Development of a 3D tissue engineered skeletal muscle and bone pre-clinical co-culture platform

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Development of a 3D Tissue Engineered Skeletal Muscle and Bone Pre-Clinical Co-Culture Platform

By

Nicholas Martin Wragg

A Doctoral Thesis

Submitted in partial fulfilment of the requirements for the award of

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Abstract

Pre-clinical studies are a necessary step in the process of material and drug testing. For this, high-throughput monolayer cell cultures are conducted followed by in vivo animal experiments. However, animal use is ethically questionable and in many cases yields misleading results. In vitro three dimensional (3D) tissue engineered (TE) structures have been shown to better represent in vivo tissue morphology and biochemical pathways than monolayer cultures and are less ethically questionable than animal models. Therefore, an in vitro biomimetic musculoskeletal junction (MSKjjct) is required as a more relevant pre-clinical testbed. This thesis describes the steps taken to co-culture 3D TE skeletal muscle and bone models as a material testbed and towards an in vitro MSKjjct.

Initially, the composition of different growth and differentiation media effects on both C2C12 murine muscle precursor cells and TE85 human osteosarcoma cells was assessed. High-glucose (HG) DMEM supplemented with 20% foetal bovine serum (FBS) and 1% penicillin/streptomycin (PS) demonstrated the least relative deviation (DNA and protein concentrations) from control protocols. Subsequently, both cell types were cultured in this growth medium followed by transition to either a myogenic medium, or an osteogenic medium. Myogenic medium (HG-DMEM, 2% horse serum, 1% PS) was found to induce differentiation in both cell lines. 3D TE models were developed using these media for later co-culture.

Following this, a scale-down model was developed for collagen-based TE skeletal muscle constructs and key manufacturing variables optimised using a factorial design of experiment (DOE). Four million C2C12s/mL with 25% extension produced the most desirable myotube characteristics across scaled models. Similarly, a collagen/hydroxyapatite-based bone model was also optimised using a factorial DOE, and assessed according to RUNX2/Cbfα1 and osteocalcin/Bglap gene expression, and alkaline phosphatase activity (ALP). A one million/mL seeding density and a 2:1 ratio was found to produce the most mature cell population.

Finally, a novel platform was created to enable the simultaneous manufacture, culture and differentiation of the skeletal muscle and bone models. In a co-culture bone model, RUNX2/Cbfα1 and osteocalcin/Bglap mRNA were found to be significantly up-regulated, whilst ALP was not significantly different. Within the skeletal muscle model, evidence of fusion was observed; however, there was qualitatively less than in isolated controls and previous cultures.

This work represents the first report of the simultaneous co-culture and differentiation of 3D TE skeletal muscle and bone and represents a significant step towards a full in vitro 3D MSKjjct model.
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<tbody>
<tr>
<td>ca.</td>
<td>Circa</td>
</tr>
<tr>
<td>µL</td>
<td>Micro-litre</td>
</tr>
<tr>
<td>µM</td>
<td>Micro-molar</td>
</tr>
<tr>
<td>µN</td>
<td>Micronewtons</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BALB/3T3</td>
<td>Mouse fibroblast cells</td>
</tr>
<tr>
<td>BGLAP</td>
<td>Bone gamma-carboxyglutamic acid protein, osteocalcin</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>BMSC</td>
<td>Bone marrow stem cells</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BSL</td>
<td>Bone surface lining (cells)</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium ions</td>
</tr>
<tr>
<td>CBFa1</td>
<td>Core-binding factor subunit α-1</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese Hamster Ovary cells</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>cm&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Centimetres-squared</td>
</tr>
<tr>
<td>c-Met</td>
<td>Hepatocyte growth factor receptor protein</td>
</tr>
<tr>
<td>CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>dH&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>Distilled water</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DM</td>
<td>Differentiation medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOE</td>
<td>Design of experiment</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double-stranded DNA</td>
</tr>
<tr>
<td>EBSS</td>
<td>Earle's Balanced Salt Solution</td>
</tr>
<tr>
<td>ECACC</td>
<td>European collection of authenticated cell cultures</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra-cellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle's Minimal Essential Medium</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td>g</td>
<td>Relative centrifugal force / gravities</td>
</tr>
<tr>
<td>GM</td>
<td>Growth Medium</td>
</tr>
<tr>
<td>GRIP</td>
<td>Glutamate receptor interacting protein</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>Water</td>
</tr>
<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
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<tr>
<td>HCl</td>
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<td>HG-DMEM</td>
<td>High glucose-DMEM</td>
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<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<tr>
<td>hGH</td>
<td>Human growth hormone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>hiPSC</td>
<td>Human induced pluripotent stem cells</td>
</tr>
<tr>
<td>hOS</td>
<td>Human osteosarcoma</td>
</tr>
<tr>
<td>hrs</td>
<td>Hours</td>
</tr>
<tr>
<td>HSMF</td>
<td>Human skeletal muscle fibroblasts</td>
</tr>
<tr>
<td>HSMM</td>
<td>Human skeletal muscle myoblast</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>lhH</td>
<td>Indian Hedgehog</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IMS</td>
<td>Industrial methylated spirits</td>
</tr>
<tr>
<td>L</td>
<td>Litres</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
</tr>
<tr>
<td>mg</td>
<td>Milli-grams</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>mL</td>
<td>Milli-litre</td>
</tr>
<tr>
<td>mM</td>
<td>Milli-molar</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetre</td>
</tr>
<tr>
<td>MM</td>
<td>Maintenance medium</td>
</tr>
<tr>
<td>mm²</td>
<td>Millimetres-squared</td>
</tr>
<tr>
<td>mm³</td>
<td>Millimetres-cubed</td>
</tr>
<tr>
<td>MPCs</td>
<td>Muscle precursor cells / myoblasts</td>
</tr>
<tr>
<td>MRF</td>
<td>Myogenic regulatory factors</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MSCs</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>MUP</td>
<td>Methylumbelliferyl phosphate</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NEAA</td>
<td>Non-essential amino acids</td>
</tr>
<tr>
<td>ng</td>
<td>Nano-grams</td>
</tr>
<tr>
<td>nm</td>
<td>Nano-metres</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Centigrade</td>
</tr>
<tr>
<td>P#</td>
<td>Passage #</td>
</tr>
<tr>
<td>Pax</td>
<td>Paired box transcription factor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCL</td>
<td>Polycaprolactone</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PLA</td>
<td>Polylactic Acid</td>
</tr>
<tr>
<td>PLGA</td>
<td>Polylactic-co-glycolic acid</td>
</tr>
<tr>
<td>PMCC</td>
<td>Product moment correlation co-efficient</td>
</tr>
<tr>
<td>POLR2B</td>
<td>RNA polymerase II B</td>
</tr>
<tr>
<td>PS</td>
<td>Penicillin/Streptomycin</td>
</tr>
<tr>
<td>PU</td>
<td>Polyurethane</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RUNX2</td>
<td>Runt-related transcription factor 2</td>
</tr>
<tr>
<td>RYR1</td>
<td>Ryanodine receptor-1</td>
</tr>
<tr>
<td>sec</td>
<td>Seconds</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic Hedgehog</td>
</tr>
<tr>
<td>SOP</td>
<td>Standard operating procedures</td>
</tr>
<tr>
<td>Sox</td>
<td>Sex-determining region y-related high mobility group box Sry-related HMG box</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single-stranded DNA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TCP</td>
<td>Tissue culture plastic</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethylrhodamineisothiocyanate</td>
</tr>
<tr>
<td>Tsp</td>
<td>Thrombospondin</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
1 General Introduction

Preclinical studies are a necessary step in the process of material and drug testing before application of a substance into the human population. Animal testing is widely prevalent as a means of investigating substances for biocompatibility prior to human studies due to a genetic homology to humans (Mestas & Hughes 2004). However, their use is highly controversial and in many cases yields misleading results, such as in the use of angiogenesis inhibitors in treatment of lung cancer (Mestas & Hughes 2004; Huang et al. 2007). These discrepancies are understandable when a small genetic difference can cause a vast change in anatomy and physiology; there is a <5% difference genetically between mice and men (European Commission 2010). Therefore, changes have been made in regulations to limit animal use in scientific experimentation.

1.1 Changes in Clinical Trials

A report covering the UK from 1945 showed a significant downwards trend in animal testing from 1975 to 2000 (Figure 1.1) (UK Home Office 2015). However, since 2000 the number of animals used increased by 2% to 2.71 million over 1999. During 2013 just over 4.1 million procedures occurred, an increase of 57% from 2001 (UK Home Office 2015). This rise can be attributed to the number of genetically modified (GM) and harmful mutant animals (HM) bred for research. Excluding these, the numbers were only slightly higher than 2000 with an increase of 8% or 159,900 and have remained approximately stable since then. In 2015, 50% of procedures were experimental (decrease of 13% from 2013) with the other 50% to create/breed GM/HM animals and not used in further procedures. Of those animals used in experimental procedures, ca. 25,000 (2.3% of 1.1 million) were used in basic musculoskeletal research and ca. 2,500 translational/applied (0.6% of 402,000). 556,000 procedures were undertaken for regulatory use (including 38% for toxicity and other safety testing), an increase of 42% from 2010. This can be explained by the increase use of fish in regulatory toxicology (UK Home Office 2015).

