principle strain, leading to a more orientated, organised fibril structure (Chapter 3). This part of the study investigated the relationship between specific matrix gene regulation and the generation of force in an HDF embedded collagen model. MMPs are a family of specific enzymes used

by fibroblasts to degrade their surrounding ECM [Liota et al., 1979; Mudera et al., 2000].

Having introduced the effect of pre-strain we hypothesis that MMP expression will be regulated by it. More specifically, the expression levels of MMP-2, 3 and 9 were investigated here, as well as TIMP-2, COL-1, COL-3 and IGF-1. TIMP's are known for their inhibitory action to MMP's [Vincenti 2001; Mudera et al., 2000], and so were tested here. Gene expressions were tested for HDF's seeded in progressively stiffer collagen constructs, at the end of 24h. All expressions were plotted against the housekeeping gene, Glyceraldehyde-3-phosphate dehydrogenase (GapDH). GapDH is a catalytic enzyme involved in glycolysis and is expressed in all cells. It is shown to be consistently expressed at uniform levels and it is not affected by mechanical alterations. It is therefore used as an internal control, i.e housekeeping enzyme. Consequently it was first established that GAPDH levels were stable and independent of pre-strain treatment. Figure 43 demonstrates GAPDH levels for all the pre-strain regimes used. Figure 43a shows the expression of GAPDH for HDFs seeded with 10% FCS and figure 43b with 20% FCS. No significant difference was found between the pre-strain regimes and/or with GAPDH expression at 10% and/ or 20% FCS.
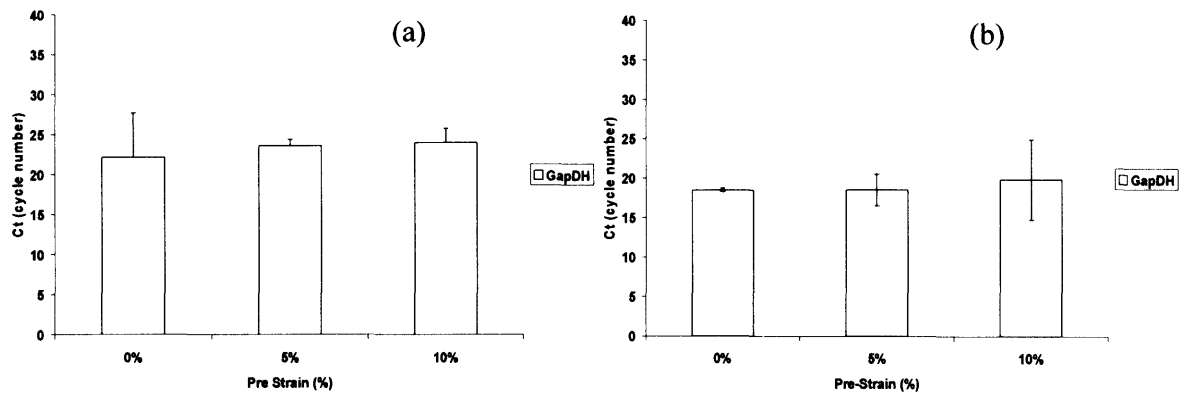
Figure 43. GapDH housekeeping gene levels are shown for 0%, 5% and 10% pre-strain. a) with 10% FCS and b) with 20% FCS. Standard deviation used for error bars.

Therefore, GAPDH can be used as a reference against which all other gene expression markers can be compared.

Changes in HDF gene expression with stiffness (in 10% and 20% FCS)

The first part of this study was to investigate the expression of the genes following 24h culture of increasingly stiffer HDF embedded collagen constructs. Genes tested for all the pre-strain regimes and FCS levels are listed in Methods (Table 4).

Figure 44, shows the changes in expression of MMP-2 (Gelatinase A) relative to GAPDH (The difference between GAPDH and test gene is plotted on the y-axis - A value of 5 indicates that the expression of the target gene was 5 times higher than the housekeeping gene) at three pre-strain regimes/matrix stiffness.

HDF's seeded within the 0% pre-strained constructs, showed significant down-regulation of MMP-2 when compared to 5% (p= 0.05) and 10% pre-strain (p=

0.0286), by 10 and 6 fold respectively. These results indicated that MMP-2 expression was sensitive to increasing matrix stiffness.
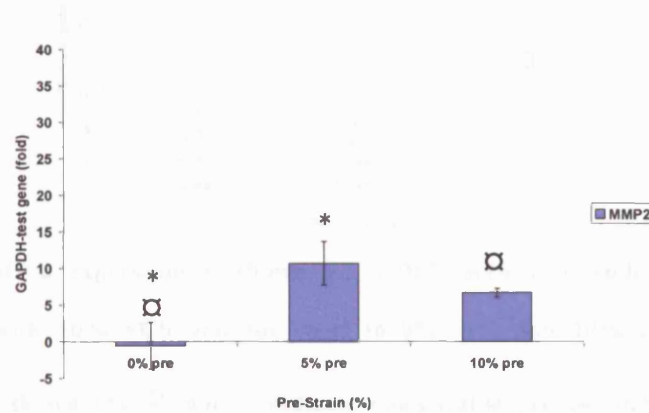
Figure 44. MMP-2 expression is shown for HDF's seeded to collagen constructs (n=3) supplemented with 10% FCS and subjected to 0%, 5% and 10% pre-strain. Standard deviation used for error bars. ⌓ and * indicate comparable groups and significance p<0.05.

TIMP-2 (Figure 45) expression was increased as matrix stiffness (pre-strain) increased, (0%-5% and 0%-10%; p= 0.05). Results suggested that TIMP-2, like MMP-2, is up-regulated by matrix stiffness, though TIMP-2 (unlike MMP-2) continued to rise when pre-strain was increased from 5% to 10% (without reaching conventional significance).
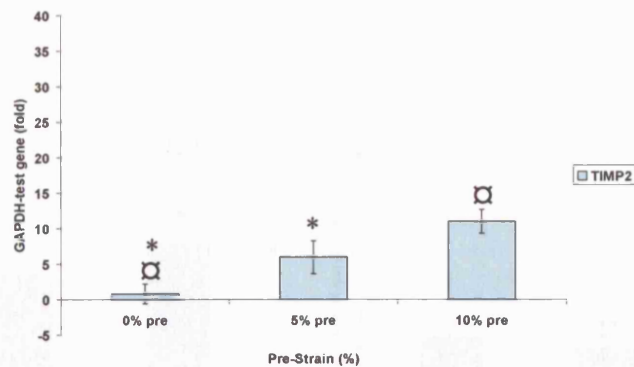
Figure 45. TIMP-2 expression is shown for HDF's seeded to collagen constructs (n=3) supplemented with 10% FCS and subjected to 0%, 5% and 10% pre-strain. Significant regulation was shown (*). ¤ and * indicate comparable groups and significance p<0.05. Standard deviation was used for error bars.

Three more genes showed significant response to increasingly stiffer matrices. COL-1 (Figure 46a; p= 0.05) and IGF-1 (Figure 46c; p= 0.05) were significantly up-regulated when 10% pre-strain was applied, compared to 0%. There was no significance between 0%-5% and 5%-10% pre-strain. COL-3 on the other hand, showed significant up-regulation only (10 fold; p= 0.05) between 5% and 10% pre-strain.

Figure 46. **(a) COL-1, (b) COL-3 and (c) IGF-1 expression is shown for HDF's seeded to collagen constructs (n=3) supplemented with 10% FCS and subjected to 0%, 5% and 10% pre-strain. Standard deviation used for error bars. □ and * indicate comparable groups and significance p<0.05.**

Results showed that the increasing matrix stiffness (due to pre-strain) resulted in significant up-regulation of ECM mechano-responsive genes (such as MMP-2, TIMP-2, COL-1, and COL -3). However, threshold in regulation of these genes appeared between 5 and 10% pre-strain. Suggesting that mechanoresponsive genes have a 'limit' to their up-regulation due to stiffness (i.e stiffer matrices will not necessarly elicit gene up-regulation).

The second part of this study involved increasing the FCS concentration to 20%. All other pre-strain parameters and analyses were as above (with 10% FCS).

Figure 47 shows the expression of MMP-2 and TIMP-2 for HDFs seeded within constructs supplemented with 20% FCS.
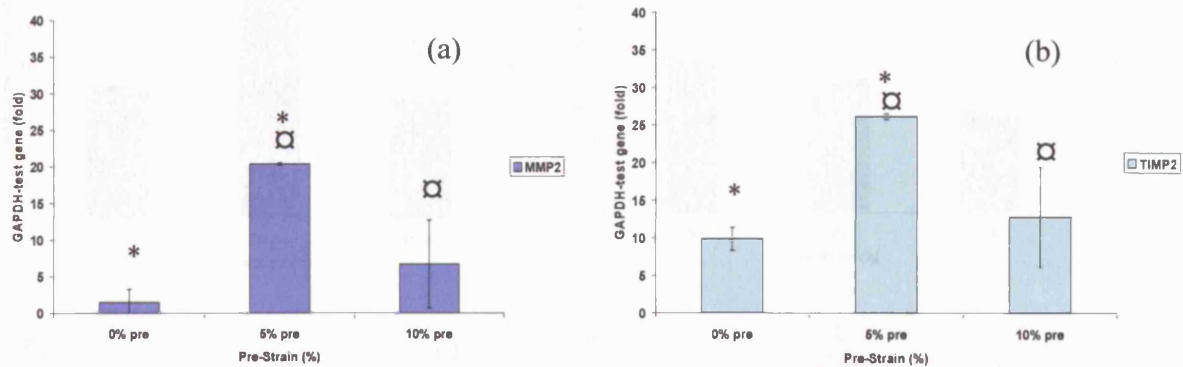
Figure 47. (a)MMP-2 and (b) TIMP-2 expression is shown for HDF's seeded within collagen constructs (n=3) supplemented with 20% FCS and subjected to 0%, 5% and 10% pre-strain. ¤ and * indicate comparable groups and significance p<0.05. Standard deviation was used for error bars.

The pattern of changes in MMP-2 (Figure 47a) gene expression, with increasing stiffness, was identical to 10% FCS. Between 0% and 5% pre-strain expression increased by 18 fold (p= 0.05), which was higher when compared to 10% FCS (10 fold up-regulation; Figure 44). Again expression fell as pre-strain increased from 5% to 10% by almost 2/3, and it was statistically significant (p= 0.05) (unlike 10% FCS). This did not mimic the 10% FCS response.

In contrast with 10% FCS, figure 47b shows the expression of TIMP-2 with 20% FCS. This time TIMP-2 showed the same pattern as MMP-2 and was significantly up-regulated between 0%-5% (p= 0.05), but down-regulated between 5 and 10% pre-strain (p= 0.05).
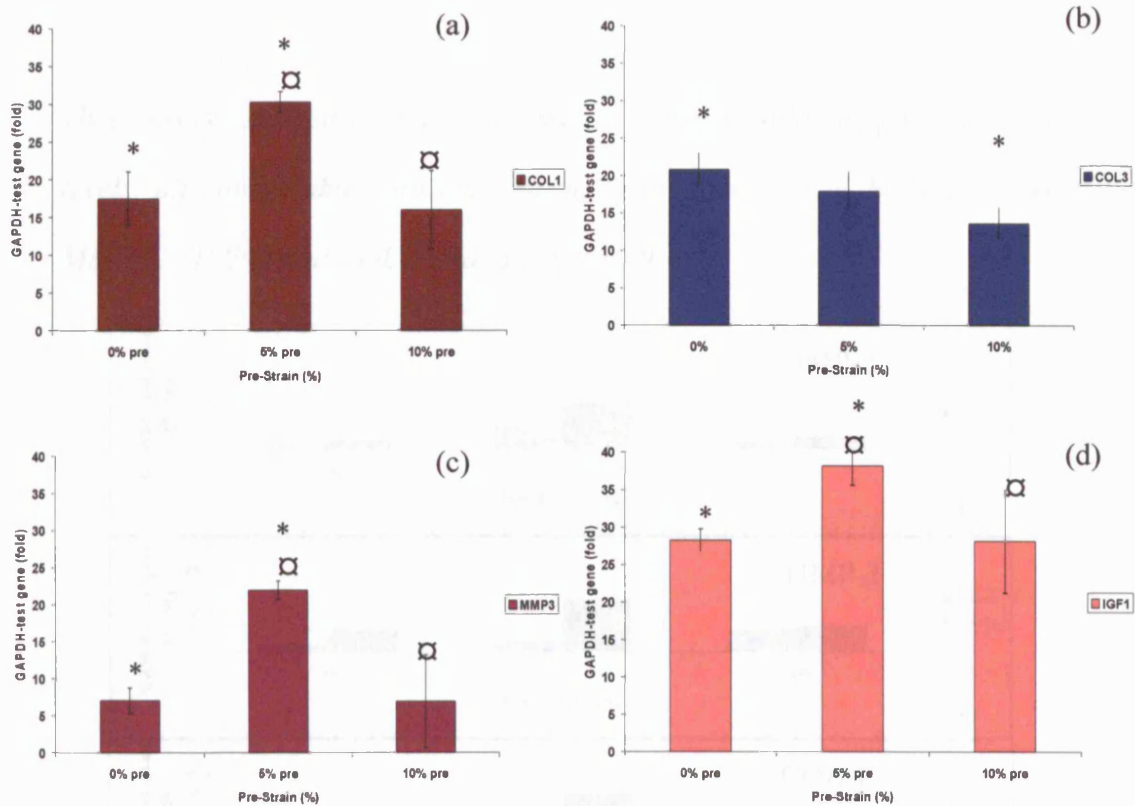
Figure 48. (a) COL-1, (b) COL-3, (c) MMP-3 and (d) IGF-1 expression is shown for HDF's seeded to collagen constructs (n=3) supplemented with 20% FCS and subjected to 0%, 5% and 10% pre-strain. Standard deviation used for error bars. ¤ and * indicate comparable groups and significance p<0.05.

Four more genes were significantly regulated due to increased matrix stiffens at 20% FCS. Figure 48a shows COL-1 expression, significantly up-regulated between 0%-5% (p= 0.05) and down-regulated between 5%-10% (p= 0.05) following exactly the same trend as MMP-2 and TIMP-2. Similar results for MMP-3 (Figure 48c; p=0.05 between both 0%-5% and 5%-10%) and IGF-1 were observed (Figure 48d; p=0.05 between both 0%-5% and 5%-10%).

Finally, COL-3 (Figure 48b), unlike at 10% FCS, showed down-regulation between 0% and 10% pre-strain (p= 0.05) indicating an inverse co-relation.

These results showed a novel combination of matrix stiffness, pre-strain and FCS levels, all controllable with our system, to regulate critical ECM genes such as MMP-2, TIMP-2 and COL-1 and -3 (Figure 49).
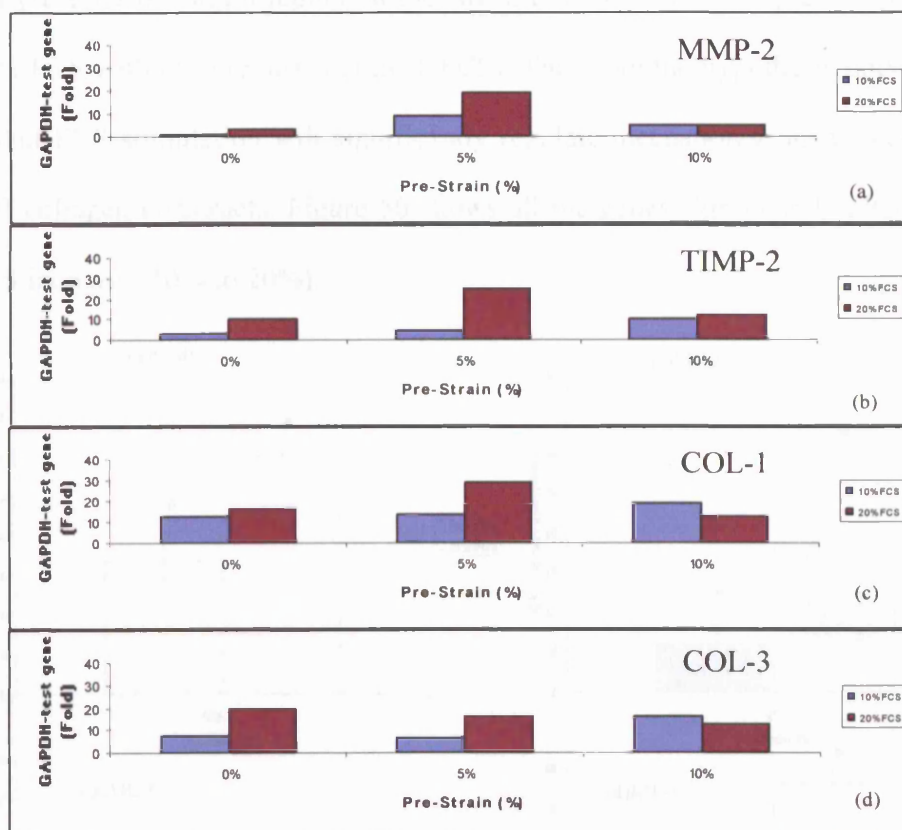
Figure 49. Genes regulation trends are shown for HDFs seeded within collagen constructs with 10% and 20% FCS: a) MMP-2, b) TIMP-2, c) COL-1, and d) COL-3.

The rest of the genes listed in table 4, showed no significant regulation when matrix stiffness was increased, i.e non mehano responsive genes.

Gene expression regulation dependent on FCS levels

Results so far showed up/down-regulation of mechanoresponsive genes when collagen matrix stiffness increased, for a) 10% FCS and b) 20% FCS. Figure 50 shows the significant up/down-regulation of the same genes; however this time we analyse each pre-strain regime at the two different FCS levels (i.e 0% pre-strain at 10% FCS with 0% pre-strain at 20% FCS). Therefore the hypothesis under test here is that FCS stimulation will significantly regulate mechanoresponsive genes within stiff collagen constructs. Figure 50 shows all the genes significantly altered, by the FCS increase (10% to 20%).
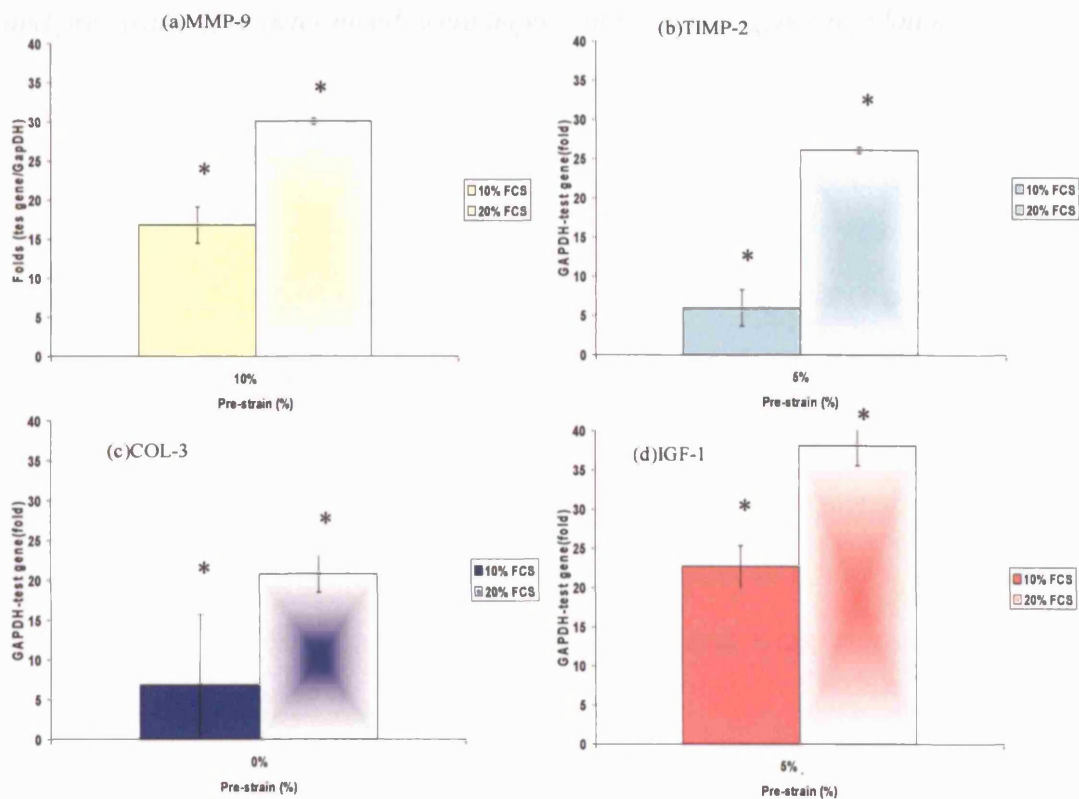
Figure 50. Gene expression is shown for HDF's seeded in collagen constructs (n=3) supplemented with 10% and/or 20% FCS and subjected to 0%, 5% and 10% pre-strain. (a) MMP-9 expression at 10% pre-strain for both 10% and 20% FCS, (b) TIMP-2 expression at 5% pre-strain for both 10% and 20% FCS, (c) COL-3 expression at 0% pre-strain for both 10% and 20% FCS, and (d) IGF-1 expression at 10% pre-strain for both 10% and 20% FCS. Significant regulation was shown (*). Standard deviation used for error bars.

