Development and Characterisation of 3D Skeletal Muscle Constructs under Defined Mechanical Regulation

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Acknowledgements

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Abstract

It has been shown that the IGF-I gene is spliced in response to mechanical signals producing forms of IGF-I which have different actions. To study the roles of environmental mechanical cues on gene programming and splicing in developing muscle, an *in vitro* 3D cell culture system was employed. C2C12 skeletal myoblasts were grown in 3D collagen cultures. The presence of this extracellular matrix component, the application of uniaxial strain (produced endogenously by the myoblasts) and a high concentration of cells was shown to enhance differentiation in these cultures.

These differentiated myotube cultures were then subjected to different regimens of exogenous mechanical strain. IGF-IEa, which initiates the fusion of myoblasts to form myotubes, was found to be constitutively expressed in myoblasts and myotubes and its expression up-regulated by a single ramp stretch of one hour duration but reduced by repeated cyclical stretch. In contrast, MGF which is involved in the proliferation of mononucleated myoblasts, that are required for secondary myotube formation and to establish the muscle satellite (stem) cell pool, showed no significant constitutive expression in static cultures, but was up-regulated by a single ramp stretch and by cycling loading. The latter types of force simulate those generated in myoblasts by the first contractions of myotubes. These data indicate the importance of understanding the physiological signals that determine the ratios of splice variants of some growth factor/tissue factor genes in the early stages of development of skeletal muscle.
## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title Page</td>
<td>1</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>2</td>
</tr>
<tr>
<td>Abstract</td>
<td>3</td>
</tr>
<tr>
<td>Contents Section</td>
<td>4 - 5</td>
</tr>
<tr>
<td>List of Figures</td>
<td>6- 8</td>
</tr>
<tr>
<td>Introduction</td>
<td>9- 64</td>
</tr>
<tr>
<td>Overview</td>
<td>9</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>13</td>
</tr>
<tr>
<td>Dystrophin</td>
<td>30</td>
</tr>
<tr>
<td>3D tissue engineering</td>
<td>32</td>
</tr>
<tr>
<td>Development of tissue engineered skeletal muscle cultures</td>
<td>37</td>
</tr>
<tr>
<td>Mechano-transduction across the cell</td>
<td>42</td>
</tr>
<tr>
<td>Insulin-like growth factors (IGFs)</td>
<td>49</td>
</tr>
<tr>
<td>Hypothesis</td>
<td>63</td>
</tr>
<tr>
<td>Aims of study</td>
<td>63</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>65- 106</td>
</tr>
<tr>
<td>Basic Methods</td>
<td>65</td>
</tr>
<tr>
<td>Real time Quantitative PCR</td>
<td>97</td>
</tr>
<tr>
<td>Results</td>
<td>107- 163</td>
</tr>
<tr>
<td>Results section 1: Collagen Material Properties</td>
<td>107</td>
</tr>
<tr>
<td>Results section 2: Development of 3D skeletal model</td>
<td>115</td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td>-----</td>
</tr>
<tr>
<td>Results section 3: Morphological characterisation of differentiating C2C12 myoblasts in 3D</td>
<td>133</td>
</tr>
<tr>
<td>Results section 4: Skeletal myotube model responses to applied mechanical loads</td>
<td>147</td>
</tr>
<tr>
<td>Discussion</td>
<td>163-209</td>
</tr>
<tr>
<td>Original hypothesis</td>
<td>163</td>
</tr>
<tr>
<td>Conclusions</td>
<td>164</td>
</tr>
<tr>
<td>Future work</td>
<td>208</td>
</tr>
<tr>
<td>References</td>
<td>210-247</td>
</tr>
<tr>
<td>Publications</td>
<td>248</td>
</tr>
</tbody>
</table>
List of figures

Introduction

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>A representative diagram of the structure of skeletal muscle</td>
<td>14</td>
</tr>
<tr>
<td>1.2</td>
<td>Diagrammatic representation of a sarcomere</td>
<td>17</td>
</tr>
<tr>
<td>1.3</td>
<td>A diagrammatic representation of the processes involved in the repair of a skeletal muscle fibre following damage</td>
<td>24</td>
</tr>
<tr>
<td>1.4</td>
<td>A diagrammatic representation of the cell cycle</td>
<td>29</td>
</tr>
<tr>
<td>1.5</td>
<td>Diagrammatic representation of cell matrix remodelling</td>
<td>34</td>
</tr>
<tr>
<td>1.6</td>
<td>A diagrammatic representation of pro-insulin</td>
<td>51</td>
</tr>
<tr>
<td>1.7</td>
<td>Schematic of the IGF-IR</td>
<td>54</td>
</tr>
<tr>
<td>1.8</td>
<td>A schematic representation of the IGF-I gene</td>
<td>60</td>
</tr>
</tbody>
</table>

Materials and Methods

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>The three tissue layers of skin</td>
<td>66</td>
</tr>
<tr>
<td>2.2</td>
<td>Collagen gel set-up</td>
<td>69</td>
</tr>
<tr>
<td>2.3</td>
<td>A schematic diagram showing the contraction of the myoblast/myotube collagen culture</td>
<td>70</td>
</tr>
<tr>
<td>2.4</td>
<td>A schematic diagram representing the set-up of the CFM</td>
<td>71</td>
</tr>
<tr>
<td>2.5</td>
<td>The CFM instrument setup</td>
<td>72</td>
</tr>
<tr>
<td>2.6</td>
<td>This picture shows the t-CFM set-up</td>
<td>73</td>
</tr>
<tr>
<td>2.7</td>
<td>A calibration curve for a force transducer</td>
<td>74</td>
</tr>
<tr>
<td>2.8</td>
<td>Patterns of cyclical load applied to myotube tissue cultures over one hour</td>
<td>79</td>
</tr>
<tr>
<td>2.9</td>
<td>The patterns ramp loading regimens applied to myotube tissue cultures</td>
<td>81</td>
</tr>
<tr>
<td>2.10</td>
<td>Pictures showing different myoblast density plated over varying time periods and the differentiation of these cells</td>
<td>85</td>
</tr>
<tr>
<td>2.11</td>
<td>Description of experimental protocols used for PCR amplification of both IGF-Ia and MGF</td>
<td>89</td>
</tr>
<tr>
<td>2.12</td>
<td>Primers used for cDNA synthesis step and PCR step given in the 5' to 3' direction</td>
<td>90</td>
</tr>
<tr>
<td>2.13</td>
<td>Picture of the lightcycler rotor</td>
<td>91</td>
</tr>
<tr>
<td>2.14</td>
<td>Picture of dsDNA</td>
<td>98</td>
</tr>
<tr>
<td>2.15</td>
<td>The melting curve analysis for GAPDH</td>
<td>101</td>
</tr>
<tr>
<td>2.16</td>
<td>This graph highlights the cycle number at which the target starts amplifying in the logarithmic phase</td>
<td>102</td>
</tr>
<tr>
<td>2.17</td>
<td>A plot of the standards points, with a line of best fit</td>
<td>103</td>
</tr>
<tr>
<td>2.18</td>
<td>Real Time quantitative data screen</td>
<td>104</td>
</tr>
<tr>
<td>2.19</td>
<td>Melting curve analysis for MgCl run with IGF-Ia primers</td>
<td>105</td>
</tr>
<tr>
<td>2.20</td>
<td>DNA products run on an agarose gel</td>
<td>106</td>
</tr>
</tbody>
</table>
Results

Figure 3.1. Graph showing 10% strain applied to the collagen gel, over a 10 minute period

Figure 3.2. Deformation of the collagen gel lengthways results in three changes of the collagen gel

Figure 3.3. 10% strain was applied to the culture over 1 hour (10% strain per hour)

Table 3.1. A table to show the total amount of strain retained by the collagen matrix

Figure 3.4. Diagrammatic representation of one cycle of loading on the collagen gel, using the t-CFM

Figure 3.5. Graph showing 1 cycle per hour loading regimen applied to a plain collagen gel

Figure 3.6. Graph showing 5 cycle per hour loading regimen applied to a plain collagen

Table 3.2. A table showing increases in the total amount force required to deform the matrix by 1%

Figure 3.7. Comparison of typical contraction profiles from three cell types

Figure 3.8. Comparison of mean rates of contraction for three cell types

Figure 3.9. Three day mean force profile for myoblasts

Figure 3.10. Histogram showing the mean gradient for force generation (rate of change of force) over phases 1 to 5

Figure 3.11. Morphology of myoblasts in 3D collagen matrix

Figure 3.12. A collagen gel after it has been set in a rectangular mould and 24 hours after cell contraction

Figure 3.13. Alignment of myoblasts within a CFM collagen gel

Figure 3.14. Myoblasts embedded within a collagen gel on day three visualised using light microscopy

Figure 3.15. Morphology of myoblasts in different regions of the collagen gel

Figure 3.16. Levels of IGF-IEa and MGF mRNA in differentiating myoblasts in a 3D collagen matrix

Figure 3.17. Phalloidin staining of myotubes in a tethered collagen gel

Figure 3.18. A typical myotube contraction curve

Table 3.3. Statistical analysis showed the time points at which the jumps occurred

Figure 3.19. Myoblasts on day 3 after being seeded into the collagen gel

Figure 3.20. Unidirectional alignment of myoblasts is shown

Figure 3.21. Light micrograph of myoblasts on day 3

Figure 3.22. Low magnification electron micrograph to show two nuclei in a myotube with complete membrane degradation between the fused cells in the fusion zone

Figure 3.23. Alignment of both myotubes and collagen fibrils (CF) within the
10 day culture

Figure 3.24. The elongated, aligned myotubes within the collagen gels exhibited banding in their nuclei

Figure 3.25. Alignment of myotube in day 10 gel along the line of principal strain (double-headed arrow), with banding of nuclei and a large amount of golgi apparatus

Figure 3.26. Sarcoplasmic Reticulum (SR) displayed in myotube

Figure 3.27. The effect of cyclical loading on IGF-IEa mRNA expression in myotube cultures

Figure 3.28. These data show the effect of a 1% strain applied to myotube cultures over a 12-hour period, at 1, 5 and 10 cycles per hour

Figure 3.29. The effect of ramp loading on IGF-IEa mRNA expression in skeletal myotube cultures

Figure 3.30. The effect of ramp loading on MGF mRNA expression in skeletal myotube cultures

Figure 3.31. Effect of application of different strain rates on the expression of IGF-IEa and MGF mRNA

Figure 3.32. The effect of different mechanical loading regimens on GAPDH expression levels in skeletal myotube cultures

Figure 3.33. Changes in levels of IGF-IEa and MGF mRNA, up to 11 hours after 1-hour ramp load (stretch) was applied to skeletal myotube cultures

Figure 3.34. The effect of increasing strain on IGF-IEa and MGF proportions in both cyclical and ramp loaded cultures

Discussion

Figure 4.1. The rate at which 10% strain is applied affects the permanent deformation of the lattice due to the elastic properties of the collagen

Figure 4.2. This representation of the changing ratios of the IGF-IEa and MGF splice variants shows how the proportions of each isoform are affected by increasing strain rates applied using two different loading regimens

Figure 4.3. A Diagram showing the lariat splicing model

Figure 4.4. Mechanisms of myoblast fusion
Introduction

Overview

Of the three muscle tissues in the human body, the development of skeletal muscle is one of the best understood both in cellular and molecular terms. One of the reasons for this understanding is the ability to culture cells and study their behaviour. The fusion of mononucleated myoblasts to form multinucleated myotubes in vitro culture has allowed for the identification of transcription factors, growth factors and cell-cell interactions, which control the process of differentiation.

Elements of this development have however been over-simplified through the culture of myoblasts in a 2-dimensional environment and on a mechanically rigid plastic substrate, as this is not the native 3-dimensional environment in which these cells normally reside. By culturing myoblasts within a 3-D environment crucial aspects of skeletal muscle development can be studied, and in turn may provide clues to engineering pieces of muscle, which may or may not need to be functional (Grinnell 2003).

Cultured within a blood plasma clot, fibroblasts vary in shape from stellate through to bipolar depending on the fibrous network orientation (Weiss, P. 1959). This dependence on the physical environment emphasises the changing and reliant interaction of the two components, the cells and matrix. In a 3D environment cells are free to move in different planes and directions, without the restriction a 2D environment provides. Chemical gradients are able to operate in different planes. Cell receptors lose much of their polarity (dorsal/ventral), and the force vectors on the cell alter diametrically.

By culturing cells in a 3D matrix, the geometry of integrins and focal adhesions on the cell surface can be mimicked (Grinnell et al. 1999). In turn this affects the geometry of cell-matrix and cell-cell interactions, therefore affecting the resultant tissue in two different ways. Most cells are anchorage dependent and by changing the way in which
cells interact with their surrounding matrix, the morphology is altered. As cell-to-cell interaction is crucial for the process of cell fusion, an altered 3D geometry of cell-to-cell contacts will no doubt affect this process. Myoblasts signal between the other cells and their surrounding matrix, and it is possible that signalling differences between the 2D and 3D environment will affect the growth, further differentiation and tissue architecture.

The culturing of skeletal myoblasts in 3D has been explored by some groups, through attempts to engineer pieces of muscle tissue (Vandenburgh et al. 1989, Okano et al. 1997, Dennis et al. 2000). Some 3D muscle cultures have relied upon primary myoblast cultures synthesising their own matrix to form 3D tissue structures, whereas other groups have embedded cells in scaffolds, including the use of collagen lattices. The process of culturing cells in 3D facilitates a means to measure isometric contractile properties of the cells (Eastwood et al. 1994). This is done by measuring the contraction of the gross piece of engineered tissue, and therefore inherent properties of the matrix or scaffold must be considered. The application of defined uni-axial strain to tissue cultures provides an additional feature, which is difficult to apply to 2D cultures. There are systems whereby 2D cultures are grown on silicone membranes, which can be stretched. However, with these systems uniform strain is difficult to apply to the whole culture (Banes et al. 1985). The application of mechanical strain allows the effects of this one variable to be tested with respect to recreating exercise on muscle in vivo, in a controlled manner.

Skeletal muscle tissue growth (hypertrophy and hyperplasia) occurs following mechanical stimulation or exercise. Exercise also results in the growth of bone and cartilage. A mechanical signal is perceived by a cell or tissue and then translated into a growth or differentiation process. This process is not well understood. Recent work mapping molecular connections between integrins, the cytoskeleton and the nucleus of a cell has provided an insight into the ‘hard-wiring’ of a cell (Ingber 1997). The tensegrity theory of how a cell maintains an internal pre-stress is an exciting way of viewing the
cell. Work in this field has extended to mechanically tug individual integrins on the cell surface, which result in mechanical distortion of not only the cell nucleus, but even effect chromosome alignment (Maniotis et al. 1997 (a)). This theory goes some way in explaining one possible mechanism for the transfer of mechanical signals to a cell. By culturing myoblasts and myotubes within a 3D environment, it will be possible to further study the effects of exercise on cells.

Most growth and differentiation of cells and tissues is facilitated by growth factors. Through signalling from the hypothalamus, Growth Hormone (GH) is released from the pituitary gland and signals to the liver, which in turn releases the insulin-like growth factors (IGF’s) into the body. The effects of GH are therefore perpetuated through the actions of the IGF’s (Stewart and Rotwein 1996). This GH axis is the most extensively studied system in the body and has well known effects on growth of tissues in the body. However the IGF’S do also have autocrine and paracrine effects in the body as well as their systemic regulation. IGF is therefore associated with local tissue growth, especially in response to exercise (Yang et al. 1996, McKoy et al. 1999). The application of mechanical strain to a tissue-engineered culture may therefore be directly studied by looking at the changes in IGF expression. Within this area is the interest in the use of alternate gene splicing from which different proteins may be derived.

The use of a bioreactor in which 3D tissue engineered cultures were mechanically loaded using precise regimens was employed to study changes in the splicing of the IGF-I gene, in response to the mechanical signals. The benefits of such a system included a closed environment in which 3D muscle cultures or ‘organoids’ were stretched under defined rates of stretch and defined strains (Eastwood et al. 1998). 3D tissue muscle cultures used to study the effects of mechanical stimulation offers a new approach to the study of exercise induced muscle hypertrophy. The application of controlled precise mechanical loading to tissue cultures allows for a greater understanding of the effects of different strain rates on growth and the regulation of growth factors in response to such stimulation. The central question in relation to IGF-I, is whether in developing and regenerating muscle IGF-I isoform regulation is solely.
Introduction

...programmed, and to what extent environmental mechanical cues can change this programming.
**Skeletal muscle**

Skeletal muscle is a huge tissue in the body, and one of the three classes of muscle, also comprising cardiac muscle and smooth muscle. All of these muscle cells differ in their function, structure and development. Skeletal muscle is formed of myofibres, which are formed by the fusion of mononucleated myoblasts. These myoblasts align and undergo terminal differentiation to form multinucleated myotubes, it is these myotubes which then fuse to form myofibres (Clark et al. 1997, Seale et al. 2000). Skeletal muscle controls almost all the voluntary action in the body. It is found during embryonic development and retained over the lifespan of an animal. This muscle adapts and changes as an animal grows and matures.

**Skeletal muscle anatomy**

Skeletal muscle is composed of bundles of long cylindrical multinucleated cells. The contraction of this muscle is quick and forceful and usually under voluntary control. The cell membrane of muscle cells is termed the sarcolemma, and these cells are surrounded by a distinct collagenous external lamina, also known as the basal lamina.

Skeletal muscle is formed from bundles of long muscle cells. *In vivo* muscle fibres are large, up to 2-3 cm long and 100 µM in diameter. Each cell is a syncytium, with many nuclei in a common cytoplasm. Approximately 80% of skeletal myofibre cytoplasm is occupied by myofibrils, which consist of aligned sarcomeres. The muscle nuclei are located at the periphery of the fibres, including the satellite cells, which is in contrast to cardiac fibres where nuclei are found in the centre of the cells. Each muscle fibre is surrounded by connective tissue, and this is termed the endomysium. Bundles of endomysia are in turn surrounded by connective tissue termed the perimysia, which in turn are encapsulated by the epimysium. This external sheath of each anatomical muscle is composed of dense connective tissue and is often referred to as the fascia. The
connective tissue surrounding the fibres allows for movement between fibres and transmits force to the tendon. Blood vessels and capillaries penetrate through the connective tissue, and provide the muscle with nutrients. The muscle tapers at the extremities, where myotendinous junctions are formed. In these regions collagen fibres from neighbouring tendon are found to lie among the in-foldings of the muscle sarcolemma or basal membrane. Satellite cells are found to reside under the endomysium or basal lamina, in the muscle fibres. These are activated when muscle fibres are damaged. They proliferate and are then recruited to add nuclei to the fibre.

![Figure 1.1. A representative diagram of the structure of skeletal muscle, with the schematic diagram showing where the section was taken. Modified from Jungquira and Carneiro, 1980.](image-url)
Muscle contraction

Much of the muscle fibre cytoplasm is made up of myofibrils, which in turn are made up of units called sarcomeres. These myofibrils are the contractile elements of the skeletal muscle. Sarcomeres generate contractile forces by means of an organised filament system based on actin and myosin, as does all muscle. The appearance of muscle fibres visualised by staining with haemotoxylin and eosin using light microscopy, shows cross striations which are formed by alternating light and dark bands. The dark bands are found to be made up of thick filaments, which are anisotropic, i.e. they are birefringent in polarised light, whereas the light bands are found to be made up thin filaments, which are isotropic, i.e. they do not alter polarised light. The darker bands are termed ‘A’ bands, whereas the lighter bands are termed ‘I’ bands, a dark transverse line, termed the ‘Z’ line, bisects each I band.

Sarcomeres consist of partially overlapping thick and thin filaments. During contraction, the sliding of thick (mostly myosin) and thin (actin, tropomyosin and troponin) filaments occurs, thereby shortening and lengthening of the sarcomeres, the thick and thin filaments do however retain their original length. The sliding filament hypothesis is accepted as the method by which the muscle fibres generate force for contraction (Overview in Bray, 1992). When the sarcomere shortens or lengthens, the two sets of protein filaments, the thin and thick filaments, slide past one another, and therefore generate a change in the tension generated and sarcomere pattern. Similar to that found in non-muscle cells, the myosin filaments possess cross-bridges (myosin II heads), which make attachments to adjacent actin filaments during muscle contraction. The myosin head is tightly bound to the actin filament. When a muscle is actively contracting ATP molecules bind to the myosin head, and lead to conformational changes of the actin binding domain, which in turn causes the myosin head to be displaced. This leads to hydrolysis of the ATP, and part of the energy release is coupled to the production of movement. This is a conversion of chemical energy to mechanical energy. This ‘power stroke’ results in the release of inorganic phosphate and ADP, and the myosin head
returns to its rest position. The myosin head is gradually pushed along the actin filament towards the positive end of the filament, the Z-line.

In vertebrate striated muscle two accessory proteins, tropomyosin and troponin further regulate this interaction. These proteins prevent the cyclical interaction between myosin and actin in resting muscle. The isoform of tropomyosin found in skeletal muscle consists of two subunits wound together forming a coiled structure. Tropomyosin is found situated in the groove of the actin helix, and this blocks any actin-myosin interaction. During muscle activation there is an increase in cytoplasmic Ca$^{2+}$. This Ca$^{2+}$ binds to troponin and this results in a change in its conformation which moves tropomyosin out of its blocking position, permitting the interaction and movement of myosin along the thin filaments. Thus the contraction of skeletal muscle is Ca$^{2+}$ sensitive, an additional feature of the control of muscle contraction.
Introduction

Negative end of the actin filament.  
Positive end of the actin filament.

Myosin cross bridges

Z-disc

Thick filament - Predominately Myosin

Thin Filament - Actin Tropomyosin Troponin

Sarcomere

Figure 1.2 Diagrammatic representation of a sarcomere, with thin and thick filaments, which move along each other in the sliding filament theory (a) The unphosphorylated state (b), compared to the phosphorylated state (c), where there has been movement of myosin heads along the actin filaments.

Regulation of skeletal muscle development
The superfamily of basic helix-loop-helix (bHLH) transcriptional regulators has over 400 members, active in many developmental processes including both myogenesis and neurogenesis (Atchely et al. 1997). These bHLH proteins are classified into five subfamilies. The important regulatory factors involved in myogenesis, known as myogenic regulatory factors (MRF’s) all belong to subfamily A. These all specifically bind to the E box sequence 5’-GAGCTG-3’ with high affinity and they also have a conserved configuration of amino acids at residue 5, 8 and 13 of the bHLH domain. These MRF’s also lack a leucine zipper motif (Atchely et al. 1997). The MRF’s were identified through the discovery of the actions of a dominant acting myogenic transcription factor, MyoD. This transcription factor was identified when non-muscle cells were fused to myoblasts and muscle specific expression was activated in the non-muscle nuclei of the heterocaryons (Blau et al. 1983, Wright et al. 1984). The subtractive cDNA approach was then employed to identify the transcription factor as MyoD (Davis et al. 1987). MyoD is a bHLH factor capable of inducing myogenic gene expression in non-muscle cells. Myf5, Myogenin and MRF4 were later identified as members of the MyoD family, also known as the MRF’s.

The MyoD family of bHLH transcription factors is required for the commitment and differentiation of embryonic myoblasts during differentiation. The specific MRF expression program during satellite cell activation, proliferation and differentiation is analogous to the program manifested during the embryonic development of skeletal muscle.

*Embryonic action of MRF’s*

Skeletal myogenesis begins in the somites. It is here that multipotent mesodermal cells commit to the myogenic lineage. Myogenic cells are derived from the mesoderm of a developing animal embryo. The paraxial mesoderm segments into different somites, where 31 pairs of somites are retained. Each somite divides into three components, the
sclerotome (forms the axial skeleton/vertebral column and develop some sensory receptors), the dermatome (forms the dermis of the skin and develops some sensory receptors) and the myotome (differentiates into myoblasts). It is in the latter that muscle fibres are formed and the myoblast fusion occurs to form primary fibres. Some myoblasts fail to fuse and instead remain close in proximity to the myofibres, and eventually the fibres are encapsulated by the basal lamina, with the unfused myoblasts encapsulated. In adult phenotype muscle there are fused differentiated fibres and a resident population of unfused myoblasts, which are termed satellite cells. Signals from the neural tube, notochord and paraxial mesoderm are involved in regulating somatic myogenesis (Cossu et al. 1996).

The four vertebrate MRF’s have distinct spatio-temporal expression patterns. In the developing mouse embryo, Myf5 is the first MRF to be expressed (Ott et al. 1991, Tajbaksh et al. 1996). As the development of the embryo progresses, Myf5 expression spreads through the whole myotome, and is subsequently down regulated as the myotome matures. MyoD expression is expressed in a different manner to Myf5, as it is detected through the myotome by day 11 and is maintained throughout development (Sassoon et al. 1989, Faerman et al. 1995).

Myf5 expression in the developing limb bud is detected between day 10 and day 12 post coitum, this occurs together with the expression of MyoD (Ott et al. 1991).

Myogenin is detected 12 hours after Myf5 in the myotome, and it’s expression spreads as development progresses, and is maintained throughout foetal life and is subsequently down-regulated postnatally (Sassoon et al. 1989, Ott et al. 1991). MRF4 is expressed transiently between day 9.0 and 11.5 in the myotome and is subsequently re-expressed in the muscles of the embryo on day 16, from here on it is the most abundant MRF postnatally (Bober et al. 1991, Hinterberger et al. 1991, Hannon et al. 1992).

The importance of the four MRF’s has been highlighted through studies where targeted gene disruption of each MRF in mice has shown irregularities in myogenesis.
Introduction

Embryonic roles of MyoD

MyoD deficient mice were born viable with no abnormalities in their skeletal muscle, be that sarcomere ultrastructure, fast/slow fiber ratio or muscle specific gene expression (Rudnicki et al. 1992). MyoD mutant mice did however show an increase in Myf5 expression postnatally (Rudnicki et al. 1992). Heterozygous MyoD mutant mice show a 1.8 fold increase of Myf5, whereas homozygous MyoD mutant mice show a 3.5 fold increase. Development of some epaxial muscle was normal, whereas hypaxial muscle development was delayed by about 3.5 days in homozygous MyoD mutant mice (Kablar et al. 1999, Kaul et al. 2000).

Embryonic roles of Myf5

Homozygous mutant Myf5 mice display no overt abnormalities in their skeletal muscle and unlike in MyoD mutant mice, and no up-regulation in any of the other MRF’s is noted (Braun et al. 1992). Epaxial myogenesis of these Myf5-/- mutants is delayed, and is rescued by MyoD expression, whereas there is no effect on hypaxial myogenesis (Kablar et al. 1999, Kaul et al. 2000). The respective delay in epaxial and hypaxial muscle development in the embryo in Myf5 and MyoD mutant mice supports a multiple lineage hypothesis, where Myf5 regulates the formation of epaxial muscle development, and MyoD regulates hypaxial muscle development (Kablar et al. 1998). There does appear to be some crossover of roles though, as no overt abnormalities were found in either mutant, this suggests that Myf5 and MyoD can compensate for each other.

MyoD and Myf5 relationship

Myf5/MyoD knockout mice were generated to study whether they could functionally compensate for one another (Rudnicki et al. 1993, Kaul et al. 2000). Once born, these mutant mice were devoid of any skeletal muscle and myoblasts, they remained immobile and died shortly after birth. The spaces normally occupied by skeletal muscle were
instead occupied by adipose and loose connective tissue (Rudnicki et al. 1993). There was also found to be disruption between the motor neurons from the spinal cord to the brain during the development of the Myf5/MyoD mutant mice, which highlights the connection between nervous development and myogenesis (Kablar et al. 1999). In the case of Myf5 \( ^{-/-} \)-MyoD \( ^{+/+} \) mutant mice, these are viable, and to some degree normal. In the case of Myf5 \( ^{+/+} \)-MyoD \( ^{-/-} \) mutants, these are not viable and have a substantial reduction in muscle fibre number, suggesting that MyoD mutants require two functional Myf5 alleles to compensate functionally (Rudnicki et al. 1993). This suggests that although there is some degree of overlap between the function these two factors play during development, they are not functionally equivalent.