Despite a recent upwards trend in total animal usage, the overall decrease in animal-based toxicology procedures from 1975 can be attributed to the adoption of a concept which was first proposed by Russell and Burch in 1959 (Lee-Parritz 2013). They stated that all animal experiments should incorporate, as far as is possible, the Three R’s (3Rs): Replacement, reduction and refinement.
These are defined as follows:

- **Replacement** - of conscious living vertebrates by non-sentient alternatives
- **Reduction** - in the number of animals needed to obtain information of a given amount of precision
- **Refinement** - of procedures to reduce to a minimum the incidence or severity of suffering experienced by the animals used.

![Figure 1.1: Experiments or procedures commencing each year (1945-2015) (UK Home Office 2015)](image)

1. Experiments started under the Cruelty to Animals Act 1876.
3. Following the transposition of European Directive 2010/63/EU into UK law, scientific procedures completed under the revised Animals (Scientific Procedures) Act 1986.

- The 1987 total includes experiments started under the 1876 Act as well as procedures started under the 1986 Act.
- * The data collection methodology changed in 2014, which resulted in some under-reporting for that year (see introductory notes for more information)

The inclusion of the 3Rs has seen the development of cell culture as a standard means for testing biocompatibility before animal tests. In the case of novel materials, *ISO 10993: Biological Evaluation of Medical Devices 2009* is now the internationally recognised assessment protocol. This is used before animal/human trials can be approved. ISO 10993 employs the use of Chinese Hamster Ovary (CHO) and BALB/3T3 mouse fibroblast cells as recommended cell lines in monolayer when conducting these tests.
1.2 The Modern Clinical Trial
To take a new molecular entity through development to market will costs in excess of $800 million (typically ca. $1 billion) (Dickson & Gagnon 2004; Mestre-Ferrandiz et al. 2012; Breslin & Driscoll 2013). Half of the drugs taken to Phase III clinical trials fail and only 12% of compounds entering human trials continue to commercialisation (Huang et al 2007). Therefore, animal studies along with monolayer cell culture experimentation have a clear deficit in predicting adverse effects in human studies. Considering that cell culture use is mainly animal derived and in monolayer, this outcome is not surprising. Murine models for testing were used in 73.3% of procedures in 2015 and whilst 99% of mice genes are homologous to humans (UK Home Office 2015), extrapolation of indicative results does not necessarily translate correctly to human subjects, due to anatomical differences governed by the 1% genetic difference or epigenetic influences.

In an effort to apply the 3Rs to preclinical tests whilst maintaining or increasing a translational relevance to humans, development of three-dimensional (3D) tissue-engineered human models has intensified and become more representative of in vivo tissues when compared to current monolayer cultures (NC3Rs 2011; Kalman et al. 2015; Smith et al. 2012). However, culturing a 3D tissue is considerably more time consuming and costly than monolayer culture (Justice et al. 2009; Breslin & Driscoll 2013).

Despite the advantages, 3D culture is noticeably absent internationally as a substitute to animal tests caused by low repeatability and reproducibility, which is mainly due to the use of bespoke systems, or a failure to amend regulation to reflect this advancement. Additionally, current examples of 3D tissue engineered models are generally aimed at physiological/pathophysiological studies and are not widespread, nor are they generally applied in a predictive manner.

1.3 Prevalence of Musculoskeletal Injury and Disease
The economic burden of musculoskeletal conditions is increasing with an rise from £3bn (2003/04) to £5bn (2010/11) spent by NHS England per year treating these conditions, with an additional £3.75bn spent on trauma and injury (Dept of Health UK 2012; Scottish Government 2016). In the UK, soft tissue injuries and conditions have been estimated to account for approximately*:

- 1/3 million hospitalisations
- 1.5 million bed days
- 1 million outpatient visits
- 3 million emergency room visits
- 1.9 million sports injuries per year

* British Orthopaedic Foundation 2012
Currently, severe musculoskeletal tissue damage is surgically repaired with the aim of restoring function regardless of form. Any resulting damage caused directly from the surgery or to other areas from use as grafts, reduces functionality and can increase the likelihood of further local injury (van der Linden-van der Zwaag et al. 2004; Sahoo et al. 2007; Font Tellado et al. 2015). Therefore, more effective solutions to enhance repair and regeneration techniques are needed.

To this end, a biomimetic in vitro musculoskeletal junction is envisaged as a more relevant preclinical testbed for drug and material investigations. This is in order to facilitate a greater healing potential in medicine, as well as providing a model to investigate the basic biology of musculoskeletal tissue and the mechanisms that underpin injury and disease. This thesis describes and assesses the co-culture of three dimensional (3D) tissue engineered skeletal muscle and bone models. It is hoped that this will enable the testing of novel tendon replacement materials and attachment techniques towards an in vitro tissue engineered musculoskeletal junction.
2 Literature Review: In Vivo Musculoskeletal System and In Vitro System Model Development

In order to manufacture a limb musculoskeletal test-bed for pre-clinical studies, it is important to understand the developmental process that leads to the formation of skeletal muscle and bone in vivo. This will provide an insight into the potential structural and biochemical characteristics needed to generate this tissue system. Specific developmental processes, for example, myoblast fusion and osteoblast differentiation/calcification, are recapitulated in post-natal regeneration and remodelling and can therefore be utilised in vitro for tissue engineered constructs. This thesis concentrates on the co-culture of 3D tissue engineered skeletal muscle and bone as an initial step towards a full in vitro limb musculoskeletal junction that may also be used as an additional screening step in toxicity tests or as a model for basic biological research.

The following review details the developmental biology in the early stages of tissue formation and also the tissue’s structure and function, as well as the interaction of skeletal muscle and bone tissue in vivo, followed by a description of current monolayer and three-dimensional tissue engineered co-culture systems.

2.1 Musculoskeletal System

The origin of limb musculoskeletal junctions lie in the early stages of embryogenesis (Gilbert 2007). During gastrulation, the third stage of embryonic development, cells migrate to form three germ layers; the ectoderm, which forms the epidermis and peripheral nervous system; the endoderm, which forms the epithelium of the digestive tube and associated organs including lungs; and the mesoderm, from which the blood and blood vessels, heart, kidneys, bones, muscles and connective tissues derive (Gilbert 2007).

During the next phase of embryonic development, organogenesis, the neural tube (formed from the ectoderm) acts as a separator between the two halves of the developing embryo. On either side, the mesoderm divides into the axial (notochord), intermediate, paraxial and the lateral plate mesoderms (Gilbert 2007).

Through the influence of a protein, Noggin, that antagonises BMP-4 activity (bone morphogenic protein 4), the cells of the paraxial mesoderm form somites (Tonegawa & Takahashi 1998), simultaneously migrating to the gathering of neural folds at the centre of the embryo. Each somite is able to form the vertebrae and rib cartilage; the musculature of the ribs and back as well as the ventral body wall; and the dermis through the commitment of cells to the sclerotome, myotome and dermatome respectively (Buckingham et al. 2003).
2.2 Skeletal Muscle

2.2.1 Early Development

Except for craniofacial muscle, all embryonic skeletal muscle derives from the paraxial mesoderm (Grefte et al. 2007). The dermatome and the myotome form after the sclerotome and is sometimes collectively referred to as the dermomyotome before determination of the individual layers (Buckingham et al. 2003; Christ & Ordahl 1995; Grefte et al. 2007). Once the myotome becomes distinct, it can be said to have two components:

- Primaxial (epaxial) region from which the trunk muscles develop
- Abaxial (hypaxial) region from which the limb muscles develop

Muscle progenitor cells delaminate from the epithelium of the abaxial region of the myotome and migrate toward the developing limb bud (Buckingham et al. 2003). During this development, limb skeletal muscle experiences a cascade of key influencing proteins (Figure 2.1), which induces the initial delamination, through the expression of c-Met transcribed by Pax3, and migration to the limb field through the presence of hepatocyte growth factor (HGF) (Dietrich et al. 1999). Once in the limb bud, the progenitor cells are influenced by a family of proteins known as myogenic regulatory factors (MRFs) that govern the determination and differentiation of the precursors to the myogenic lineage (Hawke & Garry 2001). Determination of the progenitor cells is characterised by an expression of Myf5 and MyoD (Buckingham et al. 2003). Over-expression of these factors can induce commitment to a myogenic cell fate in non-muscle lineages (Choi et al. 1990; Tapscott et al. 1988). Differentiation to myoblast and subsequent fusion to multinucleated myotubes is induced by expression of myogenin and MRF4 (Ott et al. 1991).

![Figure 2.1: Embryonic Pathway of Limb Skeletal Muscle.](image)

Cell progression from the myotome to the differentiation of limb bud skeletal muscle fibres (Adapted from Buckingham et al. 2003)
2.2.2 Structure
Skeletal muscle represents the largest organ system within the body – approximately 25-35% of the mass of women and 40-50% of men (Bianchi & Martinoli 2007). Mature muscle is characterised by a hierarchical structure in which repeating contractile units of myosin and actin, called sarcomeres, are formed into groupings of fibres that comprise the main muscle belly (Figure 2.2). Sarcomere assembly requires a change in intracellular architecture, where the nuclei and organelles are relocated to the periphery of the fibre to allow for the uninterrupted formation of the contractile apparatus space (Sciote & Morris 2000; Berendse et al. 2003).