Figure 50a shows significant up-regulation (p= 0.05) of MMP-9, 20% FCS, at the highest stiffness. In contrast, when 20% FCS was present, COL-3 (Figure 50c) was significantly up-regulated (p= 0.05) at the lowest in stiffness constructs, i.e 0% pre-strained. Similar results, due to 20% FCS, were shown for TIMP-2 and IGF-1 on Figure 50b and 50d respectively, where were both significantly up-regulated following 5% pre-strain (p= 0.05 and p=0.05).

Results here showed differential genes regulation between two different serum levels, highly dependent on pre-strain/stiffness levels. A *combination of FCS levels and pre-strains (pre determined) were important for matrix genes regulation.*

Discussion

Knowledge of how fibroblasts, in soft connective tissues, respond to external mechanical forces applied to extracellular matrix is limited. However, it has been suggested that fibroblasts which generate contractile forces are responsive to external mechanical loading [Brown et al., 1998; Eastwood et al., 1994; Grinnell, 1994; Delvoye et al., 1991].

This study was based on cell seeded collagen constructs as a model to investigate the effect of collagen stiffness in terms of cellular contraction and molecular mechanoresponsive genes, given that pre-strain significantly alters the collagen stiffness [Karamichos et al., 2006].

Our result suggest that fibroblasts regulate the force generated on adjacent collagen fibrils in response to stiffness of the ECM, i.e there is a feedback control to matrix stiffness. This is in general agreement with the early identification of tensional homeostasis [Brown et al., 1998] and is consistent with the idea that fibroblasts generate forces to monitor and control ECM material/ECM mechanical properties [Bishoff et al., 2003]. Here we quantified the forces generated by the cells seeded in collagen ECM and showed that stiffer matrices resulted in generation of smaller quantifiable forces by the host cells.

Prajapati et al [2000] demonstrated strain dependence of HDFs in this 3-D system in terms of protease expression (MMP's and plasminogen activator). Mudera et al [2000] identified a sophisticated relationship between force vector and cell alignment operates to regulate gene expression of key matrix degrading enzymes. Increases in a range of proteins including tenascin and collagen levels in response to tension have

been used and studies have suggested that cells respond to altered strain in their matrix by localized, proportional strengthening of the cytoskeleton linkages, allowing stronger force to be exerted on the integrins [Choquet et al., 1997].

There have been a number of studies [Katoh et al, 2001, Totsukawa et al., 2000] looking at different pathways of cell contractile activity such as myosin light chain kinase, and Rho kinase. More importantly it has been reported, that less organized ECMs results in insufficient cell – matrix interactions and ultimately in translocating collagen fibrils [Grinnell et al., 2002]. Also focal adhesions only form after high degree of remodelling occurs [Grinnell et al., 2002]. As our results suggest cells will manipulate/contract less stiff matrices, organising these collagen fibrils. Applied forces (pre-strain) in turn would be expected to help stiffen the ECM surrounding these cells which may have implications in physiological terms.

That is, resident fibroblasts generate tensile loads on their immediate ECM in order to achieve internal homeostasis. Forces generated by the host cells eventually lead to stiffer material properties. Recent studies by Marenzana et al [2006] suggest that over larger periods, fibroblasts have the ability to 'fix' this new material stiffness into the collagen matrix permanently. This dual stage stiffening and fixing of fibrillar collagen represents the predicted basis of ECM remodelling [Tomasek et al., 2002].

In literature cell-matrix attachment has been reported, such as the involvement of RGD-bonding integrins to force generation and to cytoskeletal structure [Sethi et al., 2002]. We have shown in Chapter 3 [Karamichos et al., 2006] that cells are viable and will not attach/contract a collagen matrix following FCS starvation. FCS is

known to contribute towards cell contraction and FCS percentage increase can lead to increased contractile forces. Here we showed that cells will recover from the FCS starvation and are still be able to contract the matrix, though after a significant time delay (post FCS addition; CIT).

Conclusions

▶   Increasing collagen matrix stiffness and fibril alignment, using pre-strain, led to generation of significantly lower forces by embedded HDFs.

▶   Increasing collagen matrix stiffness led to a significant increase in contraction initiation time.

▶   Doubling of FCS (%) had no significant effect on contraction forces **except** in non pre-strained constructs.

▶   Doubling of FCS (%) led to a significant increase in CIT.

▶   Matrix remodelling gene regulation (MMPs and COLs) were shown to have a threshold between 0% and 10% pre-strain, suggesting that optimal matrix stiffness, around 310Pa (for the levels of stiffness tested in this study), is required for maximum output and/or expression of specific genes.

Chapter 5: hBMSC cellular and molecular responses to increasing collagen matrix stiffness

Over the last decade, interest has grown in human Bone Marrow Stem Cells (hBMSCs) as an important area of TE/regeneration due to their ability to differentiate to multiple cell lineages. Furthermore, hBMSC's can, under certain physiological and experimental conditions, be induced to become adipocytes, chondrocytes, and osteoblasts [Prockop, 1997; Pittenger et al., 1999].

After establishing the responses of a well described, committed, cell-type (HDF), to pre strain our aim was to compare these with mechanical responses of progenitor cells (hBMSC) in progressively stiffer matrices. The hypothesis here was that hBMSC seeded within increasingly stiff matrices will generate smaller contraction forces, similar to HDFs (Chapter 4), i.e. display fibroblastic mechanistic behaviour.

hBMSCs were obtained by bone marrow aspirates from six healthy patients (free of infectious diseases) in this study (as described in Methods) and stained for surface marker proteins (Introduction;Table 1) CD14, CD31, CD34, CD44, and CD105 (Figure 51). Red staining (right column) indicates that hBMSCs were positive. No stain represents negative stain. As reported in literature [Barry et al., 2004; Pittenger et al., 1999; Jorgensen et al., 2003], hBMSCs stained negative for CD14, CD31, and CD34 and positive for CD44 and CD105. This test was used to identify/clarify these cells as hBMSCs.
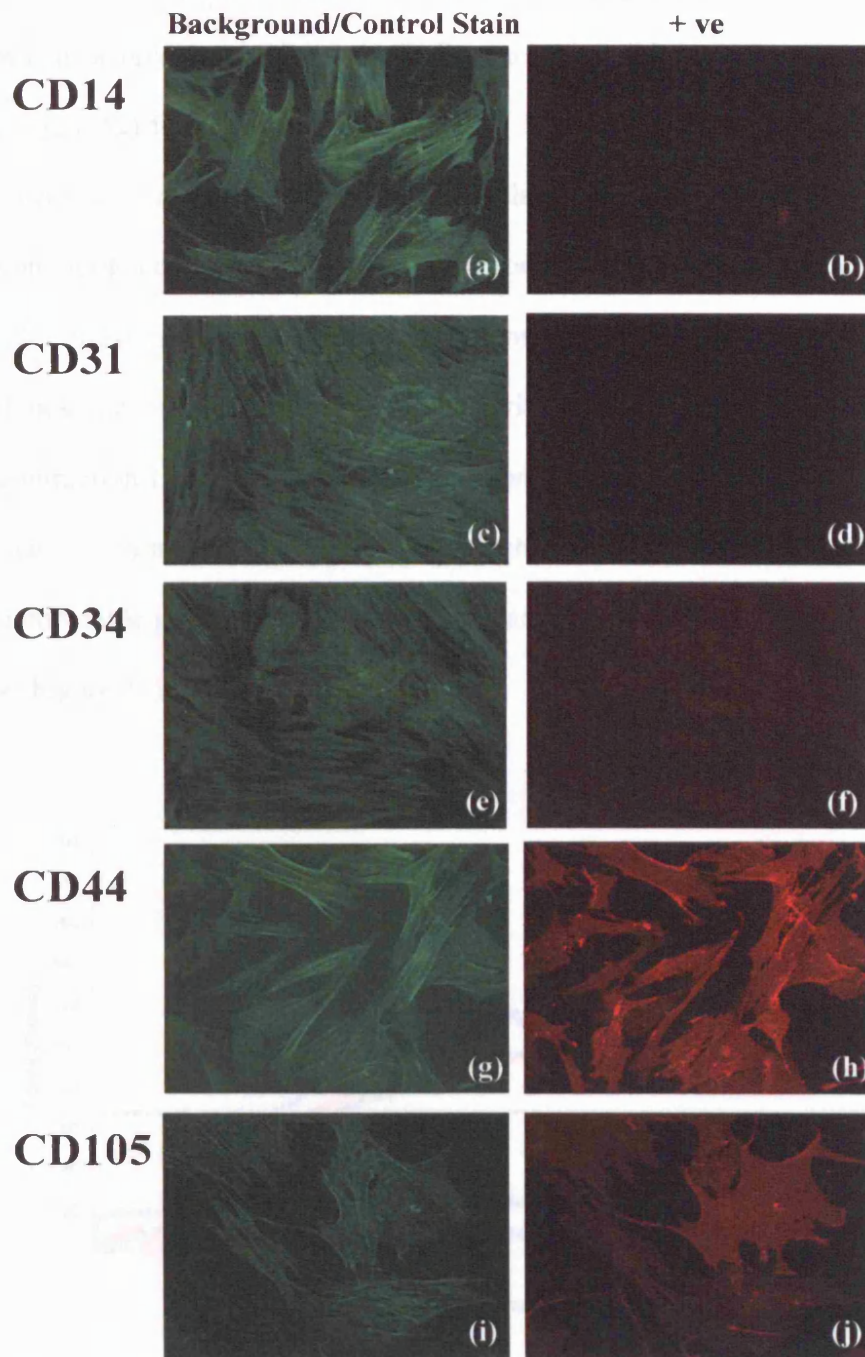
Figure 51. hBMSCs static culture were stained for (a) Negative Control (b) Stained with CD14, (c) Negative Control (d) Stained with CD31, (e) Negative control (f) Stained with CD34, (g) Negative Control (h) Stained with CD44 and (i) Negative control (j) Stained with CD105

hBMSCs (1mil/ml) were then seeded within collagen constructs and force generation was measured (over 24h) for cells from 6 different donors. Force generation patterns (Figure 52) fell into two distinct groups: 1) High contractile and 2) Low contractile. There was no clear sex or age correlation between these two groups. Highly contractile cell lines started generating force rapidly (<2h), producing peak forces of 90-120 dynes. In contrast the weakly contractile cell lines generated a total of 20-50 dynes (i.e. <50% force) with characteristically prolonged delays in the onset of contraction (12-14h). In two of these forces generation was barely detectable (less than 20 dynes) by the system. The high contraction cells generated force with a comparable pattern to HDFs, with similar final force levels (80-100 dynes; Chapter 4; Figure 33).
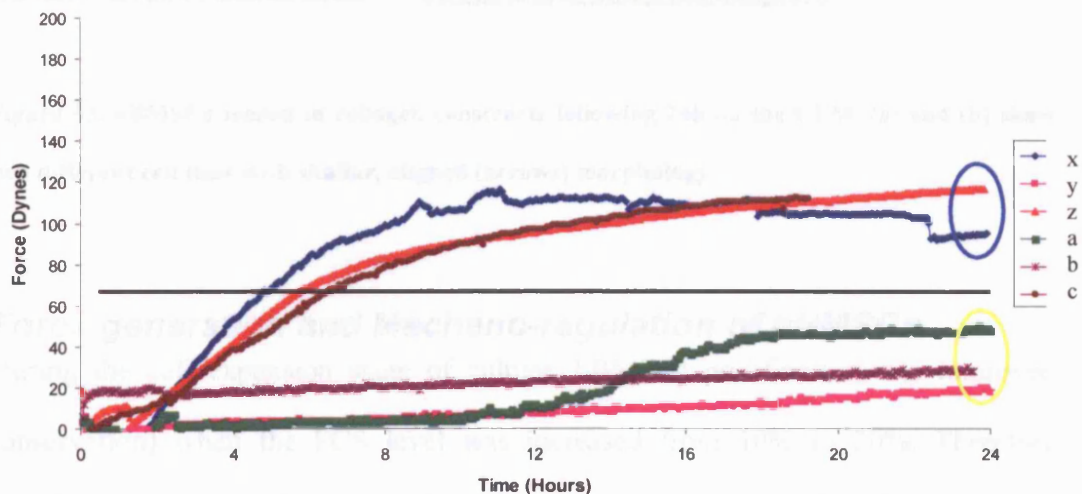
Figure 52. hBMSC's contraction profile for 6 different cell lines. 'High' and 'low' contractile cell lines are separated by an arbitrary black line (65 dynes) and final force generated indicated by a blue circle, for high contractile cell lines and a yellow circle for low contractile cells.

It is currently assumed that differences in force generation were related to some aspect of donor physiology. Previous chapters showed that HDFs will generate less force with increased collagen stiffness and the aim here was to test whether hBMSCs responded in a similar way. Therefore, only the high contractile cell lines were used in subsequent studies.

All cell cultures appeared similar morphologically (Figure 53) at the end of the contraction period with similar cell shape (elongate) for both groups.
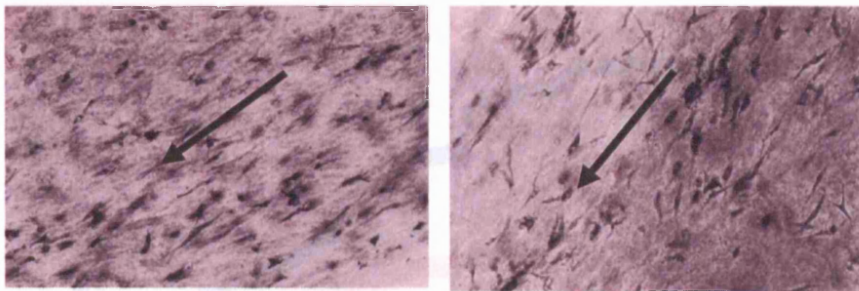
Figure 53. hBMSCs seeded in collagen constructs following 24h on the CFM. (a) and (b) show two different cell lines with similar, aligned (arrows) morphology.

Force generation and Mechano-regulation of hBMSCs

During the cell expansion stage of culture, hBMSCs proliferation rate improved (observation) when the FCS level was increased from 10% to 20%. Therefore hBMSCs were cultured in two different groups: a) with 10% FCS and b) with 20% FCS. It was concluded that cells expanded at different FCS levels might show a different mechano-response and so pre-strain experiments were repeated at both FCS concentrations. In each case, 10% and 20% FCS levels were also used for cell force generation monitoring.

hBMSC tested in 10% FCS final concentration generated peak force similar to HDFs (Chapter 4; Figure 33), producing lower peak force as the matrix stiffness increased (140, 40 and 5 dynes for 0%, 5%, and 10% pre-strain respectively). Forces generated between 0%-5% and 0%-10% pre-strain were significant different (Figure 54; $p < 0.05$).
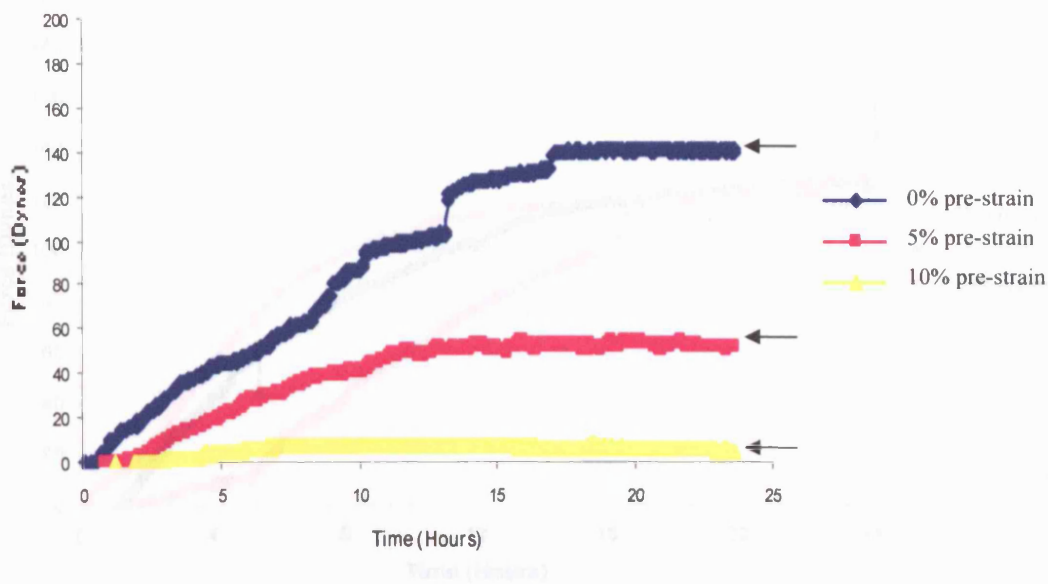
Figure 54. Contraction force profiles generated by hBMSC seeded constructs, with 10% FCS, following 0%, 5% and 10% pre-strain (n=3 each). Black arrows show final force reached by seeded cells.

Similar to the results shown previously (Chapter 4), when HDFs were seeded in stiffer collagen constructs, hBMSCs registered lower forces in response to stiffer matrices. This suggested similar mechanistic behaviour to fibroblasts.

However, when the hBMSCs response was tested at 20% FCS levels, increased force generation was shown at the stiffer matrices (Figure 55). Total force generated (at the end of 24h – Black arrow) was the same for all pre-strain levels (135dynes) indicating that a rate-limiting component of the FCS is critical in determining how cells respond to matrix stiffness.
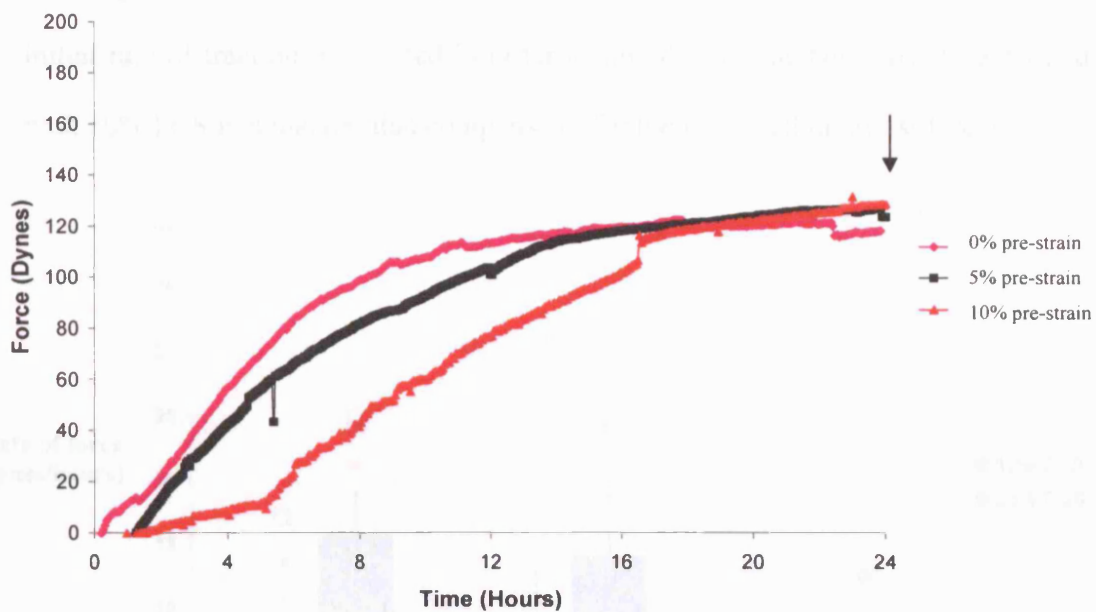
Figure 55. Contraction force profiles generated by hBMSC seeded constructs, with 20% FCS, following 0%, 5% and 10% pre-strain (n=3 each). Black arrow show final force reached by seeded cells.