**Embryonic role of Myogenin**

Unlike the MyoD \( ^{-/-} \) and Myf5 \( ^{-/-} \) mice, myogenin mutant mice have major skeletal muscle abnormalities and die perinatally (Hasty et al. 1993, Nabashima et al. 1993). A significant reduction in both skeletal muscle and diaphragm thickness was found in myogenin mutants. It has been suggested that myogenin is required for myogenesis and thoracic skeletal development as both muscle hypoplasia and thoracic skeletal defect are observed in myogenin mutant mice (Vivian et al. 1999). Skeletal muscle was studied in myogenin mutant mice, and it was found that there was a substantial reduction in myofibre density and there was extensive disorganisation of the muscle (Rudnicki et al. 1993). Mice with disruption to the myogenin gene do contain normal numbers of proliferating myoblasts, however they fail to differentiate, suggesting myogenin may have a role in the transition from myoblasts to differentiated myotubes (Nabeshima et al. 1993).

This normal number of myoblasts would therefore suggest that myogenin is not required for lineage commitment, but instead differentiation. Work both in vitro and in vivo has found that myogenin is up-regulated during the differentiation of skeletal myoblasts, which would suggest that myogenin is a secondary acting MRF (Sassoon et al. 1989, Wright et al. 1989). However myogenin does not appear to have an essential role for
myoblast differentiation in vitro. Myogenin / Myf5 and myogenin/ MyoD mutants did not reveal a reduction in myoblasts, just disorganised muscle tissue, therefore revealing there is no functional overlap between myogenin and either Myf5 or MyoD (Rawls et al. 1995). The primary MRF's, MyoD and Myf5 are important in the assignment of myoblasts to the myogenic lineage, whereas myogenin is a secondary MRF acting further downstream.

**Embryonic roles of MRF4**

MRF4 is linked to the Myf5 gene. Both MRF4 and Myf5 are found on chromosome 10, and they are linked in a head to tail orientation. Therefore when MRF4 null mice were generated, the possible affects on the Myf5 sequence may have played a significant role in irregularities in myogenesis, as targeted disruption of MRF4 affected Myf5 in a cis-acting mechanism (Floss et al. 1996, Yoon et al. 1997). Due to this there have been differences in the mutants produced from different groups. Mutant MRF4 mice tend to die perinatally, with severe defects in axial myogenesis (Patapoutian et al. 1995). A reduction in the expression of other MRF's is found on day 10 pc, in particular this is poignant, as even heterozygous MRF4 mutant mice exhibit this (Patapoutian et al. 1995). Between day 9 and day 11pc, which is associated with the first wave of MRF4 expression in normal development, there is found to be some deficit in myotome development, however by day 14 this development appears normal.

**Postnatal myogenesis and actions of the MRF's**

Skeletal muscle satellite cells were first described by both Katz and Mauro in 1961 (Katz, 1961, Mauro, 1961). Katz observed single cells, which were intimately associated with muscle fibres in the frog. These cells were reported as being in “hypoectolemmal” contact with intrafusal fibres, as they were described as being encapsulated within the muscle fibre.
Introduction

Later, Mauro also noted cells which were closely associated with extrafusal myofibres, and he termed them satellite cells (Mauro, 1961). This was based on their position relative to muscle myofibres and their distinct morphology (Mauro, A. 1961). Satellite cells adhere to the surface of myotubes prior to the formation of the basal lamina, the satellite cells thus lie beneath the basal lamina. Satellite cells are a lineage of myogenic progenitor cells, which are responsible for the growth and repair of adult muscle (Seale et al. 2000). In normal adult muscle, satellite cells are quiescent but are activated in response to stress (Appell et al. 1988, Bischoff 1994). They are activated in response to a diverse range of stimuli including injury, exercise or denervation (Grounds, 1998). There are a number of different mechanisms proposed which explain the activation of satellite cells. These include the inflammatory response, with a role for leukocytes in satellite cell activation (Jesse et al. 1998). Also included are the possible roles played by macrophages and some cytokines. As well as the role of satellite cells in muscle growth, following exercise hypertrophy of muscle occurs. Mature muscle adapts to any new functional length by adding or removing sarcomeres in series (William and Goldspink, 1973, Tabary et al. 1972). During post-natal growth, skeletal muscle fibres are able to elongate by serially adding new sarcomeres to the ends of existing myofibrils (Goldspink. 1964).

Work done in culture has determined that satellite cells are morphologically different to myoblasts. Satellite cells are spindle shaped when they withdraw from the cell cycle, whereas embryonic myoblasts remain rounded (Cossu et al. 1980). Satellite cells contain little cytoplasm, have abundant plasmalemmal vesicles and have oval and heterochromatic nuclei (Bischoff 1994). The population of satellite cells has been well studied in rodents, and as the rodent matures the satellite cell population decreases from 32% down to 5% (Gibson et al. 1983, Grounds, 1998). Once activated satellite cells proliferate and are termed myogenic precursor cells, these continue to proliferate, and once this population is great enough, the cells fuse back into the myofibril to increase its diameter.
Little MRF expression has been found in quiescent satellite cells. In fact the only MRF to be found in quiescent satellite cells is Myf5 (Smith et al. 1994, Beauchamp et al. 2000). The first MRF’s to be activated following satellite cell activation, but just prior to proliferation are, as expected, MyoD and Myf5, the primary factors (Smith et al. 1994, Cooper et al. 1999). The MRF expression pattern in postnatal growth during satellite cell activation is analogous to the program during embryonic development in skeletal muscle.
Introduction

Postnatal role of MyoD

Following experimentally induced muscle injury, MyoD is rapidly up regulated within twelve hours (Megeney et al. 1996). Skeletal muscle from MyoD mutant mice has a reduced capacity for regeneration following muscle injury (Megeney et al. 1996). Although the number of satellite cell is normal, it is found to be proliferation of these satellite cells that is affected by the loss of MyoD (Megeney et al. 1996). For further study of MyoD deficient skeletal muscle regeneration, primary cell cultures were taken from this MyoD mutant adult hind-limb muscle (Sabourin et al. 1999). When placed under conditions suitable to induce differentiation of wild-type myoblasts, MyoD^{-/-} cells continue to proliferate, displaying a differentiation defect (Sabourin et al. 1999, Cornelison et al. 2000). MyoD^{-/-} satellite cells also fail to up-regulate MRF4 expression, and have reduced levels of m-cadherin (Cornelison et al. 2000). Previous work has suggested a crucial role for m-cadherin in cell-cycle withdrawal and myoblast fusion (Irintchev et al. 1994, Zeschnigk et al. 1995).

Studies in MyoD deficient adult skeletal muscle have revealed a delay in myotube formation, which is thought to be related to an extension of proliferation of myoblasts (White et al. 2000). In these studies there was found to be no overall impairment of fusion of the myoblasts, although there was a delay. This correlates well with the theory of a reduced regenerative capacity for a short period following injury in post-natal skeletal muscle, but no long-term affect on terminal differentiation. This knowledge has led to the use of MyoD deficient myoblasts being used as donor myoblasts during Myoblast Transfer Therapy (MTT) (Smythe and Grounds, 2001). MyoD deficient myoblasts are able to promote successful migration into host muscle during MTT as the MyoD deficient myoblasts have an enhanced proliferative capacity and a delay in their fusion.

Another interesting feature of MyoD^{-/-} cells is the increased expression of Insulin-like growth factor (IGF-I) (Seale et al. 2000).
Introduction

It has been suggested that there is a role for MyoD as a negative regulator for IGF-I. MyoD is critical in the progression of satellite cells through the differentiation program. Interestingly, although inefficient, some muscle regeneration does occur in MyoD deficient skeletal muscle, and this may again be due to some functional compensation by Myf5, this does, however, remain to be proven.

Postnatal role of Myf5

The role of Myf5 in regeneration of skeletal muscle remains elusive. It has been proposed that Myf5 may be involved in maintaining the satellite cell pool in adult muscle (Sabourin et al. 1999). It has also been found that post injury, up to 20% of satellite cells express Myf5 and not MyoD, which suggests a role for Myf5 in activating the satellite cell developmental program (Cooper et al. 1999).

Cell cycle control in myogenesis

There are four main stages involved in the cell cycle; G1, S, G2 and M. Phase G1 (first gap phase), S, and G2 (second gap phase) are termed interphase. This period is generally a time of high metabolic activity and in particular, in the S phase, DNA is synthesised and replicated. Phase M is the period of mitosis, which includes the processes of prophase, metaphase, anaphase and telophase.

During prophase, the chromosomes have already been replicated in the S phase, and here the nuclear membrane begins to break down, and a new structure, the spindle apparatus is formed. This apparatus consists of spindle fibres stretching to the two poles of the cell, thus forming an axis.
During metaphase the nuclear membrane is completely lost. Spindle fibres attach to the centromeres (the point at which two chromatids are attached) of the chromosomes and change their orientation and move them to the central portion of the cell. In anaphase there is a separation of the chromosomes at the centromeres generating daughter chromosomes. This is followed by the contraction of the spindle fibres resulting in opposite daughter chromosome moving towards different poles. During telophase the two sets of daughter chromosomes are positioned at opposite ends of the pole, and a nuclear envelope develops between the different sets of chromosomes.

It is during phase M that cells divide to generate two daughter cells. As the cell cycle is tightly regulated, if growth factors are removed, cells exit the cell cycle and enter a quiescent stage, which is termed G0. For the successful differentiation of skeletal myoblasts, they permanently exit the cell cycle during the G1 phase, and do not re-enter following the addition of growth factors (Konigsberg et al. 1960, Gu et al. 1993). Throughout the differentiated state of myoblasts, additional blocks prevent them from re-entering the cell cycle.

Differentiation occurs in steps, the first involves the terminal cell cycle arrest of myoblasts, this is followed by activation of muscle specific genes, including the MRF’S, and finally myotube formation. Terminal cell cycle arrest for differentiating myoblasts occurs during the G1 phase, and during this period two major events occur simultaneously. The first is the activation of the MRF’s. The activity of MyoD in particular has been studied during cell cycle arrest. The second is the inhibition of the G1-S phase transition. Although MyoD is found in proliferating myoblasts, its activity is inhibited through its direct interaction with negative regulatory factors (Neuhold and Wold, 1993). These negative regulatory factors are a family of proteins, known as the Id family, which lack the basic DNA binding domain, thus rendering factors such as MyoD into complexes unable to bind DNA (Neuhold and Wold, 1993). MyoD is also found to be highly phosphorylated in proliferating myoblasts, whereas it is unphosphorylated at the onset of terminal differentiation (Kitzmann et al. 1999). This has led to the

Introduction
Introduction

suggestion that MyoD may have a role in mediating terminal differentiation of myoblasts (Huang and Thayer, 2002).

It has been suggested that MyoD interacts with another protein, retinoblastoma susceptibility protein (RB), and together they mediate terminal cell cycle arrest of myoblasts (Gu et al. 1993). RB is a member of a protein family of tumour suppressors, which have been found to regulate the activity of S-phase transcription factors of the ERF family. It is this family which in turn regulates the expression of genes, which are required for cell cycle progression (Sidle et al. 1996).

During the study of terminal differentiation of skeletal myoblasts in mice, levels of RB were dramatically increased during differentiation, which suggests a role for RB in growth arrest of myogenic cells during differentiation (Endo et al. 1992). RB is found to be hypo-phosphorylated during muscle differentiation, and in this form it suppresses growth, this can be directly compared to proliferating myoblasts in which RB is found hyper-phosphorylated (Gu et al. 1993). It has been further demonstrated that the hypo-phosphorylated form of RB in association with members of the E2F family results in G1 cell cycle arrest.

It is thought that RB and MyoD interact cooperatively to ensure successful differentiation. In RB deficient cells, the expression of MyoD results in the formation of defective myosin heavy chain through the differentiation process (Novitch et al. 1996). This defective program of differentiation is not found when other members of the RB tumour suppressor family, such p107 or p130 are knocked out of cells. These observations all suggest a role for RB as crucial in terminal withdrawal of myoblasts from the cell cycle, and interactions with MyoD to regulate some muscle specific genes.
Introduction

Myoblasts cease proliferating - permanent exit of myoblasts from cell cycle - onset of differentiation. MFR4 and myogenin play a role in terminal differentiation.

MyoD and RB

Figure 1.4. A diagrammatic representation of the cell cycle. In the G1 phase myoblasts exit the cell cycle to differentiate and fuse.
**Dystrophin**

Dystrophin is a 427-kDa protein and is divided into four domains, which include an N-terminal-binding domain, a central rod shaped triple-helical domain and a unique cysteine rich C-terminal domain (Ehmsen et al. 2002).

In skeletal muscle dystrophin associates with various membrane associated proteins to form the Dystrophin Associated Protein Complex (DAPC). This complex is critical for the integrity of skeletal muscle fibres. The DAPC is thought to play a structural role in the linking the actin cytoskeleton to the extra-cellular matrix (ECM), which helps to stabilise the sarcolemma during repeated cycles of contraction and relaxation. The DAPC also transmits force generated from the muscle sarcomeres to the ECM (Petrof et al. 1993), as well as protect muscle cells from contraction-induced damage (Weller et al. 1990). In experiments where the anterior tibialis muscles of normal and *mdx* (dystrophic) mice were subject to lengthening contractions, there was found to be significantly higher levels of necrosis in the *mdx* muscle fibres (Weller et al. 1990). The deficiency of dystrophin is thought to render the sarcolemma more susceptible to suffer focal breaks. This evidence suggests a role in for dystrophin in the mechanical integrity of muscle fibres.

The DAPC complex consists of dystrophin, the dystroglycans (α and β), the sarcoglycans, sarcospan and α-dystrobrevin (Durbeej and Campbell, 2002). Mutations in each of these components have been implicated in a number of muscle diseases, in particular Duchenne Muscular Dystrophy (DMD) (Cohn et al. 2000). Dystrophin binds
Introduction

to cytoskeletal actin and β- dystroglycan (trans-membrane protein), this in turn binds to
the peripheral membrane protein α- dystroglycan, which binds to laminin-2 in the basal
lamina, thus the complex links the ECM to the sub-sarcolemmal cytoskeleton. It is link
to the cytoskeleton that gives dystrophin the potential to be involved in any mechano-
transduction pathway. In particular, it has been found that in dystrophin deficient mice,
following mechanical stretch there was reduced activation of the stretch sensitive p38
mitogen-activated protein kinase (Kumar et al. 2004). This data indicates a role for
dystrophin as a load-bearing element in skeletal muscle fibres. In terms of cellular
studies, the role of dystrophin in mechano-transduction in skeletal myoblasts and
myotubes may be an important means of regulation, which may extend to the role of this
protein in the development of skeletal muscle.
3D tissue engineering

Understanding aspects of cell behaviour in terms of changing morphology, biochemistry and genetics has all has been made possible by our ability to remove cells from their native environment and grow them under conditions which are suitable for their intense scrutiny. It is obvious that by changing the environment in which cells reside, we undoubtedly change aspects of their behaviour. The balance, which must be established, is between the ease of study of cells and provision of an environment more realistic to a cell than a Petri dish.

The majority of cell culture work is carried out in 2D, with cells grown on flat, stiff plastic surfaces. These cultures are not only easy to maintain, but easy to visualise and study. With this ease has come a much greater understanding of fundamental cell biology.

In 1924 mesenchymal cells were grown on glass cover slips (Lewis and Lewis, 1924). It was noted that these flattened cells displayed fibres, termed ‘stress fibres’. This feature was not however noted in situ apart from where tissue activation in the form of wound repair and fibrosis occurred as a result of damage. This is one of the limitations of tissue culture, that by removing cells from their native environment you alter their responsive behaviours. In particular the obvious change is a cell residing in a 3D environment will attach, spread and behave differently from the 2D environment in which it is grown in
Introduction
tissue culture. Since a cell gains attachment sites all around it’s membrane (isotropic attachment) and loses its harsh dorso-ventral polarity.

The 3D physical environment in which many cells reside is of critical importance in determining the morphology of a cell. In a 3D blood clot, fibroblasts display various morphologies ranging from bipolar to stellate which are dependant upon the fibrous network of the clot, thereby displaying the interdependence of the cell and its immediate physical micro-environment (Weiss, 1959). This suggests a dynamic, responsive and iterative process in which small differences in the physical environment in which cells reside influences the shape and geometry of the cells themselves. In a 2D system where cells are unable to exert any influence on the non-compliant plastic substrate on which they are grown, aspects of this cell behaviour are inevitably concealed.

Culturing cells in 3D was described over 30 years ago (Bell et al. 1979). Fibroblasts were cultured in native collagen gels, and certain previously unreported phenomena were observed. A major example of this was cell-mediated contraction of the collagen matrix in which they were embedded. By culturing cells in an extra-cellular matrix, it is possible to re-create some of the native 3D environment in which cells receive spatial cues and the substrate deforms. The changing geometry of focal adhesions, cell surface integrins as well as cell-cell contact, all influence cells behaviour (Grinnell. 2003). The difference in spatial cues coming to a cell from a 3D, random, compliant collagen fibrin
mesh as opposed to stiff flat plastic could not be greater. Its impact should not be underestimated.

In 3D matrices, the traction exerted by cells during their migration induces not only translocation of the cells, but also remodelling of the matrix, as the forces generated move both cells and collagen fibrils (figure 1.5).

Figure 1.5 Diagrammatic representation of cell matrix remodelling. In compliant 3D structures when the cellular component exerts forces, the matrix component...
Introduction

either exerts a reactive force against it (greater stiffness) or is itself deformed (remodelled).

In a standard 2D culture the only dynamic factor is the cells, as the plastic is non-deformable. In a 3D culture, not only can the surrounding environment affect the cell phenotype, but the cells can also affect their surrounding environment, i.e. the matrix in which they are embedded. It provides a dynamic scenario in which both components of the culture are able to exert influence on the other. It is likely, in vivo, that this feedback interaction is crucial.

A major benefit of culturing in 3D collagen matrices is the ability to study and quantify cell generated endogenous tension. Cells in vivo are seldom found floating, and collagen networks are almost always tethered to surrounding structures under some element of tension.

A culture force monitor has been developed to quantify the isometric tension developed by the resident cells within a collagen matrix (Eastwood et al. 1994). By using this system it is possible to measure endogenous forces generated by different cell types. Collagen lattices respond to any tractional forces that are generated by cells during the various stages such as attachment and migration within the collagen fibrils.

The contraction of the collagen matrix by cells occurs through different signalling pathways, and is not very well understood. Cell contraction of collagen matrices is not
Introduction

possible without extra-cellular components and proteins present in nutrient media in
which the collagen gels are bathed. Within the serum added to media are known
components, which trigger contraction, including LPA (lysophosphatidic acid) and
PDGF (platelet derived growth factor). Most work is based on fibroblasts, in which both
PDGF and LPA act on receptors leading to auto-phosphorylation, with promotion of
contraction (Grinnell et al. 1999).
Introduction

Development of tissue engineered skeletal muscle cultures

It is understood that complex mechanical forces generated in the growing embryo play a crucial role in organogenesis. The differentiation and development of skeletal myoblasts in vitro is very different to that in vivo, as many aspect of the signalling are lost. This includes the missing nervous and vascular systems. However, the contribution of 3D extracellular matrix through which alignment occurs and the generation of mechanical tension, which is transmitted to skeletal muscle elements, are also key factors. Within developing muscle complex patterns of mechanical loading are applied to the skeletal myoblasts and myofibrils through the elongating skeleton and by foetal movements. To study some of the effects of mechanics on skeletal myoblasts in vitro, 3-dimensional cell culture has been employed to help mimic some of the actions of a 3D environment on myotubes. This also aims to advance the field of tissue engineering muscle, be it for tissue replacement or pharmaceutical testing.

Okano et al. (1997) used a 3-dimensional type I collagen gel, into which C_{2}C_{12} myoblasts were embedded to produce engineered muscular tissues. This was among the first where tissue engineered muscle constructs were implanted in vivo to study their adaptation to the body. It must be emphasised that in the Okano study no functional role of the implanted muscle was studied.

The requirements for a functional hybrid muscle tissue were set out to be a high density of multinucleated myotubes with a high degree of orientation in one plane. This was achieved using a process of cellular packing through centrifugation, which helped increase the cell density in the collagen gels (Okano et al. 1998 (a)). Cyclic stretching was also employed which resulted in a highly orientated hybrid muscular tissue in which both the cells and the collagen fibres were aligned in the direction of stretch (Okano et al. 1998 (a)). Further to this, a capillary network was introduced to the muscular tissues. This was done by implanting the in vitro muscular tissue grafts into nude mice (Okano et
al. 1998 (b)). Grafts were inserted into subcutaneous spaces on the backs of nude mice, and four weeks after implantation, a dense capillary network was formed in the vicinities and on the surface of the graft.

**Application of mechanical tension to muscle cultures**

In early studies by Vandenburgh *et al.* (1983 onwards) a 3-dimensional model of skeletal muscle was developed, thus providing a 3D environment for myoblast growth, with the additional feature of application of mechanical strain to the muscle culture. Co-cultures of skeletal myoblasts and fibroblasts were isolated from avian pectoralis muscle, and cultured on collagen coated elastic substratum. Within 7 days the monolayer of skeletal myoblasts detached from the substratum and remained attached only to stainless steel mesh at either end of the culture (Vandenburgh *et al.* 1989). The application of mechanical loading of these muscle cultures was then possible, as well as monitoring over a period of weeks.

Following plating of myoblasts (from between 18-54 hours), a unidirectional stretching pattern was applied at a rate of 0.35mm per hour for up to five days, and was done so to simulate *in vivo* bone elongation during development. This rate of movement was comparable to the most rapid state of limb elongation during development in the chick embryo (Ede *et al.* 1971). This period was termed phase I, during which mechanical stimulation resulted in two- to fourfold longer myotubes in culture when compared to controls where un-orientated myotubes in static were grown in static culture conditions (Vandenburgh *et al.* 1991). It was noted, however, that myotube diameters remained similar in both groups. This observation is consistent with the theory that lateral fusion of myotubes is inhibited; instead they fuse in an end to end manner (Clark *et al.* 1997). Following this period, phase II consisted of a quiescent state and phase III was repetitive stretch/relaxation deformation of between 5-20% of the substratum. This was in addition to further unidirectional stretch. These patterns were designed to simulate some of the complex perinatal muscle movements initiated *in vivo* as the skeleton grows (Landmesser *et al.* 1984).
Mechanical stimulation of these muscle cultures resulted in cell proliferation, myotube orientation and myotube longitudinal growth. This was done through the actions of growth factor alterations in the cells growth media (Vandenburgh et al. 1983). Substratum stretching resulted in a 61% increase in total cell nuclei, and 103% increase in myotube nuclei (Vandenburgh et al. 1989). It was also found that pre-stretching the collagen coated substratum resulted in increased orientation of the myotubes, and increased length.

Although the molecular mechanism by which mechanical stimuli are transduced into known biochemical events associated with growth are not known, it has been proposed that cytoskeletal alterations may be involved as they are closely coupled to growth associated cell shape changes (Folkman et al. 1978). It is likely that cell surface integrins are attached mechanically to the cytoskeleton of the cell, and in turn to the nuclei, right down to the nucleoli of a cell. Therefore mechanical deformation of the extracellular matrix (ECM) can in theory produce direct deformation (and potentially stimulation) of the nucleus (Maniotis et al. 1997 (a)).

It is known that at the extremities of muscle, there is myotendinous junction formation. This transitional region is found to have collagen fibres of tendon inserted in foldings of the basal lamina (Junquira and Carneiro, 1980). Staining of static muscle cultures for tenascin revealed a random distribution (Chiquet et al. 1984). It is true that interstitial fibroblasts synthesise most of the tenascin (the myotendinous antigen) found in muscle tissue, and this serves as a marker for myotendinous junction development (Chiquet et al. 1984). The ends of the mechanically loaded skeletal muscle cultures stained for tenascin (Vandenburgh et al. 1991(a)). This corresponds well with the region of the muscle construct under the greatest amount of tension in the mechanical cell stimulator and indicates the possible role of mechanical loads in initiation of myotendinous development of muscle in the embryo in vivo.
Certainly in the current model it was found that myotubes were able to synthesise and secrete high molecular weight factors into their medium, which in turn were able to stimulate growth related processes in other myotubes (Vandenburgh et al. 1983). During experiments it was possible to divide the growth processes into two parts, serum independent and serum dependent changes. It was found that initial growth related biochemical alterations induced by myotube stretch in vitro, depended upon events intrinsic to myoblasts, whereas subsequent stretch induced growth of myotubes was highly dependent upon the presence of serum containing medium (Vandenburgh et al. 1983). In particular, there was continued and enhanced growth of myotubes following the addition of both insulin and IGF-I to cultures (Vandenburgh et al. 1991 (2)). In cultures, where no mechanical stimulation was applied, myotubes were embedded in collagen gels. The addition of insulin and IGF-I induced rapid and pronounced increases in skeletal myofiber hypertrophy, and this included increased numbers of nuclei in myofibrils, by up to 87% per mm, increased cellular proliferation and neonatal myosin heavy chain accumulation by 2.9 fold after 4 days of incubation with IGF-I.

Among the different groups who engineer muscle, there are known factors that affect the growth and myogenesis of these cultures. Dennis et al. have engineered 3D skeletal muscle tissue ‘myooids’ from different primary muscle cultures (Dennis et al. 2000, 2001). Their sources of primary myoblasts included adult mouse, neonatal mouse and adult rat. Alongside these co-cultures of mouse cell line myoblasts and fibroblasts have also been used in muscle cultures (Dennis et al. 2000, 2001). Co-cultures of the myoblast and the fibroblast cell lines were done to mimic the cell combination found in the primary cultures. Also it is known that fibroblasts provide important cues, promoting myooid formation (Dennis et al. 2001). It seems likely fibroblasts were important in part at least, to produce extracellular matrix to mechanically support the myooid itself. Another study has indicated that by removing fibroblasts from primary cultures, actual myotube formation is prevented in vivo (Lewis, M. Personal communication, 2003). The successful formation of myotubes in vitro from primary cultures of pure myoblasts is only possible through addition of certain factors known to be released by the fibroblasts (Lewis, M. personal communication, 2003).
Each cell system was grown to form myooids, similar to those produced by Vandenburgh et al (Dennis et al. 2001). Monolayer cultures of cells eventually formed their own extracellular matrix which detached from the substratum and spontaneously rolled up to form cylindrical structures, which is probably the function of fibroblast contraction of the collagen network. Within a two week time frame the central portion of the structure was filled with fused differentiated myoblasts, and an outer ring of fibroblasts was present. The different contractile properties of the myooids were compared, both isometric and induced by electrical stimulation.

The main difference was found between the myooids produced from primary cultures and those made using cell lines. Whereas the C2C12-fibroblast co-cultures contracted singly and sporadically, myooids produced from primary cells contracted regularly and vigorously as a syncytium (Dennis et al. 2001). The C2C12-fibroblast myooids had a greater cross-sectional area, had a higher rheobase (i.e. required a larger stimulus amplitude) and thus lower excitability than any of the primary cell sources. In addition C2C12-fibroblast myooids also generated the least amount of force when excited by transverse electrical stimulation. As reported by Vandenburgh et al. (1989, 1990, 1991), the major limitations of these tissue engineered cultures was the lack of further progression of myotubes to express adult phenotype in terms of sarcomere structure, excitability and contractility.
Introduction

Mechano-transduction across the cell:

The tensegrity model

There has been much interest in the role mechanics plays in the tissue growth, development and function. It is known that mechanical forces play an important role in the growth of muscle and skin, as well as the maintenance of cartilage and bone. The exact mechanisms through which a physical force is converted to change chemical activities inside the cell are unknown, as are the mechanisms through which physical forces affect tissue development. One theory is that the cell is a complex, well-structured, hierarchical entity, in which it is hard-wired in a way that a physical force applied to any one part of the cell, is successfully relayed to the whole cell right down to the nucleus (Ingber, 1997). The details of how this force is then converted to produce the multitude of different processes in the cell remain elusive.