![Figure 2.2: Schematic of the hierarchical structure of skeletal muscle](image)

Sequentially arranged actin and myosin filaments are arranged within a single muscle fibre causing cell organelles to locate to the periphery of the fibre. These fibres are arranged in bundles as a fascicle. Multiple fascicles are further arranged into bundles forming the skeletal muscle belly.

Once skeletal muscle precursor cells (MPCs), or myoblasts, have fused into multinucleated myotubes, the manufacture and organisation of these sarcomeres takes place within the myotube cytoplasm, the sarcoplasm, to align along the axis of tension (Gillies & Lieber 2011). These myotubes mature into muscle fibres and are arranged as bundles within an extra-cellular matrix based sheath, the perimysium, as a fascicle. Separating each fibre within the fascicle is the endomysium. Groupings of multiple fascicles, which make up the muscle as a whole, are contained within a double layer of connective tissue called epimysium (Gillies & Lieber 2011).

Skeletal muscle fibres are classified as a post-mitotic tissue and cannot inherently regenerate themselves. As a result, peripherally to these fibres are remnant precursor cells called satellite cells, which are responsible for the maintenance of the muscle fibres during repair (Sinanan et al. 2006). They sit in a quiescent state (defined by a low cytoplasm to nuclei ratio) until activated, whereupon they undergo asymmetric mitosis, forming a cell capable of muscle fusion and a precursor cell that returns to a quiescent state (Morgan & Partridge 2003).
The majority of the extra-cellular matrix (ECM) is comprised of collagen (Mudera et al. 2010). As a visco-elastic material, collagen allows for contraction of the muscle without damaging the gross structure of the muscle. Type I and III are the predominant types of collagen found in mature skeletal muscle, of which up to 97% is type I (Bailey et al. 2001; Kjaer 2004).

The ECM also has an important role to play in the regeneration of muscle tissue, neural transmission and myogenesis (Lewis et al. 2001). It contains secreted growth factors and proteins that are required for differentiation and proliferation of muscle cells. Remodelling of the ECM by skeletal muscle fibroblasts allows for satellite cell activation. Activation causes the satellite cell to undergo asymmetric mitosis, which creates a ‘daughter’ cell. This then expresses genes associated with the myogenic lineage and proliferates further and migrates to fuse with existing fibres to facilitate repair (Figure 2.3) (Lewis et al. 2000; Zammit et al. 2006a). The process is recapitulated in in vitro models where muscle precursor cells are seeded onto a surface or within a matrix and stimulated into fusion.

![Figure 2.3: Cellular response to injury.](image)

Once a muscle fibre has been damaged, satellite cells adjacent to the fibre are activated and undergo asymmetrical mitosis. This results in a proliferative population with skeletal muscle associated genes. These myogenic cells then migrate to the site of injury and fuse with the existing fibres to repair the damage. (Adapted from Zammit et al. 2006).

### 2.2.3 Function

As a dynamic structure that facilitates motion, the biomechanics of the muscle are complex and hinge around the proteins that make up the contractile unit of the sarcomere (Figure 2.4). Sarcomeres are aligned in series within muscle fibres (Ovalle 1987) and are comprised of two parallel filaments, actin and myosin. The interdigitation of these proteins gives rise to the striated nature of the muscle.

The sliding filament, or ‘cross bridge’, theory, initially proposed by Huxley (1974), governs the interaction of the myosin and actin to facilitate contraction. It is mediated by the proteins, troponin and tropomyosin. In a relaxed state, the troponin positions the tropomyosin such that the myosin binding sites are covered preventing actin interaction (Jones 2005; Tregear & Marston 1979).
Ca\textsuperscript{2+} ions, released by the sarcoplasmic reticulum, bind to the troponin-tropomyosin complex which translocates and reveals the myosin binding site (Gomes et al. 2002). Regulation of Ca\textsuperscript{2+} ions release is governed by nervous input, which triggers a preceding excitation-contraction coupling process cascade. This activates ryanodine receptor-1 (RYR1) channels releasing the Ca\textsuperscript{2+} ions (Sandow 1952).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{Arrangement of myosin, actin and desmin proteins within a sarcomere. Filaments of actin and myosin are bound to the z-disk by the giant filament, tinin. The z-disk consists of \(\alpha\)-actinin bound together by the intermediate filament protein, desmin. Desmin also connects each z-disk to each other. (Adapted from Baechle & Earle, 2008, and Sparrow & Schöck, 2009)}
\end{figure}

Whilst actin and myosin act as the functional proteins of skeletal muscle, desmin, an intermediate filament protein, acts as an important binding subunit. Desmin occurs at the z-line of the sarcomere and is essential for maintaining the structural integrity and therefore, the function of the contractile apparatus (Paulin & Li 2004; Shah et al. 2004). The desmin molecule binds together the cytoskeletal \(\alpha\)-actinin molecule to create the disc-like structure that the actin and myosin act against; it also binds the sarcomeres to the subsarcolemmal cytoskeleton (Paulin & Li 2004). Desmin is also implicated in the regeneration of skeletal muscle. The absence of this protein causes delays and disruptions to repairs, often causing abortion of the process and a lack of desmin presence during development causes a fundamental myopathy to occur (Paulin & Li 2004; Shah et al. 2004). In addition to desmin, tinin (a giant filament greater than 1.0\(\mu\)m in length) binds the m-line to the z-line through the myosin filament. Through this binding, tinin limits the extension of the sarcomere, contributing to the inherent stiffness of the muscle (Sparrow & Schöck 2009).
2.3 Bone

2.3.1 Early Development
Bone forms in two different ways: Intramembranous ossification and endochondrial ossification. The first, intramembranous ossification, occurs by a direct transformation of mesenchymal cells into bone (Gilbert 2007). It is responsible for flat bones, such as the patella, and also for adding width to the periosteum in the long (limb) bones (Bianchi & Martinoli 2007). The second is endochondral ossification, where the bones of the trunk and rear of the cranium, as well as the long bones, are developed (Gilbert 2007). Limb bone formation by endochondral ossification starts off as a soft model formed from cartilage from mesenchymal stem cells (MSCs) from the lateral plate mesoderm.

Before the cartilage model is formed, there is an initial commitment of MSCs to the cartilaginous lineage. This is stimulated by Sonic Hedgehog (Shh), which induces nearby sclerotome cells to express the Pax1 transcription factor. Pax1 triggers a cascade effect which depends on external paracrine and internal transcription factors (Cserjesi et al. 1995; Šošić et al. 1997). Once the MSCs are committed, they concentrate into compact nodules and then differentiate into cartilage synthesising chondrocytes. The bone morphogenetic protein (BMP) family causes an expression of the adhesion molecules N-Cadherin and N-CAM as well as transcription factor Sox9 (Hall & Miyake 1995). The adhesion molecules are responsible for the accumulation and maintenance of the chondrocyte nodules and Sox9 is responsible for the activation of several transcription factors which cause an upregulation of the genes that encode collagen-II and aggrecan; critical components in the maintenance of cartilage (Olsen et al. 2000).

This cartilage is subsequently replaced by bone in five distinct stages after initial determination (Mackie et al. 2008). First, chondrocytes which secrete cartilage-specific ECM components start to rapidly proliferate (Gilbert 2007). Once the chondrocytes have reached a certain density, they increase their volume to become hypertrophic chondrocytes. To enable the calcification, these chondrocytes alter the matrix, adding collagen X and increasing the levels of fibronectin. During this stage, the metabolism and mitochondrial energy potential of the chondrocytes are modified due to a change from aerobic to anaerobic respiration (Shapiro et al. 1982). Hydroxyapatite, a small calcium carbonate crystal, is generated by small vesicles and secreted into the ECM, which mineralises the cartilaginous matrix (Genge 1997). The hypertrophic cells then die although the specific associated process is not fully understood. Death through apoptosis due to the alterations made within the cell processes by apoptogens from various sources, including apatite formation, was reviewed by Adams & Shapiro 2002 and Gibson 1998, although observations seem to suggest that this process is morphologically distinct from apoptosis. An alternative autophag cell death has also been
proposed, in which cytoplasmic components are degraded in lysomes (Mackie et al. 2008; Mizushima 2007).

In the final phase, a vascular network invades the cartilage model, which is induced by VEGF (vascular endothelial growth factor). The apoptotic chondrocytes are surrounded by differentiating osteoblast precursors that encircle the cartilage model and a remodelling period begins (St-Jacques et al. 1999). The mineralisation of the ECM regulates the induction of osteoblasts and, therefore, the remodelling that occurs. This remodelling causes an increase in the VEGF content, which creates more blood vessels around the disappearing cartilage. These vessels bring in osteoclasts and chondroclasts that remove the remnants of the chondrocytes. The osteoblasts then begin to secrete bone onto the partially degraded matrix and segregate the calcified cartilage matrix (Gilbert 2007).

During the aforementioned hypertrophic stage, the chondrocytes secrete Indian Hedgehog (Ihh) causing immature cells to produce RUNX2, also known as core-binding factor subunit α-1 (CBFa1), inducing the formation of osteoblasts at a later stage (St-Jacques et al. 1999) (Figure 2.5). RUNX2 allows the cell to secrete bone matrix but stops full differentiation to osteocytes. These osteoblasts then become responsive to Wnt signalling which upregulates the transcription factor Osterix that causes bone formation (Nakashima et al. 2002; Nakamura 2007). The vascular network present in the forming bone, along with the segregation of the cartilage matrix, the chondroclasts and bone remodelling osteoclasts (Mackie et al. 2008), forms a cavity where the bone marrow begins to form (Mundlos & Olsen 1997; Olsen et al. 2000). Further mineralisation and remodelling of the bone continues throughout life as a response to loading. Osteocalcin, a bone gamma-carboxyglutamic acid protein encoding gene (also known as BGLAP), has been shown to be a factor in regulation of the mineral deposition phase of osteoblast differentiation. As mineral deposition takes place in late stage osteoblast maturation, osteocalcin is regarded as late stage marker of osteoblast differentiation (Lian & Stein 1992).