In conclusion, a) total force is insensitive to increased matrix stiffness and b) initial rate force remains sensitive (contraction phase 12-24h) and cells are sufficiently activated at 20% FCS to compensate for increased matrix stiffness.

Furthermore, significant differences (described below) were shown in the initial rate of force generation 0-4h (i.e. traction phase) and the appearance of CIT with increased pre-strain (with 20% FCS), which suggests that the stiffness-dependent response was still present.

When 20% FCS was used, total force generation was increased at stiffer matrices, reaching the same level (135 dynes) as the 0% pre-strained constructs. Here the initial rate of traction was tested in order to investigate whether cells are activated with 20% FCS in a manner that compensates for the increased matrix stiffness.
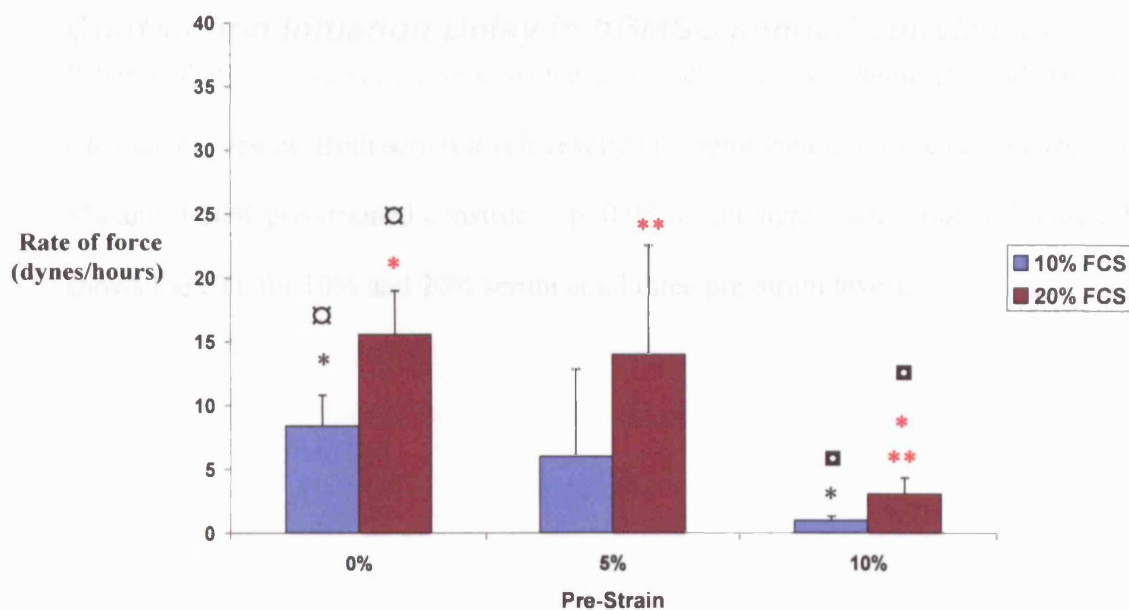
Figure 56. Rate of force generation is shown (0-8h) for hBMSCs seeded within increasingly stiffer collagen constructs with FCS at t=0h and t=1h (n=3 each). ⌷, ▫ and * indicate comparable groups and significance p<0.05.

Figure 56 shows the initial rate of traction (0-8h) for all the groups tested here. Significant rate differences were shown for 10% FCS between 0% and 10% pre-strain (p=0.05), while 20% FCS showed significance between 0%-10% and 5%-10%

pre-strain (p=0.05 for both). When the two FCS concentrations were compared (10% and 20%) significant differences were found at non pre-strained constructs (p=0.05) and also at the highest stiffness (10% pre-strain; p=0.05).

Two key conclusions can be drawn here; a) FCS is responsible for promoting cellular contraction (i.e post 10-12h) rather than traction which remains responsive to matrix stiffness independent of serum levels. b) hBMSCs are differentially responsive to serum levels when compared to HDFs.

Contraction Initiation Delay in hBMSC seeded constructs

When hBMSCs were seeded in collagen constructs, CIT was again (as with HDFs; Chapter 4) present. Both serum levels resulted in significant delays (CIT) between 0-5% and 0-10% pre-strained constructs (p=0.05 for all significant groups). Figure 57 shows the CIT for 10% and 20% serum at all three pre-strain levels.
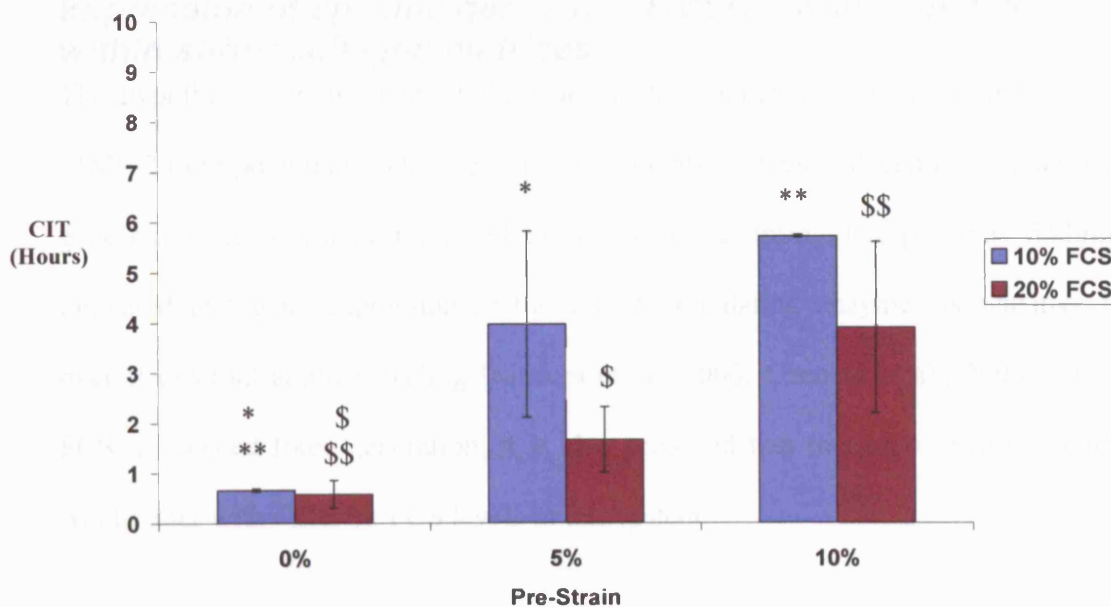
Figure 57. CIT is shown for hBMSCs seeded with 10% and 20% FCS, at the three different stiffness regimens (n=3 each). * and $ indicate comparable groups and significance p<0.05.

Based on the contraction profiles (Figure 55), *increased serum (%) significantly affected stiffer constructs (5 and 10%) stimulating equal peak on force generation to levels in non pre strained constructs, hence abolishing the inhibitory effect of matrix stiffness on peak contraction force generation.* It was assumed here that the traction phase was important in order for the hBMSCs to 'recover' their contractility at stiffer matrices.

Molecular Outputs

Expression of specific genes by hBMSC's when seeded within stiffer collagen matrices

The hypothesis for this part of the study is that selected MMP (2,3, and 9) and

TIMP-2 (and potentially other key matrix elements such as collagen types I and III)

gene expressions will be regulated by levels of pre-strain since previous findings

indicated that gene expression of these ECM regulating enzymes is sensitive to

overall external applied loading [Mudera et al., 2000; Cheema et al., 2005]. Since

FCS influenced force generation, it is also proposed that mechano sensitive genes

would alter with different FCS levels in this system.

Figure 58 shows an unchanged level (no significant differences), as shown for

HDF's previously, of the housekeeping gene GapDH, for hBMSC's in collagen

constructs (n=3) independent of the pre-strain levels with both (a) 10% and (b) 20%

FCS. Gene expression did not differ significantly between the cell lines here and so

could be used as a reference gene.
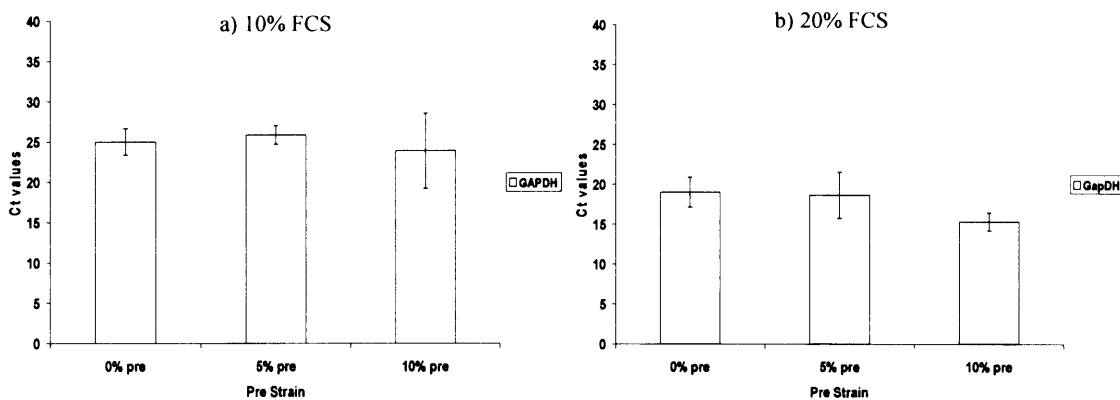
Figure 58. GapDH housekeeping gene levels shown for 0%, 5%, and 10% pre-strain for both (a) 10% and (b) 20% FCS. Standard deviation was used for error bars.

hBMSCs seeded in collagen constructs with 10% FCS

hBMSCs seeded in 5% pre-strained constructs, showed significant (10 fold) up-regulation of MMP-2 (Figure 59a; p= 0.05), when compared to unstrained constructs (0% pre-strain). However, additional pre-strain (10%) resulted in significant down-regulation of the MMP-2 (p= 0.05) by 8 fold. MMP-3 (Figure 59b) showed the same trend, in response to pre-strain, up-regulated at 5% pre-strain (p=0.0286) and down-regulated at 10% (p=0.05). MMP-2 and MMP-3 genes showed the highest expression at 5% pre-strain, indicating either peak sensitivity to matrix stiffness or a cell response to local loss of matrix stiffness. MMP-2 regulation was similar to that shown already for HDF's (Figure 44, Chapter 4).

Figure 59c shows the expression of COL-3 gene over the pre-strain regimes tested. COL-3 expression increased significantly (12 fold) with increasing matrix stiffness (0-5% and 0-10%; p= 0.05) upto a maximum at 5% (though not falling at 10%). This increased expression suggests that COL-3, like MMP-2 and -3, is up-regulated by matrix stiffness.
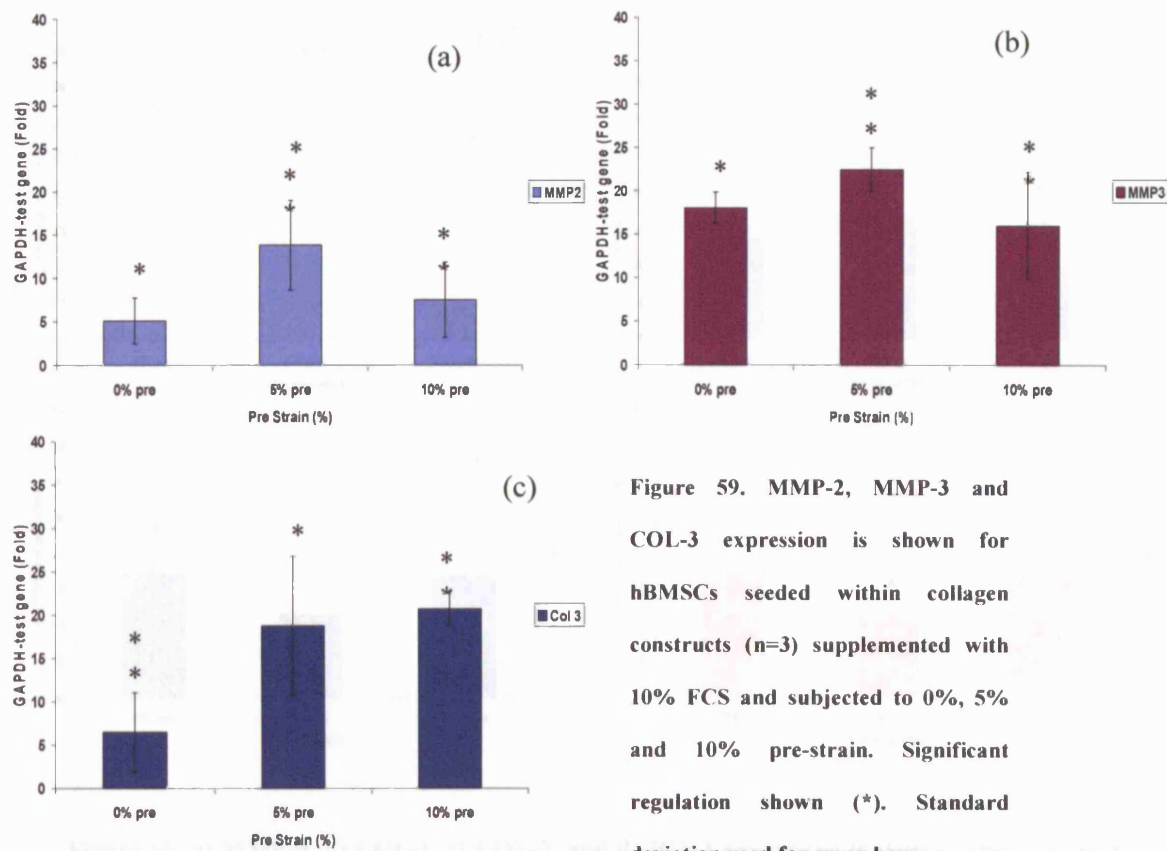
Figure 59. MMP-2, MMP-3 and COL-3 expression is shown for hBMSCs seeded within collagen constructs (n=3) supplemented with 10% FCS and subjected to 0%, 5% and 10% pre-strain. Significant regulation shown (*). Standard deviation used for error bars.

hBMSCs showed similar MMP-2 regulation (i.e. up-regulation at 5% and down-regulation at 10% pre-strain) with HDFs (Chapter 4) though TIMP-2 and COL-1 expression was not significantly altered with hBMSC seeded collagen constructs, indicating a cell lineage dependence.

When 20% FCS was tested, as already described for HDFs, several genes were differentially regulated. Figure 60a, shows significant up-regulation of MMP-9 at 5% pre-strain (14 fold; p= 0.039) and was down-regulated (15 fold; p=0.039) with further strain (10% pre-strain). 0% pre-strain did not significantly differed when compared to 10% pre-strain.
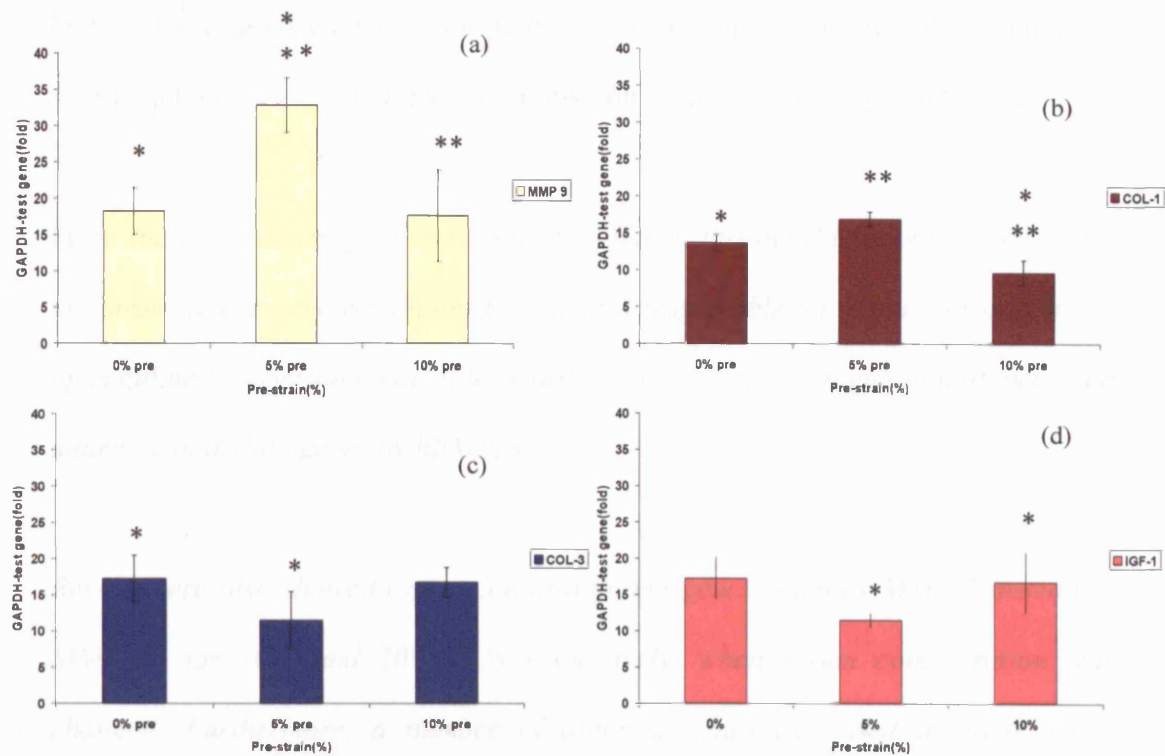
Figure 60. a) MMP-9, b) COL-1, c) COL-3, and d) IGF-1 expression is shown (after 24h on t-CFM) for hBMSC's seeded, with 20% FCS, in collagen constructs (n=3) and subjected to 0%, 5% and 10% pre-strain. Standard deviation is used for error bars. Significance is indicated by (*)

In addition, Figure 60b and 60c shows regulation of COL-1 and COL-3 in response to stiffer matrices. COL-1 expression at 10% pre-strain was significantly down-regulated (7 fold) compared with 0% and 5% pre-strain (p= 0.0286 and p= 0.05 respectively). COL-3 (Figure 60c) showed down-regulation (5 fold; p= 0.05) at 5% pre-strain compared to 0% pre-strain. Hence, changes in collagen gene expression in response to matrix stiffness were small but tended towards reduced expression with increased stiffness. hBMSCs showed, similar COL-1 gene regulation as HDFs, however COL-3 regulation was reversed (compared to HDFs) at stiffer matrices.

This would suggest cell lineage dependence on collagen genes regulation with 20% FCS and increased matrix stiffness. IGF-1 expression responded with a small but significant increase (p= 0.05) at 10% pre-strain (Figure 60d) compared to 5%.

From the above findings (Figure 60) *there was a threshold between 0% and 10% pre-strain where gene regulation for enzymes responsible for matrix remodelling is up-regulated, suggesting that stiffer matrices may trigger up-regulation of increased matrix remodelling genes by hBMSCs.*

Results here also showed expression of different genes (such as MMP-3 instead of MMP-9), for 10% and 20% FCS respectively, when serum concentration was changed. Furthermore, a number of different genes expressed different, when compared to HDFs (Chapter 4), suggesting a cell lineage dependence in response to matrix stiffness and FCS levels.

Discussion

ECM orchestrates changes in cellular behaviour [Phillips et al., 2003; Ingber 1998].

Mechanical forces transferred to the cells together with the biochemical ECM

composition regulate the balance within any tissue. Cells on the other hand will

generate forces in response to different mechanical or chemical signals in order to

control their shape and behaviour [Ingber 1998].

Reorganisation of the ECM by the cells, in response to forces has been reported in

literature [Eastwood et al., 1998; Mudera et al., 2000; Karamichos et al., 2006].