Eukaryotic cells are made up of an intricate molecular framework, which is termed the cytoskeleton, and this is composed of interconnected microfilaments, microtubules and intermediate filaments, within a viscous cytosol (Heuser and Kirschener, 1980, Fey et al. 1984). It is known that the cytoskeleton mechanically stabilises the cell. The components of the cytoskeleton provide a scaffold for the cell, holding it in shape and resisting cell shape deformation. The cytoskeletal filaments are crucial in the generation and opposition of mechanical forces.

Previous models of the cell have assumed that the primary load bearing element of the cell is the microfilament network that lies beneath the cell membrane (Albrecht-Buehler, 1987). The tensegrity model, however, differs in that it offers an alternative view of the mechanical significance and importance of the cytoskeletal filaments in terms of their role in cell structure and mechano-transduction. The tensegrity model predicts that the
Introduction

cytoskeleton of a cell behaves like a discrete mechanical network that spans across the cell surface and through the cytoplasm (Ingber, 2003 (a)).

If the cell is a pre-stressed tensegrity structure, then any stress or tension applied to a local, will result in global structural re-arrangements. Due to the interconnection and relationship between all individual components of the tensegrity structure, if one parameter is altered then each element must change the orientation and spacing relative to one another to maintain the inherent stress (Ingber, 2003 (a)). This model is therefore different to conventional models of the cell as depending upon which surface structure has stress applied to it locally, it may result in directed deformation of the complete structure. There are differences in the transmembrane surface molecules of a cell. There are those which physically couple extra-cellular anchors to internal cytoskeletal features such as extra-cellular matrix molecules, local stress applied to these provide paths for mechanical stress transfer into all internal components of the cell. In contrast, there are transmembrane receptors, which dissipate stress locally and therefore do not submit signals to the internal components of the cell.

Experiments have been devised to test the tensegrity model. A method of micromanipulation was developed by applying shear stress to receptor bound magnetic microbeads. The beads were bound to cell surface receptors (Wang et al. 1993). Following application of stress the extracellular matrix receptor, integrin β1, induced both focal adhesion formation and a stiffening response in the cell (Wang et al. 1993). Non-adhesion receptors were also manipulated and did not result in any stiffening of the cell. These results suggest that cell surface adhesion molecules such as integrins act as mechanoreceptors and as they are linked to the internal cytoskeleton they transmit mechanical signals to other cytoskeletal components (Wang et al. 1993). In comparison when transmembrane acetylated -low density lipoprotein (AcLDL) was stressed using magnetic twisting there was minimal resistance to mechanical distortion (Wang et al. 1993).
Cell-surface adhesion receptors thus provide a greater degree of mechanical coupling across the cell surface compared to other transmembrane molecules. Integrin dependant stiffening was partially inhibited through disruption of either microfilaments, microtubules or intermediate filaments. This stiffening was completely inhibited through the disruption of all these cytoskeletal components (Wang et al. 1993). It can therefore be concluded that although the cytoskeletal filaments impart mechanical stiffness, this is not determined by one component of the filaments, and instead microfilaments, microtubules and intermediate filaments are all crucial in defining the mechanical properties of the cell (Wang et al. 1993).

A major pre-determinant of the cellular tensegrity model is the concept of cellular pre-stress, crucial to cell shape stability (Ingber, 2003 (a)). Mammalian cells are known to have an inherent isometric tension. These isometric forces have been measured using the culture force monitor, among other instruments to quantify cell-generated forces (Eastwood et al. 1994, Kolodney and Wyslomerski, 1992). There has also been work to sever cultured cells from the substrate to which they are attached using micro-surgical techniques, in such experiments it was found that cut edges retracted spontaneously (Pourati et al. 1998). The tensegrity force balance, therefore, must ensure the cell remains at a level of stress inherent within any given cell, this is crucial for both cell shape and mechanical stability.

Once anchorage dependent cells are attached to any given substrate, it becomes crucial for the cell to establish a tensegrity force balance between the cytoskeletal filaments and the surrounding extracellular matrix (ECM). A pertinent comparison of the interaction between anchorage dependent cells and the adhesion substrate is that of the stability of a spider’s web and the tree branches to which it is attached (Ingber, 2003(a)). The mechanical properties of the tree branches are crucial to the stability of the spider’s web. Cells are attached to any substrate through discrete regions, which contain clustered integrin receptors as well as immobilised signal transduction molecules to name a few, and these regions are termed focal adhesions (Burrige et al. 1988, Plopper et al 1995, Miyamoto et al. 1995). The mechanical properties of the substrate to which cells are
attached are crucial in determining cell shape of anchored cells, in particular this refers to the ability of the substrate to withstand compression (Burridge et al. 1988, Plopper et al. 1995). It is thus possible to view these local ECM regions as external support elements, which resist cytoskeletal tensile forces and integrate into the tensegrity force balance (Ingber 2003(a)).

Chemical disruption of components of the cytoskeletal filaments has shown possible functions each of these components may play in the tensegrity model. Disruption of the microfilaments and intermediate filaments results in a decrease in cell traction on ECM adhesions, whereas disruption of the microtubules results in increased cell traction on the ECM substrate (Danowski, 1989, Kolodney and Wyslomski 1992, Wang et al. 1995). It has been proposed that if all the cytoskeletal filaments were similarly tensioned then disruption of any component would result in a similar manner, and any disruption would be evenly dissipated throughout the cell, instead when microtubules are disrupted there is cell traction and the force is therefore transferred to the cell’s adhesive anchors, and internal stress is re-established by this movement.

Other work had previously implicated a chemical mechanism is more likely to control this increase in cell traction following microtubule disruption as opposed to mechanical action through a tensegrity force balance, as this action activates MLC activation (Kolodney and Elson, 1995). Recent studies have shown that in experiments where microtubules are disrupted and MLC phosphorylation does not change, there is still increased cell traction on ECM (Wang et al. 2002).

The tensegrity model proposes a hierarchical view of the cell. Instead of a model where the disruption of one molecular support element results in the collapse of the whole cell, a model is proposed where multiple force balances are in place, so that if one part of the cell is disrupted there are other subgroups to maintain the structure, while one subgroup may be repaired (Ingber 2003 (a)). This prevents any disruption to the higher order of the cell. In the case of a nucleated cell, it is proposed there would be a force balance within the nucleus, this would be mechanically coupled to the other cytoskeletal
filaments within the cell, but still distinct. This model would provide the basis of any system within a system. For example in the human body, the bones and muscle provide the basic tensegrity force balance to stabilise the body, however, each organ has it’s own pre-stress and force balance, even further, the cells within each specific organ will have their own tensegrity force balance (Chen and Ingber, 1999, Omens and Fung, 1990). This model therefore proposes how cells, tissues and other biological structures could have integrated mechanical behaviour due to tensegrity architecture (Ingber, 1993).

Recent insights into the molecular connections between cell surface integrins, cytoskeleton filaments and nuclear scaffolds indicate a mechanism for transfer of mechanical signals through cells (Maniotis et al. 1997 (a)), as well as an integrated response by cells following mechanical stress. Demonstration of mechanical linkages between cell surface integrins and different components within the cell membrane provide evidence for the importance of mechanical signals in many cellular processes. Through manipulating microbeads attached to integrins, researchers were able to observe reorientation of cytoskeletal filaments and nuclei distortion (Maniotis et al. 1997(a)). This distortion of cytoskeletal filaments resulted in changes in the nucleus shape. It is known that within the cell cytoskeleton and the nucleus there are many enzymes and substrates which mediate different chemical processes, which are crucial to the cell/nucleus shape as long as they remain insoluble. When they are utilized, and they become soluble, and thus no longer provide structural support. It is thus likely that mechanical forces may play a role in the utilization of this ‘solid-state’ biochemistry, utilizing structures for mechanical support in mechano-transduction, and providing signals to these components as necessary cell regulation when they are needed no longer for mechanical support, instead for necessary chemical roles.

In experiments where the nucleoplasm or chromosomes were physically removed from cell nuclei, through the use of micromanipulation, it was found that the remaining nucleoli and chromosomes were sequentially removed (Maniotis et al. 1997 (b)). A continuous elastic thread physically connected these elements, however mechanical coupling between mitotic chromosomes and the surrounding cytoplasm appeared to be
mediated by microfilaments (Maniotis et al. 1997 (b)). These finding appear to suggest that within the nucleus, DNA and associated protein structures and cytoskeletal networks function as a well connected, structurally unified system.

As the nucleus is the ‘regulatory hub’ of the cell, a direct mechanical signal here could mean the cell responds almost simultaneously to the stimuli. It is even possible that there are direct linkages to the nucleoli, chromosomes and even possibly genes. Where integrins were pulled, there was found to be changes in the nucleus. In particular the observation of rotation of the mitotic spindle axis and partial separation of the chromosomes. Although more work needs to be done to prove this, it remains an elegant system for providing the link in application of mechanical strain causing a direct effect on the influence of cell behaviour. A clearer picture of mechano-transduction in cells is now emerging and there is an increasing realization that local mechanical signals are important in initiating developmental gene expression.

An exact, well-defined mechanism of how cells and organs perceive and respond to mechanical signals is not known. Although the tensegrity theory takes a complete overview of the cell, as a hierarchical system, it remains a theory, and there are other pieces of information and theories as to how mechano-transduction is achieved in a cell.

It has been found that mechanical forces are delivered through ECM proteins and integrins (Ko et al. 2001). There are stretch sensitive ion channels in the membranes of cells, which are activated in response to stretch, and with varying outcomes in terms of the release of stretch sensitive ions. For example, the influx of $\text{CA}^{2+}$ ions through specific calcium channels in the plasma membranes of skeletal myoblasts in response to stretch (Kumar et al. 2004).

As well as stretch sensitive ion channels, Gap Junctions are an important component of successful mechano-transduction in cells. Gap junctions are trans-membrane channels, which also allow the passage of ions and other small molecules into cells. It is known that mechanical loading, in the form of cyclic stretch, helps to regulate gap junctional
communication between osteoblastic cells, which is done through the localizing of connexin 43 (Ziambaras et al. 1998). Cell-cell interactions are also important in mechano-transduction pathways. Adheren junctions transmit mechanical forces directly between cells, and have been found to coordinate tissue-remodeling responses to physiological forces during development (Bellousov et al. 2000).

All these components create an appreciation of how complex the process of mechano-transduction is, and how further work will result in a greater understanding.
Insulin-like growth factors (IGF’s)

Growth hormone (GH) is a simple protein of 22kDa. It contains only one intra chain S-S bond and is relatively unstable. GH is secreted by the anterior pituitary (in response to signalling from the hypothalamus), and signals to the liver. This specifies the release of secreted insulin-like growth factors (IGF-I and IGF-II) into the blood and thus defining the liver as an endocrine gland (Stewart and Rotwein 1996). The original ‘somatotropic axis’ described in the 1960’s was described as being solely comprised of the hypothalamus, the pituitary and the liver, where the hypothalamus was thought to be the control centre regulating the secretion of GH from the pituitary (Reichlin 1960).

The biological actions of GH include longitudinal bone growth and remodelling, osteoblast proliferation and bone deposition and resorption, skeletal muscle and liver growth, as well as IGF-I synthesis (Butler and LeRoith 2001).

As the understanding of the somatotropic axis has increased, the details of the GH system have been revealed as more complex than first shown. It is now clear that the IGF’s are regulated in an autocrine/paracrine manner and not solely regulated systemically through the actions of GH. As well as this, there is evidence to suggest that GH has some direct effect on growth of tissues such as the liver, mediated by the GH receptor not involving IGF-I (Liu et al. 1999). Further to this, GH effects are through the actions of growth factors other than IGF-I. GH modulates basic fibroblast growth factor (bFGF) in cartilage, as well as regulating epidermal growth factor (EGF) in the kidney and liver (Ekberg et al. 1989). However, the most common mechanism is via the induction of IGF’s, which are the mediators of the tissue growth response.
Introduction

The insulin like growth structure

The insulin like growth factors show sequence homology with insulin and provide evidence for a common evolutionary origin of both insulin and the insulin like growth factors (Rotwein 1991). The A and B chains of both insulin like growth factor I and II share approximately 50% of sequence homology with the A and B chains of Insulin (Rinderknecht et al. 1976, 1978).

The discovery of insulin in 1921 by the researchers Frederick G. Banting and Charles Best initiated a new era in non-operative medicine, whereby pure forms of insulin were administered to patients suffering from diabetes. Proinsulin consists of a chain of 86 amino acids that permit the alignment of three pairs of disulphide bonds. Insulin is derived from proinsulin, by cleavage of the C peptide. Following this cleavage, the A (21 amino acids) and B (30 amino acids) chains are held together by two of the three disulphide bonds. The binding of insulin to its receptor leads to the uptake of glucose to the cell.
Introduction

Disulphide Bonds

Figure 1.6. A diagrammatic representation of proinsulin, with three parts of an amino acid chain, and three disulphide bonds. This protein is cleaved to form insulin; the ‘C’ chain is cleaved leaving chain A and B, which are left, connected by the disulphide bonds.

Within skeletal muscle tissue there is much evidence to suggest that GH independent actions of IGF-I are responsible for much of the growth and adaptation of this tissue to the environment.

The GH and IGF systems are elegantly simple, the discovery of this system was fragmented, and in particular the IGF field originated in three different areas of biomedical research. The first major find in this field was in 1957 by Salmon and Daughaday. They studied the effects of GH on cartilage growth in rats. They discovered that the effects of GH occurred through a serum factor, which they identified as ‘sulfation factor’ (Salmon and Daughaday, 1957). Although this was subsequently
changed to somatomedin C in 1972, and later IGF-I in 1978, the reason for the
designation of the term sulfation factor was that during the cartilage growth the assay
used monitored the incorporation of radio-labeled sulfate into the ECM (Salmon and
Daughaday, 1957, Daughaday et al. 1972, Rinderknecht et al. 1978 (a)).

The second major find, running concurrently with the aforementioned, was the need by
many laboratories to identify a specific component of serum. It was found that by
neutralizing the serum with antibodies against insulin, known effects of insulin
associated with metabolism were not abolished; therefore suggesting a different factor
was present in the serum with similar effects (Froesch et al. 1963, Megyesi et al. 1974).
These components in serum were termed nonsuppressible insulin-like activity, or
NSILA's, and were later termed the insulin-like growth factors (IGF's) to signify the
overlapping function these substances had to insulin (Rinderknecht et al. 1976).

The third area of research from which insights into the actions of the IGF's were
gathered was the observation that rat liver cells cultured in vitro were able to secrete
factors into their surrounding media, which enhanced cell proliferation (Pierson et al.
1972).

Research within these three disparate areas has resulted in the beginnings of a
comprehensive understanding of the IGF's and their functions. IGF-I and IGF-II were
purified and sequenced, and found to have 70% homology (Rinderknecht and Humbel,
1978 (a) and (b)).

**IGF receptors**

The actions of IGF-I and IGF-II are transduced and mediated through the binding of
these factors to specific receptors. It has been established that the IGF's bind to the
insulin receptor with a very low affinity, and instead have separate distinct receptors to
which they have greater affinity (Megyesi et al. 1975, Rechler et al. 1980). These have
been termed type I or IGF-I receptors and type II or IGF-II receptors. The IGF's bind
with high affinity to cell surfaces of the IGF receptors. The IGF-I receptor has greater
than 50% amino acid identity to the insulin receptor (Ullrich et al. 1980). This may be a
reason for why the IGF's are sometimes found to bind to the insulin receptor, be it with
a lower affinity (Florini et al. 1996).

The type I insulin-like growth factor (IGF-IR) is a transmembrane tyrosine receptor.
This is activated following binding to the IGF-I ligand; this then mediates the cellular
responses associated with IGF-I action e.g. cellular proliferation, growth and
differentiation. This sequence of the IGF-IR gene is found on chromosome 15q25-q26 in
humans (Ullrich et al. 1986). The IGF-IR is a hetero-tetrameric glycoprotein, which is
composed of two identical extracellular ligand binding α subunits (of 706 amino acids),
and two β subunits (of 627 residues) (Ullrich et al. 1986). These are held together by
disulphide bonds, which maintain the receptor heterotetramer. The α subunits contain a
cysteine rich IGF binding site, known as the ligand-binding region of the receptor.

IGF-IR is a member of the tyrosine kinase growth factor receptor family, and through
the binding of the IGF-I ligand to this receptor it is activated. This binding results in a
conformational change of the receptor, which leads to autophosphorylation of some of
the tyrosine residues in the β subunit. These critical residues serve as recruiting sites for
specific cytoplasmic proteins. In the case of both the IGF-IR and insulin receptors,
intermediary signaling proteins are required, and this is most notably the insulin receptor
substrate (IRS-1). This protein is approximately 180-kDa, and contains 21 tyrosine
residues which can serve as phosphorylation sites (Myers et al. 1994).

Through interacting directly with the IGF-IR or insulin receptor, the IRS-1 proteins
serve as mediators between these receptors and downstream signaling molecules (Sun et
al. 1992, Myers et al. 1993). For example, the binding of the adaptor, Grb2 to IRS-1
results in a cascade of events, whereby initially there is recruitment of these proteins to
the cell membrane, this then leads to the stimulation of the proto-oncogene p21 ras, which in turn stimulates the mitogen activated protein kinase pathway (Davis et al. 1994). This then results in the induction of a variety of biological effects, which includes regulation of gene expression.

\[ \text{\(\alpha-\beta\) disulphide bond} \]

\[ \text{\(\alpha-\alpha\) dimer disulphide bond} \]

Figure 1.7 Schematic of the IGF-IR. The two \(\beta\) subunits span the cellular membrane, and contain the tyrosine kinase domain where tyrosine and serine phosphorylation occur. The \(\alpha\) subunit contains the cysteine rich ligand binding domain.
IGF-I binding proteins

There have been six IGF binding proteins (IGFBP) identified to date, excluding the binding protein for Mechano-growth factor (a splice variant of IGF-I). It is the interaction of these binding proteins and the IGF's which represent a degree of pre-receptor regulation. The IGFBP are a structurally related family of secreted proteins which are able to bind to the IGF’s with a high affinity and modulate their actions (Jones et al. 1995). They are composed of cysteine rich amino- and carboxy-terminal domains, whereas the central portion of each IGFBP remains unique (Bach et al. 1995). The IGFBP’s vary in size from between 216 to 289 amino acids (Stewart and Rotwein, 1996).

The IGFBP are bound to IGF’s in the blood and other biological fluids, this action ensures that there is a reservoir of IGF’s in the body (Stewart and Rotwein, 1996). In particular IGFBP3 is associated with maintaining IGF’S in the circulation in conjunction with another protein called acid labile subunit (Hameed et al. 2002). The IGFBP’s also transport the IGF’s from the circulation to peripheral tissues. The IGFBP’s are able to both potentiate and inhibit action of the IGF’s. In the case of muscle differentiation, IGFBP 4, 5 and 6 can act to inhibit muscle differentiation, whereas IGFBP 5 has the additional capability of stimulating myogenesis (Florini et al. 1991, Florini et al. 1996).

IGF-I: Physiological Roles

Embryonic Development

The IGF’s play a critical role in the development of embryonic vertebrate species (Dupont and Holzenberger, 2003). Studies where homozygous IGF-I mutant mice were produced has shown the importance of IGF-I in foetal growth. IGF-I "-" mice are born with a 40% lower body weight, and display severe growth retardation as well as being sterile (Baker et al. 1993). A decline in growth rate of these embryos is found from day
13.5 onwards (Baker et al. 1993). Most of these animals do not survive long after birth, implying a critical role for IGF-I in foetal growth. The death of these homozygotes has been attributed to muscle hypoplasia and a decreased maturation of the lungs. Where both IGF-I and IGF-II are ablated, mice are born at 30% of wild type size and tended to die at birth (Liu et al. 1993). These results suggest that the IGF’s are crucial for normal embryonic development and growth. Studies using human umbilical cord blood have established a relationship between the concentration of IGF-I in this blood and foetal birth weight (Giudice et al. 1995), whereby greater levels of IGF-I are found where foetal birth weight is higher.

Post-natal growth and development

The GH-IGF-I axis plays a crucial role in postnatal growth and development. GH is able to induce the synthesis of IGF-I in multiple tissues and organs (Rotwein, 1991). Systemic control of IGF-I utilizes free and bound IGF’s circulating in the blood. In particular the systemic action of IGF-I results in the growth of many tissues. However it is becoming more apparent that IGF-I plays a role which is GH-independent, thus acting in an autocrine/paracrine fashion. Of particular interest is the manufacture of the IGF’s in non-hepatic tissues, i.e. muscle.

Studies in vivo have shown that over-expression of IGF-I in mice leads to increased myofiber density (Coleman et al. 1995). Also, addition of IGF-I and insulin results in myofiber hypertrophy of avian pectoralis muscle cells in 3-dimensional cultures (Vandevenhurgh et al. 1991).

In cellular proliferation, the role of IGF-I is found to be important in the progression of the cell through the S phase of the cell cycle (Baserga et al. 1993). The mitogenic effects of IGF-I are displayed in osteoblasts, smooth muscle cells as well as skeletal muscle cells, where addition of IGF-I results in greater proliferation (Jones et al. 1995).
Introduction

Local IGF-I- Autocrine and Paracrine actions

IGF-I action is not only regulated systemically, but also found to be locally manufactured and expressed in muscle cells. Although this is thought to be partially GH dependent in some IGF-I responsive tissues, in skeletal muscle it appears to be independent of GH (Hameed et al. 2002).

The autocrine/ paracrine functions of IGF-II became apparent in studies where rat liver cells grown in tissue culture secreted their own factors which enhanced cell proliferation (Pierson et al. 1972, Rinderknecht et al. 1978 (a), (b)). As well as this, in studies where serum-containing growth medium was removed from tissue culture, C2C12 myoblasts spontaneously differentiated (Tollefsen et al. 1989 (a) and (b)). This was concurrent with an increase in the expression of both IGF-I and IGF-II mRNA secretion into the medium, however it was found that IGF-II was greater than IGF-I.

The effects of autocrine expression of IGF-I in C2C12 myoblasts has also been studied where over-expression of this factor results in enhanced differentiation of myoblasts to form myotubes, and an increase in the myogenic regulatory factors, myogenin and MyoD (Coleman et al. 1995, Yang et al. 2003). When compared to cultures where exogenous IGF-I was added to myoblasts, the sustained autocrine expression was a greater myogenic stimulus (Colemen et al. 1995). This autocrine action has been shown not to leak into the general circulation, therefore providing only local action. Autocrine expression of the IGF’s contributes to myotube formation in the developing embryo (Hannon et al. 1992). Both IGF-I and IGF-II were detected in the front limb bud by day 9.5 dpc (days post coitum).

The autocrine actions of the IGF’s are also apparent in injured and regenerating muscle, furthermore when these actions are inhibited, the regeneration of the muscle is hindered. IGF-I and IGF-II autocrine expression is found in muscle following injury in rats (Jennische et al. 1992). Both the mRNA and protein levels of IGF-I in these injured rats increase and where antibodies to IGF-I are added to these animals, there is a significant
decrease in the number of regenerating myofibers, and a reduction in the diameter of these fibers (Lefaucheur and Sebille, 1995).

**The IGF-I gene**

The IGF-I gene is composed of 6 exons, located over 85 kb of genomic DNA on chromosome 12 (Jansen et al. 1983, Brissenden et al. 1984, Tricoli et al. 1984, De pagter-Holthuizen et al. 1989). It is now known that the IGF-I gene is spliced to yield different isoforms, which have different modes of action. The exons are separated by intervening intron regions of between 1.5 to greater than 21 kb (Rotwein, 1986).

Human IGF-I clones were isolated from a human liver library, and the study of these clones resulted in the characterization of two different IGF-I cDNAs, IGF-IEa and IGF-IEb (Rotwein, 1986). These cDNAs had identical 5' ends, and were expressed in liver, however both cDNAs predicted an additional unique carboxyl-terminal extension in the peptides (Rotwein, 1986). As there is only one IGF-I gene within the human genome, on chromosome 12, this finding was one of the first to suggest that alternative RNA splicing may play a role in IGF-I gene expression. In addition to this, the exons encoding the 3'ends for both the characterized IGF-IEa and IGF-IEb were on the same single contiguous strand of DNA, therefore providing conclusive evidence that each variant of IGF-I is encoded by the same gene (Rotwein, 1986).

Whereas human prepro-IGF-IEa and -IEb mRNA is the result of an alternative 3’ sequence, two similar mouse mRNAs result from the presence or absence of a 52 bp insertion (Bell et al. 1986). Alternative RNA splicing results in the synthesis of two types of mouse IGF-I precursor, and the difference is found in the -COOH terminal ‘E’ peptide (Bell et al. 1986). Studies of the mRNA have shown that the IGF-IEb peptides synthesized by either human or mouse are not homologous (Bell et al. 1986).
Introduction

There is also alternative expression of exons 1 and 2, the leader exons, in the IGF-I gene to give rise to different IGF-I mRNAs that are able to encode distinct IGF-I precursors, and are also processed differently in vitro (Simmons et al. 1993). In IGF-I sequences where exon 1 is the leader exon, the IGF-I mRNA is termed class 1, whereas IGF-I sequences where exon 2 is spliced as the leader sequence are termed class 2 mRNAs (van Dijk et al. 1991). The significance of the alternative splicing of the two leader exons is not well understood, and further study of the expression of these different splice variants will help to decipher their different roles. There are different expression patterns of class 1 and class 2 IGF-I mRNAs. In the rat class 1 IGF-I mRNAs are most predominantly found in non-hepatic tissues during both pre-natal and post-natal development (Adamo et al. 1991, Hoyt et al. 1988). In comparison to this, post-natally it is found that class 2 IGF-I mRNAs are predominant in the liver (Adamo et al. 1991, Hoyt et al. 1988).

In rodent muscle alternative splicing of IGF-I gene generates two IGF-I mRNA species which encode IGF-I peptides with alternative carboxyterminal E peptides (Lowe et al. 1988, Jansen et al. 1983, Rotwein et al. 1986, Yang et al. 1996). Whereas in humans, three splice variants of IGF-I have been characterized (Hameed et al. 2002). Due to the discovery of each splice variant, the terms are somewhat confusing, as the IGF-IEc variant in humans was called MGF, whereas in rodents, the IGF-IEb variant is known as MGF. IGF-I is synthesized as a precursor molecule that is then processed after translation, to give the mature peptide, which is biologically active.
Introduction

Schematic representation of the IGF-I gene:

Figure 1.8 A schematic representation of the IGF-I gene. The exon are represented as boxes, whereas the line represent intronic regions, with broken lines representing different splicing patterns. The IGF-IEb isoform contains an extra 52 base pair insert in exon 5.
Introduction

The first splice variant is the main isoform produced by the liver, which is also expressed in other tissue, including muscle. The second isoform contains an extra 52 base pair insert in exon 5, this is referred to as IGF-IEb. This was cloned by Yang et al. (1996), and has been termed Mechano-growth factor (MGF). In the case of humans, this isoform is termed IGF-IEc, and a 49 base pair insert is found in exon 5.

The main difference between IGF-IEa and MGF is the cleaved E-domain. Work is currently being undertaken to elucidate the function of these ‘E’ peptides. It has been shown that cleaved E peptides play an important role in aspects of muscle development. There is sequence homology for IGF-I through evolution, and therefore the maintenance of sequence homology for the ‘E’ peptides would suggest some biological function which has been necessary (Wilson et al. 2001). If this were not the case, the need for maintaining ‘E’ peptide homology would be less critical. Specific regions of the E peptide domain of pro-MGF have been shown to be mitogenic in bronchial epithelial cells (Siegfried et al. 1992). Work done by Hill and Goldspink has extrapolated a function of the cleaved ‘Eb’ peptide from MGF (Hill et al. 2003 (a), (b)). The MGF gene, which in effect is only different from the IGF-IEa gene through the ‘E’ domain sequence, has been shown to enhance stem cell activation. Following muscle damage in rats, the autocrine splice variant MGF (IGF-IEb), was rapidly expressed, and has been implicated in the activation of satellite cells (Hill and Goldspink, 2003).