**Figure 2.5:** Hypertrophic chondrocytes secrete Indian Hedgehog (Ihh) which induces pre-ostoblast cells to produce RUNX2/CBFa1 which causes differentiation to mature osteoblasts. Wnt signalling causes an upregulation of Osterix expression which causes deposition of bone matrix.
2.3.2 Structure

There exists a number of different types of osseous tissue and these can be described by either their location along the bone, their specific structure or their microscopic nature (Mangham & McClure 2007). A fibrous membrane, known as the periosteum, lines the outside of all bones except at the joints of the long bones (Figure 2.6) (Clarke 2008). This serves as an attachment site for tendons and muscles as well as providing a blood supply. Below this is the first layer of bone tissue, the subperiosteal layer. Then there is the cortical layer which forms the outer shell of the bone (Figure 2.7), enveloping small blood vessels within the bone, known as Volkmann’s and Haversian canals. The marrow cavity is encapsulated by the medullary layer, which is, in turn, lined by a thin layer of tissue known as endosteal bone (Mangham & McClure 2007).

![Figure 2.6: Structure of the long bone.](Adapted from Cummings, 2009)

The type of bone is categorised by its density and mechanical properties (Figure 2.6). Compact or cortical bone is dense and possesses an internal vascular network and is found in the main body of bone (Clarke 2008; Weiner & Wagner 1998). Trabecular or cancellous bone is a soft sponge-like tissue with a large surface area to mass ratio. In contrast to compact bone, trabecular bone lacks an internal blood supply and gains nutrients from adjacent vessels. Trabecular bone is generally found at the terminus of the long bones, proximal to joints, and within vertebrae (Bianchi & Martinoli 2007).

The microscopic nature of the bone changes as a response to injury and the repair process. Normal bone consists of plates of bone matrix laid down in organised sheets by osteoblasts. This lamellar bone provides a strong structure all dimensions (Nakamura 2007; Mangham & McClure 2007).
After injury, early deposits of bone are disorganised and are therefore weaker. This woven bone is slowly replaced over time with lamellar bone to recreate the normal structure (Newsham-West et al. 2007; Paxton et al. 2012).

Within osseous tissue, four types of cell exist to maintain the bone structure. Osteoblasts lay down the extra-cellular matrix in organised lamellae. Whilst laying down the bone matrix, some osteoblasts entomb themselves inside the matrix in lacunae to become osteocytes. These form an intercellular network throughout the bone to regulate turnover of the matrix (Gitajn & Rodriguez 2006). To remodel the calcified matrix in response to external forces to present the strongest structure, osteoclasts are needed to degrade the bone before osteoblasts deposit a reorganised tissue. Approximately 10-15% of osteoblasts become encompassed in the matrix, leaving the remainder to become bone surface lining (BSL) cells as part of the endosteal lining or die (Mangham & McClure 2007). The major components of the calcified matrix include 25% organic matrix [including cellular material (2.5%)], 5% water, and 70% inorganic material including hydroxyapatite crystals. The majority of the organic material is Type I collagen with a smaller portion of Type XI (Clarke 2008; Mangham & McClure 2007).

![Figure 2.7: Hierarchical structural organisation of bone. Bone consists of hydroxyapatite crystals laid upon collagen molecules arranged in layers and bundled into fibres. These fibres are remodelled into tight regular structures in compact bone and looser patterns in trabecular bone (Adapted from Rho et al. 1998).](image)

**2.3.3 Function**

The ultimate function of the skeleton is to provide a rigid structure to protect vital organs and to facilitate movement. Bone itself is designed to provide maximal mechanical strength whilst maintaining the minimum mass (Rho et al. 1998). The long bones house the marrow and therefore supports haematopoiesis (the act of producing blood components) and consequently play an important role in immune system replenishment. It also acts as a store of calcium, phosphate and small amounts of lipid material as well as acting as a source for these minerals (Mangham & McClure 2007).
2.4 Musculoskeletal Attachments

To create a platform for the development of an *in vitro* musculoskeletal junction and for the testing of novel materials, it is important to understand the characteristics of the skeletal muscle and bone attachments. This will inform key parameters that should be taken into account to enable attachments in the future.

2.4.1 Myotendinous Junction

There is very little literature on the specifics of early development and integration of tendon and skeletal muscle. Studies on injuries at the myotendinous junction have a tendency to focus on the muscle tears found proximal to the junction and not at the site or in the tendon near to the junction. A study of maturation of the myotendinous junction by Ovalle (1987), provides the basis for current understanding of the architecture of the junction itself.

2.4.1.1 Development

Myotendinous junction (MTJ) structural development is not one that occurs just in the first few days of embryogenesis, but is one that occurs over the early years of life as the structure follows the development of the surrounding tissue (Ovalle 1987). The mechanisms that fuel the early connection within the developing embryo are not fully understood (Schweitzer et al. 2010). Initial integration with the muscle occurs through the positioning of the muscle fibres arriving at the target tendon cell. This relies heavily on the location relative to the muscle cell and the timing of the coupling (Schweitzer et al. 2010). The overall mechanism behind the recognition of the tendon precursor, the migration of the muscle to that target cell and the integration of the two, is not yet known (Schweitzer et al. 2010). However, possible mechanisms for this migration are the interaction of the transmembrane protein Kon-tiki (also known as Perdido) with tendon specific αPS1βPS integrin receptor (Estrada et al. 2007). Kon-tiki/Perdido also interacts with Grip (glutamate receptor interacting protein) to mediate myotube projection and attachment in the *Drosophila* embryo (Estrada et al. 2007) and also muscle’s sensitivity to the extra-cellular matrix protein, Tsp (Thrombospondin). Tsp receptors are not expressed on the membrane of the advancing muscle, but the accumulation of integrin receptors promotes a response to Tsp and a further increase in integrins (Gilsohn & Volk 2010).

2.4.1.2 Structure

The structure of the myotendinous junction changes with age. Shortly after birth, bundles of muscle fibres are dove-tailed into highly crimped dense tendon tissue (Figure 2.8). The myotubes at this junction display classic signs of muscle differentiation, where large nuclei with prominent nucleoli are positioned at the periphery of the fibre. Within the tendon fibres, fibroblasts are situated in close proximity to the junction. These cells are typically large and spindle-shaped and orientated in a
specific direction. At the end of the muscle fibres, a corrugated texture forms, allowing for the invasion of the muscle territory by the tendon fibrils (Ovalle 1987).

Figure 2.8: Neonatal myotendinous junction
Dense, crimped tendon tissue is dovetailed with skeletal muscle bundles. Terminal extensions of the sarcomeres (denoted by □) can be seen inserting into tendon. Nuclei are located peripherally within the muscle (denoted by ->) and fibroblasts (denoted by △). Scale bar 20µm. Image reproduced with permission, Ovalle 1987.

By adolescence, the muscle and tendon are heavily inter-digitated with extended incursions of myofibrils and tendinous tissue (Figure 2.9). Abundant fibroblasts are apparent within the collagen arms of the tendon. Muscle fibres are well developed with obvious sarcomere development orientated parallel to the longitudinal axis of the fibre. Each sarcomere terminates at the Z-line where multiple strands diverge to enter the cytoplasmic processes at the sarcolemma (the muscle cell membrane) along the lateral walls of the cytoplasmic extensions (Ovalle 1987).

Figure 2.9: Adolescent myotendinous junction
Terminal extensions of the sarcomeres (denoted by □) can be seen inserting into tendon. Nuclei within the muscle tissue are denoted by -> and fibroblasts are denoted by △. Scale bar 20µm. Image reproduced with permission, Ovalle 1987.

In the mature myotendinous junction, there is a distinct decrease in nuclei profiles within the tendon (Figure 2.10). Within the muscle, the sarcomeres are numerous and arraigned regularly. Rather than the obvious dovetailing at the neonatal junction or the long extensions into the opposing tissues of
the adolescent junction, the mature myotendinous junction exhibits a jagged intersection where the collagen fibrils of the tendon penetrate into the surface of the myofibres (Nakao 1975; Charvet et al. 2012). Cellular features of the muscle remained conserved from the adolescent profile but with a reduction in non-contractile features, for example, Golgi apparatus (Ovalle 1987).

Figure 2.10: Mature myotendinous junction
Collagen fibrils can be observed penetrating the myofibres creating a jagged interface. Nuclei are located peripherally within the muscle (denoted by ->) and fibroblasts (denoted by □). Terminal extensions of the sarcomeres (denoted by □) can be seen inserting into tendon Scale bar 20µm. Image reproduced with permission, Ovalle 1987.

With this structure in mind, skeletal muscle constructs should possess as many myotubes as possible with as great alignment as possible to facilitate attachment to potential tendon materials/constructs.