However the means by which cells sense these forces is not well understood. Despite

a number of studies (discussed below) contribution of cells embedded within

collagen constructs to matrix properties is uncertain. For example, Bellows et al

[1982] suggested that alignment could be generated within 24h by uniaxially

tethering the constructs and allowing them to generate tension. Cell alignment has

also been reported [Eastwood et al 1998; Mudera et al 2000] due to external uniaxial

mechanical loading, and due to contact quidance following collagen fibril alignment

using magnetic fields [Guido et al.,1993] suggesting that integrins act as

mechanoreceptors and transmit mechanical signals to the cytoskeleton. Ingber

[1998], also suggested that mechanical signals may be mediated simultaneously at

multiple locations inside the cell through force-induced rearrangements within the

actin-based cytoskeleton.

Interactions of cells with the ECM are essential in almost all biological processes

including wound healing and scar formation. Cells present within various ECMs

receive specific signals/information, normally triggered by ECM mechanical

function/state via the transmembrane receptors, known as integrins. These receptors,

in sequence, are connected to a complex of structural and signalling proteins forming the focal adhesions (structural and signalling proteins; FA) that anchor the actin stress fibers to the cell membrane. The regulation operated by the integrins on the cell phenotype is, at least in part, conditioned by the mechanical rigidity of the ligand [Lambert et al., 2001]. The resistance offered by the support applies tension on integrins, increases the stiffness of the cytoskeleton [Wang et al., 1993], the strength of the integrin-cytoskeleton linkage [Choquet et al, 1997] and the assembly and signalling activity of focal adhesions proteins [Pelham et al., 1997].

However, the investigation of collagen based materials physical properties are even more complicated because a) collagen concentration or crosslinking will vary material properties [Torres et al., 2000] and therefore may elicit differential response from the cell type seeded within and b) cell type differences may have significant effect on material properties. Mechanotransduction in mesenchymal stem cells is said to be mediated through structures that link cells to ECM, such as focal adhesions that develop in cells cultured on a rigid substrate [Izzard et al., 1976; Burridge et al., 1988].

This study has concentrated on 3D collagen constructs and hBMSCs seeded within those constructs showed differential responses when FCS % was altered. Previous chapters (Chapter 4) showed that HDFs responded to increased stiffness by generating lower contraction. Importantly, this effect is abolished in hBMSCs when FCS concentration was increased to 20%. The question arising is what levels of force are needed in order to elicit hBMSC response which are similar with both 10% and 20% FCS. One potential way to test this is to increase cell number and keep the FCS levels low, and investigate their responses again at molecular, contraction forces, and

morphology level. Alternatively, higher or different in magnitude strains have to be applied and tested for differential hBMSC responses.

The mechanism by which fibroblasts regulate the contraction of 3D collagen matrices has been shown to vary according to growth factor stimulus, mechanical environment, and the differentiation state of the cells [Grinnell et al., 2006]. Physiological agonists platelet-derived growth factor (PDGF) and lysophosphatidic acid (LPA) both have been shown to stimulate collagen matrix contraction, even though these agonists have opposite effects on the movement of cellular dendritic extensions within the matrices. PDGF increases their extensions; LPA causes their retraction [Grinnell, 2003].

Further studies have emphasized the bipolar morphology of cells [Elsdale et al., 1972; Tomasek et al., 1984], which might be a response to the LPA in serum. Unpublished data have shown that progressive, step-wise, addition of FCS to cell seeded collagen construct will give an instant increase in force generation. Similar to what it is shown in this study using the traction phase as reference. It may therefore be possible that serum (and mainly LPA in serum) has similar effect to contraction forces generation as growth factors such as TGF-b [Brown et al., 2002].

Conclusions

▶ Stiffness increase led to lower peak contraction forces generated by embedded hBMSCs, identical to HDFs.

▶ Increased FCS (%) significantly affected stiffer constructs (5 and 10%) stimulating identical peak contraction forces to levels in non pre strained constructs, hence abolishing the inhibitory effect of matrix stiffness.

▶ Similar to HDFs, hBMSCs showed CIT in both serum levels (10 and 20%), suggesting a matrix stiffness dependence, and not a cell lineage dependence.

▶ MMP-2 and COL-3 were mechano-responsive in patterns similar to HDFs suggesting that matrix stiffness will modulate matrix remodelling genes.

Chapter 6: HNFF cellular and molecular responses to increasing collagen matrix stiffness

HNFF responses to pre-strain

This section of the study was designed to test the hypothesis that Human Neonatal

Foreskin Fibroblasts (HNFFs) respond by generating lower contraction forces, to

increasing matrix stiffness, i.e a similar response to HDF. The HNFF response to

FCS starvation, as previously shown using HDF, was also tested here. FCS was

added as before at 1h and force generated was recorded for an additional 23h (as

described in Methods). Experiments were run over 24h and constructs processed for

RT-PCR.

The same pre-strain regimes of 0%, 5% and 10% were used. Figure 61 below shows

HNFF responses to pre-strain without serum starvation.
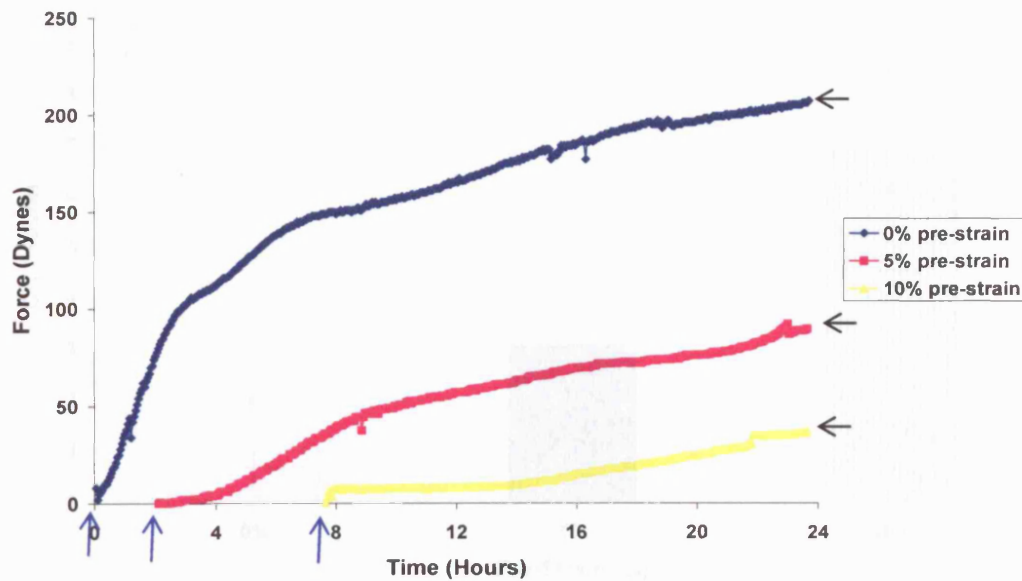
Figure 61. Contraction force profiles generated by HNFF seeded constructs, with 10% FCS, following 0%, 5% and 10% pre-strain (n=3 each). Black arrows show final force reached by seeded cells and blue arrow shows CIT levels.

HNFF demonstrated the same force generation response to increasing matrix stiffness, as HDFs. When FCS was present from the start the peak force generated was reduced from 200 dynes at 0% pre-strain to 90 and 35dynes for 5% and 10% pre-strain respectively. As for HDFs, the fall in peak (24h) force generated was significant between 0% and 5% as well as 0% - 10% pre-strain (falling by 50% and 82%: p=0.05 for both).
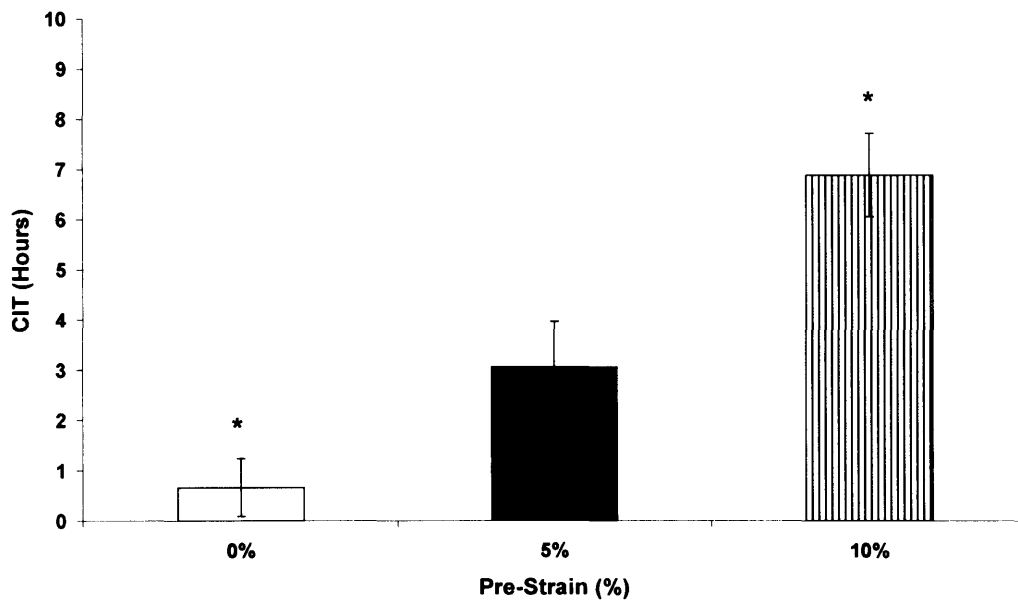
Figure 62. CIT is shown for HNFF seeded constructs at the three different stiffness regimens (n=3 each). There was a significant difference in CIT between 0%-10% pre-strain (*; p<0.05).

HNFFs showed significant delay in force generation (i.e CIT; Figure 62). CIT was only significantly different (p=0.0265) between 0% and 10% pre-strained constructs, in contrast to HDFs where CIT was significant different at 5% pre-strain as well as 10%. *These results showed that CIT is pre-strain (i.e stiffness) dependent.*

124

HNFF's contraction altered by serum starvation

HNFFs were subjected to FCS starvation (as with HDF; Chapter 4) to determine the effect on force generation and CIT. Figure 63 shows HNFF contraction under 0%, 5% and 10% pre-strain, following 1h FCS starvation and replacement.
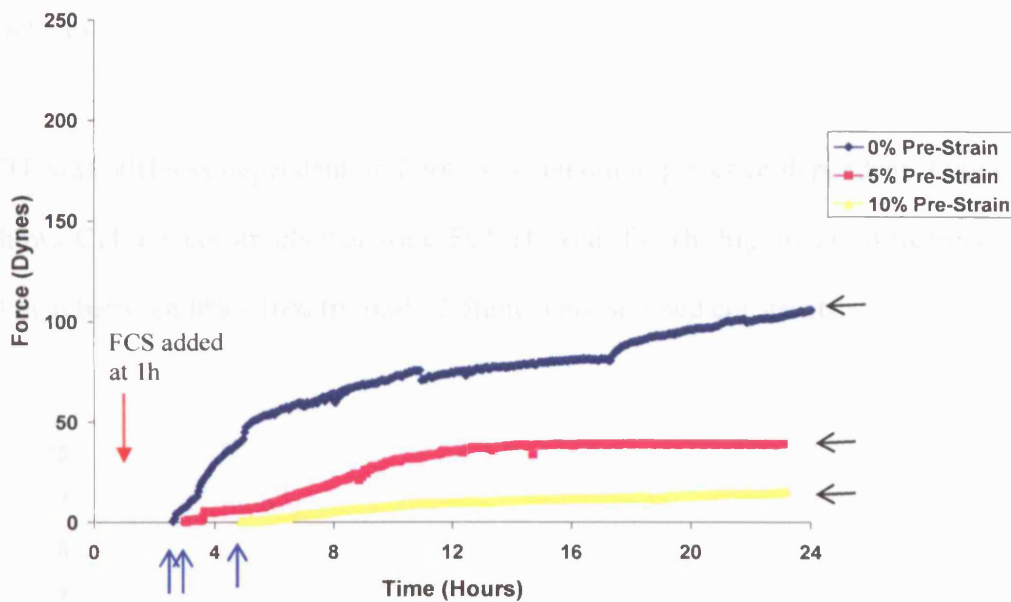
Figure 63. Contraction force profiles generated by HNFF seeded constructs, following 1h FCS starvation for 0%, 5% and 10% pre-strain (n=3 each). Black arrows show final force reached by seeded cells and blue arrow shows Contraction Initiation Time levels.

There was a significant (p=0.05), incremental reduction in total force generation with increasing matrix stiffness following FCS starvation, similar to that with HDFs (Figure 36). At 0% pre strain peak force generation was 100 dynes, decreasing to 39 and 12.3 dynes at 5% and 10% pre-strain respectively (Figure 63-Black arrows). Importantly, when cells were FCS starved (for 1h), total peak force for 0%, 5% and 10% pre-strained constructs were significantly lower (all three; p=0.05) compared to constructs with FCS at t=0h.

Critically, forces generated by HNFFs, with 20% FCS presence, were progressively lower with increase in matrix stiffness. This is similar to HDFs but different to hBMSCs (where total peak force was the same at all stiffness levels) suggesting that serum levels increase will affect force generation differentially between cell lineages.

CIT was stiffness dependent and not FCS starvation/presence dependent. Figure 64 shows CIT for constructs that were FCS starved, for 1h. Significant difference was shown between 0% - 10% (p<0.05; 2.5times) pre strained constructs.
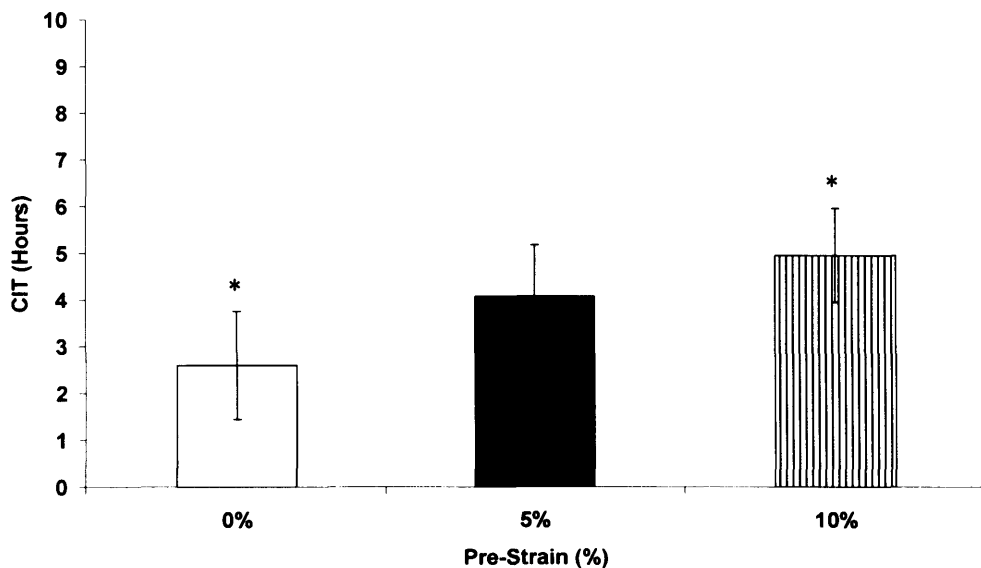
Figure 64. CIT is shown for HNFF seeded constructs following 1h serum starvation, at the three different stiffness regimens (n=3 each). There was a significant difference in CIT between 0%-10% pre-strain (*; p<0.05).

When HDFs were tested, CIT was significant higher at lower matrix stiffness (i.e 5% pre-strain) as well as 10% pre-strain. *This indicates that different cell lineages will have different 'threshold' of CIT, when matrix stiffness is increased.*

Tractional forces generated by HNFFs
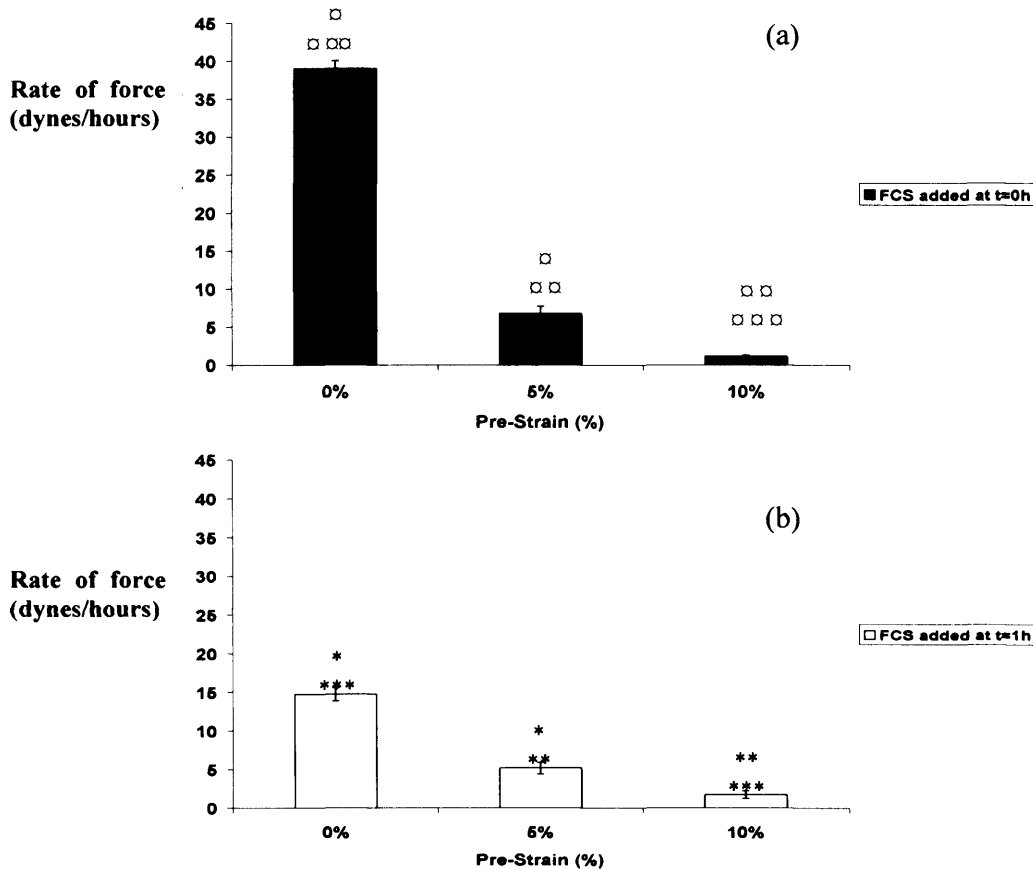
Figure 65. Rate of force generation is shown (0-8h) for HNFFs seeded within increasingly stiffer collagen constructs with (a) FCS at t=0h and (b) FCS at t=1h (n=3 each). □ and * indicate comparable groups and significance $p<0.05$.

When FCS was present from t=0h, non-strained HNFF constructs showed a significant (Figure 65a) 32 and 39 times faster (in terms of rate between 0-8h) contraction rate than constructs with the higher stiffness (5 and 10% pre-strain respectively; both $p<0.05$). FCS starvation, for 1h, showed similar trends with 10 and 12 times faster contraction rate between 0%-5% and 0%-10% pre-strained constructs (Figure 65b; $p<0.05$) respectively. Contraction rates were significantly different in both FCS presence and FCS starved constructs between 5% and 10%

127

pre-strain (both p<0.05). When constructs with FCS presence compared to the serum starved ones, all 0%-5%, 0%-10% and 5%-10% pre-strained constructs were significantly different (all p<0.05) by 25, 5 and 2 times faster.