Yang et al. have also established that C2C12 cells transfected with MGF tend not to differentiate and carry on in a proliferative state, this is opposed to C2C12’s transfected with IGF-IEa, where the cells initially proliferate but quickly differentiate to form multinucleated myotubes (Yang et al. 2002). Hence the work confirms that the E peptide form the cleaved MGF mature peptide appears to have a mitogenic and proliferative effect, as opposed to a differentiative effect. The study of these two different isoforms which result in different E peptides being manufactured is therefore important, as it may
provide clues as to how these different isoforms are regulated, especially in such a mechano-sensitive tissue.
Introduction

Hypotheses:

Embedding C2C12 skeletal myoblasts at a concentration in excess of one million cells per ml, into a 3D collagen gel, with application of uniaxial tension will result in fusion of myoblasts to form multinucleated myotubes.

Application of both ramp and cyclical loading regimens to 3D skeletal myotube cultures will result in up-regulation of both isoforms of IGF-I, IGF-I\text{Ea} and MGF.

Objectives:

Establish basic operating conditions, while characterising the performance or behaviour, of a 3D mechanically defined muscle model.

Aims of study:

1) Characterise the changing contractile and morphological properties of myoblasts in a 3-dimensional collagen matrix, over a seven-day period.
2) Study the changing growth factor mRNA expression within the constructs over seven days. Establish patterns of both IGF-I splice variants over a seven-day time span.
3) Test and monitor the effects of defined mechanical loading regimens on seven-day constructs. Regimens include cyclical and ramp loading at varying strain rates.
4) Define the changes in IGF-I\text{Ea} and MGF mRNA in the muscle constructs following strain application, both in terms of absolute changes and respective ratios of the two splice variants.
5) Characterise the morphological changes in the muscle constructs up to day ten, for an indication of further maturation.
Materials and Methods:

Basic Methods

Cell Culture:

Media:
Dulbecco's modified eagle's medium (DMEM) (Gibco, Paisley, UK), was supplemented with 10% Foetal Calf Serum (FCS), (First Link, West Midlands, UK), 2mM glutamine (Gibco, Chemicals), penicillin/streptomycin (1000u/ml; 100μg/ml) (Gibco Chemicals), and termed 'complete' DMEM. All cells were maintained in this media, with 5% CO₂.

Sub-culturing Cells:
Cells were expanded. 1 passage was equivalent to each cell doubling. For Human Dermal Fibroblasts and Rabbit Bladder Smooth muscle cells, cells used in experiments were between passage 4 and 10. It has been shown that once cells pass passage 10, there is a decrease in cell proliferation and force generation (unpublished observation). Flasks containing cells were first washed 3 times with 0.1M Phosphate Buffered Saline (PBS), and then cells were removed from the substratum, by using trypsin (0.5% in 5.3 mM EDTA). This was incubated for about 5 minutes at 37°C. Adherent cells were then released; the action of trypsin was inhibited by further addition of the serum-containing medium. Cells were then re-plated at lower concentration, to allow expansion.
**Human Dermal Fibroblasts:**

Human dermal fibroblasts were explanted from normal human skin obtained from fresh mammary reductions. The skin was received as pieces of tissue, comprising three tissue layers, as shown below in figure 2.1. The layer of fat was scrapped off the sample, and the remaining two layers are explanted. Skin was cut into small pieces (3mm x 3mm) and placed dermis side down on a flask. This ensured that the dermal fibroblasts were the first cells to migrate out from the tissue. The explants were dried on to the flask floor for half an hour in a dry incubator and then flooded with complete DMEM.

![Diagram of skin layers](image)

**Figure 2.1.** The three tissue layers of skin. The epidermis comprises mostly of keratinocytes, whereas the dermis comprises mostly of dermal fibroblasts. By scrapping off the fat, the dermis layer was exposed, allowing the migration of dermal fibroblasts when placed on a culture flask.

2 to 3 weeks after explantation, cells were visibly migrating from the tissue, and attached to the bottom of the flask. Once cells had grown to about 50% confluence,
explants were removed, by displacing the samples with sterile glass tips, and washed away with sterile PBS (0.1M); the remaining cells on the flask were passaged as usual.

**C₂C₁₂ Skeletal Myoblasts:**

C₂C₁₂ immortalised mouse skeletal muscle cells were obtained from the European collection of cell cultures (ECACC, Centre for applied microbiology & research, Salisbury, Wiltshire SP4 0JG, UK).

This mouse cell line is a diploid sub-clone. These cells are able to proliferate and differentiate rapidly, to form multi-nucleated myotubes, which are contractile and express characteristic muscle proteins. Cells were plated and once flasks were approximately 80% confluent, cells were passaged as usual.

**Rabbit Bladder Smooth Muscle Cells:**

Bladder Smooth Muscle Cells were explanted from rabbit bladders. The epithelium and underlying lamina propria constitute the mucous membrane, and this layer was scrapped from the tissue sample. The remaining sheet of smooth muscle cells was then chopped into small pieces (3mm x 3mm) and placed down on a flask. Within 10 days, cell migration was seen from the samples, which comprised mainly of 2 cell types, the smooth muscle cells and fibroblasts. To remove fibroblasts from the culture, early cell passages were treated with Monoclonal Mouse anti-Rat Thy-1 (MAS 027, Harlan Scientific Ltd, UK). This antibody labelled fibroblasts, nerve cells and stem cells, and on addition of complement (Rabbit Complement, Sigma), these cells were discarded (Ahmed et al. 1999), and smooth muscle cells remained
3-Dimensional collagen gel model:

Cell populated 3D collagen lattices

The cell type to be studied was seeded into collagen gels. In this case C₂C₁₂ myoblasts were seeded into 3D collagen gels. Collagen lattices were set in a silicon elastomer mould (2.5 x 7.3 x 1.5 cm) between 2 polyethylene mesh floatation bars. For collagen lattice preparation, 0.5 ml of 10X Eagles MEM solution (Gibco, Paisley, UK) was added to 4ml of rat-tail type I collagen (First Link) (in 0.1M acetic acid, protein concentration = 2.035 mg/g), neutralized with 5M NaOH, until a colour change (yellow to cirrus pink) was observed (Eastwood et al. 1994, Prajapati et al. 2000). This gel preparation was added to the cell suspension. To produce a 5ml gel, 5 million cells were suspended in 0.5ml of complete DMEM and cast between the floatation bars (figure 2.2). This gel was then set in a humidified incubator with 5% CO₂. Within 20 minutes the gel set, and was physically detached from the base of the mould and floated in media (figure 2.4). After gelling, the collagen lattice was tethered to the CFM (Eastwood et al. 1994); cell contraction was measured using a force transducer (Measurements group, Basingstoke, England). For mechanical loading of the culture, the collagen gel was tethered to the t-CFM (figure 2.5).

Culture Force Monitor (CFM):

Collagen gels were cast between 2 floatation bars, which were in turn attached to stainless steel wire A-frames. These A-frames facilitated a connection between the collagen gel and the CFM system. As the collagen gel containing cells contracts, the floatation bars are pulled in. Thus rendering the gel taut. The region directly adjacent to the floatation bars is termed the ‘delta’ zone, and here cell contraction is less
evident, with no alignment of cells along the line of endogenous tension (Eastwood et al. 1998). This is due to the stiffness of the bar, which restricts any contraction.

Figure 2. Collagen gel set-up. The collagen gel was cast between two stiff floatation bars. The region adjacent to the bars marked by the broken line highlights the delta region. The stiffness of the bar is imparted onto the collagen gel, thus rendering minimal strain (Eastwood et al. 1998).

One A-frame was hooked to a fixed point, and the other to a force transducer, transmitting the cell contraction of the collagen gel to the force transducer. The analogue output signal was amplified, digitalised and processed through ‘Labview’ program (National Instruments, Berkshire, England), to give real-time profiles of force generation.
Materials and Methods

A-Frame Collagen Gel Floatation Bar

(a) Contracted Collagen Gel (24 hours)

(b) Figure 2.3 A schematic diagram showing the contraction of the myoblast/myotube collagen culture. (a) The collagen gel is set between two floatation bars, in a rectangular shaped well. One A-frame is attached to a fixed point, while the other is attached to a force transducer. (b) As the collagen gel contracts the bars are pulled inwards. It is important to note that the stiff bars on either end of the gel prevent contraction of the gel in the delta zones, compared the centre of the gel where much contraction is evident.
Materials and Methods

The diagram below shows how the collagen gel attaches to the CFM system, which provides the link between the gel contraction and displacement of the force transducer.

Figure 2.4 A schematic diagram representing the set-up of the CFM. (Courtesy Dr. N. Wilson Jones.). The contracting collagen gel pulls in the floatation bars, one of which is attached to the force transducer. The force is transmitted to a microprocessor; the signal is amplified and converted for analysis using ‘Labview’ software. Contraction profiles plotted over 24 hours, with data collected at a rate of 1000 reading per second, and recorded at 1 reading per second.
Materials and Methods

The floating collagen gel thus provided a near friction-free system whereby no other factors interfered with the contraction data acquisition.

Figure 2.5 The CFM instrument setup. In this picture it is possible to see the floating collagen gel in the mould (A), attached at both ends to floatation bars. One floatation bar was attached to a force transducer, through which gel contraction could be transmitted to a microprocessor (B), while the other floatation bar was attached to a fixed point (C). The CFM is used in a humidified, 37°C, CO₂ incubator. Therefore the open culture system employed required dedicated incubators.
Materials and Methods

Figure 2.6 This picture shows the t-CFM set-up. The additional feature in this set-up is the stepper motor, which is attached to the base (arrow). This motor can be programmed via a computer, to apply varying mechanical loads to the collagen gel by moving the base uni-axially. The programming was done through ‘X-Ware 4’ software (Micromech, Braintree, UK) (Eastwood et al. 1998). Loading programs were designed to apply ramp and cyclical loading, at varying strain rates.

CFM Calibration

The CFM and t-CFM were calibrated once a month. The force transducer from each machine was positioned horizontally, and was calibrated against weights between 0.5 and 30 mg. Each weight was placed on the force transducer; the data was recorded for five minutes, and then averaged. The force transducer recording from each
Materials and Methods

weight. The force transducer recordings were then plotted against the weight converted into force (dynes/10^5 Newton's). It was important that linearity was shown; therefore an R^2 value close to 1 was expected. If the R^2 value fell below 0.95 for a particular force transducer, it was not used. Also important to take from the calibration curve was the calibration factor (the value needed to multiply the readings).

\[ y = 2.5268x \]
\[ R^2 = 0.9976 \]

Figure 2.7 A calibration curve for a force transducer. The force transducer reading was a reading of the voltage after displacement of the force transducer occurred following the addition of the weights. There was a relationship between the force applied by the weights (in dynes (10^-5 Newton's)) and the voltage reading. From this relationship, both a calibration factor and an R^2 were derived, where the R^2 value indicated how good the linearity was.

Tensioning Culture Force Monitor (t-CFM) set-up.

Collagen gels were cast in the same way as for the CFM set-up between 2 flotation
Materials and Methods

bars, which were in turn attached to stainless steel wire A-frames. These A-frames allowed a connection between the collagen gel and the t-CFM system, loading and monitoring. One A-frame was fixed and the other attached to a force transducer (Eastwood et al. 1996). The analogue output signal was amplified, digitalised and processed through 'Labview' program (National Instruments, Berkshire, England), to give real-time force contraction profiles. In addition, a computer-controlled micro stepper motor, acting in a plane parallel to the tethered axis of the collagen gel enabled exogenous loads to be applied to the muscle cultures. The myoblasts generated an endogenous tension through the collagen gel, and against the tethered attachment bars. Hence a balance was formed between the endogenous tension and the restraining bars and force transducer. Controls included cell-free 3D collagen gels and tethered collagen gels without external loading (endogenous loading only). Myotube cultures were given a twelve-hour period to generate any further endogenous tension, and then loading was applied over a period of twelve hours.

Loading of myotube cultures using the t-CFM:

Programs using the X-Ware software were designed to apply certain loads to the cultures. The stepper motor used to load the cultures was linked to the base of the CFM stage. Each revolution of the motor related to a certain distance by which the stage moved. This was measured by programming the motor to turn 4000 steps (which was known to be one revolution) and measure the distance the stage moved. It was found that;

1 revolution = 0.5 mm displacement
Thus, by applying 14 revolutions (negative 56,000 steps), 7 mm of displacement of the stage was possible. For application of 10% strain, a 7 cm collagen gel was stretched to 7.7 cm (strain applied during ramp loading). 1% strain (applied during cyclical loading) required programming the motor to apply 0.7 mm of displacement to the construct. The motor was programmed to apply -5600 steps or 1.4 revolutions. This steps required to deform the matrix were denoted by ‘D’ when programming the t-CFM. The second feature important in determining how this load was applied was the required velocity, denoted by ‘V’, over a known time span denoted by ‘T’.

This was determined by dividing the number of steps by the total time in seconds.

I.e. we require 10% strain to be applied over 12 hours (12 hour ramp regime).

12 hours is 43200 seconds, the T value

10% deformation = 14 revolutions, which is achieved by loading 56,000 steps (D-56000).

\[
\frac{\text{Distance}}{\text{Time}} = \frac{\text{Steps (D)}}{\text{Seconds (T)}} = \frac{-56000}{43200} = -1.2963 \text{ steps per second}
\]

The velocity is expressed as revolutions per second. -4000 steps are required to achieve one revolution; therefore the steps per second are converted to show the revolutions per second.

Therefore \[
\frac{-1.2963}{-4000} = 0.000324 \text{ revolutions per second (V)}
\]

After these components of the loading regimen have been determined, the t-CFM motor was programmed. Certain features remained constant i.e. the acceleration rate
and the mode normal.

An example for the ramp loading (12 hour ramp regimen) is given;

ION ; Energise drive
T43200 ; Time delay (wait for 12 hours)
A1 ; Acceleration rate (sets acceleration at 10 revs/sec^2)
V0.000324 ; Velocity
MN ; Mode normal, sets the mode to normal preset distance.
D-56000 ; Distance, measured in steps (start loading 10% strain)
G ; Go, make a move.
N ; End loop

Specific programmes were written for each regimen applied. There was an additional feature for the application of cyclical load.

An example for cyclical loading (5 cycles per hour regimen) is given;

ION ; Energise drive
T43200 ; Time delay (wait for 12 hours)
L12 ; Loop, commands between L & N are repeated
L5 ; Nested loop (in this case 5 cycles per hour)
MN ; Mode normal
A1 ; Acceleration rate
V0.00778 ; Velocity
D-5600 ; Distance, measured in steps (1% strain per cycle)
G ; Go
T180 ; Time delay (in seconds)
D5600 ; Negative distance; unload construct.
Cyclic loading:

The 3 regimens used consisted of 1, 5 and 10 cycles/hour, over the last 12 hours of the culture. Each cycle consisted of four equal phases (15, 3 and 1.5 minutes duration for the 3 respective cycle regimes). Phase 1 was application of 1% strain over a fixed time; phase 2 consisted of constant strain; phase 3 was release of the applied strain (unloading); phase 4 was again no movement.

The cyclical loading was conducted over a twelve-hour period. Clearly the 1 cycle/hour regimen therefore applied and removed strain linearly over 15 minutes (total of 12 cycle); the 5 cycles per hour regimen consisted of 60 cycles, each comprising four 3 minute phases; 10 cycles per hour regimen comprised 120 cycles, involving four 1.5 minute phases. 1% strain was applied for all three regimens (i.e. the total displacement of 0.7 mm over a total myotube culture length of 70mm). 600 μNewtons of force was required to achieve this 1% strain (giving loading rates of 40, 200 and 400 μN/minute and strain rates of 4, 20 and 40 %/hour for the three regimens.
Materials and Methods

Figure 2.8 Patterns of cyclical load applied to myotube tissue cultures over one hour, the regimens were continued for twelve hours in total. (a) The 1 cycle per hour regime consisted of four phases, seen in the graph. (b) The 5 cycle per hour regime, over a twelve-hour period consisted of a total of 60 cycles. (c) The 10 cycle per hour regime, over a twelve-hour period consisted of 120 cycles.

Ramp loading:

There were three ramp regimens, applied over the last 12 hours of culture. Each ramp regimen consisted of application of 10% strain (7mm of displacement over the 70mm culture) requiring an applied load of 6000 pN. This ramp was applied over three different time periods. For the first ramp load regimen a 10% strain was applied over
Materials and Methods

a twelve-hour period at a rate of 0.83 % strain/hour (12 hour ramp). The second ramp load regimen was the same 10% strain but applied over a one-hour period, at a rate of 10% strain/hour, the collagen gel was then held at this strain for the remaining eleven hours in culture (1 hour ramp). A third ramp regimen was the 10% strain applied over a ten-minute period, at a rate of 60% strain/hour, the muscle culture then being held in the position under the same tension for the remaining eleven hours and fifty minutes (10 minute ramp).
Figure 2.9 The patterns ramp loading regimens applied to myotube tissue cultures. (a) 12-hour ramp regimen 10 % strain (7 mm displacement) was applied to the cultures over a 12-hour period. The necessary rate to achieve this strain was 0.83% strain per hour. (b) 1-hour ramp regimen: 10% strain (7mm displacement) was applied to the cultures over a 1-hour period. 10% strain per hour rate was applied to achieve this load. (c) 10-minute ramp regimen: 10% strain (7mm displacement) was applied to the cultures over a 10-minute period. 60% strain per hour was applied over 10 minutes to achieve this load.
Cell morphology:

To observe morphological changes of cells as they contracted collagen gels, specimens were fixed at various time points. Gels were first washed in 0.1M phosphate buffer then fixed in 2.5% gluteraldehyde in 0.1M phosphate buffer for 1 hour at 4°C, washed twice in fixative free buffer and stained in toluidine blue for 5 minutes (0.4% toluidine blue and 1% sodium borate in distilled water), with destaining and storage in phosphate buffer. Collagen gel lattices were studied by stereomicroscopic examination on an ‘Edge’ High Definition stereo Light Microscope (Edge Scientific Instrument Corporation, Los Angeles, CA) (Greenberg and Boyde, 1993).

Myotube collagen gels were fixed in the same way, washed in 0.1M phosphate buffer and stained for F-actin accumulation with FITC Phalloidin (Sigma-Aldrich, Dorset, U.K.). Briefly, this stains the cytoskeletal actin in the cytoplasm of cells. After fixing, gels were washed in 0.1M phosphate buffer, and cells membranes were permeabilised with 0.5% Triton X-100 (Sigma, Poole, Dorset, U.K.) for 10 minutes. After further washing, gels were incubated with rhodamine phalloidin (Molecular Probes, at a 1:50 dilution). After final washing in phosphate buffer, gels were mounted on glass slides with glycerol and sealed with cover slips (Faulstich et al. 1993). Confocal imaging of cytoskeletal actin was performed using the Noran-Odyssey confocal laser-scanning microscope.
Transmission Electron Microscopy:

Transmission Electron Microscopy was employed to study the differentiating myoblasts in collagen gels. The benefit of this technique was the high-resolution images, which showed myoblast alignment and the details of membrane fusion. The fusion was particularly of interest, in terms of cell-membrane contact.

Specimens of collagen gel were washed in 0.1M phosphate buffer, fixed in 2.5% gluteraldehyde in 0.1M phosphate buffer for 1 hour at 4°C. This was followed by two phosphate buffer washes and a secondary fixation with 1% osmium tetroxide in a 0.1 M cacodylate buffer, for 1 hour at room temperature. The specimens were then dehydrated through an increasing acetone series. The final series was 100% acetone, which was then infiltrated with 1:1 acetone: araldite CY212 resin overnight in a specimen rotator. The resin was then replaced by fresh resin twice, each change for a minimum of 3 hours. Once this procedure was complete, specimens were embedded in araldite CY212 resin and blocks were polymerised at 60°C for 18 hours. Semi thin (1μm) sections were cut from the specimens using a Reichart-Jung Ultracut E ultramicrotome and floated onto distilled water and collected on glass slides. Sections were stained with toluidine blue (0.4% toluidine blue and 1% sodium borate in distilled water), to select areas where myoblasts and myotubes were present. Ultra-thin sections (80-100nm) were cut using a Diatome diamond knife, floated onto distilled water, collected on copper grids and stained with 2% uranyl acetate and lead citrate for 10 minutes in each solution. The stained sections were viewed on a Phillips CM12 electron microscope. (All the reagents used in the TEM procedure were from Agar Scientific Ltd., Essex, England).
Differentiation of skeletal myoblasts

Known factors influencing myoblast differentiation:

i) High density of myoblasts within the matrix

ii) The presence of extra-cellular matrix, either self-generated or supplemented

iii) Application of tension to the myoblasts

iv) Reduction in the serum level within the media, i.e. depletion of serum factors

In the appropriate media C₂C₁₂ myoblasts are known to spontaneously differentiate to form multi-nucleated myotubes. This is in contrast to primary cultures, where the presence of resident fibroblasts in the culture is crucial to myoblast differentiation. The studies for this have been done using 2D cultures. It is known that passive stretch of cultured myotubes results in an increase in myotube length. Thus stretch, much like growth factors and hormones, is known to stimulate the growth of skeletal myotubes in vitro (Vandenburgh. 1983). By leaving collagen gels tethered to the floatation bar, endogenous tension was generated within the collagen gel.

Myoblasts were seeded at increasing concentrations (per ml of gel), and left for different time periods (figure 2.10), serum levels in the media dropped from 10% to 2% FCS, thus promoting differentiation. Cell-generated endogenous tension in collagen gels promotes differentiation of myoblasts (Vandenburgh et al. 1988), therefore gels were kept tethered. It was established that 4 million cells/ml of gel, over a period of 5 days lead to optimum differentiation (figure 2.10). For myotube
cultures, a 5ml collagen gel was seeded with 20 million cells and allowed 5 days in culture before force generation profiles were plotted.

![Figure 2.10](image)

Figure 2.10. Pictures showing different myoblast density plated over varying time periods and the differentiation of these cells. It was established using that 4 million cells over 5 days produced optimal differentiation.

All myoblasts and myotubes were aligned along the principal axis of strain through the gel.

**RNA extraction and PCR procedure:**

**RNA isolation:**

Total cellular RNA was isolated from the stretched cell populated collagen lattices and those kept under endogenous tension (in CFM model), using the Qiagen Rneasy
kits (Qiagen, UK), all components in this procedure were provided in the kit. Some alterations were made to the protocol supplied with the kit.

Myotube gels were flash frozen in liquid nitrogen, and stored in a −80°C freezer. This was done to prevent degradation of RNA by RNases in the culture media. 500μL of ‘RLT’ Lysis buffer was added to each frozen gel (Rneasy kit, Qiagen, UK). This buffer contained guanidine isothiocyanate, which is highly denaturing and also immediately denatures RNases in the samples. The gel was dissolved in this buffer over a 40-minute period at room temperature, the resulting solution was then aspirated using a 21G needle to disrupt the cell membranes, to release the RNA contained in the sample. From this point on the commercial assay protocol was followed. Equal quantities of 70% ethanol were added to each sample, and mixed well, by pipetting; this provided the appropriate conditions for RNA binding to the membrane in the spin column. 700 μl of this mixture was pipeted into a spin column (provided) sitting in a 2-ml collection tube, and centrifuged for 15 seconds (7,000 g). If the total amount exceeded 700μl then further aliquots of 700 μl were added to the column, and centrifuged. The provided spin column comprised of a silica-gel membrane in the collection tube, when the mixture was centrifuged the RNA bound to the membrane. The flow through was then discarded and 700 μl of ‘RW1’ buffer was pipetted into the column, and centrifuged for 15 seconds (7,000 g). This buffer helped to wash away contaminants. The flow through was again discarded, and the collection tube was changed. 500 μl of ‘RPE’ buffer was then pipeted onto the column, and centrifuged for 15 seconds at 7000 g. This procedure was repeated with one change, the column was spun for 2 minutes, this buffer helped wash away
contaminants. The final step was to elute the RNA from the column membrane. The collection tube was changed, and 50μl of RNase-free water was pipetted onto the membrane and then centrifuge for 1 minute at 7000 g. The resulting elute contained the RNA.

**Measurement of RNA quality on the spectrophotometer:**

1 μl of the resulting elute of RNA was measured using the Genespec I (Naka instruments, Japan) spectrophotometer. Ratios of 260 nm (absorption of nucleic acids) over 280 nm (absorption of proteins present in the sample) were expected to be between 1.8 and 2.0. This indicated good quality RNA, i.e. the correct size (above 200 bases). The total yield per sample varied between 0.2μg and 5μg, therefore 0.5 μg of all RNA was used in the following cDNA synthesis, so that the greatest number of the samples could be included in the study without compromising the efficiency of the cDNA synthesis.

**cDNA Synthesis:**

This step synthesised a complementary strand of DNA to the RNA in the sample, which was then amplified using the polymerase chain reaction. The total RNA was reverse transcribed into cDNA using Expand reverse transcriptase (Roche Diagnostics, East Sussex, UK). IGF-I and MGF first strand cDNA was synthesized in a 40 μl reaction volume containing 0.5μg of RNA. 1μl of primer (10pM/μl) was added to each sample (diluted in 19 μl of water). The primer
Materials and Methods

contained the complementary sequence to both regions 5'TTGGGCATGTGTCAGTGTGG 3' (synthesised by Sigma Genosys, Cambridge, UK). For PCR of GAPDH, random primers were used to generate the cDNA instead of specific primers. This 20 µl mix was heated to 70°C for 10 minutes in a water bath. Following this 20 µl of the following reaction mix, using the 'Expand reverse transcriptase' kit (Roche Diagnostics, East Sussex, UK), was added to each sample, and incubated at 40°C for 2 hours in a water bath: 8 µl first strand buffer, 4 µl 0.1 M DTT (dithiothreitol) 5µl dNTPs (deoxynucleoside triphosphates) (5mM), 1µl RNAse inhibitor (Roche), and 2 µl superscript (50 units/µl). The superscript enzyme propagates the reaction. The primers bind to target sites (upstream of the IGF-IEa and MGF regions), and dNTP's provide the additional bases required to produce the complementary strand. Once the incubation period was over, samples were heated at 95°C for 2 minutes, to denature the enzyme.

PCR amplification of IGF-IEa and MGF

Polymerase Chain Reaction (PCR) was performed to amplify the cDNA present. Experimental protocols are described in table 2.1. For amplification of the IGF-IEa and MGF mRNA the same forward primers were used, and are described in table 2.2 (Sigma Genosys). Quantitative PCR was performed using the ROCHE LIGHTCYCLER, with the Fast-start DNA SYBR green detection kit (for IGF-IEa)(Roche diagnostics), and the DNA master SYBR green I kit (Roche Diagnostic), for MGF. When detecting IGF-IEa the reaction mix contained: 2.0 µl enzyme, 0.8 µl MgCl2 (2.5 mM), 1µl forward primer (7pM), 1µl reverse primer (7pM), 13µl water and 2µl cDNA (final reaction volumes of 20 µl per glass capillary). The same
reaction mix was used to detect MGF, primers were used at a 10pM concentration. Capillaries were then placed into the lightcycler rotor (figure 2.11).

The fluorescently labelled SYBR green enzymes bind to the minor groove of double stranded DNA. As the cDNA was amplified, the increase in fluorescence was detected by the lightcycler, Lightcycler software 3.3 (Roche Diagnostics) was used for fluorescence quantification, through the second derivative analysis mode. Product specificity during the PCR was verified by melting the products, and the results were analysed using a melting curve program using the Lightcycler software 3.3 (Roche Diagnostics).