2.4.1.3 Function
This interface represents the primary site of force transmission and also the first boundary for losses in this force (Charvet et al. 2012). Therefore, the function of the myotendinous junction is to transfer this force as efficiently as possible to the tendon to reduce any possible damage to the musculature from stress points.

2.4.2 Osteotendinous Junction (Enthesis)
The osteotendinous junction, or enthesis, is the attachment site of tendon into bone. Due to the various types of muscle and attachments, tendinous insertions into bone were initially identified by the attachment site. Biermann (1957) and Knese & Biermann (1958) categorised these types as ‘diaphyseal-periosteal attachments’ and ‘chondro-apophyseal attachments’ meaning attachment to the periosteum on the shaft of the long bones and to the softer bone at protrusions, for example, at the epiphysis at either end of the long bones (Benjamin et al. 2006). These terms were subsequently found to be specific to the long bones and did not apply to all tendinous insertions (Benjamin et al. 2002).

Later work by Woo et al. (1989) attempted to broadly classify enthesis type by the absence or presence of a periosteum at the attachment site, hence by direct or indirect attachment, respectively. Modern day terminology categorises entheses according to the tissue facilitating
attachment: Fibrous or fibrocartilaginous insertions (Benjamin & Ralphs 1998; Benjamin et al. 2004). This is roughly equated to the work done by Woo et al. (1989), but allows for more specific descriptions and more flexibility in tendon location. Specifically, not all direct attachments to the bone require a fibrocartilaginous enthesis (Benjamin et al. 2006). Literature pertaining to fibrous enthesis development and architecture is severely limited. François et al. (2001) note that descriptions of structure are often restricted to the fibrocartilaginous enthesis “as if there were no other”. This is possibly due to the prominence of the fibrocartilaginous enthesis in medical texts. The fibrocartilaginous insertion is more susceptible to injuries and therefore of more interest in healing and structural studies.

2.4.2.1 Development

As for the development of the myotendinous junction, the initial attachment and association of tendon and bone in the limbs is not fully understood. However, the same mechanism is apparent in which cell-to-cell targeting occurs at arriving tendon precursors. In the trunk tendons, bone-tendon association is made during somite compartment determination with the regulation of scleraxis (Scx) expression by both the myotome and sclerotome. In limb buds, tendon determination occurs regardless of muscle or cartilage markers (Schweitzer et al. 2010). During early attachment, the fibrocartilaginous enthesis attaches directly to the cartilage of the developing bone (Claudepierre & Voisin 2005). As the cartilage undergoes endochondral ossification, the tendon cells undergo metaplasia into cartilage cells which secrete fibrocartilage. Once completed, these two processes result in a clear smooth transition from tendinous material to bone (Shaw et al. 2008; Gao et al. 1996). A potential developmental reason for the difference in attachment type, as opposed to possible mechanical reasons, is that the type of bone development, either endochondral or intramembranous ossification dictates the attachment type. However, the dual nature of some attachments renders this unlikely (Benjamin et al. 2002).

2.4.2.2 Structure

Fibrous enthesis

Fibrous entheses are made up of dense fibrous connective tissue and are associated with some of the largest muscles in the body (Benjamin et al. 1986; Benjamin et al. 2006). Fibrous insertions can be further grouped into periosteal and non-periosteal attachments. Periosteal entheses spread the forces over a large area, reducing the effects of stress caused by the muscle, and are anchored by extensions called Sharpey’s fibres (Sverdlova & Witzel 2010; Milz et al. 2002; Paxton et al. 2012; Benjamin et al. 2002). These tendons are often short to cope with the large forces and the limited amount by which the tendons of this type can stretch.
There is a general lack of literature sources on the cellular and molecular makeup of the fibrous enthesis, thought to be due to the low prevalence of injury and therefore low medical interest, however parallels can be drawn from the ligament enthesis. The development of an in vitro musculoskeletal junction could provide the opportunity to study this area in more detail. Matyas et al. (1990) described the five layered tibial enthesis of a rabbit lateral ligament. The first layer consists of connective tissue, leading to a layer of densely packed collagen fibres that blend with the fibrous layer of the periosteum. This is followed by a layer of loose connective tissue which merges with the osteogenic layer of the periosteum. The lower layers are of cortical bone although the architecture changes with age. The fifth layer, the deeper cortical bone, is initially remnants of cartilage and primary bone. This later gets remodelled as lamellar bone. The fourth layer develops as skeletal maturity nears, causing layers two and three to ossify. However, the parallels drawn for the fibrous enthesis may not be relevant. Various glycosaminoglycans (GAGs) present in this are also present in fibrocartilaginous enthesis (Ababneh et al. 1998). This idea is conserved in periosteal fibrous enthesis, where age can cause the insertion to become ossified with skeletal maturity (Matyas et al. 1990). However, evidence exists that fibrous periosteal attachment can persist throughout life (Hems & Tillmann 2000).

**Fibrocartilaginous Enthesis**

The fibrocartilaginous enthesis is characterised by its four distinct zones. Dense fibrous tissue from the tendon slowly changes into an avascular region of uncalcified fibrocartilage. The tissue then abruptly changes to a thin region of calcified fibrocartilage which forms the connection to bone (Figure 2.11) (Bedi et al. 2012).

**Figure 2.11:** Histology of the enthesis attachment. Each zone is shown with the tidemark clearly visible between calcified and uncalcified fibrocartilage zones. (Adapted from Bedi et al. 2012)
Between the two fibrocartilage regions lies a basophilic line called a tidemark. If the insertion to bone occurs close to articular cartilage, the tidemark continues across both. There is a high proteoglycan content at the tidemark and its relative linearity allows a low-stress attachment of soft tissue to calcified tissue, whilst enabling force transition with little loss (Benjamin & McGonagle 2001).

To facilitate a strong attachment, the connection from the calcified fibrocartilage to the subchronal bone is highly irregular. This signifies an anatomical change similar to the myotendinous junction and the true site of the soft tissue attachment. The calcified region also acts as a barrier to blood diffusion from the bone (Maffulli et al. 2005). Boyde et al. (1995) and Vajda and Bloebaum (1999) show that calcified fibrocartilage in the iliac crest and the upper end of the femur is mineralised to a greater extent than the surrounding bone (Benjamin & McGonagle 2001).

Cells within the uncalcified zone are arranged in longitudinal rows with collagen fibres arranged in between (Benjamin & Ralphs 1998; Benjamin & McGonagle 2001). Fibroblast cells found in the enthesis are more rounded than typical fibroblasts and lack sheet-like processes that characterise fibroblasts found elsewhere. The isolated nature of these cells within the cartilaginous matrix means cell-to-cell communication between these tendon fibroblasts and osteocytes responsible for communicating organisation and remodelling signals in the adjacent bone is blocked (Benjamin & Ralphs 1998). Aggrecan within the uncalcified cartilage attracts water into the tissue. The pressures within this region allow water to act as an incompressible substance and stiffen the matrix to allow superfluous movement in the enthesis to pass into the surrounding soft tissue (Waggett et al. 1998).

Osteological structure at the enthesis
The particular characteristics of the enthesis, for example, the vascular barrier of the calcified fibrocartilage zone and the linearity of the tidemark, are reflected in bone markings. In healthy attachments, the bone is smooth with a well-defined contact area and shows no vascular intrusion (Benjamin et al. 1986). The subchronal bone beneath the enthesis acts as a plate allowing deformation with tendon loading.

Sharpey’s fibres
Sharpey’s fibres are a dense matrix of collagenous fibres that extend directly into the trabecular bone beneath entheses. The specific nature of these fibres is under considerable debate. François et al. (2001) consider Sharpey’s fibres to be exclusive to fibrous entheses. They characterise them within the context of the vertebral column as a contingent of tightly woven collagen fibres around which bone forms. Along with Benjamin et al. (2006), they believe that the collagen fibres that penetrate through the tidemark at the fibrocartilaginous enthesis and penetrate through the
calcified fibrocartilage should not be viewed as Sharpey’s fibres. Haines & Mohuiddin (1968) describe calcified fibrocartilage as a recognised form of bone in German literature. Matyas et al. (1990) supports this idea and, therefore, collagenous intrusions into this region can be viewed as variations of Sharpey’s fibres.

Any models aimed towards recreating the enthesis must therefore demonstrate the mineral component of bone whilst maintaining an ability to be remodelled to facilitate the specific structures of the enthesis transition. Therefore, a hydrogel/mineral hybrid construct is most suitable because each component individually fulfils these requirements (Murugan & Ramakrishna 2006; Kim & Kim 2014; Henson & Getgood 2011; Oryan et al. 2014).

2.4.2.3 Function

The function of the tendon enthesis is multi-purpose. It must anchor the muscle to the bone via the tendon in a strong fashion whilst providing a smooth transition for the contractile force of the muscle to effect movement. This attachment may occur in close proximity to joints and therefore, must be supple, whilst not eroding the strength of the osseous tissue it is connected to.

A secondary purpose for the enthesis was suggested by Knese and Biermann (1958); that the enthesis can act as a growth plate for apophyses at the insertion sites into bone (Gao et al. 1996). A developmental study by Gao et al. (1996) of the femoral attachment of the medial collateral ligament seems to support this idea. Cartilage formed during embryogenesis is replaced during endochondral ossification by the fibrocartilage of the enthesis.