This is a reverse response by HNFFs compared to HDFs (Chapter 4). Here FCS starvation led to significantly slower contraction rate, where for HDFs FCS starvation led to faster responses. *Indicating that FCS starvation is cell lineage dependence, i.e rates of contraction forces generated by seeded cells (after FCS starvation) will depend on the cell lineage itself.*
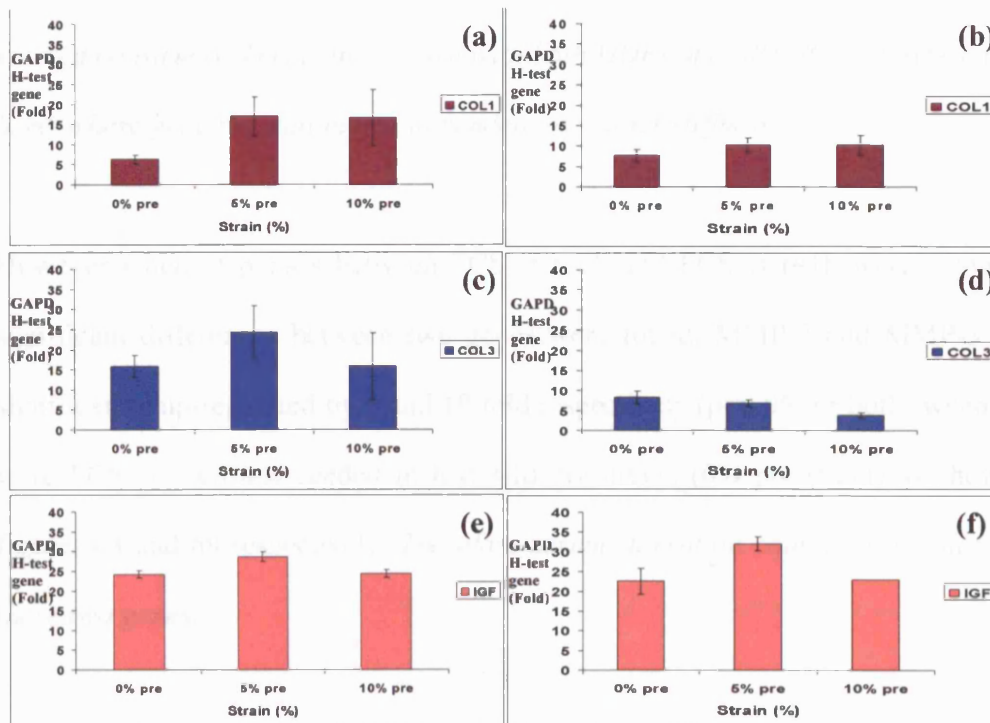
Figure 67. Gene expression levels shown for both with and without FCS starvation. Expressions for cells not starved are: (a) COL1, (c) COL3 and (e) IGF1. Expressions for cells starved for 1h: (b) COL1, (d) COL3 and (f) IGF1. Standard deviation used for error bars.

Despite the fact, those genes expression, did not reach conventional statistical significance, **trends** showed (Figure 66) that MMP-2 and MMP-3 up-regulated when FCS was present at t=0h (Figure 66a and 66c respectively; arrow), with a peak expression at 5% pre-strain showed when cells were FCS starved (Figure 66b and 66d respectively; arrow)

Concluding this part of the study, expression of the marker genes tested here, was independent of matrix stiffness (i.e pre-strain) for both FCS supplemented and FCS starved constructs. This is in contrast with both HDFs and hBMSCs cell types, tested here, where gene regulation was dependent on matrix stiffness.

However when responses between FCS at t=0h and FCS at t=1h were compared, significant differences between two genes were found. MMP-2 and MMP-3 were significantly up-regulated by 5 and 10 fold respectively (p=0.05 for both) when cells were FCS starved and seeded in less stiff constructs (0% pre-strain) as shown in figures 68 and 69 respectively. *Therefore serum starvation caused up-regulation of these two genes.*
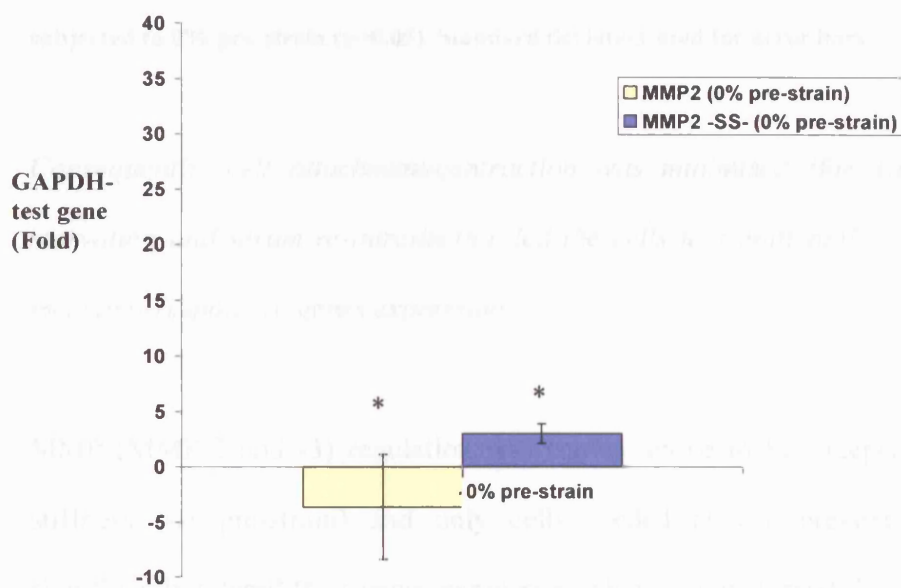
Figure 68. MMP-2 gene expressions for constructs (n=3) with FCS and without FCS starvation, subjected to 0% pre-strain (p=0.05). Standard deviation used for error bars.
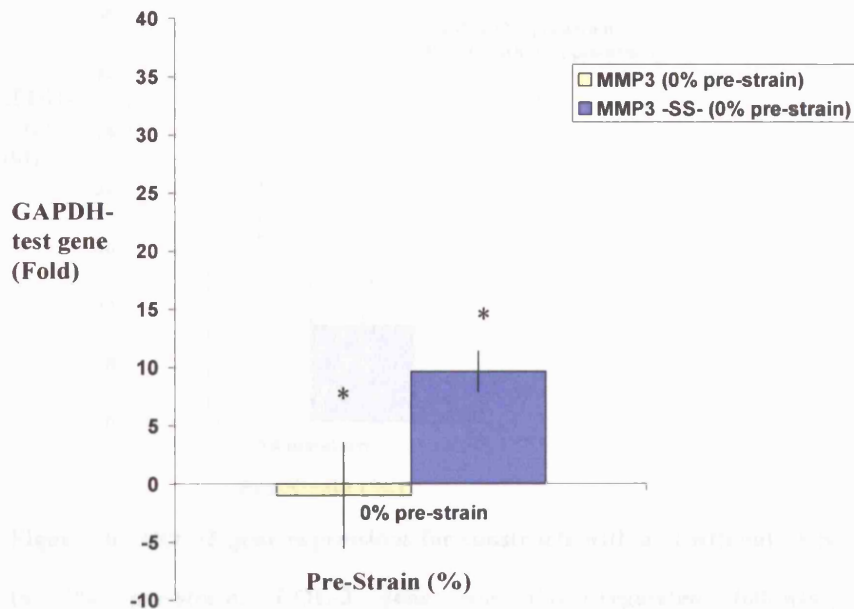
Figure 69. MMP-3 gene expressions for constructs (n=3) with FCS and without FCS starvation, subjected to 0% pre-strain (p=0.05). Standard deviation used for error bars.

Consequently, cell attachment/contraction was minimised (for 1h) using serum starvation and serum re-introduction led the cells to significantly increase specific mechano-responsive genes expression.

MMP (MMP-2 and -3) regulation was shown above to be independent of matrix stiffness (i.e pre-strain) and only cells seeded at 0% pre-strained constructs significantly altered their genes expression when serum starved. In contrast, COL-3 was significantly (p=0.05) down-regulated when cells were FCS starved for both 0% and 5% pre-strained constructs (Figure 70 and 71).

132
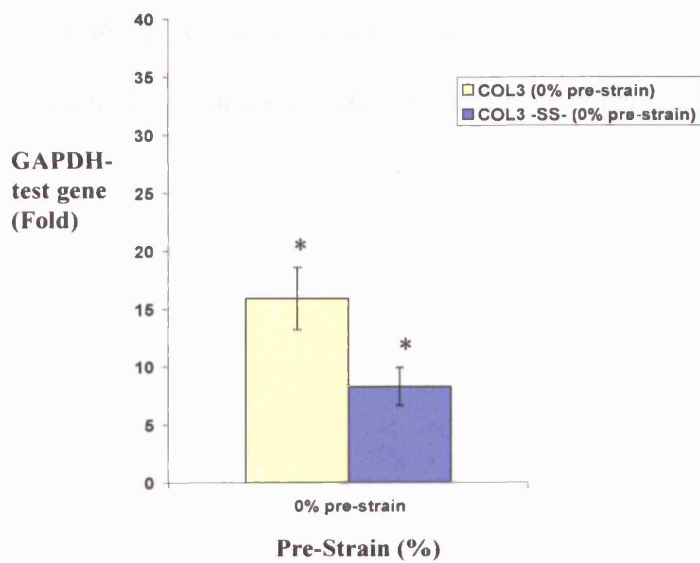
Figure 70. COL-3 gene expressions for constructs with and without FCS starvation, subjected to 0% pre-strain. COL-3 gene was down-regulated following serum starvation (p=0.05).Standard deviation used for error bars.
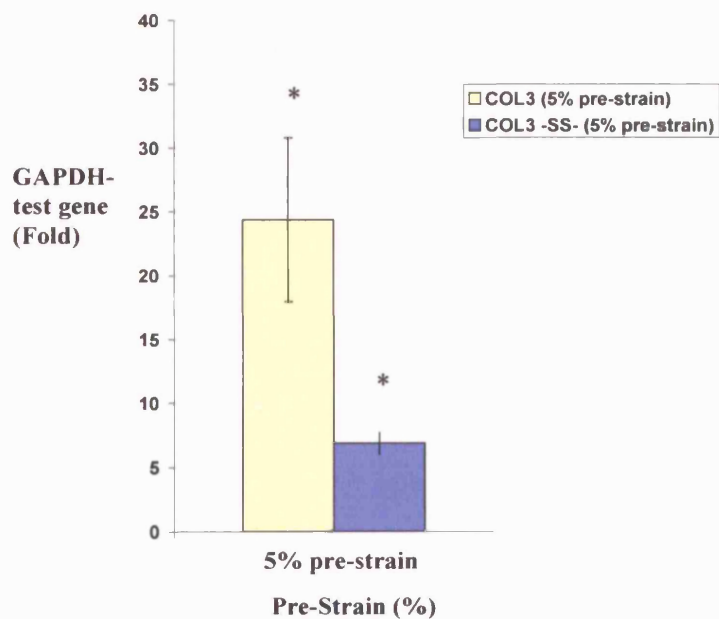
Figure 71. COL-3 gene expressions for constructs with and without FCS starvation, subjected to 5% pre-strain. COL-3 gene was down-regulated following serum starvation (p=0.05). Standard deviation used for error bars.

COL-3 expression was FCS dependent at 0% and 5% pre-strained constructs. However, when the constructs were pre-strained by 10%, COL-3 expression was not altered significantly, *indicating an internal (cellular) threshold above 5% pre-strain.*

Discussion

This study was based on cell seeded collagen constructs as a model to investigate the effect of increasing matrix stiffness on cell force generation and its effect on molecular mechanoresponsive genes. This is based on the findings that uniaxial pre-strain significantly alters the collagen stiffness, as shown in Chapter 3 [Karamichos et al, 2006].

These results suggest that fibroblasts regulate the force generated on adjacent collagen fibrils in response to stiffness of the ECM, i.e there is a feedback control to matrix stiffness. This is in general agreement with the early identification of a tensional homeostasis [Brown et al., 1998] and is consistent with the idea that fibroblasts are cells generating forces to monitor and control ECM material/ECM mechanical properties [Bishoff et al., 2003]. Here we have quantified the amount of forces generated by the HNFF cells seeded in collagen ECM and showed that stiffer matrices resulted in a reduced generation of quantifiable forces by the resident cells, as with HDFs and hBMSCs shown earlier.

It is important in this respect to consider the influence of different cell lineages as different fibroblast types may have different responses to increasing ECM stiffness. Fibroblast is a generic term for cells resident in and maintaining a wide range of collagen matrix types. It would seem inevitable that cell sensing and responses must differ if they are to maintain different material properties. A 'signature' of each cell lineage may be achieved which will be useful in guiding specific cellular responses (i.e cell seeded constructs which aim to mimic different tissues). Our results here suggest similar response to ECM stiffness by different cell types (HDFs, hBMSCs and HNFFs) in terms of early (24h) force generation but crucially different responses

in terms of the detail of their gene expression, which may have long term implications.

COL-3 gene expression in HNFFs showed a synergistic inverse relationship which reached the lowest expression values at 310Pa construct stiffness. HNFFs showed FCS dependence rather than stiffness dependence. MMP-2 and -3 were both up regulated when cells were FCS pre-starved for 1h. In contrast, COL-3 was down-regulated (as with HDFs at 5%), indicating opposite regulation of MMPs and collagen. Furthermore, there was an FCS dependence on COL-3 expression, since it was the only gene significantly down-regulated, in 310Pa stiff constructs (5% pre-strain). Functionally COL-3, in dermis, is thought to act as an early stage provisional fibre element in remodelling, repair and growth of the ECM.

This study has also shown that cells will recover from the FCS starvation and are then able to generate forces, which are significantly higher at 0% pre-strained constructs, though with a longer CIT. CIT measure is important in this respect as it appears to reflect the ability and rate of cells to generate tractional forces (early stages) as they spread and move (Force rates; Figures 44a and b). Hence factors reducing cell spreading (reduced FCS, increased matrix stiffness and density) resulted in delayed traction force generation.

The increased stiffness of a Tissue Engineered construct by means of external mechanical stimuli will have to be tailored to elicit predictable cellular responses. Furthermore, FCS levels will have to be taken into account on rate as well as initiation time of generation of cellular contractile forces.

Conclusions

▶   Increased collagen stiffness led to generation of lower contraction forces by the seeded HNFFs.

▶   HNFFs recovered from the FCS starvation and generated significantly lower forces at the lowest stiffness constructs.

▶   CIT was present in FCS starved/non starved constructs, showing similar responses to HDFs and hBMSCs (Chapter 4 and 5), suggesting matrix stiffness dependence.

▶   HNFFs showed no ECM stiffness dependence as far as gene regulation is concerned, indicating that altered regulation on mechano-responsive genes are possible even in relatively similar cell types (HDFs vs HNFFs).

▶   MMP-2 and MMP-3 gene expressions were up-regulated and COL-3 down-regulated at less stiff constructs following FCS starvation.

▶   COL-3 was also down-regulated at 310Pa stiff constructs with no further significant regulation at higher stiffness.

Chapter 7: HDF specific mechanoresponsive gene regulation: Stiffness and ramp loading dependence

HDFs gene regulation dependent on stiffness and ramp loading regimes

Previous chapters have shown that different cell types (HDF's, HNFF's, and hBMSC's) will have a differential response, in terms of contraction force, to external mechanical strain, when seeded in increasingly stiffer constructs. Contraction forces generated by embedded cells and molecular outputs at the end of 24h were investigated as well as the effect of FCS presence/absence (within these increasingly stiffer constructs) in terms of contraction forces and molecular outputs.

It has been reported in literature (as discussed later) that different loading regimes can regulate specific genes within cell seeded collagen constructs. The final part of this study was to combine the pre-strain regimens with specific ramp loading regimens (see Methods, Figure 19 and 20) and investigate the molecular outputs.

HDFs and hBMSCs were seeded in collagen constructs and pre-strained, as described before (see Methods, Figure 19 and 20), by 0% and 5% pre-strain. 10% pre-strain was omitted as contraction forces were not detectable for all the cell types tested here. Constructs were cultured with 10% and 20% FCS (as with pre-strain experiments) on the t-CFM for 12h before two different ramp loading regimes applied: (a) 10% strain over 1h, and (b) 10% strain over 12h. Figure 71b shows exmples of these loading regimes. All the experiments were stopped and processed for mRNA extraction at the end of 24h.
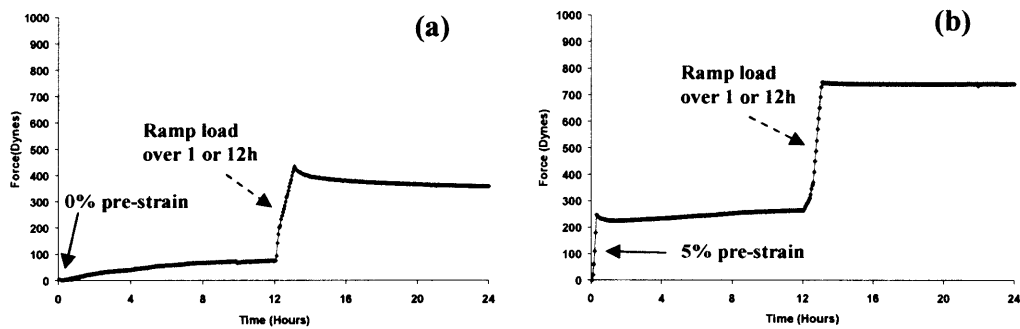
Figure 71b. Loading regimes shown, used for both HDFs and hBMSCs. a) 0% pre-strain applied following by 10% ramp load at 12h (over 1h or 12h depending on experiment). b) 5% pre-strain applied following by 10% ramp load at 12h (over 1h or 12h depending on experiment)

In order to investigate the molecular outputs we split the data into three different comparable groups:

a) Stiffness dependent

b) Rate dependent

c) FCS dependent

Groups are summarised in table below:

HDF's	10% FCS	10% FCS	20% FCS	20% FCS
Ramp Load	10% over 1h	10% over 12h	10% over 1h	10% over 12h
0% pre strain	Group 1	Group 2	Group 3	Group 4
5% pre-strain	Group 5	Group 6	Group 7	Group 8

Table 5. Four different groups of pre-strain and ramp loading were used for each FCS supplement levels (10% and 20%). For 10% FCS: (Group 1) 0% pre-strain and 10% over 1h, (Group 2) 0% pre-strain and 10% over 12h, (Group 5) 5% pre-strain and 10% over 1h, and (Group 6) 5% pre-strain and 10% over 12h. For 20% FCS: (Group 3) 0% pre-strain and 10% over 1h, (Group 4) 0% pre-strain and 10% over 12h, (Group 7) 5% pre-strain and 10% over 1h, and (Group 8) 5% pre-strain and 10% over 12h.

In terms of contraction, both cell types in all groups showed no measurable contraction (at the end of 24h) following the ramp loading regimes.

Effect of stiffness on ramp loaded collagen constructs

This part of the study was conducted to investigate the effect of ramp load on gene regulation in increasingly stiffer HDF seeded collagen constructs. The hypothesis here was that introduction of two different ramp loading regimes will regulate MMP and COL genes, in increasingly stiffer matrices. Table 6 below shows the Groups compared here.

Stiffness	Group 1 vs Group 5
	Group 2 vs Group 6
	Group 3 vs Group 7
	Group 4 vs Group 8

Table 6. Groups which are different in stiffness but identical in ramp rate and FCS levels were compared for specific gene regulation as shown. Group 1, 2, 3, and 4 are subjected to 0% pre-strain where Groups 5, 6, 7, and 8 to 5% pre-strain.

Molecular outputs were compared for significant regulation depending on the stiffness (i.e initial pre-strain) when ramp load was applied (10% over 1h and/or 12h). Genes tested here were the same as listed before (Table 4; Methods); MMP-2, -3, -9, TIMP-2, COL-1, -3, and IGF-1.