<table>
<thead>
<tr>
<th></th>
<th>IGF-IEa</th>
<th>MGF</th>
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<tbody>
<tr>
<td>Denaturation</td>
<td>95°C for 10 minutes</td>
<td>95°C for 30 seconds</td>
</tr>
<tr>
<td>Amplification</td>
<td>95°C (15 second)</td>
<td>95°C (1 second)</td>
</tr>
<tr>
<td></td>
<td>58°C (5 seconds)</td>
<td>58°C (3 seconds)</td>
</tr>
<tr>
<td></td>
<td>72°C (25 seconds)</td>
<td>72°C (19 seconds)</td>
</tr>
<tr>
<td>Melting Curve Program</td>
<td>70°C - 95°C</td>
<td>68°C - 95°C</td>
</tr>
<tr>
<td></td>
<td>(Heating rate=0.1°C/second)</td>
<td>(Heating rate=0.1°C/second)</td>
</tr>
<tr>
<td>Cooling step</td>
<td>40°C</td>
<td>40°C</td>
</tr>
</tbody>
</table>

Table 2.1 Description of experimental protocols used for PCR amplification of both IGF-IEa and MGF.
### Table 2.2. Primers used for cDNA synthesis step and PCR step given in the 5' to 3' direction. The resultant size of the product is also given.
DNA purification for standard curve data points:

PCR was performed using specific primers to either the IGF-IEa sequence or the MGF sequence (methods described in PCR amplification). A 2% agarose in 1% TAE (Tris Acetate Electrophoresis) buffer, was set in a gel electrophoresis tank. The gel also contained 0.1% Ethidium Bromide, which bound to the DNA and fluoresced under Ultra Violet light, thus visualisation of the amplified DNA was possible. The gel was cast with wells embedded at one end. Once floated in 1% TAE buffer (containing 0.01% Ethidium Bromide), the wells were placed on the negatively charged side and a current was passed through the gel, thus the negatively charged...
DNA was pushed to the positively charged side of the gel. The required bands were then excised from the agarose gel with a razor blade after being visualised with medium UV light. The agarose gel was melted at 70°C. The DNA was then purified using a ‘Wizard PCR preps’ purification kit (Promega, Southampton, UK). The purification was done using a vacuum manifold. Once DNA was purified, the DNA was measured using a spectrophotometer. This provided accurate amounts of DNA of the required products and these could be diluted to provide accurate amounts for the standard curve amplified alongside the samples during quantitative PCR.

Optimisation techniques:
Quantitative PCR is a highly sensitive technique, requiring extensive optimisation. The reliability and reproducibility of the technique is dependent upon the expression levels of the target genes being studied. Genes which are abundant, such as GAPDH are easier to use, whereas MGF, which is expressed at relatively low levels is more difficult to quantify. GAPDH, which has been used as a control for traditional RT-PCR was also used as a control in the work using real time RT-PCR.

Statistics:
Statistical analysis of data (minimum of four samples per loading regimen, six for static controls) was performed using the non-parametric, ANOVA testing (Kruskal-wallis), using Prism (Prism, Graphpad software, San Diego).
**Materials and Methods**

**Primer design for RT-PCR and quantitative PCR:**

Criteria for primer design

1) Size of product: Shorter products amplify more efficiently. For IGF-IEa and MGF, the products were 300 and 350 bp respectively, whereas for GAPDH the size amplified was 480 bp.

2) The optimal length for primers is between 15-25 bp.

3) The GC content should be between 40-60%.

4) The forward primer for IGF-IEa and MGF is the same, whereas the reverse primers were specific. For IGF-IEa the reverse primer spans across exon 4 and 6, whereas for MGF, the primer spans across exon 5 and 6.

**Primer Sequences**

IGF-IEa sequence and location of primers (highlighted):

Organism: *Oryctolagus cuniculus* (Rabbit)

Title: Partial cDNA sequence of rabbit insulin-like growth factor 1 (IGF-I)

```
1 atgattacac ctacagtaaa gatgcgcctc ctgctcttctt cgcatctctt ctacctggcc
61 ctctgcttgc tcaccttcac cagctcggcc acagccggac cggagacgct
tgcggtgtct
121 gagctggtgg atgctcttca gttcggtgtgt ggagacaggg
gctttatatt caacaagccc
181 acaggatacg gctccagcag tcggagggca cctcagacag
241 ttccggagct gtgatctgag gaggctggag atgtactgtg
 cacccctcaa gcccgaanag
301 gcagcccgct ccgtccgtgc ccagcgccac accgacatgc
ccagactca gaaggaagta
```
Materials and Methods

MGF sequence and location of primer (highlighted):

Organism: *Rattus norvegicus* (Rat)

Title: Sequence of two rat insulin-like growth factor I mRNA's differing within the 5' untranslated region

The 5' region of both IGF-IEa and MGF is the same, and it is the specificity of the 3' region in which these different isoforms differ. The reverse primer for IGF-IEa spans
Materials and Methods

across exon 4 and exon 6, whereas the reverse primer for MGF spans exon 5 and 6. Therefore each target is specific for the particular isoform.

GAPDH sequence and location of primers (highlighted):

Organism: Mus musculus (Mouse)

Title: Glyceraldehyde-3-phosphate dehydrogenase mRNA is a major interleukin 2-induced transcript in a cloned T-helper lymphocyte
1021 catggcctac atggcctcca aggagtaaga aacccctggac
cacccacccc agcaaggaca
1081 ctgagcaaga gaggccctat cccaactcgg ccccccaacac
tgagcatctc cctcacaatt
1141 tccatcccag acccccataa taacaggagg ggcctaggga
gccctcctcta ctctcttgaa
1201 taccatcaat aaagttcgcct gcacccac
Real Time Quantitative PCR

Quantitative PCR allows the accurate quantification of a particular gene sequence in a sample. The total RNA is extracted from a sample. The component of interest is the messenger RNA, which is being manufactured by the cell in response to the intervention applied. By using specific primers to the mRNA, cDNA was synthesised by incubating the RNA with the primers and reverse transcriptase enzyme, described in the cDNA synthesis methodology. This cDNA was then amplified to form double stranded DNA (dsDNA), during the PCR reaction. Using quantitative PCR the dsDNA was detected. There are several methods of detection which include the SYBR green system, which was used in this case (Roche Diagnostics). SYBR green is a fluorescent labelled component which binds to the minor groove of double stranded DNA, as it is amplified during the PCR reaction.
Material and Methods

Figure 2.12 Picture of dsDNA. The major and minor grooves are annotated. It is the minor groove to which SYBR green binds.

As the dsDNA was amplified, binding of these components increases, and the corresponding increase in fluorescence is detected by the Lightcycler. Standards of known quantities of DNA were made and amplified alongside the samples in the lightcycler. A standard curve was derived from the readings of known quantities of DNA and the samples compared to the standard curve produced. Due to slight variations in the handling of PCR products every time a set of samples was studied, a set of standards was run concurrently, to give precise quantification.
Standard curve:

Standards containing known quantities of the DNA to be amplified were first produced. Using the specific primers to the product to be studied (i.e. IGF-IEa, MGF or GAPDH); a positive control sample was amplified using those primers using PCR. The products were then run on a 2% agarose gel, and the correct band (i.e. correct size of product) was excised from the gel. This DNA was then purified (‘Wizard PCR preps’ purification kit, Promega, Southampton, UK), and the total yield and concentration was measured using a spectrophotometer. The DNA was then diluted to give various concentrations of the DNA.

For example:

10 ng/µl of IGF-IEa product is purified. This is then diluted to give a series of varying concentrations.10 ng/µl, 1 ng/µl, 0.1 ng/µl, 0.01 ng/µl etc

These standards were amplified together with the samples. A standard curve was derived from the data, and the samples were quantified by comparison. It was important to ensure that all samples quantified had values which fell within the range established by the standard curve, if the samples fell outside the range, PCR was performed again, altering the concentration of the standards accordingly. In this way every single sample was accurately quantified. The reading of fluorescence was an arbitrary reading. For direct comparison with different products being amplified, the exact copy number per sample was needed. In this way the two different isoforms of IGF-I, IGF-IEa and MGF, could be quantified in copy number, and compared.
Materials and Methods

Equation for calculation of copy number from the concentration:

The exact copy number of a particular mRNA sequence was calculated from the quantitative PCR concentrations. The following

3.375 x 10^-8 ng/µl of IGF-IEa.

Therefore, we multiply this number by 10^-9 to establish the number in grams.

3.375 x 10^-8 x 10^-9 = 3.375 x 10^-17 grams

The size of the product amplified is multiplied by the weight of ssDNA (which is represented in grams/mol).

This calculation is 300 bp (size of product for IGF-IEa) x 330 (weight of ssDNA in grams/mol) = 99000 grams/mol

Therefore we divide our final weight by the weight of each molecule of IGF-IEa.

\[
\frac{3.375 \times 10^{-17} \text{ grams}}{99000 \text{ grams/mol}} = 3.409 \times 10^{-22} \text{ moles}
\]

This then calculates the number of molecules in the sample, which is then multiplied by the Avogadro constant: 3.409 x 10-22 x (6.23 x 1023) = 212.38

Therefore 212 molecule were present in 2 µl of a 40µl solution containing 0.5 µg of RNA. To calculate the copy number per µg of RNA, 212 x 20 x 2 = 8495 copies/µg RNA.

Melting curve analysis:

This function was performed to check that one product was amplified. Each PCR product has a specific melting temperature, therefore if there had been non-specific binding, multiple peaks were observed. The only exception is the curve for the
Materials and Methods

melting products of the primer dimer. In this case the GAPDH product melts at 90.5°C and the primer dimer melts at 77.5°C. This form of analysis is also important as a mechanism for detecting any contamination in the experiment, as the melting curve analysis for the water is visible. The only peak in the example given (fig. 2.13) was that of the primer dimer, no other product was amplified, therefore ensuring that there was no contamination of the experiment.

Figure 2.13 The melting curve analysis for GAPDH. There are two main peaks in this curve. The first at around 77.5 (primer dimer) and the other at 90.5
Materials and Methods

Analysis of amplification:

The analysis of amplification is crucial in determining the quantity of target in the sample. This function shows the cycle number at which the PCR product begun to amplify (the logarithmic phase). It was this measurement that was crucial to the quantification of samples as the cycle number at which the samples start their phase of logarithmic amplification, this was then measured against that of the standard curve.

Figure 2.14 This graph highlights the cycle number at which the target starts amplifying in the logarithmic phase. After the logarithmic phase of amplification, a plateau in the amplification is observed.
Standard curve, logarithmic phase: The standard curve must have an $R^2$ value that is as close as possible to 1, with 0.95 being the minimum. The $R^2$ value denotes the percentage of variation in the dependant variable (for each specific point on the standard curve) accounted for by the independent predictor variables (the determined line of best fit). In this case the $R^2$ value was 0.98. This was done to ensure the accuracy of the standard curve. If the points did not produce a reliable standard curve, then the samples falling within the curve would not be accurately quantified.

Figure 2.15 A plot of the standards points, with a line of best fit. The $R^2$ value of 0.98 indicates the points are close to the line of best fit, thus producing an accurate standard curve.

Quantification of PCR product. The standard curve is plotted, and the cycle number at which the PCR product begins its exponential amplification is crucial. The cycle number at which the samples begin this phase is thus compared to that of the...
standards, and calculated using this data. It is important here that no contamination in the water sample is found.

Figure 2.16 Real Time quantitative data screen, including the cycle number at which fluorescence was detected for each sample.

Quantitative PCR method:
To optimise the quantitative PCR, reactions were carried out using different concentrations of Magnesium Chloride (McGill). It was then possible to use the correct amount to give the most efficient PCR product. The main factors in deciding the correct concentration of MgCl are a single peak in the melting curve (indicative of one product being amplified) and the smallest amount of primer dimer possible.
Materials and Methods

(indicated by the peak at approximately 78°). MgCl is required for the reaction, but in large quantities can actually lead to increased levels of primer dimmer. Incorrect concentrations of MgCl can also lead to the amplification of more than one specific product, leading to multiple peaks.

Figure 2.17 Melting curve analysis for MgCl run with IGF-IEa primers is shown here. The melting temperature for the IGF-IEa product was approximately 89°C. Where the MgCl concentration is not optimal two peaks are observed, however where a concentration of 2mM MgCl was used, a single melting peak was observed.

Once the correct MgCl concentration was established for a set of primers, this concentration remained constant. In addition to the quantitative PCR data, PCR products were visualised by running the DNA through an agarose gel, alongside marker DNA to compare the sizes of bands. The correct DNA size can be seen here.
Figure 2.18 DNA products run on an agarose gel with a DNA ladder indicating the size of the bands. The bands show the product size of the IGF-IEa amplified, 300 bp. The first six wells are the standards, the next four wells are samples, and the last is a negative template control.
Results

The aim of this work was to study the 3D skeletal muscle culture in stages over a 7-day period, as it developed. Initially the changes in contractile properties of myoblasts as they fused to form multinucleated myotubes was studied and over the same time frame changing morphology and changes in growth factor mRNA expression were observed.

Results section 1:

Collagen material properties

Following a period of 6 days of maturation, myotube cultures were mechanically loaded in the t-CFM bioreactor. It was important to establish some of the material properties of the collagen, to equate loading regimes to the actual mechanical properties of the collagen gel.

The myotube cultures were formed by seeding myoblasts into collagen gels. The collagen gels contained approximately 2% collagen in acetic acid; therefore collagen represents a relatively small proportion of the material, the majority being water. Collagen gels are visco-elastic materials whose response to deforming loads combines both viscous and elastic properties.

Perfectly elastic materials deform under stress, and return to the original shape and size when the load is removed. A viscous material however, after being subjected to a deforming load, does not recover its original shape and size when the load is removed. The collagen gel is a material whose properties lie in between that of an elastic or viscous material, hence visco-elastic.
**Results**

**Ramp loading**: The rate at which strain is applied results in stiffening of the collagen matrix.

Strains of up to 10% deformation (during ramp loading) were applied, and the rates of deformation applied varied from 0.83% per hour to 60% per hour. In the case of ramp loading, the load was applied over varying times. When the load was applied rapidly (over 10 minutes), the dynamic stiffness was increased such that this loading stopped; the material became more compliant and was unable to retain the entire applied load. In essence the collagen gel underwent stress relaxation as a result of its visco-elastic properties (figure 3.1). The stress relaxation (unloading) occurred within three hours of the end of loading. The deformation retained by the gel was approximately 5.5% (55% of the original load applied). The rate of strain application in this case was 60% per hour (over 10 minutes).

As tensile load is applied to the collagen gel, the gel elongates so the core is compressed (i.e. the collagen fibres are pushed together). This is the origin of the dynamic stiffening at high rates of strain, as shape change is too rapid for water to move in response. As a result the apparent stiffness increases but the material continues to deform (i.e. stress relaxation) after loading, as water content is re-distributed. This is typical visco-elastic behaviour. The difference between applied strain (10%) and actual measured displacement (of the CFM force transducer) reflects the highly compliant gel properties, representing reorganisation of the internal structure and shape (figure 3.2).

Therefore the collagen gel relaxed when the loading finished. In this case only 5.5% of strain was retained by the collagen gel, as the remaining 4.5% was lost.
**Results**

Figure 3.1 Graph showing 10% strain applied to the collagen gel, over a 10-minute period (60% per hour). Following the ramp load, a final force of 3500 \( \mu N \) is retained, equivalent to an actual strain of 5.5% (deformation).

Figure 3.2 Deformation of the collagen gel lengthways results in three changes of the collagen gel. (i) Compression deformation of the gel as it becomes narrow...
at its waist (ii) movement of water (iii) alignment of collagen fibrils. These are consequences of high material compliance.

The effect of strain rate was apparent when the same 10% strain was applied to a collagen gel over a one-hour period (i.e. 10% per hour). In this case a far higher proportion of the applied strain was retained (i.e. stress relaxation was reduced) and so a greater load was retained by the material (figure 3.3).

![Figure 3.3](image)

**Figure 3.3.** 10% strain was applied to the culture over 1 hour (10% strain per hour). A total of 6000 μN (force) was required to produce 10% strain in the collagen gel. Stress relaxation under this loading was reduced and so final strain was greater than under rapid loading (60% strain per hour).

It is known that the response of a viscoelastic material is affected by the duration and time for which the load is applied, hence the rate at which load is applied i.e. viscoelastic behaviour is strain-rate dependant. It was expected from the collagen gels
Results

tested, that the greater the rate of strain application, above 10% strain per hour, the
greater the dynamic stiffness of the collagen material. In the case of the 10-minute
ramp (60% per hour) and 1 hour ramp (10% strain per hour) loading, the predicted
increase in final strain was seen at lower rates (figure 3.1 and 3.3). This behaviour
complicates the way in which external loading of cultures is likely to affect resident
cells.

<table>
<thead>
<tr>
<th>Type of mechanical loading</th>
<th>Percentage of Stress relaxation</th>
<th>Final actual strain in the material (10% applied)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 minute ramp</td>
<td>45%</td>
<td>5.5%</td>
</tr>
<tr>
<td>1 hour ramp</td>
<td>17%</td>
<td>8.3%</td>
</tr>
</tbody>
</table>

Table 3.1. A table showing the total strain retained by the collagen matrix. Due
to the visco-elastic property of the collagen, the percentage of strain retained
was lowered, reflected in the retained strain (right hand column).

Cyclical load: Increasing the frequency of cyclical load results in stiffening of
the matrix

Strains of up to 1% deformation were applied during cyclical loading. The frequency
of cyclical loading was varied from 1 cycle per hour to 10 cycles per hour; this
resulted in an increase in the rate at which strain was applied. The strain rates varied
from 4% per hour (1 cycle per hour) to 40% per hour (10 cycles per hour).
Results

Each cycle consisted of four equal phases, (i) loading of collagen gel, (ii) constant load on collagen (iii) unloading of collagen gel (iv) no strain application, held at baseline level.

![Diagram of cycle](image)

Figure 3.4 Diagrammatic representation of one cycle of loading on the collagen gel, using the t-CFM. Phase 1 consists of load application, and after being held at 1% strain (phase 2) the collagen gel is unloaded (phase 3), and finally held at baseline endogenous tension (phase 4). Each cycle was repeated 1, 5 or 10 times per hour over a 12-hour period depending on the regime used. Each phase of the cycle was of equal duration.

The time of each cycle varied depending on the cyclical regimen applied. Where the 1 cycle per hour regimen was performed each cycle lasted the complete 1-hour, and each phase was 15 minutes. Compared to the 10 cycles per hour regimen, where each cycle lasted 6 minutes, with each phase maintained for 1.5 minutes.
Results

A-cellular collagen gels were mechanically loaded in the t-CFM. By increasing the cycling frequency the matrix stiffness increased. This was evident, as a greater amount of force was required to apply the same strain.

In figure 3.5, a one cycle/hour-loading regimen was applied. The mean force across the construct did not change over time, therefore with this frequency, matrix stiffening was not observed, as reported previously (Eastwood et al. 1998).

Figure 3.5 Graph showing 1 cycle per hour loading regimen applied to a plain collagen gel.

In comparison, when a 5 cycle per hour loading regimen was applied, the force required to apply a 1% strain is increased greatly. An extra 50 μN of force is required to deform the matrix by the last cycle applied. This was further increased when the 10 cycle per hour regimen was applied.
Figure 3.6. Graph showing 5 cycle per hour loading regimen applied to a plain collagen gel. 1% strain is applied during each cycle, as the cycling increases a greater amount of force is applied to create the same strain.

<table>
<thead>
<tr>
<th>Type of load applied</th>
<th>% Increase in force applied between first and last cycle.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 cycle/hour</td>
<td>0%</td>
</tr>
<tr>
<td>5 cycles/hour</td>
<td>8%</td>
</tr>
<tr>
<td>10 cycles/hour</td>
<td>12%</td>
</tr>
</tbody>
</table>

Table 3.2. A table showing increases in the total amount force required to deform the matrix by 1%. This percentage is the increase in addition to the 600 μN required to apply 1% strain to a plain collagen gel.
Results

Results section 2:

Development of 3D skeletal model

Endogenous tension developed by skeletal myoblasts in comparison to other cell types:

Comparison of typical contraction profiles generated by the three cell types studied showed significant differences in peak force attained, initial contraction rate (the first 100-600 μN of force generated), and the time point at which contraction of the matrix began (figure 3.7). Whilst the peak force attained was the first distinct difference between the cell types, more interestingly, the instantaneous force generated at the key time point of 8 hours (when force generation was evident) was 70 μN, which was the baseline level of force generation for myoblasts compared with 870 μN for human dermal fibroblasts (approximately 12 fold greater), and 2010 μN for smooth muscle cells (approximately 29 fold greater).
Results

Figure 3.7. Comparison of typical contraction profiles using five million cells from three cell types. C2C12= immortalised mouse skeletal muscle myoblasts, HDF= human dermal fibroblasts, SMC= mouse smooth muscle cells. The contraction profiles were obtained by seeding five million cells into a 5ml collagen gel. The contraction of the collagen matrix by resident cells was measured using a CFM instrument over a 24-hour period immediately following gelling. In this example, the instantaneous force generated was determined at 8 hours. Each force-time profile is the mean of 5 separate experiments around which variation was less than 20%.

The initial contraction rate was determined over the first 100 and 600 μN of force generation. Dermal fibroblasts and smooth muscle cells had the highest and effectively the same initial contraction rates at 8 ± 1.1 and 7.4± 0.4 μN per minute,
respectively. These were 40% higher than for skeletal myoblasts at 4.8 ± 0.4 μN per minute (figure 3.8). This high initial contraction rate was maintained for over 8 hours in smooth muscle cell cultures, giving characteristically elevated inflexion point and peak force. Onset of contraction of the matrix by each cell type differed considerably. Smooth muscle cells and dermal fibroblasts both generated force immediately (100 μN in the first half hour), however in myoblast cultures there was an initial lag in force generation, lasting 6.2 ± 1.8 hours (during which the total force produced was less than 100 μN).

![Graph](image)

**Figure 3.8.** Comparison of mean rates of contraction from between 100-600 micronewtons (i.e. on an initial force basis) for three cell types. C2C12 = immortalised skeletal muscle myoblasts (n=4, SD ± 1.4), HDF = human dermal
fibroblasts (n=3, SD ± 1.9), SMC= rabbit bladder smooth muscle cells (n=3, SD ± 0.7).

Long term monitoring of the development of force by these 3D cultures (0-68 hours) is shown in figure 3.9. The contraction profile was divided into discrete regions of different gradients within the profile, which were termed phases. After rapid contraction over the first 12 hours (phase 1 and 2) force increased along two shallower and significantly different gradients (phase 3 and 4). Phase 5 was a plateau of steady state force maintenance (57-68 hours). Detailed analysis of gradients using 4-hour periods clearly showed that the gradients of phases 2 to 5 were distinct from each other, rather than part of a continuous curve (figure 3.10). In effect phases 3 and 4 represent a 45 hour ramp gradient (increasing the total force by 60%) followed by a complete halt to the increase.
Figure 3.9. Three day mean force profile for myoblasts (n=3), showing increasing force within 5 phases for 20 million cells. The arrows denote changes in phase of contraction.
Results

Figure 3.10. Histogram showing the mean gradient for force generation (rate of change of force) over phases 1 to 5, which were identifiable from the graph (phase 1 = 0-4 hours; 2 = 4-12 hours; 3 = 12-34; 4 = 34-57 hours; 5 = 57-66 hours). Each phase appeared to have a distinct slope from the previous phase, which were significantly different. A T-test was performed between each neighbouring phase. Mean gradients were calculated (± SD) from the instantaneous gradients every 4 hours through the time course (due to the short time for phase 1, 1 hour time points were taken), and this is shown by the decreasing rate of contraction (phases 2-5). * = P<0.01, ** = P<0.001
Delay in myoblast-matrix attachment

Possible causes of the 6-hour lag period were suspected to relate to the ability of cells to attach, spread and extend cell processes, therefore cell morphology was examined over this time course (figure 3.11). Between 0 and 6 hours post-seeding, myoblasts in collagen gels remained rounded with little extension of cell processes, in contrast to the behaviour of dermal fibroblasts over the same time period in collagen gels (Eastwood et al. 1996, Talas et al. 1997) and consistent with delayed force generation. Extension of cell processes became evident from 6 to 8 hours over which period cell shape changed to become stellate.

Figure 3.11. Morphology of myoblasts in 3D collagen matrix. (3.11a) Myoblasts in collagen remained rounded 1 hour post-seeding. (3.11b) 4 hours post-seeding
Results

there was very little change. (3.11c) 8 hours post-seeding some cell processes became visible. (3.11d) 24 hours post-seeding, many cell processes were visible. Cell clumping had also become evident.

Uni-axial tension leads to alignment of myoblasts

Where contracting gels were tethered uni-axially (in the CFM) cells became predominantly bipolar after 24 hours under tension, and aligned parallel with the long axis of the gel (figure 3.12). Apparent in the collagen gel is the contraction of the collagen matrix, which was indicated by the tapering along the sides. Random cell alignment was found in the delta zone of the gels where there was non-directional strain, immediately adjacent to the stiff floatation bars (figure 3.13b) (Eastwood et al. 1998).
Figure 3.12a. A collagen gel immediately after it has been set in a rectangular mould. 3.12b. 24 hours after cell contraction by the resident myoblasts within the collagen gel. Shortening of the collagen gel is restricted by the stiff bars on either side of the gel, contraction is evident along the sides if the gel, indicated by the arrow.
Figure 3.13. Alignment of myoblasts within a CFM collagen gel 24 hours after being cast. (3.13a). Randomly aligned myoblasts in a collagen gel. The two arrows indicate the stellate multi-directional nature of the cell processes. (3.13b). Aligned myoblasts in a collagen gel. Note the axis of principal strain is shown by the double-headed arrow, along the long axis of the gel. The two single headed arrows highlight the direction of the cell and cell processes.

Within the contracted collagen matrix, there was an internal control, where the embedded myoblasts were not under the influence of the principal line of strain through the gel, these regions were termed the delta zones, and were directly adjacent to the stiff floatation bars. The majority of myoblasts within these regions died after three days, stressing the importance of tension to the survival of the myoblasts.
Results

(figure 3.14). The myoblasts, which survived in this region, aligned along the bar, and used this feature to provide the necessary tension.

Within the collagen gel it was important to establish myoblast morphology within the different regions over the complete seven-day period for which myotube gels were cultured. Primarily the regions studied were the delta zone and central zone. Day three myoblasts appeared to not only be aligned, but also had long cellular processes aligned to the strain. There was a small percentage of myoblasts, which appeared rounded, which is indicative of cell death. On day three myoblasts were aligned along the principal line of strain through the gel (figure 3.14a), however in the delta zone cells appeared rounded, with little cell processes and no alignment to the strain applied to the gel (figure 3.14b). This was somewhat different to the delta zone in day 1 gels, here the myoblasts appeared more stellate, with evidence of cell processes.
Results

Figure 3.14b

Figure 3.14. Myoblasts embedded within a collagen gel on day three visualised using light microscopy. (a) Myoblasts appeared to be aligned along the line of principal strain (double-headed arrow), with long cellular processes. (b) The majority of myoblasts appeared rounded and non-aligned. Some stellate myoblasts were evident.

After seven days the appearance of myoblasts within the different regions of the gel had changed. In the central zones, the myoblasts were all aligned, and in the cases where this alignment was end to end, fusion of the myoblasts was apparent (figure 3.15b).
Figure 3.15. Morphology of myoblasts in different regions of the collagen gel. (a)

Within the central zone, myoblasts aligned along the principal line of strain.
Results

(double-headed arrow) applied to the collagen gel. (b) Alignment of the myoblasts to the principal line of strain is shown (double-headed arrow). Myoblasts align end to end, and fusion is evident (F) between the cells. No lateral fusion between myoblasts was observed. (c) Within the delta zones of the collagen gels, no alignment was observed. The majority of myoblasts appeared rounded, indicative of cell death.