2.5 Muscle and Bone Interactions

In 2010, Liu et al. postulated that skeletal muscle may have a larger role in bone healing than just the initially supposed vascularisation and remodelling according to load (Wolff’s law), although Urist first realised skeletal muscle’s ability to induce bone growth from demineralised bone implants into muscle in 1965 and 1970 (Eastwood, Mudera, et al. 1998; Liu et al. 2010). This hypothesis was based on the potential for adjacent skeletal muscle to be capable of providing cells that can contribute to bone formation (Sinanan et al. 2006). Further evidence was shown through prevention of contact between the healing bone and adjacent muscles and compromised healing from quadriceps atrophy (Hao et al. 2012; Harry et al. 2008).

In addition to the physical and direct cellular interactions, secreted factors have also been shown to influence skeletal muscle and bone. The human growth hormone/insulin-like growth factor (hGH/IGF1) axis is a fundamental factor of bone health in its essential role in bone growth maturation and maintenance, through the enhancement of Wnt-dependant activity (Locatelli &
The hGH/IGF1 axis is also an important factor in promoting skeletal muscle cell growth and influencing fusion (Perrini et al. 2010). Proteins associated with this axis are secreted by both osteoblasts and skeletal muscle precursor populations and would therefore be influences in any system which includes both tissues. Differentiated skeletal muscle has also been shown to produce extra-cellular ATP which is broken down into pyrophosphates (Martinello et al. 2011). In bone, alkaline phosphatase is produced to assist in calcification during osteogenesis by reducing pyrophosphates to phosphates in the creation of hydroxyapatite (Orimo 2010; Golub et al. 1992) and so skeletal muscle co-culture with bone could enhance calcification.

Within some in vitro cell models, osteoblast-like cell lines (including osteosarcoma cells) are used in place of primary osteoblasts to develop cultures without donor variation from primary sources (Pautke et al. 2004; Clover & Gowen 1994). These cultures do not maintain all of the characteristics of primary osteoblasts but have been shown to conserve features necessary for modelling purposes (Clover & Gowen 1994; Pautke et al. 2004). For example; in terms of influencing factors in a co-culture, transforming growth factor β1 (TGFB1) that is present during osteoblast differentiation and bone formation, has been shown to be secreted by osteosarcoma cell lines and can act as an inhibitor of myogenic differentiation (Franchi et al. 1998). TGFB1 has also been shown to increase proliferation of myogenic cells and enhance contractility in tissue engineered skeletal muscle models (Katagiri et al. 1994; Liu et al. 2001; Furutani et al. 2011; Weist et al. 2013).

In addition to the above, inflammatory factors, such as interleukin-6, -7 and -15 (IL-6, IL-7 and IL-15), and myostatin have also been implicated as influencing agents in both skeletal muscle and bone and can be present in diseases such as myositis ossificans, where skeletal muscle begins to calcify (Davies et al. 2015). More in depth reviews on the skeletal muscle and bone interaction are offered by Sartori & Sandri (2015) and Hamrick (2012). In a musculoskeletal co-culture system, these secreted factors may induce characteristics not normally observed in single engineered constructs, for example, longer time to differentiate, and so individual constructs should be developed so to enable flexible culture conditions, such as long term culture periods.
2.6 *In Vitro* Musculoskeletal Co-culture systems

Orthopaedic trauma and degeneration is most commonly associated with soft tissues, most prominently at interface points, such as the myotendinous and osteotendinous junctions (Benjamin et al. 2006; Maffulli et al. 2005; Yang & Temenoff 2009; Lu et al. 2010; Rothrauff & Tuan 2014). Current strategies for repair involve surgical intervention and rehabilitation, although these methods do not result in the true re-establishment of the tissue/tissue interface and can result in re-rupture (Brassart et al. 2008; Hohendorff et al. 2008; van der Linden-van der Zwaag et al. 2004; Sawadkar et al. 2013). This has led to the creation of new materials to assist in the regeneration of damaged or diseased tissues (Scott Taylor & Shalaby 2013; Young et al. 1998; Butler et al. 2008; Saxena et al. 1999; Fan et al. 2009; Hukins et al. 1999; Caliari et al. 2011; Ratner et al. 2013; Davis et al. 1992; Hench & Paschall 1973). Prior to commercialisation, these new materials must first go through a series of pre-clinical and human clinical trials before regulatory approval (Johnson et al. 2001; Garimella et al. 2013; Stavropoulos et al. 2015; Correia et al. 2014; Schoen & Levy 2013). Current pre-clinical toxicology testing utilises a high-throughput approach of monolayer cell biochemical or gene expression assays to screen new technologies for adverse effects (Peters et al. 2009). However, studies have shown that the final effects on target tissues and associated systems cannot be fully anticipated (Vandenburgh 2010; Nam et al. 2014; European Commission 2010; Mestas & Hughes 2004). This results in numerous unsuitable technologies continuing through to later human trials wasting time and resources as well as causing an ethical conundrum (up to 88% of drugs fail in human trials)(Kintisch 2012).

3D tissue-engineered approaches to regenerative medicine not only offer functional alternatives for replacement or assisted regeneration of damaged/diseased tissues but can act as an additional screening element for pre-clinical testing. Many different 3D models developed towards pre-clinical testbeds currently exist; for example, skeletal and cardiac muscle (Vandenburgh et al. 2008; Eschenhagen & Zimmermann 2005), tendon/ligament (Shearn et al. 2011), tumour (Fischbach et al. 2007) and liver (Ebrahimkhani et al. 2014; Sivaraman et al. 2005; Khetani & Bhatia 2008). Although each of these models produces a desired characteristic that may allow for translation to human trials, each ignores the neighbourhood effect of interlinked tissues and systems. This interlinkage is especially important within the musculoskeletal system.

The complexity of the musculoskeletal system lies in the individuality of each tissues function. Bone is a rigid structure required to support the mass of the human body. Its cellular component is responsible for healing and remodelling according to external influences (Gitajn & Rodriguez 2006; Sommerfeldt & Rubin 2001). Skeletal muscle’s cellular body provides an active function, generating a contractile force to enable movement (Huxley 1974; Ostrovidov, Hosseini, et al. 2014; Ostrovidov,
Ahadian, et al. 2014), which then transitions into the passive functionality of the tendon. In contrast to the muscle, tendon tissue is predominantly extra-cellular matrix (ECM) and its elasticity, through its crimped structure and inherent material characteristics, acts as a buffer between the skeletal muscle and bone (Benjamin et al. 2006; Benjamin et al. 2002; Lin et al. 2004; Cheng et al. 2014; Calve et al. 2004; Wang 2006). It has been demonstrated that a deficiency in one of these tissues can have negative effects on the whole system (Benjamin et al. 2002; Shah et al. 2013; Sartori & Sandri 2015; McCullagh & Perlingeiro 2014; Office of the Surgeon General (US) 2004). Therefore, to understand the impact of new regenerative techniques aimed at a single tissue, for example, tendon reattachment suture material, the effect on the interlinked neighbourhood tissues should also be assessed. As a result, in order to provide a relevant testbed to assess regeneration techniques, multiple tissue culture systems are necessary in the future of pre-clinical testing.

Co-culture systems have been used across a wide number of disciplines to allow for the study of multiple tissue population interactions (Dietze et al. 2002; Rieger et al. 1987; Mikos, Herring, Ochareon, Elisseeff, et al. 2006), improving culture conditions (Ekwueme et al. 2015; Ostrovidov, Ahadian, et al. 2014; Hinds et al. 2013; Kalman et al. 2015) and material studies (Agrawal & Ray 2001; Bitar et al. 2004). Monolayer co-cultures especially have allowed for these studies to be free from the complexities inherent in situ observations. This review focusses on the various co-culture solutions which have been used to investigate the independent and varied needs of the adherent cellular populations of the musculoskeletal system.

2.6.1 Monolayer co-culture models
Monolayer co-culture systems allow for the isolation of cells from interactions with the surrounding extra-cellular matrices, immunological systems and outside molecular interferences. Within regenerative medicine, the main motivations for monolayer co-culture are to study cell-cell interactions towards the creation of an in vitro biomimetic tissue (Cooper et al. 2004; Miki et al. 2012; Paschos et al. 2014), to understand how a desired cell process will affect a target system (Jiang et al. 2005; Choi et al. 2013; Dodson et al. 1997; Goetsch et al. 2014) and to enhance the culture of a desired population (Beier et al. 2011; Kino-oka et al. 2013). Monolayer cell cultures have the advantage of simplicity; in that they are relatively easy to set-up, low cost in comparison to other systems, such as 3D tissue engineered models, and enable high-throughput. A number of systems reflecting the above aims have been developed around two concepts: Direct and indirect culture; each enabling various different types of inter-cell signalling (Doorn et al. 2012).
Direct culture is where each population of cells is placed into a single culture vessel without any separating boundaries between cultures (Figure 2.12). This allows for all types of inter-cell signalling. Typically, monolayer co-cultures are seeded onto tissue culture plastic (TCP) or glass slides, although these surfaces can be modified through surface patterning or coating to add an element of cellular control (Shah et al. 2012; Anselme et al. 1999; Martin et al. 1995; Pittenger 2008; Song et al. 2004; De Bari et al. 2003). The simplest way to affect direct culture is to mix the cell types of interest at the required culture ratio and then seed them onto a surface (Beier et al. 2011). By varying the ratio of cell types, the relative signalling biases can be controlled. However, interpretation of these cultures must take into account metabolic differences as well as any potential dilution effects from having a mixed population which makes a true comparison to in vivo tissue difficult. The degree of direct interactions can further be influenced by micro-patterning (Bhatia et al. 1997; Ostrovidov, Ahadian, et al. 2014) or through the use of a temporary barrier, which is removed or degrades after or over a period of time (Wang et al. 2007). Micro-patterning can influence the type of cells that attach to particular areas of the surface out of preference of a specific molecular substrate, thereby restricting cell adhesion whilst enabling cell contact at the surface interfaces. A temporary barrier allows the cells to adhere before cell migration facilitates contact.