Fast ramp loading (over 1h) was used for Groups 1 and 5. Group 5 though had an increased initial stiffness (310Pa) at t=0h. When gene regulation was quantified here results showed the following; MMP-2 and COL-1 were significantly ($p < 0.05$) down-regulated (Figure 72a and 72c; 12 and 16 fold respectively) at stiffer constructs,

140

while TIMP-2 and COL-3 (Figure 72b and 72d; $p<0.05$) were significantly up-regulated at the same group of stiffness, indicating an inverse co-relation. IGF-1 was also up-regulated (Figure 72e; $p<0.05$) in stiffer constructs; similar with previous results i.e up-regulation at higher stiffness (>5% pre-strain).
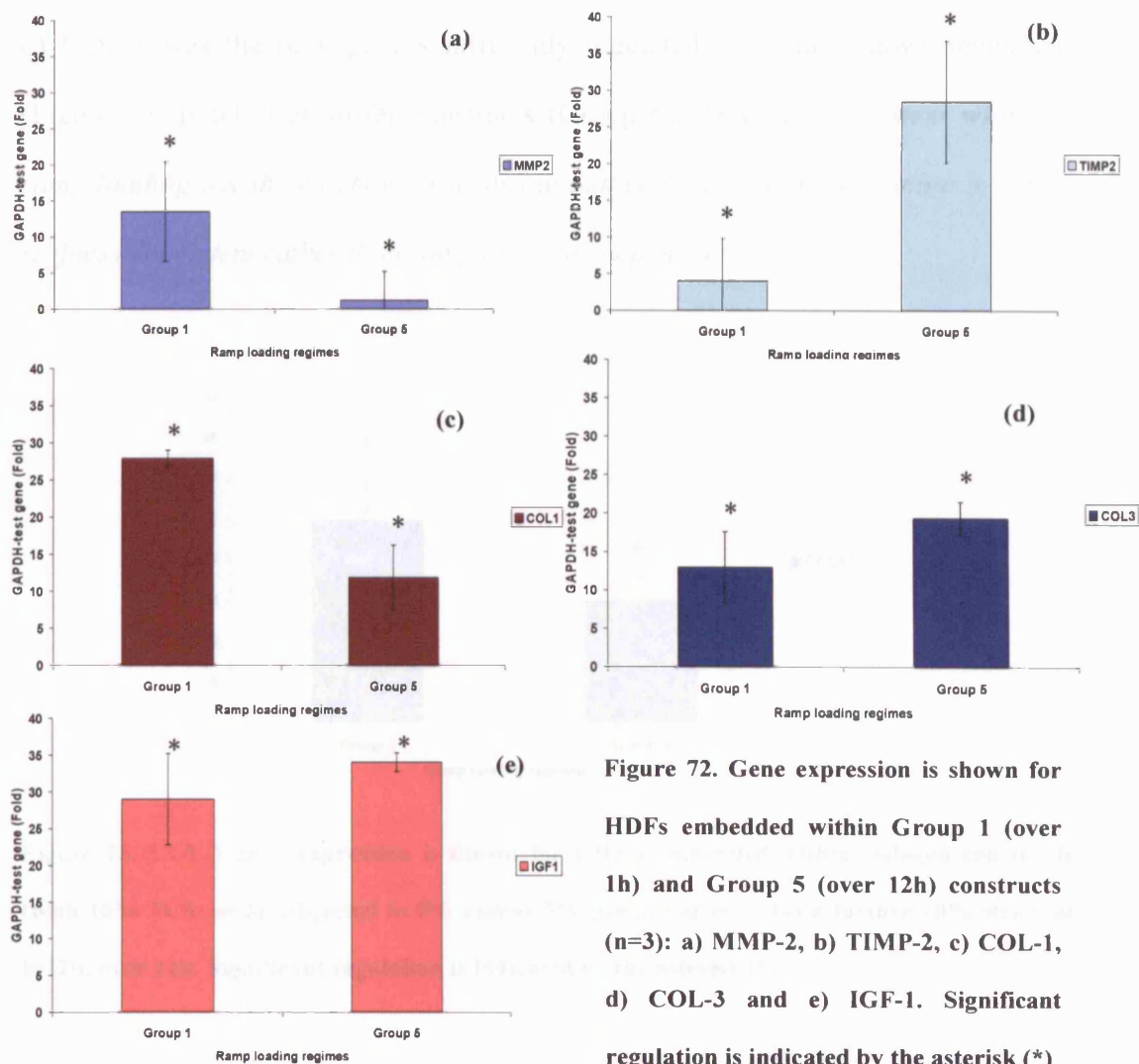
Figure 72. Gene expression is shown for HDFs embedded within Group 1 (over 1h) and Group 5 (over 12h) constructs (n=3): a) MMP-2, b) TIMP-2, c) COL-1, d) COL-3 and e) IGF-1. Significant regulation is indicated by the asterisk (*)

It is important here to note that MMP-2 and TIMP-2 showed inverse correlation between Group 1 and 5. Similar correlation was shown between COL-1 and -3 at the same Groups. These results indicated that matrix genes expression can be controlled with a combination of stiffness and fast/slow ramp loads; however the balance of the two is necessary to achieve predictable results.

In contrast, when slow ramp loading was used for both stiffness levels (Group2 and 6) COL-1 was the only gene significantly regulated, showing a down-regulation (Figure 73; $p<0.05$) at stiffer constructs (Group 6). *This was consistent with fast ramp loading (as shown above) suggesting that collagen I gene regulation is highly stiffness dependent rather than ramp load rate dependent.*
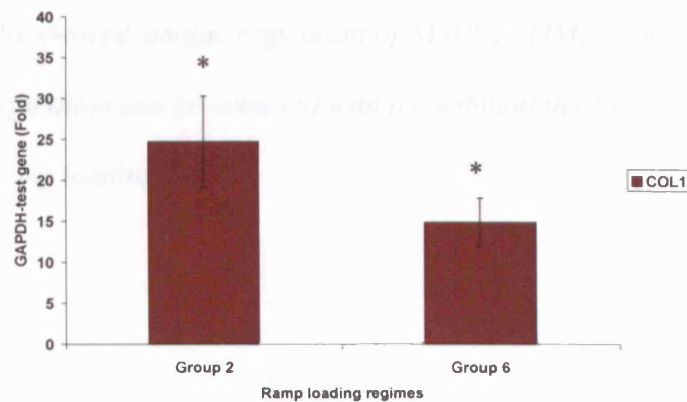
Figure 73. COL-1 gene expression is shown for HDF's embedded within collagen constructs (with 10% FCS; n=3) subjected to 0% and/or 5% pre-strain and also a further 10% strain at t=12h, over 12h. Significant regulation is indicated by the asterisk (*)

Effect of stiffness on ramp loaded collagen constructs (20% FCS)

Ramp loading experiments using HDFs were repeated with 20% FCS, as described previously, in order to compare and investigate any changes in gene expressions due to FCS levels elevation. Firstly, stiffness effect is investigated when 20% FCS is present. Again there was no contraction following the application of ramp loading regimes.

Figure 74a and 74b shows, as with 10% FCS, significant inverse correlation between MMP-2 and TIMP-2; MMP-2 gene was down-regulated ($p < 0.05$) and TIMP-2 up-regulated ($p < 0.05$). Identical results with 10% FCS were shown with IGF-1 here (Figure 74e) which was significantly up-regulated at stiffer constructs. However, the ramp load was applied slowly (over 12h) and not fast (over 1h) as with 10% FCS. *These results showed similar regulation of MMP-2, TIMP-2 and IGF-1, suggesting that gene regulation can be achieved with a combination of serum levels (ex. 10% or 20%) and ramp loading rates.*
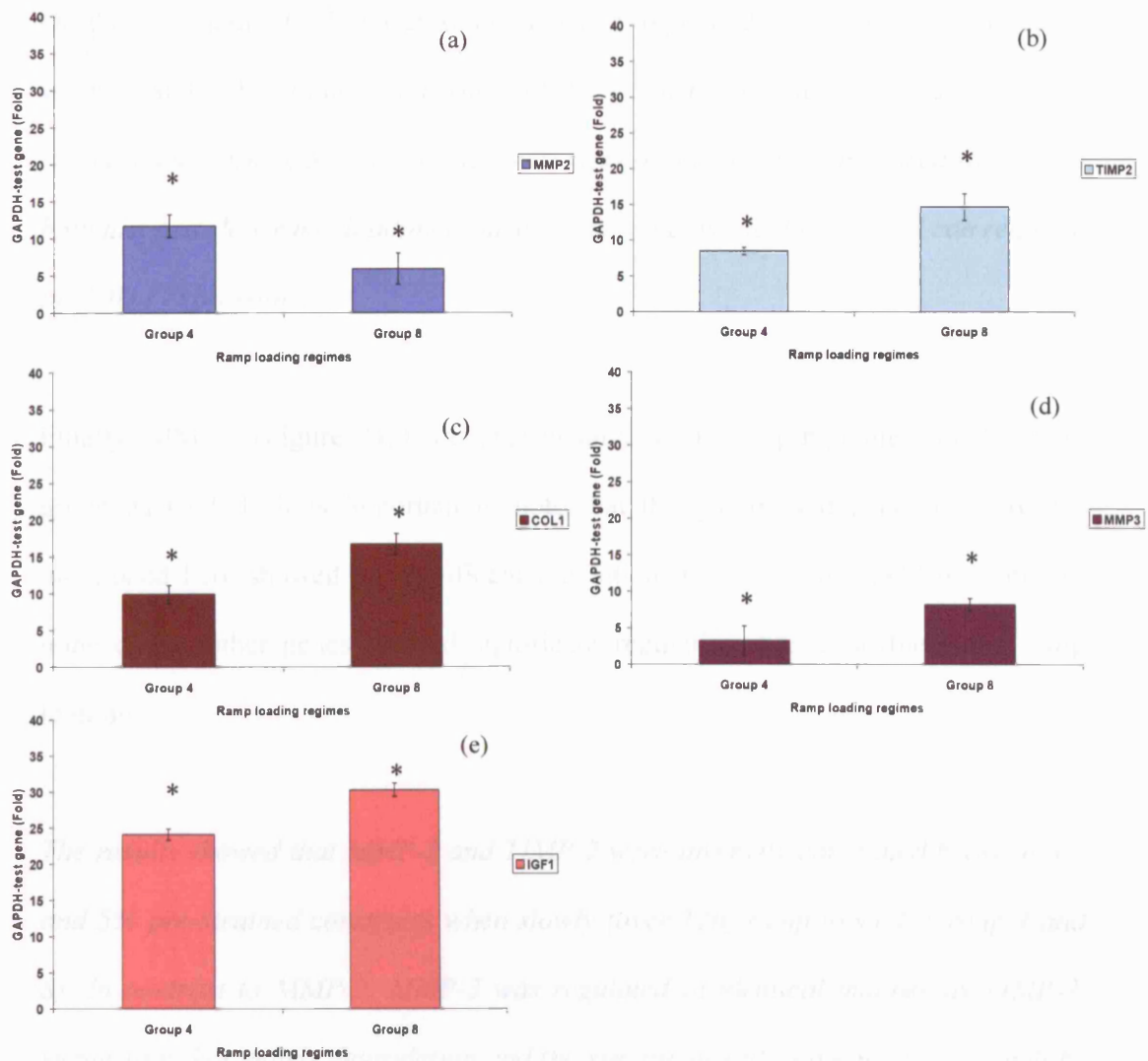
Figure 74. Gene expressions shown from HDF's embedded within collagen constructs (with 20% FCS) subjected to 0% and or 5% pre-strain and also a further 10% strain at t=12h, over 1h and/or 12h (n=3). (a) MMP-2 following ramping over 12h, (b) TIMP-2 following ramping over 12h, (c) COL-1 following ramping over 12h, (d) MMP-3 following ramping over 12h, (e) COL-1 following ramping over 12h, and (f) COL-3 following ramping over 12h

On the other hand, COL-1 was significantly up-regulated (p<0.05) due to increased

stiffness and a slow ramp load (over 12h). *COL-1 was regulated in opposite manner,*

compared to 10% FCS, where COL-1 was down-regulated with increased stiffness in

both fast and slow ramp loading regimes, indicating that factors in FCS can regulate

in COL-1 expression.

Finally, MMP-3 (Figure 74d) was significantly (p=0.05) up-regulated for the same

group as COL-1. It is important to note that the groups and genes that are not

mentioned here showed no significant regulation, i.e when 20% FCS was present

none of the other genes showed significant regulation due to stiffness and ramp

loading.

The results showed that MMP-2 and TIMP-2 were inversely correlated between 0%

and 5% pre-strained constructs when slowly (over 12h) ramp loaded (Group 4 and

8). In contrast to MMP-2, MMP-3 was regulated in identical manner as TIMP-2,

suggesting that matrix degradation and the specific metalloproteinase which will be

responsive to external loads are very much dependent on serum levels.

Effect of ramp loading rate for collagen constructs

Results showed genes up/down-regulated by the effect of increased stiffness and

ramp loading regimes. Here we investigated the effect of the ramp loading rate on

gene expression. Genes that were not significantly regulated are not presented here.

Groups compared are listed in Table 7.

Rate	Group 1 vs Group 2
	Group 3 vs Group 4
	Group 5 vs Group 6
	Group 7 vs Group 8

Table 7. Groups which are different in ramp rate but identical in stiffness and FCS levels were compared for specific gene regulation as shown. Group 1, 3, 5, and 7 are subjected to fast ramp load (over 1h) where Groups 2, 4, 6, and 8 to slow ramp load (over 12h).

Figure 75 shows significant down-regulation (p=0.05) of TIMP-2 at slow rate when compared to fast rate (Group 6 and 5 respectively) when HDFs were seeded within stiff constructs (5% pre-strain).
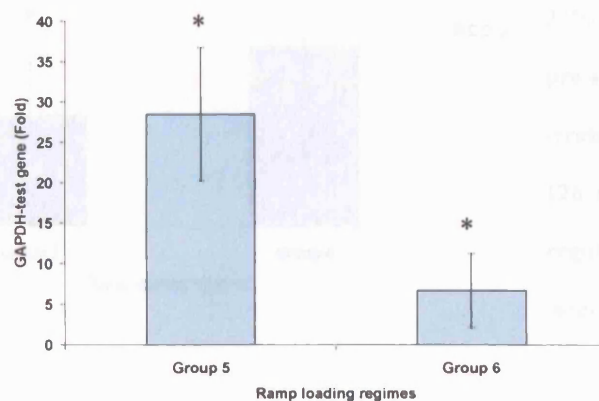
Figure 75. TIMP-2 gene expression is shown for HDF's embedded within collagen constructs (with 10% FCS; n=3) subjected to 5% pre-strain and also a further 10% strain at t=12h, over 1h and/or 12h (Group 5 and 6). Significant regulation is indicated by the asterisk (*)

TIMP-2 expression was consistently up-regulated with 10% and 20% FCS when constructs were pre-strained at t=0h (i.e matrix stiffness dependent) at Figure 72 and 74. However here, TIMP-2 showed down-regulation in response to ramp load rate. Crucially, MMP-2 was not significantly different (i.e independent of ramp rate)

when the same groups were compared, in contrast to Figure 72 and 74 where MMP-2 was regulated in an opposite manner to TIMP-2. *Indicating that specific metalloproteinase are very much dependent on the rate on which the ramp loads is applied.*

Lastly, the ramp load rate when 20% FCS was present only affected COL-3. COL-3 showed up-regulation with slow ramp load in 0% pre-strained constructs (Figure 76; $p < 0.05$).
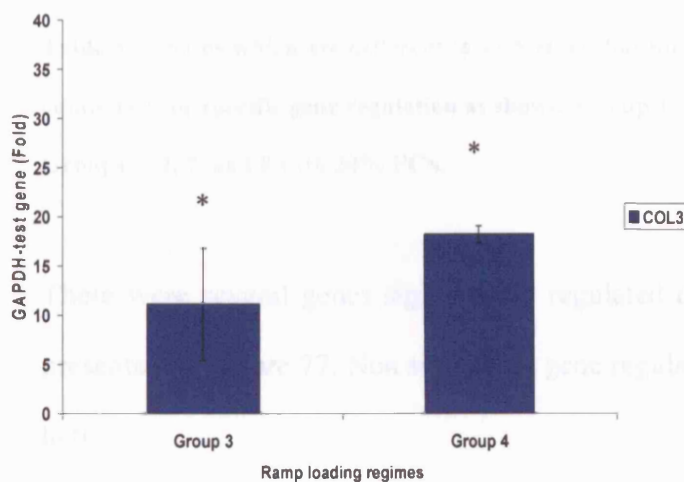
Figure 76. COL-3 gene expression is shown for HDF's embedded within collagen constructs (with 20% FCS; n=3) subjected to 0% pre-strain and also a further 10% strain at t=12h, over 1h and/or 12h (Group 3 and 4). Significant regulation is indicated by the asterisk (*)

Effect of FCS doubling on cell seeded collagen constructs (10% vs 20%)

The final part was to investigate gene regulations due to FCS level elevation. The groups compared here are listed on table 8.

FCS	Group 1 vs Group 3
	Group 2 vs Group 4
	Group 5 vs Group 7
	Group 6 vs Group 8

Table 8. Groups which are different in FCS levels but identical in stiffness and ramp rate were compared for specific gene regulation as shown. Group 1, 2, 5, and 6 were with 10% FCS where Groups 3, 4, 7, and 8 with 20% FCS.

There were several genes significantly regulated due to doubling of FCS, and are presented on Figure 77. Non significant gene regulation is not discussed or presented here.

Expression of MMP-2 and TIMP-2 were both significantly up-regulated (Figure 77a and 77b; $p < 0.05$ both) with 20% FCS, when no pre-strain and a slow ramp load was applied (Group 4). In contrast, MMP-3 and COL-1 (Figure 77c and 77d) were significantly down-regulated (both $p < 0.05$) at the same Group when 20% FCS was present.

It is important to note that this is the only time where MMP-2 expression was up-regulated, when ramp loading was applied, indicating that FCS levels are important regulators of specific matrix genes.
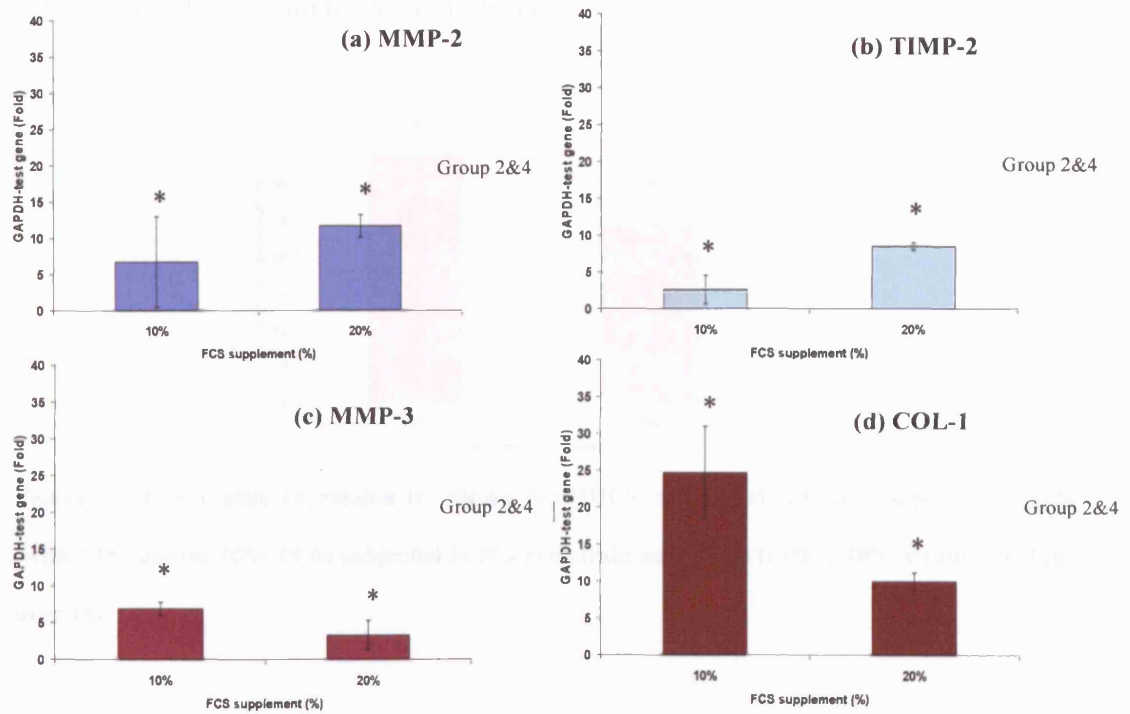
Figure 77. Gene expressions shown from HDF's embedded within collagen constructs (with 10 and/or 20% FCS) subjected to 0% pre-strain and also a further 10% strain at t=12h, over 12h (n=3). (a) MMP-2, (b) TIMP-2, (c) MMP-3, and (d) COL-1.

149

Finally, IGF-1 is shown here (Figure 78) to be stiffness dependent when the two serum levels were separately investigated. Figure 78 shows significant down-regulation, with 20% FCS, (p=0.05) of IGF-1 expression at 5% pre-strained constructs and fast ramp loaded (Group 7).
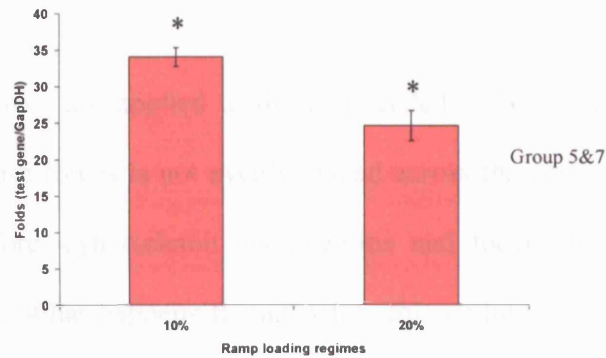
Figure 78. IGF-1 gene expression is shown for HDF's embedded within collagen constructs (with 10% and/or 20% FCS) subjected to 5% pre-strain and also a further 10% strain at t=12h, over 1h.