IGF-1Ea and MGF mRNA expression over a seven-day study

Real time Quantitative PCR was used to determine the levels of IGF-1Ea and MGF mRNA expression in the developing model tissue, over the 7-day period in culture and under uni-axial tension. During this cell fusion and maturation period, mRNA levels were monitored and underwent a transient elevation of both IGF-1Ea and MGF gene expression on day 3. This brief peak in expression fell back almost immediately to basal levels (day 4) and remained constant until assessment of force generation on day 6 (figure 3.16). From day-2 to day-3 there was a 36-fold increase in IGF-1Ea expression and a 77-fold increase in MGF expression.
Results

Figure 3.16. Levels of IGF-IEa and MGF mRNA in differentiating myoblasts in a 3D collagen matrix. (3.16a) IGF-IEa mRNA expression increased 36 fold from day-2 to day-3.6b. (3.16b) MGF mRNA expression increased 77 fold from day-2 to day-3. The 1st and 2nd measurements are duplicate runs.

Morphology and contractile properties of the 3D skeletal muscle culture

The next stage of this study aimed to characterise the development of a 3-D model neo-tissue consisting of aligned, differentiating myoblasts maintained under uni-axial load (figure 3.17). Here it was evident from cytoskeletal phalloidin staining (of cytoskeletal actin) that fusion of myoblasts had occurred (figure 3.17a). Figure 3.17b illustrates the network of myotubes formed, with alignment clearly visible, along the uni-axial strain through the gel. Cultures were maintained under tension (hence maintaining cell alignment) over periods of up to 6 days, in order to promote fusion to myotubes.
Results

Figure 3.17. Phalloidin staining of myotubes in a tethered collagen gel. In (3.17a) arrows indicate the unstained nuclei of the myotube. The lower magnification picture (3.17b) shows the myotube network, and the arrow indicates the direction of uni-axial strain applied to the gel.

The force generation profile over 24 hours for such myotube rich gels (post 6-days) was distinct from that of myoblast gels (3.18). The maximum force produced in myotube gels varied from 59-132 μN per million cells seeded (mean peak force of 106 ± 16). This compared with an equivalent force generation by myoblast gels of 228 ± 21 μN per million cells (range between 180- 320 μN per million cells). Force generation for myotube gels was characterised by a series of rapid contractions, or jumps, typically at 2 time points in the analysis, after the zero time jump.
Figure 3.18. A typical myotube contraction curve. The total force produced, in this case was 1150 μN. Jumps evident at (i) 0 time point – not observed in all samples, (ii) First jump, (iii) Second jump.

The rapid contractions were at 2.1 ± 0.28 hours (range= 1.3-2.9 hours- 95% C.I.) and 7.0 ± 0.45 hours (range= 6.0-7.8 hours, 95% C.I.). The time of onset of the jumps was constant between runs (n=4), but the total force generated in each jump varied considerably (table 3.3). This effect appeared to be independent of the force generated by each jump, and may represent a form of synchronous contraction.
Table 3.3. Statistical analysis showed the time points at which the jumps occurred (only occurred twice in 24 hour period), these were consistent between the replicates (n=4).

In summary: the model tissue developed over a 7-day period, the pattern of force generation changed from initially a gradual contraction of the collagen gel myoblasts, to sharp jumps in contraction for the differentiated myotube cultures. During that period cell fusion occurred, following a peak at day 3 in IGF-1 and MGF mRNA expression. This was preceded by a steady rise in force generation and followed by onset of myoblast fusion.
Results

Results section 3:

Morphological characterisation of differentiating C_2C_{12} myoblasts in 3D culture

The morphological characterisation of differentiating myoblasts in a developing skeletal muscle cultures (over ten days) was studied using transmission electron microscopy. It was possible to see the myoblasts attach to the collagen gel and align along the direction of tension applied to the gel. This alignment of myoblasts was evident by day seven. Fusion of myoblasts only occurred when myoblasts aligned end to end, where the diameter of the myotubes was one nuclei. Cultures were mechanically loaded at this stage (day seven), it was therefore important to establish the morphology of the myoblasts by day seven, as this was the stage at which the mRNA was quantified. The question asked was 'what was being mechanically loaded, myoblasts or myotubes?' An exact percentage could not be ascertained, however by day seven all myoblasts were either aligned or fused, and by day ten, no single nuclei myoblasts could be observed. Gels were studied until day 10. In this case further contraction of the gels was evident from the appearance of the collagen, as the gel was dehydrated (in the medium) the percentage of collagen fibres visibly increased. Alignment of myotubes and collagen fibres was shown.

Skeletal myoblast membrane contact, initial stage of differentiation was evident by day 3

As seen by electron microscopy, on day 3 direct membrane contact was evident (figure 3.19a and b). Cells aligned along the direction in which principal strain was applied, this was seen in figure 3.19a, where the double-headed arrow denotes the
direction in which endogenous strain developed. After the myoblasts aligned along the lines of strain, there was direct membrane contact observed between the myoblasts. This only occurred towards either end of the cell processes, and nowhere was this contact found to be lateral, whereby contact was observed between two adjacent nuclei. The distinct cell membrane (CM) surrounding the two myoblasts appears to be lost in a small part of the region between the cells where contact was evident (figure 3.19b). Here fusion was apparent (F), as the cytoplasm was continuous and no distinct cell membrane was evident. What was also apparent in the micrograph was the alignment of the mitochondria (M) along the direction of principal strain.

![Figure 3.19a](image-url)
Results

Figure 3.19. Myoblasts on day 3 after being seeded into the collagen gel. 3.19a. Two cells can be seen aligning to the direction of principal strain (double headed arrow) with a clear region of membrane contact. Here the cell membranes (CM) are distinct up to a point, and then the two membranes are lost in a small region where the cytoplasm may be beginning to fuse, denoted by the 'F' annotation. 3.19b. A close up of the region where the myoblasts are in contact. There is a region where the individual membranes of the cells become indistinct, and the cytoplasm may be beginning to fuse. Also visible in this field is the mitochondria (M), which appear to align along the lines of principal strain.

Alignment of myoblasts by day 7

Sections of collagen gel from day 7 showed alignment of myoblasts. The unidirectionality of the cells was shown clearly with evidence of cell fusion. By day seven all myoblasts had aligned along the line of principal strain (shown by double-
headed arrow), as all nuclei (N) were also aligned along this strain. It must be noted that although bundles of myoblasts aligned together, no lateral fusion was observed between these, as there are clearly spaces (S) between neighbouring myoblasts.

Figure 3.20. Unidirectional alignment of myoblasts is shown. The principal strain running through the gel (parallel to it's long axis) is indicated by the double-headed arrow. It is also possible to see that there are spaces between the
adjacent cells (S). 3.20b Nuclei (N) of myoblasts are aligned in the direction of tension application.
Results

Lower magnification pictures display starkly the difference between myoblasts in a day 3 (figure 3.21a.) gel versus a day 7 gel (figure 3.21b). It is evident from the pictures that there is further alignment of cells in the day 7 gel, and more fusion. The alignment of nuclei (N) can be seen more clearly in the day seven gels, as all the nuclei were aligned along the lines of principal strain.

Figure 3.21a. Light micrograph of myoblasts on day 3. Direct cell membrane (CM) contact between myoblasts is evident. Some nuclei (N) alignment is
Results
evident, however in regions were fusion (F) is occurring this alignment is less apparent. 3.21b. Light micrograph of myoblasts on day seven. Myoblast alignment is evident, the alignment of nuclei (N) is also apparent, in the case of every single cell. There appears to be fusion (F) of myoblasts at the ends, of the cell processes, where cell membranes become distorted. Nowhere is there any evidence of adjacent myoblast fusion, only where myoblasts align, and fusion is end to end.

Myoblast fusion evident by day 7

As well as myoblast alignment by day 7, myoblast fusion to form myotubes was also observed. Myotube formation was evident as indicated by a growing number of elongated multinucleated syncytia. Myotubes were long narrow structures and only one nuclei wide (diameter). In all the sections observed, no myotube was observed where the diameter was two nuclei wide; consistent with the idea that myoblast fusion does not entail the lateral fusion of the myoblasts. Higher magnification pictures showed that the ‘fusion zone’ between myoblasts had lost all of the membrane separating the two cells, with complete cytoplasmic intermingling. Within this region there was a large amount of sarcoplasmic reticulum (SR). These sarcoplasmic reticulum are specialised smooth endoplasmic reticulum, which store CA$^{2+}$, and when this CA$^{2+}$ is released muscle contraction occurs.
Figure 3.22a. Low magnification electron micrograph to show two nuclei in a myotube with complete membrane degradation between the fused cells in the fusion zone (FZ). 3.22b. A higher magnification shows no membrane between the cells in the fusion zone (FZ). In the fusion zone it is possible to see a large number of sarcoplasmic reticulum (SR).
Results

3D cultures by day 10

Cultures were kept tethered for a further 3 days until they had been maturing for ten days. By this stage no single nuclei myoblasts were observed in the collagen gels.

Alignment of myoblast/myotube and collagen fibres

The myotubes in 3D collagen cultures, which were 10 days old, appeared to be very long and remained aligned in the direction of endogenous tension running through the gel (figure 3.23). The alignment of collagen fibres in the gel was also evident. The appearance of an increased percentage of collagen fibrils on day 10 indicates further dehydration of the collagen gel. Although the collagen gel was embedded in media, due to further contraction of the collagen by resident myoblasts and myotubes, the density of collagen fibres increased. The myoblasts consolidated the collagen gel through contraction-orientated compaction. This essentially means that the collagen gel is contracted by the cells and this leads to the loss of water. In figure 3.23, an increase in the collagen fibre density is visible, the result of consolidation by the myoblasts and myotubes.
Figure 3.23 Alignment of both myotubes and collagen fibrils (CF) within the 10-day culture. The increased amount of collagen fibrils within the day 10 gel was a result of consolidation of the collagen by the myoblasts and myotubes, which resulted in dehydration of the collagen gel. The alignment of nuclei (N) corresponds to the line of principal strain through the collagen gel (shown by the double headed arrow). It is also possible to see alignment of collagen fibrils to the line of principal strain.
Results

Presence of banding in healthy myotube nuclei

By day ten the myotubes present in the collagen cultures had very long nuclei, up to 10μM long. All the myoblasts were differentiated, as no single nuclei myoblasts were present in any sample. The myotubes remained aligned along the line of principal strain. The appearance of banding (B) in the nuclei of the myotubes was evident in all of the cultures. The number varied from one band to three bands per nuclei. It was unknown whether this was fusion of nuclei within a myotube.

Figure 3.24a
Figure 3.24 The elongated, aligned myotubes within the collagen gels exhibited banding in their nuclei. 3.24a. A myotube with an elongated nuclei (N). There are 2 bands (B), which are evident and split the nuclei into 3 parts. The myotube and nuclei are aligned along the principal strain (double headed arrow) within the collagen gel. 3.24b. A picture taken at higher magnification, where the banding (B) can be seen.

Myotubes within collagen cultures, which had been left to mature over a ten-day period, displayed features of active cells, i.e. cells that were actively producing protein. Present in these myotubes were Sarcoplasmic Reticulum (SR) and Golgi complexes, which are involved in the secretion of many exocrine and endocrine proteins.
secretions. Numerous mitochondria were also found around the cell, all indications of a healthy and active cell.

Figure 3.25. Alignment of myotube in day 10 gel along the line of principal strain (double-headed arrow), with banding of nuclei and a large amount of golgi apparatus (G). The myotube with banded nuclei (B) were healthy cells, not undergoing apoptosis, as synthetically active cells were indicated by the extensive sarcoplasmic reticulum (SR), mitochondria (M) and golgi apparatus (G).

Also apparent was the large amount of granular (or rough) sarcoplasmic reticulum (SR) (figure 3.25). Here it is apparent that alongside the SR is golgi apparatus, and a
large number of mitochondria (M). All of these features indicate that the myotubes are healthy, it was therefore hypothesized that the nuclear banding seen was nuclear fusion. Although this has not yet been reported in muscle in vivo, it is possible that this is an early stage phenomenon seen by virtue of the accessibility of this model, or alternatively, a non-physiological response to the in vitro culture environment.

Figure 3.26 Sarcoplasmic Reticulum (SR) displayed in myotube. Smooth endoplasmic reticulum, also known as sarcoplasmic reticulum. These are specialised ER in muscle cells which store CA^{2+}. Golgi apparatus (G) was also visible. All these features denote a healthy cell in a state of protein manufacture.
Results

Results section 4:

Skeletal myotube model responses to applied mechanical loads

After producing a 3D in vitro model of early skeletal muscle development, it was possible to then apply various mechanical loading regimens to the cultures, and observe changes in regulation of IGF-I\(\alpha\) and MGF. The mRNA expression of IGF-I\(\alpha\) and MGF were established as the muscle culture developed. Once this characterisation was complete, it was possible to mechanically load the cultures and observe changes in the mRNA expression of IGF-I\(\alpha\) and MGF. The absolute changes were of as much importance as the changes in respective ratios of the splice variants.

Various mechanical loading regimens were applied to skeletal muscle cultures in the t-CFM bioreactor. Changes in regulation of IGF-I\(\alpha\) and MGF mRNA expression were observed, depending on the type of mechanical loading regimen (cyclical or ramp), total strain application and rate of strain application. The baseline levels of IGF-I\(\alpha\) and MGF mRNA were established in cultures, where no force was externally applied. The only force acting on the myotubes was the endogenous tension generated by the cells themselves.

Effect of cyclical load on mRNA expression levels of IGF-I isoforms in 3D skeletal muscle cultures

The first mechanical loading regimen to be studied was cyclical loading, where the frequency of cyclical loading was altered, this was compared with cultures with no external loading (static). Interestingly, there was a relatively high basal expression of
Results

this species under static load. Application of a 1 and 5 cycle/hour loading regimen resulted in a 36% and 46% decrease in the expression of IGF-IEa respectively, whilst at 10 cycle/hour loading regimen there was a 90% decrease in IGF-IEa mRNA expression (figure 3.27). Hence IGF-IEa mRNA expression was inversely related to the number of cycles applied. These differences all reached statistical significance compared with static loaded basal expression.

![Figure 3.27. The effect of cyclical loading on IGF-IEa mRNA expression in myotube cultures. 1% strain was applied to skeletal myotube organoids over a 12-hour period, at 1, 5 and 10 cycles per hour. IGF-IEa mRNA was measured at the end of this 12-hour period. No strain was applied to the control cultures, maintained under endogenous tension with no external loading. Figures show](image-url)
mean copy number (n=3), with bars indicating ± SEM. Asterix shows statistical significance of treatments compared to static loading (* = P<0.05, ** = P<0.001).

For 1 cycle/hour loaded cultures the mean copy number was 10309 (n=3, SEM ± 1412), the mean copy number for 5 cycle/hour-loaded cultures was 8684 (n=3, SEM ± 414), and for 10 cycle/hour-loaded cultures, the mean copy number was 1544 (n=3, SEM ± 889). Therefore the expression of IGF-IEa decreased when the cells were subjected to cyclical strain.

In contrast to the high baseline levels of IGF-IEa mRNA expression, MGF mRNA expression in static loaded cultures was negligible (mean of <100 copy numbers/µg RNA), very close to the limit of detection by quantitative PCR. It is important to emphasise that IGF-IEa and MGF mRNA levels were measured from these same mRNA samples, representing a measure of the differential response of these two splice variants to the same pattern of loading. Application of the slowest cyclical loading (1 cycle per hour) stimulated expression of MGF mRNA by 807 fold over the basal. Increasing load (5 and 10 cycles per hour) gave progressively less effect, with a 79 fold and 0 stimulation respectively. Again this suggests that a highly sensitive threshold strain response.
Results

Figure 3.28 These data show the effect of a 1% strain applied to myotube cultures over a 12-hour period, at 1, 5 and 10 cycles per hour. No strain was applied to the control cultures, which were maintained under endogenous tension. Here the mean copy was 50 (n=3, SEM ± 34). MGF mRNA was measured after this 12-hour period. Figures show mean copy number (n=3), with bars indicating ± SEM. Asterix shows statistical significance of treatments compared to static loading (* = P<0.05).

Effect of ramp loading on mRNA expression levels of IGF-I isoforms in skeletal muscle cultures

For ramp loading regimens, the total 10% strain was applied as a single movement and IGF-IEa mRNA expression was measured in triplicate cultures (figure 3.29). It is important to note that the same non-loaded baseline data is shown as for figure 3.27,
but expressed on an axis that is compressed by 250-fold to accommodate the maximal expression level. Hence the same basal level (16, 143 copies/µg RNA) appears trivial. Application of a slow (12 hour) ramp load produced a 3.5 fold increase over basal IGF-IEa mRNA expression. Strikingly an intermediate ramp loading (1 hour) produced a 1800 fold increase in expression whilst the fastest loading rate (10 minutes) resulted in a small (2 fold) fall in expression of IGF-IEa mRNA expression. This is consistent with a marked threshold response to strain rate.

Only three different strain rates were studied in the ramp loaded samples, therefore a pattern was difficult to establish, there was however a trigger response elicited following the 1 hour ramp load, where the rate of load applied was 10% per hour (1-hour ramp regimen) were applied. Mechanical loading of cyclical regimens elicited a steady decline in IGF-IEa mRNA expression, as the frequency of cycles was increased. These responses clearly show that the type of mechanical load applied to cultures was important in determining changes in IGF-IEa mRNA expression. In other words, it mattered to the cell response that strain was applied continuously as a single increasing tensile load (stretch) rather than a series of strains in alternate direction (cyclical loading).

Figure 3.29 shows a relatively high level of expression under static load, such that strain sensitive responses operated to alter that basal level of expression.
Figure 3.29. The effect of ramp loading on IGF-IEa mRNA expression in skeletal myotube cultures. 10% strain was applied to skeletal myotube cultures over different periods, either 10 minutes, 1 hour or 12 hours. Cultures were kept in the t-CFM bioreactor for 12 hours, after which IGF-IEa mRNA levels were measured. Where the 10% ramp was applied over a 10-minute time-span (60% per hour), the mean copy number of IGF-IEa mRNA was 9622 (n=3, SEM ± 720), where 10% strain was applied over 1 hour (10% per hour), the mean copy number was 2911109 (n=3, SEM ± 1602857). 10% strain applied over 12-hour period (0.83% per hour) resulted in IGF-IEa copy number of 56049 (n=3, SEM ± 15244). Asterix shows statistical significance of treatments compared to static load (* = P<0.05).

Figure 3.30 shows the effect of slow, intermediate and fast ramp loading on MGF mRNA expression, demonstrating a similar threshold response, again to the intermediate (1 hour) loading rate. This was a similar pattern to the response with
Results
cyclical load, with optimal stimulation at 10% strain per hour of load, compared to 4% per hour with 1 cycle per hour of loading. The effect of ramp loading was, however more profound than cyclical load, by approximately 3.5 orders of magnitude above the basal level. The 12-hour ramp load (0.83% strain per hour) resulted in no change in levels of MGF mRNA expression, whereas 10% strain per hour (the 1-hour ramp) resulted in an increase in the MGF mRNA expression of over 6000 fold; the greatest up-regulation found in the study. Further increase in strain rate to 60% strain per hour (the 10 minute ramp) resulted in a much smaller 110-fold increase. Note that the basal or housekeeping level of MGF was very low, giving the effect of enormous rate of over stimulation and making MGF by far the most sensitive of the two IGF-1 splice variants to loading.

![Figure 3.30](image)

Figure 3.30 The effect of ramp loading on MGF mRNA expression in skeletal myotube cultures. 10% strain was applied to skeletal myotube cultures over different periods, either 10 minutes, 1 hour or 12 hours. Where the ramp stretch was applied over a 10-minute time-span (60 % per hour), the mean copy
number of MGF mRNA was 5548 (n=3, SEM ± 2566), where the ramp stretch was applied over 1 hour (10% per hour); the mean copy number was 302952 (n=3, SEM ± 22641). The ramp stretch applied over 12-hour period (0.83% per hour) resulted in no MGF mRNA expression.

The threshold response of myotube cultures to different applied strain rates was studied by charting the different strain rates applied and the corresponding IGF-IEa and MGF mRNA expression levels (figure 3.31a and 3.31b). In the case of ramp loading, strain rates in the order of 10% per hour resulted in the greatest up-regulation of expression of both IGF-IEa and MGF, falling back to basal levels at high rates (60% per hour). In the case of cyclical loading, the optimal applied strain rate was 4% per hour, which produced a dramatic up-regulation of MGF. Only IGF-IEa mRNA expression differed from this pattern in that cyclical loading at all strain rates resulted in decreased mRNA expression. It was also notable that ramp type strains produced much greater effects on mRNA expression than comparable strain rates applied as cyclical loading. In the case of cyclical loading a similar threshold response was noted with the MGF mRNA.

![Figure 3.31a](image_url)

*Figure 3.31a*
Figure 3.31. Effect of application of different strain rates on the expression of IGF-IEa and MGF mRNA. The base data in figures 3.21-3.24 was converted to represent the effect strain rate had on the expression of IGF-IEa and MGF. (a) 1% strain was applied during each cycle of the cyclical load regimen, representing: 4%, 20% and 40% strain per hour for the 1, 5 and 10 cycles/hour respectively, applied in phase 1 of each cycle (i.e. 1% strain over 15, 3 and 1.5 minute periods respectively). (b) The 10% strain applied over the three regimens represents: 60% strain/hour over the 10-minute period (10 minute ramp); 10% strain over the 1 hour period (1-hour ramp); and 0.83% strain over the 12 hour period (12-hour ramp). Each ramp thus loaded a total of 10% strain.
Results

Effect of mechanical loading on GAPDH mRNA levels in skeletal muscle cultures

GAPDH levels were determined in 1 sample per loading regimen studied (fig. 3.32). This was important, as GAPDH is known to be a housekeeping gene and often used as a reference gene. It has been found that GAPDH expression is not altered by mechanical stimulation. As expected therefore, GAPDH levels were not significantly altered between loading regimens, this also confirmed that they were not having major damaging effects on the cells and highlighting the mechano-sensitive nature of the two IGF-I splice variants tested here. The co-efficient of variation between these samples was less than 10% across the range of loading patterns.

![Graph showing GAPDH levels](image)

Figure 3.32. The effect of different mechanical loading regimens on GAPDH expression levels in skeletal myotube cultures. This shows the values for
Results

GAPDH mRNA. 1 sample per loading regimen was tested (n=1). The coefficient of variation between these samples was less than 10%.

Stability of IGF-IEa and MGF mRNA levels over a 12-hour period

After application of ramp load over a 1-hour or 10-minute period, cultures remained held at 10% strain over 11 hour or 11 hours and 50 minutes (respectively) after load was applied. Whilst the time for ramp loading varied, all cultures were left in the t-CFM bioreactor for 12 hours, i.e. the time-point at which mRNA was extracted from samples and analysed for IGF-IEa and MGF mRNA. Consequently it was important to establish the stability of IGF-IEa and MGF message over the complete 12-hour period. This was to discount any possible effect of progressive loss over time due to the instability of the RNA or incomplete expression.

The 1-hour ramp period was used to test this at time-points 0, 4, 8 and 11 hours after load was applied (fig. 3.33a and 3.33b). Both IGF-IEa and MGF mRNA expression responded in a similar fashion. At 1 and 4 hours after loading, the levels of IGF-IEa and MGF mRNA measured were the same as the control but by 8 hours expression had increased in both (by 100 and 2000 fold, respectively). Between 8 and 11 hours after loading, both IGF-IEa and MGF reached a plateau in the levels of mRNA expression and remained constant, demonstrating a high level of message stability over the experimental time course.
Figure 3.33. Changes in levels of IGF-IEa (3.33a) and MGF (3.33b) mRNA, up to 11 hours after 1-hour ramp load (stretch) was applied to skeletal myotube cultures. These figures show the expression curves for both IGF-IEa mRNA and MGF mRNA (initiated by a single ramp stretch) were similar, even though absolute values were different. Up to 4 hours after ramp load was applied, levels for IGF-IEa mRNA and MGF mRNA were the same as baseline levels. Then
Results

from 4-8 hours mRNA expression increased. Between 8 and 11 hours a plateau in the levels of mRNA were shown.

Changes in IGF-IEa/MGF ratio following mechanical loading

The rate of IGF-IEa to MGF mRNA expression in the cultures changed with increasing strain rate. Again the responses were very different for cyclically loaded and ramp loaded samples (figure 3.34a and 3.34b), indicating a profound distinction in differential expression. The application of ramp regimens was studied first. The lowest strain rate (8.3 μN/minute/12-hour ramp) did not elicit a change in the expression ratio compared to static loaded cultures (100/0 IGF-IEa /MGF). As the strain rate was increased the IGF-IEa/MGF mRNA ratio changed. The proportion of MGF mRNA gradually increased until it was 91/9 (IGF-IEa/MGF mRNA), when 100 μN/minute of strain was applied. At the greatest strain rate applied, 600 μN/minute, the proportion of MGF mRNA was further increased to 67/33 (IGF-IEa/MGF). The cyclically loaded cultures displayed a different response to increases in strain rate, in terms of changes in ratio. The lowest strain rate applied (1 cycle/hour= 40 μN/minute), resulted in a greater proportion of MGF mRNA expression at 20/80 (IGF-IEa/MGF). As the cycling frequency, and strain rate were increased the MGF mRNA proportion gradually decreased. The final ratio was 100/0 (IGF-IEa/MGF) when 400 μN/minute of strain were applied. The increase in strain rate resulted in changes in ratios of the IGF-IEa and MGF splice variants, therefore the type of mechanical loading was crucial to these changes.
Results

Application of increasing strain when ramp loading cultures, favoured a greater proportion of MGF mRNA expression, whereas with the cyclical loading, application of increasing strain favoured a greater proportion of IGF-I Ea mRNA expression.
Figure 3.34a. The effect of increasing strain on IGF-I and MGF proportions in both cyclical and static loaded samples. As expected, a significant load gradient was observed in the proportion of MGF. In IGF-I, a gradual increase was noted from static to 12 hours of loading, followed by a decrease in the case of 1 hour of loading.
Figure 3.34. The effect of increasing strain on IGF-IEa and MGF proportions in both cyclical and ramp loaded cultures. As strain rate was increased in cyclically loaded cultures, the proportion of MGF mRNA gradually decreased. After 1 cycle/hour of load, the ratio of MGF:IGF-IEa was 80:20, this gradually decreased to 0:100, in the case of 10 cycle/hour of loading.

As strain rate was increased in ramp-loaded cultures, the proportion of MGF mRNA expression gradually increased. After 12-hour ramp the MGF:IGF-IEa mRNA ratio was 0:100, following the 10-minute ramp load (the highest strain applied, 600 μN/minute) the ratio of MGF:IGF-IEa mRNA expression in skeletal cultures was 37:63.
Discussion

Original Hypothesis

Embedding C_{2}C_{12} skeletal myoblasts at a concentration in excess of one million cells per ml, into a 3D collagen gel, with application of uniaxial tension will result in fusion of myoblasts to form multinucleated myotubes.

By seeding four million myoblasts per ml of collagen, with the generation of uniaxial endogenous tension after tethering, resulted in fused multinucleated myotubes by day seven in culture. Fusion in these cultures was evident by day three.

Application of both ramp and cyclical loading regimens to 3D skeletal myotube cultures will result in up-regulation of both isoforms of IGF-I, IGF-I\(\text{Ea}\) and MGF.

This hypothesis was not correct, and now seems over simplified. Each isoform responded independently in response to cyclical load. For MGF this pattern of loading resulted in an up-regulation at certain frequencies at least, in comparison, this loading resulted in a complete down-regulation of IGF-I\(\text{Ea}\). The application of ramp loading resulted in similar responses for both isoforms, where at 10% strain per hour, there was optimal stimulation of both isoforms.

In addition to this, the ratio of both isoforms was influenced by the type of mechanical loading and the rate of strain application. The patterns characterised result in further hypotheses, to be tested.