Mixed cultures are created by mixing the cell populations and seeding onto an unmodified surface. Cell contact can be limited by modifying the surface. As a result internal population signalling is not as diluted. Temporary barrier cultures can be manipulated both temporally and spatially, causing cell migration to become the mixing factor.
Each of these systems have been used in musculoskeletal studies to assess a variety of interaction effects, though mixed cultures are reported most often within the literature. This is due to their ease of set-up and success in replicating aspects of in vivo biology that are most often sought in in vitro studies. An example is the creation of a neuromuscular junction by co-culturing nerves and differentiated skeletal muscle (Guo et al. 2011; Demestre et al. 2015; Eckle et al. 2014). However, the types of cell co-cultured are predominantly from a single tissue type or located at a specific junction rather than the whole tissue system.

Rather than use multiple cell lines or biopsies from different tissues, several different cell types can be obtained from a single skeletal muscle biopsy explant culture, thereby creating a direct mixed co-culture from a single source. This co-culture can be used to produce more favourable cell populations through cell sorting for tissue engineering or enhance understanding of how populations can influence each other in different concentrations. In order to facilitate the greatest myotube formation in 3D, Martin et al. (2013) has shown that a greater population balance from an explant culture favouring skeletal muscle-derived desmin positive cells is preferable (50% and 75% desmin positive cells compared to 10%). Kino-oka et al. (2013) used a direct mixed culture to test a modified surface to observe effects on human skeletal muscle myoblasts (HSMM) and fibroblasts (HSMF) in co-culture and discovered reduced stress and preferential growth of HSMM on one surface in particular.

Additionally, direct mixed cultures can be used to help understand disease states. Rieger et al. (1987) used direct mixed differentiated culture of muscular dysgenesis myotubes with spinal cord neurons to attempt to understand the loss of excitation-contraction loss in this disease state. In this co-culture, a direct mixed culture allowed for the interaction of these cell types to reform key interactions and pathways observed in vivo. Other examples include work by Demestre et al. (2015), who used myotubes and neurons derived from the same source (human induced pluripotent stem cells, hiPSC) to model the neuromuscular junction; Beier et al. (2011) who used muscle precursor cells and mesenchymal stem cells to differentiate down a myogenic lineage for use in tissue engineering and for potential use in skeletal muscle regeneration. Due to the loss of myogenicity in high passage skeletal muscle precursors, the use of multipotent stem cell populations to augment muscle forming populations has been a favoured area of study (Gunetti et al. 2012; Bajek et al. 2012; Tsai et al. 2013; Merritt et al. 2010). Direct mixed cultures, whilst simple to create, cannot replicate the spatial grouping of in vivo tissue population interactions, for example skeletal muscle to tendon, and considering the mechanical nature of skeletal muscle, this level of organisation is required to function.
By placing a temporary barrier between monolayer cultures, the interactions between cell populations can be controlled both spatially and temporally, though this technique has not been widely reported due to its set-up difficulty. Additionally, the porosity of the barrier can offer a level of control over any secreted factor transfer. Wang et al. (2007) used a temporary, permeable hydrogel barrier to initially culture indirectly for one week before removing the separator and allowing the cells to migrate into this central region to interact. This was used to study enthesis healing following rupture and suture/graft surgery although it lacked the matrix components which comprise the majority of bone and tendon structure. As an additional control measure, surface modification can be used to limit the interaction of cell populations. Rao et al. (2013) used a novel “comb” to act as both direct and indirect contact methods for myoblasts and fibroblasts cultures. By comparing both methods, determination of both juxtacrine and soluble factor signalling effects could be determined. However, surface modification requires complex processes and/or equipment which increases the cost of production and the preparation time. Other considerations such as shelf-life, durability and bioactivity also become factors (Hill & Chilkoti 2013). Direct co-culture systems are therefore suitable for adjacent tissue interaction modelling and may be appropriate for skeletal muscle-tendon, tendon-bone or skeletal muscle-tendon-bone investigations.
Table 2-1: Examples of direct and reconfigurable monolayer cultures detailing the study, culture method, cell types and culture conditions and the advantages and disadvantages of each method

<table>
<thead>
<tr>
<th>Direct/Indirect Interaction</th>
<th>Culture Method</th>
<th>Example Studies</th>
<th>Cell Type Culture Conditions</th>
<th>Advantages and Disadvantages</th>
</tr>
</thead>
</table>
| Direct                     | Culture Method | Example Studies | Mouse myoblasts seeded onto a human fibroblast substrate | Advantages:  
  • Better represents in vivo tissue than single cell culture  
  • Allows for cell-cell contact and paracrine signalling potentially enhancing culture conditions  
  • Can be used to influence the differentiation of multipotent stem cells to enhance population numbers  
  • Simple and relatively cheap assessment of interactions  
  • Allows for specific cell type interactions in isolated conditions  
Disadvantages:  
  • Only gives indications of 3D or in vivo physiology cannot translate directly  
  • Specific modelling does not account for native in vivo structure  
  • Doesn't account for extra-cellular matrix properties, i.e. stiffness |
| Mixed                      |                | (Cooper et al. 2004) | Bovine osteoblasts seeded onto a bovine chondrocyte micromass |                |
|                            |                | (Jiang et al. 2005) | Primary rat and rat cell line myoblast mixed with rat mesenchymal stem cells (MSCs) before seeding |                |
|                            |                | (Beier et al. 2011) | Human induced pluripotent stem cell (hiPSC)-derived motor neurons were seeded onto myotubes differentiated from hiPSCs |                |
|                            |                | (Demestre et al. 2015) | Mouse embryonic spinal cord and skeletal muscle slices placed onto a cover slip and cultured in a roller tube |                |
|                            |                | (Eckle et al. 2014) | Human skeletal muscle explant-derived positive and negative cells mixed before sorting and seeding into a 3D construct |                |
|                            |                | (Martin et al. 2013) | Mouse primary spinal cord neurons were seeded onto mouse primary skeletal muscle precursor-derived myotubes |                |
|                            |                | (Rieger et al. 1987) | Mouse myoblasts and bone marrow-derived MSCs mixed prior to seeding |                |
|                            |                | (Gunetti et al. 2012) | Rabbit primary bone/ligament fibroblasts mixed with bone marrow stem cells |                |
|                            |                | (Prasad et al. 2010) | Human primary monocytes were seeded onto osteogenically differentiated human primary bone marrow stromal cells |                |
|                            |                | (Heinemann et al. 2011) | Rat primary dorsal root ganglia were seeded onto 3-day cultured rat primary skeletal muscle derived cells |                |
|                            |                | (Zhang et al. 2012) | Human spinal cord stem cell-derived motor neurons seeded onto human skeletal muscle precursor-derived myotubes on a modified tissue culture plastic (TCP) surface |                |
|                            |                | (Kino-oka et al. 2013) | Human primary skeletal muscle myoblasts and fibroblasts were mixed and seed on a modified TCP surface |                |
|                            |                | (Bhatia et al. 1997) | Rat primary hepatocytes were seeded onto a patterned surface and unattached cells removed. Mouse fibroblasts were then seeded and adhered to non-patterned areas |                |
|                            |                | (Guo et al. 2011) | Mouse fibroblasts and myoblasts seeded into separate comb-like structures and then placed in contact |                |
|                            |                | (Kino-oka et al. 2013) | Calf primary fibroblast and osteoblasts were seeded either side of a permeable hydrogel barrier which was later removed |                |
| Surface modified           |                | (Wang et al. 2007) |                             |                |
| Mixed/surface modified     |                | (Rao et al. 2013) |                             |                |
| Surface modified/ reconfigurable surface |                |                             |                             |                |
| Temporary barrier          |                |                             |                             |                |

Advantages:  
• Can control spatial and temporal interaction  
Disadvantages:  
• Additional set up and manufacture costs  
• Difficult to maintain repeatability in temporary barrier cultures
Musculoskeletal cell populations can be affected by wider systems that are not directly connected (for example skeletal muscle and bone). Indirect culture enables the spatial and temporal disassociation of each cell population, thereby allowing control of the concentration and period of exposure to secreted factors. This enables for the isolation of potential pathways and observation of paracrine and endocrine signalling without the confounding effects of physical contact. As complete separation of cells is difficult in monolayer cultures, various systems have been implemented to enable this (Figure 2.13). By initially culturing cell populations on movable surfaces, such as glass coverslips, cells can be allowed to adhere before moving each of the populations to a single well for co-culturing. However, due to the difficulty in maintaining separation over time, this technique is not widely implemented in modern literature (Bogdanowicz & Lu 2013). Similarly, the culturing surface can be modified to allow independent cell population adherence with a surface which prohibits cell attachment placed in between (Rao et al. 2013). Cells can also be separated by use of a trans-well insert, which permits factors to move through a porous membrane, onto which one population of cells is seeded, although the direction of factor movement is generally one-way (Takegahara et al. 2014; Dines et al. 2007; Miki et al. 2012; Im 2014). A physical barrier which blocks cell migration, but allows soluble factor movement through or over the barrier can also enable single well segregated culture. Alternatively, each cell population can be cultured separately and one population exposed to the medium used to culture the other population (Takegahara et al. 2014).