Discussion

Cells in the musculoskeletal tissues experience a variety of mechanical stress through physical distortion (strain) of the ECM, in which cells are embedded. In addition to those forces, each individual cell will generate an internal isometric tension under which physiological processes take place [Tomasek et al., 2002].

When external forces are applied to the cell seeded ECM construct or tissue, the distribution of those forces is not evenly spread across the cell surface membranes. As discussed before, cytoskeleton via integrins and focal adhesions take on the forces transferred. What happens though when this cellular force balance is being forced to higher/lower levels? For example, in this study we used two levels of different collagen stiffness by pre-straining the constructs 0 and 5% and allowed the cells to reach internal homeostasis (as seen on the CFM by a ''plateau'' phase). However, the last part of this study aimed to disrupt this inherent force balance, by applying a ramp load after the plateau has been reached (at 12h). By doing so we wanted to investigate any alterations in cellular contraction forces and specific gene expression. It is crucial to investigate the expression of specific ECM genes such as MMPs and TIMPs, since excessive forces to constructs/tissues may lead to non-physiological responses.

When ramp loads were applied to constructs, in this study, effectively the collagen stiffness was increased further. Initial pre-strain was shown here to significantly increase collagen stiffness [Chapter 3; Karamichos et al., 2006]. Following that, 12h of cellular contraction (as described in this study) would mean a further increase in collagen stiffness until tensional homeostasis was reached. All cell types used in this study reached a 'plateau' within 8-10h therefore in terms of mechanical integrity

constructs had reached a steady-state. At 12h, a fast and/or slow ramp load was applied to these constructs which would immediately mean, that the resident cells, will reach a new internal mechanical steady-state. Despite the increased construct stiffness, imposed by ramp loading the collagen matrix, cells did not generate measurable contraction forces, suggesting that cells will respond to the increasing ECM stiffness by other means.

Despite the importance of mechanical forces in tissue homeostasis, the mechanisms how cells convert those mechanical signals into a specific response in ECM gene expression patterns are poorly understood [Renedo et al., 2005]. Imposing external forces on the ECM will lead to changes of the stress levels applied to the integrin receptors of the surface membranes of the cells and will produce physical alterations of the construct and/or tissue in terms of cellular responses. Consequently, changes of force balance across integrins will result in changes in the transcription of specific genes.

Results here showed clear mechano-sensitivty of specific genes. It is important to note that strains applied here and total matrix stiffness was relatively small, however within a loosely woven collagenous substrate [Prajapati et al., 2000] cells were shown to be sensitive to such loadings.

Rate dependence

HDFs showed significant regulation of ECM regulatory genes when a fast ramp was applied to stiff constructs, at 12h. Previous study [Prajapati et al., 2000] has shown significant up-regulation of MMPs enzyme levels when external mechanical

stimulation was applied, such as MMP-2 and MMP-9, following cyclical load. The same study also reported a down-regulation of MMP-3 where here we showed that MMP-3 can be up-regulated if FCS levels are increased.

Prajapati et al [2000] has also suggested that cells are stress-shielded by their own matrix which would agree partly with the data shown here. However, it is likely that cells hold an internal threshold where anything above that (i.e higher external loads) will initiate the reverse of the process. An example of this speculation is the peak expression of genes after 5% pre-strain and return to 0% levels when 10% pre-strain was applied. It is possible that genes which showed no significant responses here due to ramp loading, have already reached a threshold and alternative pathways may have been activated.

Previous work has shown that there are optimal levels of cell-substrate adhesion i.e, where the binding is neither too strong nor too weak [Palecek et al., 1997]. Therefore, the effect of ramp loading will be more noticeable at sites where cells are strongly attached to the ECM, as mechanical signals will be able to be transferred and ultimately translated.

The limitation of this study was that gene expression was investigated at the end of 24h and therefore differences in expression might exist at earlier points. We showed here that HDFs have a significant delay (Chapter 4) until they actually attach and therefore contract the collagen matrix. This attachment delay might be, on its own, a significant regulatory effect for specific gene expressions.

Conclusions

► Ramp loads applied to increasingly stiffer collagen constructs, at 12h, showed no further measurable contraction force generated by the seeded cells.

► Fast ramp loads applied to increasingly stiff matrices showed regulation of MMP-2, MMP-3, COL-1 and TIMP-2 genes, at the presence of 10% FCS.

► Slow ramp loads applied to increasingly stiff matrices showed regulation of MMP-2, COL-1, COL-3, TIMP-2, and IGF-1 genes, at the presence of 20% FCS.

Chapter 8: hBMSC specific mechanoresponsive gene regulation: Stiffness and ramp loading dependence

hBMSCs gene regulation dependent on stiffness and ramp loading regimes

The previous chapter showed that HDF genes, linked to functional ECM remodelling, were regulated differentially in response to a combination of changes in collagen matrix stiffness and external loading regimens. This part of the study was conducted to investigate the effect, in terms of gene regulation, of controlled combinations of the same ramp loading regimens applied to increasingly stiff collagen constructs, this time seeded with hBMSC, where multipotent stem cells respond differently when compared to terminally differentiated cells i.e. fibroblasts.

Pre-strain and ramp loading regimens were identical as described before. Briefly, two different ramp loading regimes were applied: a) 10% strain over 1h, and b) 10% strain over 12h, on constructs cultured with 10% and 20% FCS. Molecular outputs were investigated here according to the same three different comparable groups:

a) Stiffness dependence

b) Rate dependence

c) FCS dependence

There was again no force generation recorded by hBMSCs after 12h. Genes tested here were the same as stated in Table 4 (Methods).

Effect of stiffness on ramp loaded collagen constructs

The effect of ramp load was investigated on gene expression in increasingly stiff seeded collagen constructs. Real-time PCR analysis showed that there was no significant change in gene expression in relation to increasing matrix stiffness (i.e pre-strain). Results were in complete contrast to HDFs where MMP-2 and COL-1 were down-regulated and TIMP-2 and COL-3 were up-regulated under the same regimes (Chapter 7), *suggesting a differential matrix gene regulation by hBMSCs (i.e cell lineage dependence) when subjected to ramp loads.*

Effect of ramp loading rate for collagen constructs

Here we investigated specific remodelling gene regulation due to the effect of different ramp loading rate, on increasingly stiff hBMSC seeded collagen constructs. Again marker gene expressions that were not significantly altered, as listed in table 4 is not shown. Groups compared are listed in Table 7 (Chapter 7).

The differential ramp load rate, in the presence of 10% FCS, only affected MMP-2 expression. MMP-2 was significantly (p=0.05) down-regulated in 5% pre-strained constructs under slow ramp load (Figure 79: Group 6).
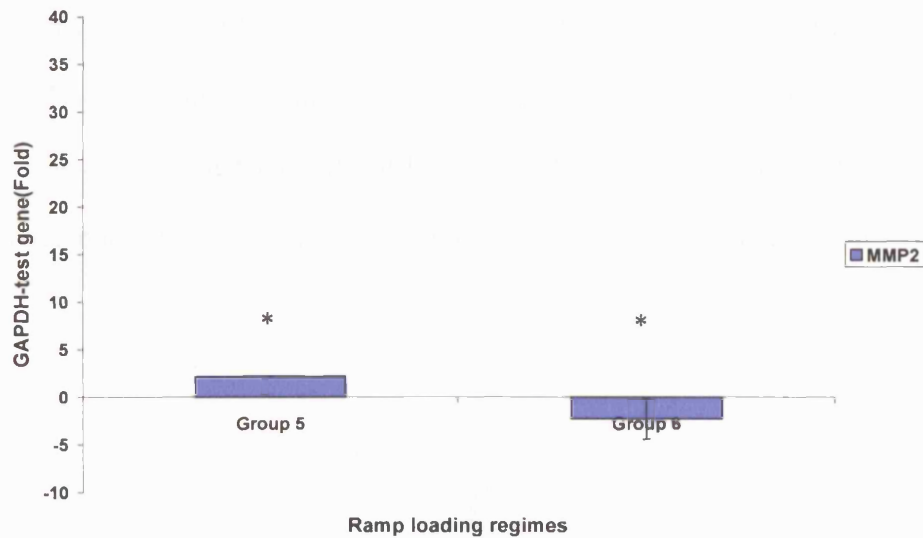
Figure 79. MMP-2 gene expression is shown for hBMSCs embedded within collagen constructs treated with 10% FCS, at 5% pre-strain followed by 1h (Group 5) and 12h (Group 6) ramp load.

In contrast to HDFs (see Figure 72) where MMP-2 was down-regulated due to increased matrix stiffness in the presence of 10% FCS, hBMSCs were sensitive to ramp loading rate in stiffer (5% pre-strain) constructs. *This suggested again a cell-lineage dependent on matrix gene regulation.*

FCS levels regulate marker gene expression

Several genes were significantly regulated when FCS levels were doubled from 10%

to 20%. The same ramp loading regimes were applied. The hypothesis under test was

that FCS levels will substantially influence mechano- responsive genes. This was

tested over both slow (over 12h) and fast ramp (over 1h) loading regimes (Figure

80).
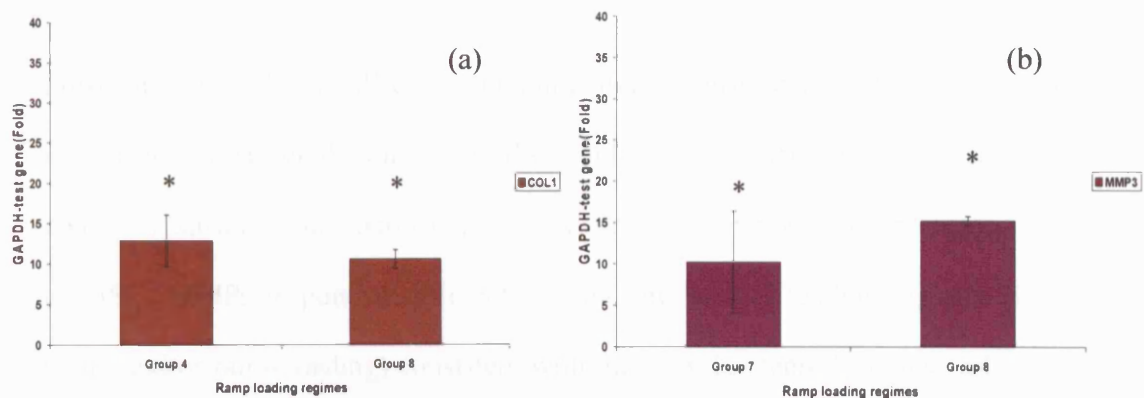
Figure 80. hBMSC's seeded in collagen constructs subjected to ramp loading over 1h and 12h.
(a) Shows COL1 regulation for 0% (Group 4) and 5% (Group 8) pre-strained constructs when
subjected to slow ramp loading (12h). (b) Shows MMP3 regulation for 5% pre-strained
constructs when subjected to both fast (1h; Group 7) and slow (12h; Group 8) ramp loading.

There was a small but significant fall in COL-1 expression (Figure 80a; p=0.05) in

response to increased stiffness (5% pre-strain) and slow ramp load over 12h (Group

8). In other words combination of the increased, 310Pa, stiffness together with a

slow ramp load led to down-regulation of COL-1, indicating that construct stiffness

is important. The previous chapter, with HDF seeded constructs identified the

complete opposite pattern of regulation of COL-1, suggesting that this apparent

matrix mechano-remodelling response is cell lineage dependent.

Figure 80b shows MMP-3 expression was up-regulated (p=0.05) with slow ramp load (over 12h) in constructs with 310Pa stiffness (i.e pre-strained by 5%). This compares with a similar MMP-3 up-regulation in HDFs under same matrix stiffness but without ramp loading. *This suggests that responses in MMP expression are cell lineage dependent. These may correlate to similar cell differences in matrix remodelling.*

Pre-strain results for hBMSC's seeded in collagen constructs in 20% FCS, showed a significant down-regulation of MMP-2 and MMP-9 between 310Pa and 380Pa construct stiffness. In contrast those two genes were not mechano-responsive in hBMSC. MMPs responded differently to the mechanical loading regimes (i.e pre-strain and/or ramp loading) consistent with the idea that there is a close relationship between matrix remodelling by cells and mechanical loads. Furthermore, the differences in gene expression, with ramp loading in the presence of 20% FCS, suggest that the sensitivity of mechano-responses in these cells is highly FCS dependent. 20% FCS presence was shown (Chapter 5) to increase the contraction rate at early stages (traction phase) compared to 10% FCS, which may correlate with the differential gene regulation shown here.

In conclusion, this indicates that cellular responses to external mechanical loads/strains can be regulated by number of factors (FCS levels, pre-strains and ramp loads). Furthermore, increased attachment at early stages (traction phase) may be leading to these responses. These effects may act through changes in gene expression of key factors in matrix remodelling.

Effect of doubling FCS levels on marker gene expressions

The effect of different serum levels (10% and 20%) on hBMSC seeded collagen constructs was investigated. Groups compared are listed in Table 4 (Methods). Several genes were significantly regulated with increased FCS.

MMP-2 expression (Figure 81a) showed a significant up-regulation (7 fold, p= 0.05) with 20% FCS when constructs were 310Pa stiff and also ramp loaded slowly over 12h. This is in contrast to HDFs where MMP-2 was up-regulated at non pre-strained constructs, though with the same ramp load rate (over 12h).
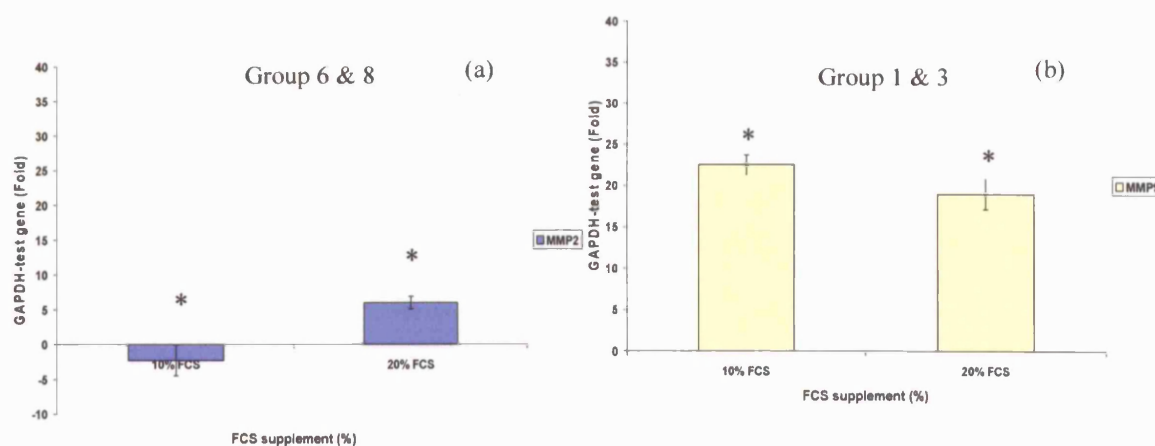
Figure 81. **Gene expressions shown for hBMSC's embedded within collagen constructs, supplemented with 10% and/or 20% FCS, following 0% or 5% pre-strain at t=0h and ramp loading at t=12h over 1h or 12h (n=3). (a) MMP2 - 5% pre-strain and 10% ramp loading over 12h and (b) MMP9 - 0% pre-strain and 10% ramp loading over 1h.**

Figure 81b shows up-regulation (3 fold, p=0.05) of MMP-9 with 10% FCS, under 0% pre-strain and a fast (over 1h) ramp load. The only time that MMP-9 was previously regulated was between the two stiff matrices (5 and 10% pre-strain) and with 20% FCS present. HDFs and ramp loads did not showed significant regulation of MMP-9. The expression of MMP-9 is not shown for most of the cell lines and

literature on its regulation is very limited (as described later), hence these findings are very important for the role of the MMP-9 on the collagen matrix degradation.

In conclusion, different levels of FCS will give differential gene regulation (MMP-2 at 10% FCS and MMP-3 and COL-1 at 20% FCS) altering the responses to mechanical stimuli as well as matrix stiffness.

Discussion

Cell-cell interactions play an important role in tissue formation and regeneration, both in embryonic and adult stages. Mesenchymal cells have been shown to improve performance of composite skin substitutes in the animal models [Gulsun et al., 2004; Kataoka et al., 2003; Spees et al., 2003]. Lacroix and co-workers [2002] have also predicted that stem cells have a considerable effect on the healing pattern and healing rate.

Experimental studies concentrating on tissue repair [Kelly et al., 2005] have shown that several physical factors such as the size and location of the defect, or the type of loading, can influence the quality, type and durability of the repair tissue. Also the local mechanical environment of mesenchymal stem cells was found to influence cellular proliferation, differentiation, and the subsequent remodelling or degradation of the repair tissues, within an osteochondral defect, as outlined by Kelly et al [2005].

The process of wound healing requires coordinated cellular activities, including phagocytosis, chemotaxis, mitogenesis, differentiation, synthesis and reorganisation of collagen and ECM [Clark, 1996]. New studies report that mobilised bone marrow hBMSCs do actively participate in skin wound healing [Fathke et al., 2004; Harris et al., 2004]. Collagen deposition and epithelial differentiation is believed to take place under conditions of mesenchymal epithelial communication [Harris et al., 2004; Maas-Szabowski et al., 2001; Aoki et al., 2004]. This makes hBMSCs good candidates for TE applications.

162

This study showed a significant regulation of genes important in ECM remodelling and turnover when stiff constructs, ramp rate and FCS levels were combined. This has major implications in the area of TE, specifically in tissues where load bearing is a major function. De Palma et al [1966] for example observed that cartilage formation in defects in non weight bearing areas occurred at a slower rate, and was quantitatively inferior to the cartilage that formed in weight bearing areas. Other studies including O'Driscoll et al [1988] demonstrated that continuous passive motion helped to heal injured rabbit knee joint articular cartilage repaired with autogenous periosteal grafts containing MSCs [O'Driscoll et al., 1988]. The question really is how and when do cells perceive these controlling loads through their dense ECM.

Stem cells are known for their unique intrinsic characteristics enabling them to control cell replacement during homeostasis and tissue repair [Daniels et al., 2001]. This makes them highly attractive for use in TE applications.

Conclusions

▶ MMP-2 was the only gene significantly regulated showing down-regulation at stiffer matrices when a slow ramp was applied, indicating overall matrix stiffness dependence as well as ramp load rate at stiff matrices, in hBMSCs.

▶ COL-1 showed stiffness dependence, with 20% FCS, when constructs were slowly ramp loaded.

▶ MMP-3 showed ramp rate dependence, with 20% FCS, at stiffer constructs.

▶ MMP-2 and MMP-9 showed differential dependence on FCS levels. At stiff matrices and slowly ramp loaded (for MMP-2) and at non-stiff matrices and a fast ramp load (for MMP-9).

Chapter 9: GENERAL DISCUSSION

Trinkhaus [1984] suggested the role of coupling between mechanical forces and tissue growth and remodelling. Many studies have now concentrated on the effects of mechanical forces such as tension, compression, and shear stress at the cell level [Edwards et al. 1999; Huang et al., 1999; Kaspar et al 2000; Tabony et al 2002]. 3D collagen constructs have been studied for many years and much is now known about contractile forces generated by fibroblasts over time in culture [Tomasek 1984; Eastwood et al., 1994; 1996; Elsdale 1972; Bell et al., 1979; Delvoye 1991; Brown 1996]. These have been put forward as models representing wound contraction and/or morphogenesis. In this study we have used the 3D collagen constructs as an in vitro model (seeded with different cell types) to investigate the effect of external mechanical forces (uniaxial tension) on the matrix properties and the molecular response of resident cells.