Increases in strain rate, during application of ramp loading of 3D muscle cultures will result in greater proportion of MGF being spliced in these cultures, in relation to the static cultures. By increasing the strain rate during cyclical loading, greater proportions of IGF-I\(\text{Ea}\) will be spliced, in relation to 1 cycle per hour loading, where IGF-I\(\text{Ea}:\) MGF ratio was 20:80.
Discussion

Conclusions

An important conclusion from this study is that there is a clear strain-rate dependence, in terms of mechanical regulation in early myotubes on growth factor gene expression and that in the case of IGF-I this involves gene splicing to give different splice variants which have different biological functions. Total IGF-I mRNA expression was optimally stimulated by 10% strain per hour (both ramp and cyclical loading regimens) (though the magnitude of stimulation was much greater for ramp loading). This would lead to muscle hypertrophy if translated to muscle in vivo. In most cases a threshold response was seen in the range of 4-10% per hour, which consistently suppressed IGF-IEa mRNA expression). It is concluded that ramp loading not only invokes a different response, but strain rate is also critical in determining the type and magnitude of mRNA expression and IGF-I gene splicing. MGF mRNA expression appears to be more sensitive to strain rate than to the type of mechanical loading.

Since IGF-I isoforms have distinct functions in myogenesis and muscle growth and these are regulated in different ways following mechanical loading, it is hypothesised that application of mechanical strain to this cell type affects the splicing of IGF-I. The mechanism for this remains unknown and it is plausible there may a direct link between mechanical loading and gene splicing. It is known that mechanical strain applied to cells can directly affect mitotic spindle formation within the nuclei, therefore if mechanical strain may have a direct and immediate affect on splicing (Wang et al. 1997). The deformation of membrane integrins and focal adhesions result in simultaneous
deformation of the cytoskeleton and the nucleus of cells, therefore this direct affect on
splicing seems less far-fetched with this knowledge (Wang et al. 1997). Recent evidence
that the mechano-transduction mechanism involves the dystrophin complex of the
cytoskeleton is suggested by the fact dystrophic muscle has an impaired ability to
produce MGF (Goldspink 2003). This function can be restored by the transfer of
functional dystrophic muscle using transfected pluripotent stem cells (De Bari et al.
2003). However this change in splicing may be the result of indirect actions of the
mechanical strain. We know the splicing mechanism is dependent upon There is a
transfer of mechanical signals to biochemical signals within the myotubes, and this
‘transfer of information’ is difficult to test directly.

As well as regulation of the IGF-I gene, the differential isoform splicing provides a
vertebrate mechanism for producing distinctly separate regulatory growth factors. The
independence of the total IGF-I expression from the differential isoform regulation
suggests two levels of control, which would provide extra sensitivity to the cell for
responses to mechanical signalling. This would imply that a complex pattern of
signalling may operate, including dependence on IGF-I Ea: MGF ratios. Identification of
this new level of ‘signal ratio’ environmental cuing not only emphasises the need to
understand better how mechanical cues affect gene expression, but also to understand
more clearly what regulates the shift from one splice variant to another. Though
evidence for this was not identified here, it seems plausible that ‘signal ratio’ shifts
could act much more rapidly than total increase/decrease of gene expression.
Discussion

There is a tendency to think in terms of all developmental processes as being programmed in the DNA of every cell except erythrocytes. There is however a growing appreciation that local environmental cues are important including mechanical loading and these may act on nearby cells and tissues through inducer molecules. The present work provides an example of how the same cell type expresses different growth factors in response to mechanical signals.
Discussion

Growth factor regulation

The complex network of local and systemic factors that co-ordinate morphology, size and function of whole organisms or individual organs is not well understood. There are distinct regulators of these factors and none are more evident than mechanical loading on skeletal muscle tissue. For example, skeletal muscle undergoes hypertrophy to adapt to overload and therefore the importance of local regulation remains an important area of study. The effects of passive stretch are also known to result in a rapid increase in skeletal muscle mass, which is known to be associated with the rapid production of new sarcomeres, added to the ends of existing myofibrils (Tabary et al. 1972, Williams and Goldspink et al. 1973). In particular the splice variants of IGF-I have been shown to have a key role in this process (Haddad and Adams, 2002, Hameed et al. 2002, Yang et al. 1996).

In vitro studies, in which exogenous loading was applied to embryonic skeletal muscle fibres, showed that mechanical stimulation resulted in the same biochemical processes associated with muscle hypertrophy in vivo (Vandeburgh 1987). Following mechanical stimulation it was previously assumed that myotubes in vitro increased the uptake of serum factors from culture media (Vandeburgh et al. 1983). By culturing myotubes in serum-free media it was then proposed that following mechanical loading, no growth of myotubes would ensue, however myotubes did increase in size (Vandeburgh et al. 1983). It was later found that internal changes (locally manufactured factors which were
unidentified) within myotubes resulted in this growth. The actions of these locally manufactured factors were thus implicated in aiding further growth and myogenesis.

3D myotube cultures in vitro have been mechanically stimulated previously. This has resulted in increased levels of myogenesis and growth of the myotubes (Vandenburgh et al. 1991, Vandenburgh et al. 1983, 1990). The strains applied to myotube cultures were higher than those applied in the current study, where approximately 20% to 40% strain was applied, the frequency was also high- 0.25 Hz or one cycle every four seconds (Vandenburgh et al. 1983). Although it was found in these studies that this mechanical stimulation resulted in increased synthesis of prostaglandins, which in turn regulate protein turnover and muscle cell growth, no attempt was made to distinguish how the different strain rates affect these changes. In these experiments myoblasts were cultured on 2D surfaces, which were stretched by 20-40%, in a uni-directional manner. The detail of mechanical stretching of these cultures was not well defined, but the 2D versus 3D configuration would make detailed mechanical comparison impossible.

According to the data presented in the current study, the high strain rates applied may not be necessary, as low strain rates resulted in even greater up-regulation of certain factors. The high rates of strain applied in previous studies may in fact inhibit the expression of certain growth factors. These data on precise strain rates and types of mechanical loading provide information about the efficient and accurate growth of tissue-engineered cultures. Certainly, an applied ramp load over one hour, at a rate of 6000μN per hour, resulted in an up-regulation of both IGF-IEn and MGF mRNA.
Discussion

Although the protein analysis was not followed up, this indicates an increase in these growth factors, which are known to result in both enhanced myoblast proliferation and differentiation (Florini et al. 1996, Yang and Goldspink, 2002). The regimens may even be manipulated to initially result in an increase in MGF, important in the proliferation of C2C12 myoblasts, by application of a cyclical regimen, such as one cycle per hour, and following this, the application of a ramp load (ideally 6000 μN per hour, for one hour), which would result in an increase in IGF-IEa, to enhance differentiation of the myoblasts.

The effect of two splice variants of the IGF-I gene were studied in the in vitro model developed. For this model the most appropriate control culture was referred to as ‘static loading’. This involved maintaining the myoblast/myotube culture without application of any exogenous load, but tethered (in the long axis i.e. uni-axially) such that cells generated their own endogenous tension, against the CFM force transducer. Unloaded collagen gels i.e. untethered, would not be an appropriate control and certainly not physiological. Almost all cells in situ are tethered in some manner and any unloaded system would be a separate experimental condition. Experimental regimens consisted of application of exogenous load, in addition to the endogenous load already generated by the cells.

For comparison, baseline levels of IGF-IEa and MGF were measured in these controls. The negligible baseline levels of MGF found here correspond well with studies done in vivo where levels of this splice variant in control muscles, which were not subjected to
mechanical stimulation, were also too low to be measured (Yang et al. 1996, McKoy et al. 1999). Basal levels of IGF-IEa were substantial and indicate that this isoform is maintained in a 'house-keeping' manner, presumably for background signalling, or as the null form of the 'ratio' signal, i.e. 1:0 (IGF-IEa: MGF), with no external load. This ratio was studied alongside the absolute values.

The IGF-IEa splice variant has not only been found in resting muscle, but also in human liver, hepatoma cells and fibroblasts (Nagaoka et al. 1991). The ratio of different splice variants of IGF-I (human IGF-IEa and IGF-IEb) has been determined in resting tissues (Nagaoka et al. 1991). IGF-IEa was 10-fold more abundant in the liver, hepatoma cells and fibroblasts. This is thought to be due to the stability of the IGF-IEa transcript in comparison to human IGF-IEb (Nagaoka et al. 1991). The basal levels of mouse IGF-I splice variants (IGF-IEa and IGF-IEb) are also different in resting muscle, with IGF-IEb expression negligible. In the case of mouse IGF-IEb (also referred to as MGF) there remains debate as to the stability of this transcript. It is thought that MGF is not glycosylated, and is therefore expected to have a shorter half-life than IGF-IEa (Hameed et al. 2000). If this is the case, then it is unlikely that MGF is regulated systemically, and instead its role as an autocrine/paracrine locally acting growth factor is more likely.

The most effective pattern of mechanical loading for increasing both IGF-IEa and MGF was the ramp regime. However, the increase in mRNA levels was highly dependant upon the rate at which strain was applied. Of the strain rates studied, 10% per hour resulted in the greatest up-regulation of both splice variants. Although it is difficult to directly link
how this ramp load might convert to physiological loading in vivo, it is more likely to resemble patterns of continuous stretch in one direction, perhaps analogous to weight lifting in vivo. This would contrast with cyclical loading, which might be analogous to in vivo loading, during running. The state of the muscle cultures is primitive, with small myotubes (up to four nuclei per myotube) and no myofibrils. There is also no basal lamina at this stage, which would be composed of laminin, fibronectin and other matrix components, to provide stability, in adult muscle phenotype this component would be crucial in both shielding and transducing mechanical signals. The myosin type is also not adult type myosin. As well as being an in vitro system, these cultures do not thus represent a perfect model for such comparisons, however the results would appear to mimic the in vivo scenario to a certain degree.

With a strain rate of 10% per hour, IGF-IEa and MGF mRNA levels were increased dramatically by 3-4 orders of magnitude each. One important question to arise from the study was why this particular regimen resulted in such dramatic changes in the expression of these isoforms. This is likely to be due to a combination of the following factors. The material properties of the collagen gel were important to take into consideration, as this formed the support matrix. The collagen matrix is a visco-elastic material (i.e. with hybrid viscous and elastic mechanical properties). In particular the elastic properties would conflict with the viscous properties. Depending on the rate at which strain was applied, different elastic responses were exhibited by the collagen matrix.
Discussion

Collagen lattice

Collagen lattice- with 10% deformation = stress relaxation

Collagen lattice- following application of strain at 10% per hour (1 hour ramp), the percentage of strain retained, 8.3%

Collagen lattice- following application of strain at 60% per hour (10 minute ramp), the percentage of strain retained was 5.5%

Figure 4.1. The rate at which 10% strain is applied affects the permanent deformation of the lattice due to stress relaxation and the visco-elastic properties of the collagen.

Where 10% per hour load was applied 83% of the load applied was retained (8.3% of the 10% applied). This time period and rate of strain application resulted in a permanent deformation of the material by 8.3%. This is in direct contrast to the loading applied at a strain of 60% per hour (i.e. 10 minute ramp, same strain), where almost 50% of the applied load was lost due to stress relaxation.
Discussion

The visco-elastic mechanical properties and stress relaxation of the collagen scaffold provide a partial explanation of the changes in IGF-IEa and MGF with 10% per hour strain as opposed to loads applied at higher strain rates. In the case of IGF-IEa mRNA levels, application of the high 60% strain per hour rate resulted in decreased levels of this isoform. Therefore the application of strain at this rate caused down regulation of this isoform.

An understanding of the role IGF-IEa plays in muscle development may provide clues as to why the ramp loading leads to increase in levels of this isoform. *In vitro* studies using cultured myoblasts have found that the Ea peptide enhances differentiation of C2C12 cells, whereas studies using MGF (the Eb peptide in mice) showed that its expression inhibits differentiation in cultured skeletal myoblasts, and enhances proliferation (Yang and Goldspink, 2002). It has been proposed that MGF enhances the initial proliferation of skeletal muscle satellite cells in response to stimuli to increase muscle nuclei for further growth of muscle fibres (Hill and Goldspink, 2003). Following the highest rate of strain application (60% per hour, 10 minute ramp regimen), although absolute levels of IGF-IEa were down, compared to MGF levels, which were up, the ratio of IGF-IEa: MGF was approximately 2:1. If we are to correlate known functions of the isoforms to the relative amounts produced, it can be postulated that both proliferation and differentiation would ensue in these cultures.
Discussion

The effects of cyclical loading were different on IGF splice variants. Whereas IGF-IEa was further decreased as the frequency and strain rate of cyclical loading were increased, this was not the case with MGF. Following two cyclical regimes, MGF levels increased. The pattern of this regulation did have similarities with the IGF-IEa expression, as increasing the frequency of cyclical load did result in lower levels of MGF mRNA.

An important conclusion can be made from this general overview, IGF-IEa expression is not up-regulated following cyclical loading, and therefore IGF-IEa expression is more sensitive to the type of mechanical stimulation. It is possible that MGF is an isoform more sensitive to the application of strain under any type of mechanical load, this would certainly relate to previous work where both electrical stimulation and stretch resulted in the up-regulation of this isoform (McKoy et al. 1999).

The importance of application of different strain rates is of particular importance with regards to the regulation of MGF mRNA expression. There appears to be a distinct strain rate threshold value at which MGF mRNA expression peaks, regardless of the type of mechanical load. Regardless of the type of regimen applied, of the strain rates tested (from between 0 and 60% strain per hour), 4 and 10% strain per hour resulted in the greatest increase in MGF mRNA levels. This sort of strain rate dependence is important, as the role of MGF is still being understood, and this may help further elucidate the types of environmental cues crucial to the regulation of this IGF-I splice variant. The limits of strain within which MGF is up regulated are quite distinct. Within the complex muscle system, this sort of dependence helps regulate many different
components. However in this experiment the IGF-I\textit{Ea} isoform was also found to be optimally stimulated following similar rates of strain application, therefore this strain rate dependence did not provide signalling for different absolute levels of IGF-I isoforms. Instead this strain rate dependence was influential in the changing ratios of the two isoforms.

**Physiological roles of IGF-I\textit{Ea} and MGF**

The difference between the physiological roles of the IGF-I\textit{Ea} and MGF isoforms needs to be addressed if the data regarding up-regulation of these factors is to be understood. Some of the physiological roles of mature IGF-I are thought to be understood, it is linked to mitogenic processes in many cell types of the body (Nagaoka \textit{et al.} 1991), and found to have myogenic effects in muscle (Florini \textit{et al.} 1996). However the mechanism of action of the cleaved ‘E’ peptides are not reasonably well understood. Many cleavage “by-products” are themselves biologically active. In the case of pro-insulin, the chain is enzyme cleaved to produce two mature insulin chains with loss of a cleaved ‘carboxy’ peptide. The cleaved ‘carboxy’ peptide prevents vascular dysfunction in diabetic rats and augments glucose utilisation in humans (Ido \textit{et al.} 1997, Wahren \textit{et al.} 1994). This indicates that the carboxy peptide is bioactive, it is reasonable then to assume that a comparable peptide from closely related IGF-I could also have it’s own physiological role. These findings imply that there is likely to be some biological function for cleavage by-products.
Discussion

There is evidence of some biological functions of the cleaved IGF-I ‘E’ peptides. The roles of the cleaved ‘E’ peptides are yet to be completely understood. It is known that the IGF-I ‘E’ peptides are conserved among vertebrate species, and although the fate of these ‘E’ peptides from the IGF-I pro-hormone are not entirely clear, it is known that free pro-IGF-II E peptide is secreted by culture cells and is present in blood (Straus and Takemoto, 1988, Daughday et al. 1992, Hylka et al. 1987). Mature IGF-I is known to function in both endocrine and autocrine/paracrine fashion and it would not be surprising if IGF-I ‘E’ peptides were not co-secreted alongside mature IGF-I, and exerted their effects via similar autocrine/paracrine mechanisms. The relationship of these two splice variants is at the crux of understanding how alternative splicing of the IGF-I exon sequence may be regulated. There are some known functions associated with the expression of the different cleaved ‘E’ peptides of mature IGF-I.

Expression kinetics of the two autocrine splice variants, IGF-IEa and MGF were studied over 7 days following muscle damage in rat tibialis anterior muscles (Hill et al. 2003). The expression of MGF was found to increase rapidly following muscle damage, and interestingly this was concurrent with an increase in M-cadherin, associated with satellite cell activation (Cornelison and Wold, 1997). The levels of MGF then gradually declined over the following six days, but over this same period there was a reciprocal increase in IGF-IEa (Hill et al. 2003). This is taken to indicate that both IGF-IEa and MGF have distinct and different roles in muscle repair. It is postulated that IGF-IEa may play a myogenic role following activation of satellite cells. Indeed C2C12 myoblasts supplemented with MGF, showed reduced terminal differentiation and increased
Discussion

myoblast proliferation, (Yang et al. 2002). In contrast to IGF-IeA, which promotes terminal differentiation of myoblasts.

Ratio of IGF-IeA: MGF in cultures

Baseline levels of IGF-IeA and IGF-IeB (MGF) in rat liver were found to be at a ratio of 77% IGF-IeA and 23% IGF-IeB (Zhang et al. 1997). Fasting of the rats shifted this differential by decreasing IGF-IeB (MGF) levels, whilst IGF-IeA levels remained the same. This resulted in increasing the proportion of IGF-IeA. It was proposed that this differential regulation was caused by post-transcriptional mechanisms such as nuclear splicing or RNA degradation to attenuate translation of the IGF-I mRNAs.

This differential change in IGF-I isoforms would result in a change in regulation of growth and function, the question arises as to why this sort of change is seen, rather than a change in the absolute levels of individual isoforms. This sort of differential regulation would result in a sensitive and fast-acting mechanism in response to changing environments. This sensitive mechanism could code for many more responses than strain rate threshold dependence, which would all be related to the differential proportion of just two of isoforms, making the system very efficient. This regulatory system would provide a more sensitive and rapidly responsive scenario, which would also be very economical in terms of gene information.
Discussion

In the case of expression of IGF-IEa and MGF in 3D skeletal muscle cultures, two parameters were considered. Each of these parameters formed the basis of two new hypotheses, deriving from the work. These parameters were the absolute amounts of the RNA of both splice variants and the ratio of the two. Through analysing the IGF-IEa/MGF ratio, it was possible to see a distinct relationship in increasing mechanical strain rates and the proportions of these two isoforms. Quite surprisingly, it was the type of strain applied, either ramp or cyclical, which was crucial in determining the changing ratio. In all cases the static control samples were in effect 100% IGF-IEa.

The first hypothesis is that threshold levels of strain result in the regulation of both IGF-IEa and MGF and it is these absolute levels of each isoform, which dictate myocyte responses. The second hypothesis derived, is that the isoforms are expressed as a balance or ratio from 0:1 to 1:0 and cell responses are coded within the ratio of their levels at any given time, with the basal maintenance ratio set at 1:0 (IGF-IEa: MGF).

Studying the effects of cyclical loading on changes in the ratio of the two isoforms, revealed the only instance in the entire study where the proportion of MGF was greater than IGF-IEa. This was evident in 3D cultures following 1 cycle per hour loading regimens. If the proportion of MGF were greater following the 1 cycle per hour regimen in our 3D cultures, then increases in proliferation would have resulted in greater numbers of myoblasts in the cultures. As the strain and cycling frequency of the applied mechanical regimens were increased, this isoform gradually decreased and an increase in the IGF-IEa isoform ensued.
Figure 4.2. This representation of the changing ratios of the IGF-IEa and MGF splice variants shows how the proportions of each isoform are affected by increasing strain rates applied using two different loading regimes. It must be noted that the greatest levels of expression of MGF are in the region of 5-10% strain rate, and when seen together with the absolute values it is possible to see that the highest levels of both these isoforms were found in this region.
However the increasing proportion of IGF-IEa as cyclical frequency increased, hides the fact that absolute levels of IGF-IEa actually decreased compared to the static control. This implies that under these conditions local growth of these myoblasts, either proliferative or hypertrophic, will not be occurring and this equates to decreased hypertrophy in skeletal muscle \textit{in vivo}. If these growth factor changes were produced \textit{in vivo} one might predict a decrease in satellite cell activation following the decrease in MGF produced after increasing the frequency of cyclical load. The decreasing MGF levels in the 3D culture would be mimicking the effect of increasing cyclical load frequency. Of the mechanical regimens studied, lower levels of both isoforms, IGF-IEa and MGF are found following cyclical loading compared to ramp loading.

If the known roles of MGF are linked with the expression levels of this isoform following mechanical stimulation, then low frequency cyclical load would result in an increase in myoblast proliferation, with less terminal differentiation, especially since basal levels of IGF-IEa are lower. If this change was also found \textit{in vivo}, then the result should be enhanced satellite cell activation. As the frequency of cyclical load is increased in vitro, however, MGF levels gradually decrease, which in vitro may result in reduced myoblast proliferation.

The effect of ramp loading on isoform ratio proportions was also studied after application. Surprisingly, in this case, the effect of increasing strain rates had the opposite effect in terms of changing ratios of the two splice variants. As the strain applied to muscle cultures was increased, the proportion of IGF-IEa decreased and MGF
Discussion

increased. Increases in MGF would be expected to result in greater proliferation of myoblasts and \textit{in vivo} would result in increased satellite cell activation (Yang et al. 2002, Hill et al. 2003). As cycling frequency increases, it is expected that this MGF effect would correspondingly decrease \textit{in vivo}.

Trying to relate this work to whole body responses may appear to be a large leap at this stage, yet these are fundamental cell controls, which are likely to impact on function. However if we consider an activity where skeletal muscle is under continual cyclical load, we can relate the actions of this mechanical load on \textit{in vitro} and \textit{in vivo} muscle. The response by myoblasts to ramp loading in 3D cultures appears to mimic skeletal muscle responses \textit{in vivo}. The absolute levels of both isoforms following ramp loading are found to be much higher, and result in both increased proliferation and terminal differentiation. Regimens \textit{in vivo} where mechanical load is applied over relatively long periods i.e. comparable to ramp loading, such as weight lifting, result in muscle growth and hypertrophy. In these cases muscle hypertrophy is occurring through increased growth of muscle fibres, with addition of further nuclei to muscle fibres occurring.

The cyclical load applied to muscle cultures involved a repetitive positive and then negative strain application, at various rates. The myotubes within these cultures experience a cyclical load, which entails frequent reversal of sign, stretch and relaxation. Skeletal muscles in the legs of long distance runners undergo cyclical loading at relatively high frequencies and yet these athletes do not undergo the muscle mass
increase as do weight lifters. If related to our model, it is possible to see increasing cyclical frequency results in a greater proportion of IGF-IEa, and falling levels of MGF. In the muscle model this might correspond to decreasing cell proliferation (Yang et al. 2002) and decrease in satellite cell activation in vivo (Hill et al. (a), (b) 2003).

Physiological systems that regulate muscle mass must therefore be able to detect and discriminate between these two different types of loading. The fundamental difference is the 'sign' of the strain. During ramp loading, only positive strain is applied, whereas in cyclical loading strain is applied along two different vectors, both positive (stretch) and negative (relaxation). Even similar rates of strain between the two regimens did not result in similar expression levels of the isoforms. This means that cells can clearly tell the difference between strain vectors as well as the rate at which strain is applied.

There was an important experimental anomaly in these series of investigations, which required us to assess the stability of IGF-IEa and MGF mRNA. The ramp loads were applied over different time periods, 10 minutes, 1 hour and 12 hours. Following the 10 minute ramp and 1 hour ramp, the muscle cultures were maintained for the full 12 hours under the load applied. The stability of IGF-IEa and MGF was measured over an 11 hour period following a one hour ramp load. It was important therefore to ascertain whether the mRNA signal diminished over the time frame studied, which was 12 hours. Within eight hours following loading, the greatest up-regulation of both IGF-IEa and MGF mRNA was measured, and this level was maintained for at least the remaining four hours studied. We can therefore be reasonably sure that there was no degradation of
Discussion

mRNA for at least 12 hours following mechanical stimulation. Beyond twelve hours the signal was not studied and therefore it was not possible to elucidate the length of time for which the signal was up-regulated. This data also shows how fast myoblasts respond to mechanical loading, in this case following ramp loading.

Gene splicing

The number of genes in an organism does not correspond with the total number of proteins synthesised, which is much higher. Through alternative processing of RNA, fewer genes result in a much greater number of protein products and greater complexity. The mechanism most widely used to enhance protein diversity is alternative splicing, which leads to the generation of multiple transcripts encoding different proteins. The differences may be small. In the case of IGF-I, different isoforms are expressed in muscle and in humans three different isoforms have been identified, resulting in the production of different ‘E’ peptides cleaved from mature IGF-I (Hameed et al. 2002). Splicing of the IGF-I exons both in vitro and in vivo is a two-step process. Step 1 is the process of cleavage of the 5’ splice site at its consensus guanosine residue. This 5’ guanosine at the intron end reacts with a residue upstream from the 3’ splice acceptor, forming a 5’ to 2’ phosphodiester bond. The transient product of this interaction is referred to as a ‘lariat’ structure. Step 2 of the process involves the release of the intron and ligation of the exons. The exact mechanisms of bringing together correct exons and promoting the second step are not well understood.
Although it is known that alternative splicing does result in an increase in the potential of generating different proteins from one gene, this study indicates a further mechanism for regulating diversity, and this can be seen by the alternative splicing of IGF-I leader exons (Yang, H. et al. 1995). Multiple transcription initiation sites define two distinct leader exons, which encode different 5' untranslated regions (Adamo et al. 1991, Jansen et al. 1991, Kim et al. 1991). These different leader exons are promoters, and result in differences in the translatability of the mature IGF-I product. Transcripts that initiate at exon 1 are widely expressed in extrahepatic tissues, whereas those initiating at exon 2 are highly growth hormone (GH) dependant, and are most commonly found in the liver.
Discussion

(Hameed et al. 2002). The alternate splicing of the leader exons therefore helps to regulate location of expression when the gene is up-regulated in an endocrine or autocrine/paracrine manner. The findings of the current study would suggest that the alternative splicing of exon 5 is sensitive to mechanical signals, and this in turn results in a different pattern of cleaved ‘E’ peptides.

The human genome has been sequenced and somewhat surprisingly there are only about 30,000-40,000 different genes as it is realised that there are many more proteins (Graveley 2001). The IGF-I gene can be spliced in several different ways and in muscle this is apparently determined to a large extent by the type of mechanical signals to which muscle cells are subjected. The potential role for alternative splicing in response to physiological signals in producing distinct isoform ratios suggests one possible route by which a relatively few gene-coded signals could act in concert with environmental cues to produce tissue diversity and dynamics. Further studies are required to determine the nature of the mechano-transduction mechanics involved in gene expression and gene splicing in relation to autocrine growth/tissue factor control of tissue development.

Future work would include monitoring of 3D muscle cultures after application of load, primarily to study the protein synthesis following up-regulation of the IGF-Ie and MGF genes. These findings will result in greater knowledge of the pattern of growth factor protein accumulation due to the mechanical environment of the muscle cultures.
Roles of IGF-I in development

The roles of IGF-I in developing muscle have been difficult to study. Most studies have focused on either in vivo studies in skeletal muscle, or removal of cells and studies in vitro. The 3D cultures developed were cultures of pure myoblasts, with no pre-existing myofibrils, which are crucial in signalling in repair of muscle adult muscle. They provide mechanical and chemical cues along which myoblasts fuse. The model developed may only be comparable to primitive, early developmental muscle formation, and therefore understanding the roles of IGF-I at this early stage was important.

Some work has been done to establish how the IGF's may be involved in foetal programming, and how their effects can influence early muscle formation. IGF-I gene and IGF-I receptor (IGF-IR) mouse knockouts had reduced body weight (Liu et al. 1993). In particular this appears to be as a consequence of a decrease in the tissue cell number, therefore hypoplasia, as opposed to changes found in cell size. The muscle groups were affected by the mutation through the decreased number of myocytes, along with a delay in the ossification of cranial and facial bones (Liu et al. 1993). The role of both IGF-I and IGF-IR in embryonic development has been shown to be GH independent, which leaves the autocrine/paracrine route of action (Baker et al. 1993). Previously, in the absence of GH and experimental ablation of the pituitary gland in mutant animals, prenatal growth was not found to be impaired (van Buul-Offers. 1983, Milner and Hill. 1987, Chad et al. 1989). IGF-I plays an important role in enhancing cellular proliferation during embryogenesis and acts through autocrine/paracrine
methods. It is unclear as to whether the different splice variants play distinct role during embryogenesis. It is reasonable to view the skeletal muscle model here as pseudo embryonic in stage and its isotype expression responds to load in a complex and reproducible manner, which would suggest distinct actions. The IGF-I splice variants in the model described here were studied in terms of their autocrine/paracrine action, although phenotype changes, such as myoblast proliferation were not studied. Regulation of IGF-I splice variants has not been studied in embryonic, developmental studies, and this regulation could be through a ratio-based signalling system proposed here.

Collagen material properties
In the late 1970's, Bell et al. described a phenomenon by which human foreskin fibroblasts, embedded in rat tail collagen lattices, were able to contract the lattice to produce what was described as a 'tissue-like' structure (Bell et al. 1979). The authors noted that the fibroblasts interacted with the collagen fibrils and resulted in a denser arrangement of the collagen, with the expulsion of water from the lattice. Direct measurement of these contractile forces in collagen has been made possible through the development of the CFM, and the difference in contractile properties of different cell types has been studied (Eastwood et al. 1994, 1996, Cheema et al. 2003).

Prior to casting in collagen lattices, cells were removed from their culture flasks by trypsin digestion, one of the possible reasons for the delayed onset of myoblast contraction. Following this treatment contraction of the collagen matrix by fibroblasts
was measured. Microscopic analysis of these fibroblasts revealed the extensive cell processes protruding from the cells. This was not true of myoblasts, which retained a rounded appearance following trypsin digestion and embedding in collagen (Cheema et al. 2003). By increasing the time that myoblasts were left in trypsin, a correlation was found where the longer the period of time myoblasts were in contact with trypsin, the greater delays in the extension of cell processes was noted. This 'trypsin effect' was not noted with other cell types (Brascamp, unpublished observation, 2001). Trypsin did not kill the myoblasts but prevented their contraction. It is suggested therefore, that this extension of cell processes is key to force generation and so contraction of collagen lattices by the embedded cells.

**Force generation in collagen gels**

Work on human dermal fibroblasts has highlighted the importance of cell-matrix attachment in the generation of contraction by cells (Brown et al. 2002, Sethi et al. 2002). By inhibiting the integrin attachment of human dermal fibroblasts to the collagen fibril substrate, through binding of free RGD-peptides to integrins, which would normally attach to ECM ligands, contraction was inhibited (Sethi et al. 2002). Interestingly the importance of cell-matrix attachment was further emphasised when growth factors known to enhance integrin synthesis and processing were added to cultures, and greater contraction of collagen lattices was observed (Brown et al. 2002). The hypothesis derived from this was that cell-process extension was crucial for contraction, and that the likely role this played was in attachment of the myoblasts to the
Discussion

matrix. Hence trypsin treatment of myoblasts may result in removal of cell surface adhesion molecules, such as cell-surface integrins.

The role of cell-matrix, in this case cell-collagen attachment, is important for fibroblast contraction. The added complexity with myoblasts is the inherent process of differentiation, with myoblast fusion, for which cell-cell attachment is paramount. Previous work regarding the effects of trypsin on myoblast cultures has shown that treatment of myoblasts with trypsin renders them able to neither aggregate or fuse (Knudsen and Horwitz, 1978. Cornell et al. 1980). This effect extends to cell-cell adhesion in myogenesis, whereby factors known to be crucial to cell-cell adhesion are removed through differential trypsin treatment (Gibralter et al. 1985, Knudsen et al. 1988).

The action of different signalling pathways causes the force generation by cells, resulting in contraction of the matrix surrounding the cells. With fibroblasts, serum factors are able to enhance the myosin –light chain phosphorylation mechanism of contraction (Yanese et al. 2000 and Parizi et al. 2000). Myosin light chain phosphohorylation controls the actin-myosin function in the cell, which is involved in the control of cytoplasmic motor function. In vertebrate muscle cells it is the Ca^{2+} troponin switch which controls contraction. This is part of the actin filaments rather than the myosin molecular motors.
Discussion

The initial phase of contraction (up to 600 μN of force) by myoblasts is related to increased cell motility, extension of processes and potentially, increased interaction with the collagen fibril matrix. This stage of the contraction has previously been implicated in force generation through traction (Eastwood *et al.* 1996, Talas *et al.* 1997, Harris *et al.* 1980).

The measurement of contraction does not necessarily indicate one particular cellular function, as it is possible that different cell actions may result in cumulative force generation. Therefore, known specific behaviours of certain cell types can help to explain the reasons for any resulting contraction. In the case of skeletal myoblasts, fusion occurs during differentiation. A pre-requisite of fusion is the proximity (contact) of myoblasts to each other and this is likely to occur with alignment and contact. It is therefore likely that the continuing contraction of the collagen matrix was due to cell traction, where myoblasts eventually aligned and came close enough to fuse.

Importantly two other experimental observations were made concurrent with this time frame, which make the continuing cell traction the likely cause of continuing force generation. The first being morphological and the second measurement of a surge in IGF-I splice variants in the culture, indicating the onset of differentiation. Morphological observations on day one reveal that cell processes had been extended and were aligned along the axis of principal strain, with little cell contact. For the myoblasts to fuse effectively, with the additional factor or the 3D arrangement, further cell locomotion or movement, or cell process extension would have been necessary, and this was noted by
Discussion
day three (72 hours). This fusion of myoblasts is not possible unless cell membrane contact is made, the observed aligned arrangement and close proximity of myobasts by 72 hours post-embedding is therefore necessary for successful fusion. One component resulting in this contraction may have been the cell-cell attachment resulting in passive contraction of collagen fibres, however another component may have been due to the ongoing cell migration up to day 3.

Work is currently underway to determine the stability of collagen lattices, and it has been shown that any degree of collagen gel manipulation results in a loss of water from the gel, i.e. an increase in collagen fibre density (Wiseman et al. unpublished observation). This water loss is thought to be irreversible, resulting in permanent deformation or remodelling of the collagen. It is therefore likely that even small cell movements such as the traction of cells within a collagen gel could induce a similar effect but on a much smaller scale, leading to contraction. This could explain the high density of collagen fibres directly surrounding the myotubes by day 3, which become even more pronounced by day 7 and day 10.

It is proposed that one of the reasons for the ongoing contraction of collagen matrices by myoblasts up to day three is due to continued migration of myoblasts. This hypothesis is further supported by a surge of two splice variants of IGF-I, IGF-IEa and MGF, measured on day three of the developing collagen cultures. IGF-I is known to have an important role in myogenesis, and is found up-regulated prior to differentiation, inducing Myogenin gene expression (Florini et al. 1991, 1996). The changing levels of expression
of two IGF-I isoforms (IGF-Ie and MGF) were measured over the same time span as the myoblasts were cultured in collagen. The surge in both isoforms corresponded with membrane contact and fusion of the myoblasts from day 3 onwards.

The differences in initial rates of contraction between different cell types emphasise the importance of understanding the behaviours of single cell types in such cytomechanical studies, before using mixed cell populations or co-cultures, for example primary muscle cultures that are co-cultures of fibroblasts and myoblasts. It is presently uncertain whether contraction of fibroblasts or myoblasts in co-culture are simply additive or if they communicate or act synergistically. Interestingly, the initial rates of contraction for dermal fibroblasts and smooth muscle cells were almost double that produced by skeletal myoblasts (with consequentially higher total force generation). Given the relative mechanical outputs of the parent tissues, this is surprising compared to the isolated single cell types. There appear to be differences in the contractile output of these early stage muscle and mature muscle. This emphasises the importance of organisational development and differentiation, which is essential for myoblast cultures to generate mechanically significant outputs. Although this study is concentrated on individual cell types and not on the functions of the tissue from which they are derived, the behaviour of cell types is likely to reflect the nature of the parent tissue to some extent. In this respect it might be expected that skeletal myoblasts would generate the greatest level of contraction, measured as force generation on the CFM. However, this is clearly not the case, therefore not an essential prerequisite for myoblast fusion to form myofibres. It is these myofibres that are associated with the high force and contractile properties of
Discussion

skeletal muscle in vivo. As expected the truly large force output of differentiated muscle (myofibrils) seems to be a function of the specialist structure of mature muscle.

Development of 3D model

Some degree of development of the 3-D culture (in terms of fusion and maturation of cells to form aligned myotubes) was promoted using the CFM system, which provided additional cues to guide myoblasts in a particular direction, as uni-axial tension was generated along the long axis of the culture. These proliferating, motile myoblasts form aligned arrays, with bipolar morphology and exhibit an inhibition to lateral fusion (Clark et al. 1997, 2002).

Although this was not directly measured, it has been shown that application of uni-axial strain results in increased proliferation of myoblasts, elongation of myotubes and alignment of myotubes (Vandenburgh et al. 1989, 1991). In the current model it was found that myoblast fusion resulted in the formation of multinucleated myotubes over a period of 5-6 days. However, the culture remained a mixture of both myotubes and myoblasts. Therefore it is reasonable to assume that those myoblasts with the elongated shape, in spatial position, i.e. end to end, and in proximity with neighbours, at the appropriate stage in their growth would fuse, whereas others would remain myoblasts.
Discussion

Figure 4.4. Mechanisms of myoblast fusion. The 3D area of the collagen gels changes in area as the gel is contracted, therefore the relative geometry and proximity of the myoblasts changes.

The 3D culture of myoblasts in collagen was a long-term study, and as such the progression of myogenesis could be observed, of particular interest was the interaction between collagen and myoblasts. Although cell-matrix adhesion is crucial for the
Discussion

progression of myogenic precursors to the post-mitotic myocyte stage, there is no
evidence to suggest that this process is directly involved in myotube formation
(Knudsen, 1990). In the presence of extra-cellular matrix in 2D culture, differentiation of
myoblasts is enhanced. Flasks are often coated in either gelatine or collagen to provide
the extracellular matrix. The disruption of cell-matrix adhesion results in reduced
myogenesis both in vitro and in vivo, thus highlighting the importance of cell-matrix
interactions with laminin (Knudsen, 1990). Disruption of adhesion of myoblasts to the
matrix components, laminin and fibronectin, have resulted in reduced myogenesis
(Knudsen, 1990). In the presence of laminin, a matrix component, rodent muscle cells in
vitro have increased cell motility, bipolar morphology and stimulated DNA synthesis
(Goodman et al. 1989). Increased levels of laminin result in enhanced myogenesis in
newborn mouse cultures (Goodman et al. 1989). Laminin and fibronectin are just two of
the matrix components found under the basal lamina of skeletal muscle fibres. Following
muscle damage myogenic precursor cells (MPC) proliferate and differentiate. Although
the exact roles for such matrix components are not completely understood, it is proposed
that such cell-matrix adhesion promotes cell migration, whereas cell-cell adhesion
molecules may promote myoblast interaction prior to and during cell fusion.

Cell-Matrix and Cell-Cell interactions

It is axiomatic that for cells to bring about 'contraction' of a collagen gel, it is essential
for them either to attach to the matrix or to each other. Although cell-matrix adhesion is
of importance to the progression of myogenesis, it is not directly involved in myotube
formation. Prior to fusion cell-cell attachment is probably more important than cell-ECM adhesion for efficient cell fusion (Wakelam et al. 1985, 1988). It is known that during the period just prior to fusion there is an increase in neural cell adhesion molecule (NCAM) and N-cadherin, which are known to be important in the process of cell-cell attachment (Knudsen 1990 (1), Maccalman et al. 1992, Zeschnigk et al. 1995). Although NCAM and N-cadherin are unlikely to be the only cell surface molecules involved in cell-cell adhesion, they have been associated with myogenesis and are regulated in different ways. NCAM is a Ca\(^{2+}\) independent adhesion molecule, whereas N-cadherin is a Ca\(^{2+}\) dependent adhesion molecule. Transfecting mouse myoblasts with a lipid-linked isoform of NCAM results in enhanced myogenesis (Dickson et al. 1990). By blocking the actions of both of these molecules, however, myotube formation was not completely inhibited (Knudsen et al. 1990(2), Knudsen et al. 1990(3)). Due to this the participation additional adhesion molecules cannot be excluded.

In terms of cell attachment, cell-cell and cell-matrix adhesion are complementary and largely mediated by distinct groups of receptors. Most cell-matrix binding is through integrin receptors. Since myoblasts undergo partial detachment from their plastic substratum during fusion in vitro forming long linear arrays, it has been suggested that cell-cell interactions are more important than cell-matrix interactions at this stage (Gu et al. 1994). In addition, myoblast integrin expression is known to change between the undifferentiated and differentiated state. Once myoblasts have differentiated their expression of integrins (\(\beta_1\)) with affinities for fibronectin is found to decrease (Duband et al. 1992, Steffensen et al. 1992). A relatively poor attachment, even of
Discussion

undifferentiated myoblasts i.e. at time zero, might explain not only why the initial rate of contraction was low, but also why the overall contraction was low compared to other cell types. However there does remain an anomaly, if contraction were mediated chiefly through integrin-matrix binding, as found in fibroblasts (Sethi et al. 2003) then there was no fall in force generation as differentiation occurred. If previous work has shown that myoblasts undergo partial detachment from their substratum during differentiation, then it would be expected this loss in cell-matrix attachment would be measured by the CFM on day three, when differentiation is beginning. Instead, in this system, we find a corresponding plateau in force generation on day three. This then is consistent with the idea that a substantial proportion of strain applied to the collagen is mediated indirectly by cell-cell attachment.

Spontaneous contracting cultures

Twitching of the myotube cultures was evident on day five of maturation. This entailed the spontaneous and synchronised slow twitching of differentiated myotubes in 3D collagen cultures. The 2-3 spontaneous contractions seen over 24 hours produced variable levels of force, similar to that described previously for electrically stimulated twitches by Dennis et al. (2000). In this study, however, there was no need for exogenous stimulation in the form of electrical stimulation. Previous work carried out as early as 1899 using skeletal, smooth and heart muscle fibres ex vivo, have shown the rhythmical contraction of these fibres, when maintained in media (Howell, W.H. 1898,
Discussion

Lingle, D.J. 1900, 1902). Even more interesting is the independence of this contraction from the influence of nervous impulses, displaying an intrinsic element of the muscle fibres. Lewis (1915) was amongst the first to culture skeletal muscle tissue from an explanted piece of embryo chick leg, in media, which included serum and dextrose (six-carbon sugar d-glucose). This culture displayed rhythmical contraction in the absence of any nervous tissue. The frequency of contraction varied between fibres, from as many as 120 contractions per minute (high), down to 3 contractions per minute (low). This in vivo developmental tissue provides insight into the developing muscle culture. The tissue-engineered piece of muscle described in the current study has shown some features of such a model and native tissues. Periodic contraction was seen in our model, though at a much lower rate than that studied from fibres ex vivo (Lewis, 1915). One of the explanations for this lower level of contraction was the state of maturation of the muscle model. On day five after embedding in collagen gels, not all myoblasts had fused, and even fused myotubes did not contain more than three nuclei per myotube. When comparing this to ex vivo myofibrils, these will have large numbers of nuclei per fibre, with distinct basal lamina surroundings, therefore with different overall structure.

It is proposed that further differentiation of the myotubes, through maturation of the cultures to form longer myofibrils, as found in other concurrent studies, would lead to changes in the frequency of spontaneous contractions or twitches, to mimic more closely those found in muscle fibres from developing muscle (Vandenburgh, 1991, Dennis et al. 2001). In studies using primary myoblast cultures, the population of fibroblasts in the culture have been crucial for successful differentiation of the myoblasts, as they provide
Discussion

additional factors to the growth media and they add to the production of extracellular matrix, which is known to enhance differentiation. In the present model, a longer period in culture, the presence of collagen, the application of uni-axial strain, enhanced by factors or supplements known to enhance differentiation, i.e. IGF-1, is hypothesised to result in further maturation of the cultures (Florini et al. 1996, Yang et al. 2002). It is further proposed that application of specific mechanical loads to the muscle cultures will enhance the production of local growth factors by the cells, and in turn result in further differentiation.

For example, application of the one-hour ramp results in increased IGF-IEa and MGF mRNA levels, as long as this mRNA successfully translates to protein, this provides a way to control growth factor regulation. The control of growth factors in a culture could then be regulated by application of mechanical loading, enhancing growth and maturation and in time the myotubes may have increased their intrinsic contractions to a level similar to the ex vivo developmental muscle fibres.

In the present work two periods of contraction were observed in muscle cultures on day five after embedding. This contraction appeared to be synchronised between all the myotubes in the cultures. The synchronised rhythmical contraction may indicate some form of communication, acting between the myoblasts/myotubes within the culture, as they all contracted together.
The force generated during each contraction was between 60 and 130 μN in force. This amount of force could not be produced by one single myoblast or myotube, and therefore was assumed to be groups of myotubes or myoblasts contracting in synchrony.

There are some options as to how this synchronised contraction occurred. This would include the possibility that the myotubes and the myoblasts within each collagen culture were physically attached, or may be due to some form of chemical communication, where due to contraction, release of any chemical may influence the surrounding cells, through possible Ca²⁺ release.

If all the myotubes in the collagen gel were physically connected, i.e. through attached cellular processes, or via collagen fibril intermediates, this physical contraction of one myotube would inevitably pull on the other myotubes it was attached to. It is possible that this could result in an amplification of the contraction, as the reaction of a myotube to a ‘tug’ or pull of part of the membrane or cytoskeleton may be contraction itself, to offset any movement. This cumulative affect may have resulted in the contractions measured. The extent of cellular processes and attachment of myotubes was different in individual cultures, which may go some way in explaining the variability in force generation of each contraction. The other conceivable mechanism through which the synchronised contraction of myotubes may occur is some form of ‘chemical release’ communication. This may include the release of growth factors.

The action of synchronised contraction, whereby single or multiple myotubes contract, and trigger other myotubes to contract suggests that some form of communication
Discussion

between all the myotubes did exist in the muscle cultures, although the nature of this communication was not further investigated. The period of increasing contraction was small and as it was unlikely that one myotube generated the entire force, the argument for a physically connected network of myotubes is strong. It is also interesting to note that following the spontaneous contraction, the force generated did not fall back, as would be expected in a twitching culture, instead it was maintained. This may have been due to physical connections between the myotubes, which seem the most likely basis for the synchronised contraction.

In the present study, the first spontaneous contraction occurred at set-up (0 time), conceivably in response to the small set-up background loading, which is routinely applied at this stage. However, for later jumps no additional outside mechanical load was given, representing an endogenous signal. In all cultures a certain proportion of myoblasts will have differentiated by day 5, this proportion of myotubes and any single myoblasts generated force within the culture, which was approximately 1500 μN. It is known that in developing tissue, mechanical tension helps to regulate the growth of different tissues. Spontaneous contractions within the culture may have represented inherent mechanical loading, applied by myotubes themselves to stimulate growth factors, especially growth factors known to be up-regulated following mechanical loading. Although a role for diffusible chemical factors cannot be excluded at present, this idea was not tested.
Discussion

The nature of the endogenous, time based trigger is under investigation. A force transducer working in the μN range was employed (Dennis et al. 2000), to measure both spontaneous baseline contractions and electrically stimulated twitches from myooid constructs (mixed primary myoblasts and fibroblasts). The baseline spontaneous contractions were measured at about 20 μN and the stimulated twitches measured up to 440 μN. In comparison the CFM force transducer is not sensitive enough to resolve forces below 50 μN, and the data acquisition used was 1 Hz, 100 times slower than Dennis et al. Since any similar baseline contractions in the myotube cultures would not have been registered on the CFM system. Developmental stage myosin heavy chain was detected in the myotube cultures on day-7 (unpublished observation), which indicates that the myotube cultures were behaving in a manner comparable with early embryonic development. Dennis et al. were able to produce myooids from adult mouse and embryonic mouse myoblast cultures, and compared the contractile properties of these two with myooids made using fibroblast and muscle cell lines (Dennis et al. 2001). The primary adult myooids displayed the greatest contractile properties and the myooids made from C2C12 cells and fibroblasts displayed lower contractile potential. It was postulated that this was due to the presence of developmental myosin as opposed to adult myosin (Dennis et al. 2001).

Morphology of differentiating myoblasts

In these collagen gels the changing morphology of myoblasts was observed as they differentiated within this matrix over ten days. In addition to generating tension in one
axis, there were regions (delta zones) within the collagen gel that were stress shielded. Due to the stiffness of the flotation bars, the regions close to these bars were prevented from contraction. Certainly on day one, myoblasts were attached and displayed cellular processes distributed in a random manner in the delta zones, however by day seven myoblasts exhibited completely rounded morphology, which suggest cell death or apoptosis in these regions. The numbers of cells within these regions was also diminished, and although not tested, there is the possibility that these cells migrated out of this region.

Cell membrane contact was evident by day three in an end-to-end manner, where no membrane fusion was evident between lateral myoblasts. Previous data highlights the importance of end-to-end fusion, to the extent that if an early differentiating cell was at an angle of 45° to another cell, the two cells did not fuse (Clark et al. 2002). In our study, by day seven, myoblasts within the collagen gel had aligned along the line of principal strain, and multinucleated myotubes were observed. At this stage no opposing cell membranes or cell junctions could be identified between adjacent myoblasts (i.e. between groups of 2 or 3 nuclei). These were taken to be myotube syncytia. Interestingly, in all examples seen, myotube nuclei were arranged in a single line, parallel with the principal axis of strain. Where end-to-end contact was not made, fusion did not ensue, instead myoblasts remained aligned.

Although the precise mechanisms of myoblast recognition are unknown, these cells are known not to fuse with other cells, including other types of muscle cells. Skeletal
**Discussion**

myoblasts do not fuse with smooth muscle cells or cardiac muscle cells, however heterotypic fusion between species of rabbit and rat myoblasts does occur (Wakelam, 1988). There must therefore be common recognition sites between mammalian species, which are not shared between different muscle cell types. One of the candidate recognition molecules for this is N-cadherin, a cell-cell adhesion molecule, which is expressed in skeletal myoblasts during differentiation. Exogenous N-cadherin added to a skeletal myoblast cell line in a three-dimensional culture promotes differentiation of the cells (Redfield et al. 1997). It is also known that in the absence of N-cadherin, differentiation of myoblasts does still occur, therefore it has been proposed that other cadherins may substitute the functionality of N-cadherin (Radice et al. 1997).

**Fusion of myoblasts in 3D**

In 2D culture it is found that high density plating of primary embryonic chick myoblasts, followed by lowering of serum levels in medium results in fusion after 40-42 hours, and this process continues for up to 18 hours (Wakelam, 1988). In the present model tissue, C2C12 myoblasts in collagen, the first observation of membrane contact was at 72 hours, comparatively much later. This suggests there may have been a change in the geometry of cell-cell contacts. The 3D nature of the collagen gels added an extra dimension for the cells to move in, this may have resulted in a decreased percentage of contact incidence. Certainly, physiologically this is more native than the 2D environment. It is known that
myogenesis occurs when myoblasts are in close contact to one another (Gurdon *et al.* 1993). This effect is known as the community effect, and is known to affect the differentiation of various cell types including embryonic stem cells and somites (Gurdon *et al.* 1993, Slager *et al.* 1993).

The alignment of myoblasts was necessary for fusion. *In vivo*, in developing muscle the cues directing the orientation of myotube formation are not well understood, however in repair of muscle, it is evident that the existing myofibril may provide cues along which the myoblasts align and fuse. These are strongly associated with basal lamina of myofibrils, and in particular some of the components e.g. laminin (Kuhl *et al.* 1986). *In vitro* in 2-dimensional cultures where cues are not provided to guide myoblasts, the orientation of myotubes is random, and often branched. It is possible to align myotube formation through providing cues such as physical grooves in culture dishes, and culturing of myoblasts in collagen gels and fibronectin gels, where the generation of isometric tension in a uni-axial plane provides a direction for orientation (Iseva *et al.* 1980, John *et al.* 1980, Turner *et al.* 1983). The strain generated in the 3D collagen lattices used in the current work provides mechanical cues along which the myoblasts aligned.

The fusion of myoblasts is key to the successful differentiation of skeletal muscle. The transition from mono to predominantly multi-nucleated muscle cells occurs through alignment, recognition and fusion of myoblasts. The importance of membrane union and the regulation of this aspect are also an important part of the process, although the exact
mechanisms of membrane fusion, not only for myoblasts but of biological membranes in
general, remains unknown. It is generally agreed that for membrane fusion to occur, a
fluid membrane state is needed. Previous studies have shown that when cells fuse, the
greatest changes in membrane fluidity occur in areas of cell contact between fusing cells
(Herman and Fernandez, 1978, 1982). This increase in membrane fluidity results from
the redistribution of surface antigens (Kaufman and Foster, 1985).

The observation of chromatin condensation within the nuclei of 10 day old myotubes
differed starkly to that noted in the earlier myotubes and myoblasts. Although the
appearance of bands of chromatin in these cultures may be due to the plane in which the
myotube was cut, it is highly unlikely that this would be present in all day 10 specimens
and none of the other cultures, especially as they were all processed in the same manner.
Therefore ruling out any processing artefact. This is likely to be a specific change in
nuclear structure between day seven and day ten. Chromatin condensation is not
indicative of unhealthy or apoptotic cells, as cells were rich in golgi, endoplasmic
reticulum and mitochondria, and protein manufacture would cease in unhealthy cells.
Discussion

Future work

The findings of the study may be extended in different areas. Of particular interest is the use of mechanical loading regimes to help enhance and increase myogenesis in tissue engineered skeletal muscle cultures. Although protein work and morphological data are not present, it is likely that the up-regulation of the mRNA of two growth factors, IGF-IEa and MGF, will result in further proliferation and differentiation of the myoblasts in 3D cultures. The 1-hour ramp regimen appeared to provide the most potent cues for stimulation of these factors in vitro.
It is also hoped that some further advances in our understanding of mechanical stimulation are made. Simply applying mechanical load is not enough, particularly when this is not physiological. A precise strain rate, which is surprisingly low compared to other groups, appeared to provide the greatest stimulation for the growth factors studied. Physiological loads were difficult to compare as in vivo, cells are embedded within highly organised and differentiated matrices. Physiological loading such as that related to walking and running would be more difficult to simulate. In muscle for example, myofibrils are encased in basal lamina, with components of laminin and fibronectin present. Application of any load to this greatly shields its cells. In the present model where cells are embedded in a pliant collagen gel, less of this stress and strain shielding occurs. Along with this strain rate dependent up-regulation, it was possible to see the effects of different types of mechanical load. Although a small selection of regimens and possible strain rates was studied, some insight has been gained.

The study of primary human myoblasts would give a much greater understanding of the effects of mechanical stimulation. In vivo the roles of MGF are thought to be associated with the activation of stem-cell like satellite cells. In the current model, if the regulation of the human myoblasts is similar to that of the C2C12's, it may be possible to differentially regulate the MGF and IGF-IcA, where levels of IGF-IcA are decreased, and MGF increased by following the 1 cycle per hour loading regimen. If this were possible the population of satellite cells may be specifically increased in human myogenic cells. In treatment for Duchenne muscular dystrophy, ideally satellite cells would be injected into muscle sites. These cells however, are difficult to obtain and
when obtained are often in small quantities. There is the possibility that by culturing these cells and applying mechanical regimens known to stimulate MGF, this growth of this population would be stimulated.

This model was also extremely basic. There was no neural input, and no effects of systemic regulation. The advantage it offers over the in vivo situation is that it is possible to alter one parameter at a time and therefore it is possible to begin to understand the mechano-transduction mechanisms of muscle cells. Muscle contraction was found to be intrinsic, and the regulation of locally acting growth factors was also completely independent of systemic effects and this emphasises the point that developmental changes are not all pre-programmed in the genome. By understanding this basic model, incremental advances can be made in the future. This may entail the addition of some nervous tissue, to create basic models for the study of neuromuscular junctions.
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