External forces (i.e strain) are critical for tissue homeostasis and elicit specific cellular responses, such as gene expression and protein production [Hinz 2006; Eckes et al., 2004; Parsons et al., 1999]. The process of converting different mechanical forces into biochemical signals and integrating these signals into the cellular responses is referred to as mechanotransduction [Huang et al., 2004]. In other words, the process of passing mechanical (external) signals into the cell (internal) involves biochemical and molecular events in the cell cytoplasm, cytoskeleton and nucleus. Mechanosensing is postulated to involve many different cellular and extracellular components such as cell-matrix adhesions and cell-cell junctions [Davies et al., 1997].

To date, very few studies have been carried out on how forces are been distributed through a collagen construct. These have included the use of magnetic twisting cytometry [Eckes et al., 1998; Wang et al. 1995] and linear force magnetocytometry [Karcher et al., 2003]. With both models though, their findings were limited to deformations and stresses localised within 10μm from the site of force application. Supporting these studies, Davies et al. [1997] has proposed that mechanotransduction occurs at the local site of force application or at sites where force reaches the cell nucleus, cell-matrix adhesions, or cell-cell junctions. The strain field is far from homogeneous and widely distributed throughout the cell and therefore, it is certain that more structures are involved such as cell-matrix attachment points (i.e. focal adhesions), as discussed later.

Other models have shown and calculated cell stiffness and/or overall construct stiffness [Wakatsuki et al., 2000] however; the limitation for these studies is that the cell-ECM system has been investigated as one, which adds to the complexity of mechanotransduction. Cell interactions with the ECM are critical for the mechanical regulation of cell activity and as well as for connective tissue homeostasis [Tomasek et al., 2002; Brown et al., 1998] though to get around the problem of complexity of the cell body contribution to collagen construct mechanics/stiffness, the author devised a system to selectively exclude the cells [Karamichos et al., 2006] so matrix stiffness can be accurately quantified.

The important aspect of this study is that it extends our limited knowledge of mechanotransduction derived from cell-matrix culture models towards understanding (1) the effect of mechanical forces on resident cells in bio-artificial matrices in vitro

(2) how mechanical forces of a certain type, amplitude, and duration can elicit specific cellular responses for potential in vitro applications in the TE area, and (3) what combinations of mechanical stimulation produces possible predictable cellular responses. Understanding of mechanotransduction is vital for successful transition from in vitro to in vivo models. It is important to identify the critical transduction process at cellular and molecular level. However, to control such process is clearly much more complicated than any single signalling molecule or even any individual transduction pathway. The same stimulus (external or intracellular) can produce an entirely different response dependent on both the chemical and the mechanical context in which signal transduction proceeds.

External forces and cellular responses

For some years now it is clear that many cell types are sensitive to mechanical forces from their surroundings [Jones et al., 1992]. The magnitude and type of forces that are needed, in in vitro ECM models, to elicit specific cellular responses is not clear. The amplitude, length, and time where these external forces are applied vary and depend very much on models/tissues under investigation. Stiffness, integrity and strength of a tissue or scaffold depend on the rate at which forces are applied and will vary widely between tissues [Frank et al., 1985].

Previous studies have used ex vivo and in vivo models to investigate the effect of tissue stretch on subcutaneous tissue fibroblast morphology [Langevin et al., 2005], highlighting the importance of mechanotransduction in determining function at cellular level.

In the first part of this study, the applied force rate was constant but the magnitude was used to regulate stiffness of the collagen matrix, which led to unique cell type responses, i.e. contraction "signature".

Focal adhesions and ECM interactions following pre-strain

The human body, from an engineering point of view, is a load-transmiting mechanism [Kenedi et al., 1975]. Human tissues such as tendons (muscle to bone) and ligaments (bone to bone) largely transmit loads across joints. Consistent with this function, their structure of aligned collagen fibres provides for load bearing primarily in one direction and contributes to highly anisotropic material properties [Lynch et al., 2003].

At the cellular scale, cells embedded in a particular tissue appear to probe the stiffness as they attach and pull on their surrounding ECM [Bischoff et al., 2003]. Such processes are highly dependent on myosin-based cytoskeletal 'motor' contractility and cellular-matrix integrin based adhesions [Discher et al 2005]. When external forces are applied, it is proposed that cells respond to the stiffness changes of the substrate (in this study collagen) by adjusting their adhesion strength, cytoskeleton and tensional homeostasis [Brown et al., 1998]. Several findings suggest that cells are more responsive to **changes** in force. Previous chapters showed that application of an initial rapid strain (pre-strain) can elicit specific cellular responses (reduced contraction), suggesting that **timing** of strain application is equally important to cellular responsiveness.

Cell adhesion to the ECM is central to development and the organisation, maintenance, and repair of tissues by providing anchorage and triggering signals that

direct cell survival, migration, cell cycle progression, and expression of differentiated phenotypes [De-Archangelis et al., 2000; Danen et al., 2003].

However, in vivo, a cell-mediated control of tension must be a far more complex balance of forces due to the composite ECM material properties (including elastin and proteoglycans) [Brown et al., 1998]. Abnormalities in adhesive interactions are often associated with pathological states, including wound healing defects [Wehrle-Haller et al., 2003; Jin et al., 2004]. Deficient healing exists when there is insufficient deposition of connective tissue matrix and the tissue is weakened to the point where it can fall apart [Langevin et al., 2005]. Therefore it is important to trigger regeneration of injured tissues/sites. Regeneration is the process that occurs when there is loss of structure and function but the organism has the sophisticated capacity to replace that structure by replacing exactly what was there before the injury. Good examples of regeneration ability are the salamander and crab, which can regenerate tissues. However, for mammals the ability to regenerate has been lost and only very limited amount of regeneration can occur at a few sites of injury [Langevin et al., 2005]. Hence, understanding how cells sense and respond to their surrounding environment is only the beginning of a potential breakthrough in tissue engineering and tissue remodelling.

A common pathway for force transmission is via focal adhesions. Integrins, which form bonds with various ECM proteins (fibronectin and vitronectin), constitute primary pathway and have been viewed as major candidates of mechanosensing [Huang et al., 2004]. On the intracellular side, proteins such as paxillin and focal adhesion kinase (FAK) tend to localise to focal adhesions [Huang et al., 2004].

These have multiple binding patterns and are very important to focal adhesion strengthening and mechanotransduction. This is comparable to doing weight lifting with one hand instead of two. Two hands will stabilise the load application and ensure good force transmission from the muscle (motor) to the bar, to complete the lift up.

Generally the accepted model for cell-ECM adhesion strength, proposed by Lotz et al. [1989], postulates a three-step sequence consisting of initial integrin-ligand binding followed by adhesion strengthening [Lotz et al., 1989]. The strengthening response arise from 1) increases in adhesive molecules along the length of the border of cell-substrate contact area (spreading), 2) receptor recruitment to anchoring sites so adhesions are stronger, by means of detachment from substrate resistance (i.e all forces will be equally distributed across the bonds, clustering; and the bonds would break at the same time), and 3) coupling of cell surface receptors leading to FA strengthening.

The mechanical aspects of adhesion remain poorly understood and in this study have not been investigated in detail. However, when using collagen ECM, it is accepted that collagen fibrils can transmit signals through integrins [Schlessinger, 1997]. Collagen is a flexible but inextensible fibrillar structure with a stiffness of 30-100 Pa as measured by a dynamic mechanical analyzer [Wang et al., 2003] and lies far lower that of a normal cell culture dish which is non flexible and with a stiffness of more than 1GPa [Wang et al., 2003]. The two of them represent the two extremes away from physiological, human tissue stiffness; however external stimulation can increase collagen stiffness and hence alter focal adhesions. Previous data has shown

that increased mechanical force to integrin-mediated adhesions increased the stiffness of the cell [Wang et al., 1993; Choquet 1997]. Based on these findings Wang et al [2003] later suggested that physical properties of collagen fibrils cause down-regulation of focal adhesion complex proteins, which in turn resulted in the disappearance of functional focal adhesions and stress fibres. This, according to the authors, may contribute to a decrease in intracellular tension. It is therefore certain that such a decrease in intracellular tension will lead to alterations in the signalling pathways and result in differential cellular responses to the new tension steady-state. Wang et al [2003] finally suggested that focal adhesion complex proteins, like FAK were decreased within 1h when cells were seeded in collagen constructs. FAK is a cytoplasmic non-receptor tyrosine kinase located close to focal adhesions and may be key to integrating signalling to cells from the ECM-integrin and growth factors. This suggests that decrease in focal adhesion complex proteins will be altered by external forces applied to collagen constructs.

Riveline et al [2001] applied mechanical force to vinculin-containing dot-like adhesions at the cell edge using a micropipette. Local centripetal pulling led to local assembly and elongation of these structures and to their development into streak-like focal contacts. They therefore suggested that integrin-containing focal complexes behave as individual mechanosensors exhibiting directional assembly in response to local force. To an extent this phenomenon is the inescapable effect of single force vectors on compliant anchored materials.

From all the above studies we can postulate the following model, on collagen stiffness and cell-adhesion (Figure 82).
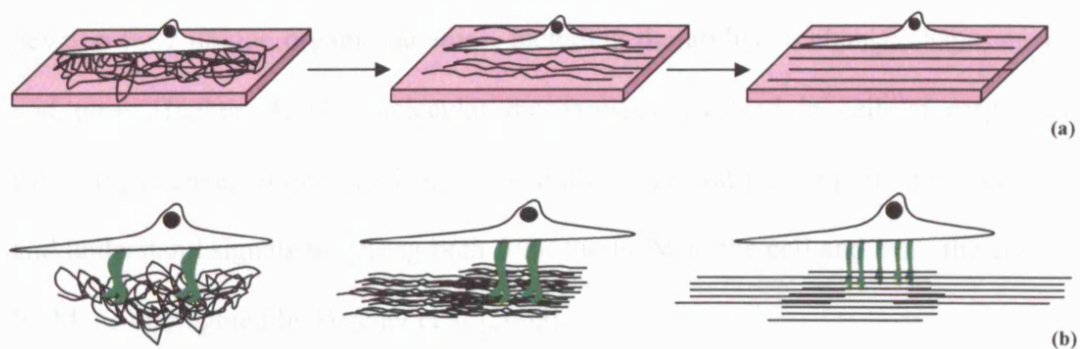
Figure 82. a) Diagrammatic representation of increasingly aligned and stiffer, cell seeded collagen constructs, using pre-strain. b) Focal adhesion model is shown. Focal adhesions get stronger as collagen matrix stiffness increase following application of pre-strain.

Collagen stiffness and alignment will increase as pre-strain increases (Figure 82a) and according to this model, resident cells will adhere to the ECM by forming stronger focal adhesions as indicated in figure 82b. Putting this into perspective, increase of pre alignment and stiffness in the collagen matrix (at t=0h) will lead to differential response by the cells, in terms of signalling pathways activated due to altered focal adhesions, following mechanical loading.

This study has tested three different cell types and many similarities in terms of responses to ECM stiffness (i.e reduced contraction as stiffness increased) were shown, however in terms of gene markers regulation differential outputs were shown, as discussed below.

From mechanotransduction to gene expression

Molecular cell biology is guided by the desire to understand how cells and tissues develop their unique organic qualities, including the ability to change shape, move and grow [Ingber, 1998]. Molecular mechanisms involved in cellular responses following changes in matrix stiffness are still unclear, but it is important to examine and understand signals travelling both from the ECM to the cell and from the cell to ECM, as highlighted by Discher et al [2005].

A variety of studies have shown the significance of external load application and their effect in tissues. Application of loads to bone [Burger et al., 1999; Hsieh et al., 2001; Rubin et al., 2000; Brighton et al., 1991; Huiskes et al., 2000], ligament [Huiskes et al., 2000; Bhargava et al., 1997], and tendon [Arnoczky et al., 2002; Banes et al., 1995; Hannafin et al., 1995] have been implicated in the maintenance of tissue homeostasis. This is thought to occur through the transfer of tissue strain to the cell cytoskeleton that, in turn, initiates a mechanotransduction signalling response [Banes et al., 1995; Ingber et al., 1995]. Mechanotransduction can be regulated outside the cell by extracellular matrix proteins and proteolytic enzymes [Laiho et al. 1989]. More specifically, proteolytic degradation of the ECM is an essential feature of repair and subsequent remodelling stages [Gailit et al., 1994]. MMPs are known for their role in wound healing, angiogenesis, embryogenesis, and in many pathological processes such as tumor metastasis [Stetler-Stevenson 2001]. Studies have shown a correlation between cytoskeletal architecture and MMP gene expression, where agents that altered the actin-based cytoskeleton produced a parallel induction of MMP-1 and MMP-3 expression [Werb et al., 1977; Aggeler et al., 1984; Werb et al., 1986; Unemori et al., 1986]. Such cytoskeletal changes are intricately associated with cell shape and substrate attachment. In addition, changes

in the cytoskeletal architecture have been reported induce changes in protease activity, although a number of cytokines have also been implicated [Werb et al., 1993]. Clearly therefore, protease production is essential in ECM remodelling. Understanding of the process and the specific proteases involved in mechano-responsive changes will be critical in in vitro and/or in vivo models.

A well studied model is the fibroblast seeded 3-D collagen lattice. Fibroblasts embedded in these constructs affect the surrounding ECM through new synthesis, deposition, and remodelling [Kurkinen et al., 1980; Welch et al., 1990]. Prajapati et al [2000] have shown the linkage between extracellular protease productions and defined mechanical loading on HDFs, concluding that mechanical loading elicited complex and substantial changes in matrix modifying proteases.

Any cell type that is embedded in these construct will be sensitive to relatively small amounts of external loading, because of the high compliance of the collagenous matrix. This study has concentrated on the effect of external mechanical loads and how this affects specific mechano-responsive genes. Significant effects on gene regulation were shown, despite the relatively small external loading (involved 0%-10% strain). In agreement, with other studies [Brown et al., 1998; Burt, 1992] suggesting that HDFs regulate any increase or decrease in matrix tension through protease production.

Implications for gene regulation at the in vivo level have been shown in a number of studies, including Dupuytren's disease [Tarlton et al., 1998], where notably significant differences in MMP levels were found which suggested the existence of a

threshold in terms of gene expression. These findings are in agreement with the data shown, in this thesis, where most of the significant genes tested showed a threshold of maximum or minimum mechano-responsiveness. These findings suggest that the external loading resulted in an increase, in the MMP levels, which would be likely to weaken the collagen fibres (eg. by depolymerization) or result in removal of non collagenous ECM elements (proteoglycan, fibronectin, laminin). In addition, Palecek's [1997] work on fibroblast locomotion has shown that there are optimal levels of cell-substrate, i.e., where binding is neither too strong to prevent retraction nor too weak to allow traction reinforcing the statement of threshold gene regulation existence.

Stem Cells and gene expression

The therapeutic efficacy of hBMSCs in tissue engineering and regenerative medicine is determined by their unique biological, mechanical and physicochemical characteristics that are yet to be fully explored. During embryonic development, physical forces exerted by hBMSCs organize ECM into a wide variety of structures and mechanical properties giving rise to different tissues [Stopak et al., 1982; Stopak et al., 1985; Bard et al., 1975; Bard 1977]. Similarly, wound contraction and remodelling of connective tissue matrix are strongly influenced, if not dominated by mechanical interactions between fibroblasts and the collagen ECM [Ehrlich 1988].

Although hBMSCs are not the major cell type in the normal dermis recent studies showed that participation of these cells in skin wound healing, e.g. by the mechanism of homing and differentiation [Kataoka et al., 2003; Korbing et al., 2003]. Aoki et al. [2004] showed that bone marrow stromal cells accelerated epidermal regeneration better than preadipocytes and dermal fibroblasts [Aoki et al.,

2004]. hBMSCs participation in skin wound healing has been further investigated by other groups, where collagen deposition, epithelial and epidermal differentiations are believed to take place under the control of mesenchymal-epithelial communication [Spees et al., 2003; Harris et al., 2004; Maas-Szabowski et al., 2001; Aoki et al., 2004].

Mechano-remodelling of collagen is a key component of the process of cytomechanical control. This comprises altered load transmission by asymmetric (fibrous) matrix structures and production/revision of that structure by resident cells, to which stem cells may contribute into wound healing process in tissue engineering [Tomasek et al., 2002].

Previous studies have shown that cytoskeleton disruption resulted in a 4-fold tether length increase in fibroblasts but had no effect in hBMSCs, indicating a weak association between the cell membrane and hBMSC actin cytoskeleton [Titushkin et al., 2006]. Despite the fact that this is a 2D observation and it cannot directly be compared to our 3D system, it may be a significant difference between hBMSCs and fibroblasts. hBMSCs in this study showed differences in response to increasingly stiffer matrices when FCS levels were increased. It will be valuable to further investigate and compare morphological changes, particularly related to the actin cytoskeleton in 3-D, when serum levels are altered.

This study has introduced a novel way of increasing the stiffness and organisation of collagen ECM. The development of the t-CFM by Eastwood et al [1998] has allowed accurate measurement of average contractile forces generated by cell populations

within 3D constructs, with or without the application of defined external loads. This study investigated the influence of matrix stiffness on3 different cell type contraction as well as effect on the regulation of key matrix-remodelling genes regulation. Differences between these effects on fibroblasts from different anatomical sites were also investigated, highlighting altered responses to similar environmental stimuli. These findings have implications in the basic understanding of cellular processes involved in normal remodelling, wound repair and related in the processes important in the engineering of soft connective tissues.

Summary

The table below summarises all the results showed in this study.

10% FCS	Force generated	Molecular Outputs	Ramp loading molecular outputs
HDFs	• Decreased at higher stiffness	• MMP2-Peak at 5% pre-strain • TIMP2-Progressive increase with stiffness • COL1-Progressive increase with stiffness • COL3-Progressive increase with stiffness • IGF1-Progressive increase with stiffness	• MMP2 down-regulated at stiff matrices with slow ramp • TIMP2 up-regulated at stiff matrices with slow ramp • COL1 down-regulated at stiff matrices with slow ramp • COL3 up-regulated at stiff matrices with slow ramp • IGF1 down-regulated at stiff matrices with slow ramp
HNFFs	• Decreased at higher stiffness	• No significant difference due to stiffness	• N/A
hBMSCs	• Decreased at higher stiffness	• MMP2-Peak at 5% pre-strain • MMP-3 Peak at 5% pre-strain • COL3- Increase at stiffer matrices	• MMP2 down-regulated at stiff matrices with slow ramp when compared to fast ramp load
20% FCS	**Force generated**	**Molecular Outputs**	**Ramp loading molecular outputs**
HDFs	• Decreased at higher stiffness	• MMP2- Peak at 5% pre-strain • TIMP2-Peak at 5% pre-strain • COL1- Peak at 5% pre-strain • MMP3- Peak at 5% pre-strain • IGF1- Peak at 5% pre-strain • COL3- Progressive decrease with stiffness	• MMP2 down-regulated at stiff matrices with fast ramp • TIMP2 up-regulated at stiff matrices with fast ramp • COL1 up-regulated at stiff matrices with fast ramp • MMP3 up-regulated at stiff matrices with fast ramp • IGF1 up-regulated at stiff matrices with fast ramp
HNFFs	• N/A	• N/A	• N/A
hBMSCs	• Remained the same at all stiffness	• MMP9- Peak at 5% pre-strain • COL1-Peak at 5% pre-strain • COL3- Lower at 5% pre-strain • IGF1- Lower at5% pre-strain	• COL1 down-regulated at stiff matrices with slow ramp • MMP3 up-regulated at stiff matrices with slow ramp compared to fast ramp load
1h FCS Starvation	**Force generated**	**Molecular Outputs**	**Ramp loading molecular outputs**
HDFs	• Decreased at higher stiffness	• N/A	• N/A
HNFFs	• Decreased at higher stifness	• No significant difference due to stiffness	• N/A
hBMSCs	• N/A	• N/A	• N/A

Table 9. Main experimental results for all three cell types (HDF,hBMSC and HNFF) are summarized here. Forces generated and molecular outputs under different conditions are shown.

Future work

• Improvements on the t-CFM hardware can be made in order to make it more user-friendly.

• Work on cell-matrix interactions, concentrating on integrins, will give a valuable inside on to how exactly cells perceive the concept of different in stiffness matrices.

• Following the gene expression study, here, protein levels can be examined for these cell types.

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