Figure 2.13: Schematic of monolayer indirect contact co-culture systems. Cell populations can be segregated through a variety of different methods. By initially culturing cell populations on items that can be moved, cells can be allowed to adhere before moving each of the cells to a single well for co-culturing. Similarly, the culturing surface can be modified to allow independent cell population adherence with a surface which cells cannot attach being placed in between. Cells can also be separated by use of a trans-well insert in which factors can move through a porous membrane onto which one population of cells is seeded. A physical barrier which does not allow cells to migrate over but soluble factors can pass through or over can also enable single well segregated culture. Alternatively, each cell population can be cultured separately and one population can be exposed to the medium used to culture the other population.
Trans-well or porous membrane/filter well inserts have been used for many years to allow segregated culture. Grobstein, in 1953, demonstrated the use of a membrane to study the morphological changes resulting from the indirect co-culture of embryonic mouse tissues (Justice et al. 2009) from which the more modern system derives. Trans-well inserts are produced with many different characteristics, including a variety of pore size and material (Justice et al. 2009) and have been widely implemented in the study of musculoskeletal system tissues focussing on different aspect of the interactions (summary: Table 2-2). Dodson et al. (1997) used a trans-well to study how inter-tissue signalling influences pre-adipocyte differentiation when co-cultured with explant-derived skeletal muscle precursor cells. This early work was followed by Dietze et al. (2002), in which a trans-well insert was used to culture differentiated skeletal muscle with adipocytes to better understand the influences of any paracrine interactions on the skeletal muscle. While this study found that the adipocytes induce rapid disturbance of insulin signalling, any potentially modulating effects from the muscle influencing the adipocytes was lost due to the single direction of signalling. Additionally these techniques required prolonged configuration assessment to achieve the desired outputs from the different membrane characteristics.

Other uses of a trans-well insert have included studies into the effects of fibroblasts on skeletal muscle differentiation and maturation via a specific pathway (Zhang et al. 2010), and osteoblast and fibroblast/bone marrow-derived MSCs interactions (Tsai et al. 2011). This demonstrates that the trans-well system has the capacity to study different pathways and mechanisms for a variety of different uses even within identical cell type interaction models. Despite the advantages of this system in ease of setup, as observed in Dodson et al. (1997), several different configurations may be required to obtain the desired results from the culture. This may include testing membranes with different characteristics, for example, porosity and stiffness, to allow successful implementation of the co-culture.

Although the trans-well/porous membrane insert is a useful tool for studying indirect interactions, alternative systems offer the application of specific mechanisms, such as mechanical stimulation, or are simpler to fit requirements. Hicks et al. (2014) used a novel indirect assay where the cell populations were cultured on two surfaces: The muscle populations on a static glass coverslip, and the fibroblasts on a flexible surface. This study isolated the gene regulation and differentiation of the skeletal muscle precursors and the strain-influenced fibroblasts to observe how secreted factors influence skeletal muscle differentiation (Hicks et al. 2014). This method of co-culture better represents in vivo extra-cellular matrix by affecting changes in surface stiffness, although the complexity of manufacture of this technique renders it difficult to apply in other laboratory studies. Goetsch et al. (2014) also studied myoblast and fibroblast culture, however, a low barrier technique
was used in this case. This study sought to replicate wound healing using a single muscle source to generate myoblast and fibroblast populations to better understand factors critical for myoblast migration in skeletal muscle injury repair (Goetsch et al. 2014). Each population was separated by a radial low wall in a centre well dish, commonly used for in vitro fertilisation, to allow the mixing of soluble factors during a scratch wound migration assay. Low barrier separation is easy to set up, cost effective (depending on barrier material or source) and can be adapted to fit different culture chambers or areas. Alternatively, Takegahara et al. (2014) used conditioned medium to expose primary skeletal muscle cells to soluble factors from a highly adipogenic cell line to demonstrate the effects of indirect interaction in vivo. This method can also be utilised to expose cells to potential material degradation particles or by-products of the manufacturing procedure, for example, hydroxyapatite particles formed through precipitation.

It is important to mention that a further level of control when co-culturing in monolayer can be provided by the type of material onto which the cells are seeded. As discussed above, standard monolayer models use TCP, coated or uncoated glass or surface modification to enhance or manipulate cellular adhesion. Several studies have shown the influence of the cellular “niche” (microenvironment surrounding cells within a tissue), which includes the influences of the extracellular matrix upon which the cells sit (review: Li & Xie 2005). In response to this, various biomaterials have been developed that are capable of influencing particular cell properties due to their specific characteristics (Anselme et al. 1999; Martin et al. 1995; Nikkhah et al. 2012; Ahmed et al. 2004a; Ahmed et al. 2004b; Bitar et al. 2004). Due to the varied requirements of the musculoskeletal system, several stratified scaffold designs have been researched. As the final outcome of this thesis is a proposed 3D skeletal muscle and bone pre-clinical testing platform, these material solutions could be proposed as the target market. Further in-depth information on these possible solutions is provided by Seidi et al. (2011).

Both direct and indirect monolayer co-culture systems have advantages and disadvantages. Each system can be used to study multiple pathways and applications although a comparison of both systems allows for the isolation of soluble factor signalling. This gives a better understanding of the relative contributions of each signalling type on gene expression and morphology. Normal in vivo skeletal muscle and bone mainly interact through secreted factors (see Section 2.5) and so indirect culture provides a more biomimetic approach for this interaction than direct culture.
Table 2-2: Examples of indirect interaction monolayer cultures, detailing the study, culture method, cell types and culture conditions, and the advantages and disadvantages of each method.

<table>
<thead>
<tr>
<th>Culture Method</th>
<th>Study</th>
<th>Cell Type Culture Conditions</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conditioned medium</td>
<td>(Takegahara et al. 2014)</td>
<td>Rat primary skeletal muscle progenitor cells were exposed to conditioned medium from both pre-adipocyte and adipocyte cells</td>
<td>Advantages</td>
<td>Isolates soluble factors.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Disadvantages</td>
<td>Ignores extra-cellular matrix effects and any modulatory effects from two-way interactions.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Conditioning time can cause build up of factors above desirable concentrations.</td>
</tr>
<tr>
<td>Separate surfaces</td>
<td>(Hicks et al. 2014)</td>
<td>Mouse myoblasts were seeded onto a stiff surface whilst human fibroblasts were seeded onto an adjacent flexible surface</td>
<td>Advantages</td>
<td>Isolation of material stiffness.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Disadvantages</td>
<td>Complex manufacturing set up.</td>
</tr>
<tr>
<td>Trans-well/ Porous membrane</td>
<td>(Miki et al. 2012)</td>
<td>Human fibroblastic stromal cells were seeded above human breast carcinoma cells</td>
<td>Advantages</td>
<td>Simple isolation of soluble factors.</td>
</tr>
<tr>
<td></td>
<td>(Choi et al. 2013)</td>
<td>Differentiating bovine adipocytes were seeded above differentiating bovine skeletal muscle precursor cells</td>
<td></td>
<td>Real-time exposure better represents in vivo conditions.</td>
</tr>
<tr>
<td></td>
<td>(Dodson et al. 1997)</td>
<td>Mouse adipocytes and explant-derived skeletal muscle precursor cells were culture in various combinations on a variety of different membranes</td>
<td></td>
<td>Can allow one- or two-way interaction.</td>
</tr>
<tr>
<td></td>
<td>(Bajek et al. 2012)</td>
<td>Rat primary muscle precursor cells were cultured above bone marrow mesenchymal stem cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Takegahara et al. 2014)</td>
<td>Rat pre-adipocytes and adipocytes were seeded below muscle progenitor cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Dietze et al. 2002)</td>
<td>Human adipocytes were seeded above differentiated muscle progenitor cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Park et al. 2013)</td>
<td>Mouse pre-adipocytes were cultured below rat myoblasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Zhang et al. 2010)</td>
<td>Chicken primary muscle progenitor cells were seeded on membranes and cultured above myoblasts or fibroblasts</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>(Tsai et al. 2011)</td>
<td>Human primary osteoblasts were seeded above human bone marrow stromal cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Barrier</td>
<td>(Goetsch et al. 2014)</td>
<td>Mouse primary myoblasts cultured inside a low radial wall with fibroblasts cultured outside</td>
<td>Advantages</td>
<td>Adaptable for different well sizes.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Disadvantages</td>
<td>Permeable wall can allow controlled diffusion.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Simple to manufacture low barrier.</td>
</tr>
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</table>
2.6.2 Three-Dimensional Biomimetic Co-culture Models

The ultimate aim of tissue engineering is to create biomimetic models of *in vivo* tissues and systems to enhance regeneration. As a by-product of this, increasingly relevant three-dimensional (3D) *in vitro* models are being developed, which can be used for applications similar to those of monolayer studies. Within musculoskeletal regenerative medicine, this mostly results in direct culture applications due to the more obviously applicable nature of these studies, for example, skeletal muscle-tendon models.

3D co-cultures can be subject to the same categorisation as in monolayer systems. Direct co-culture of tissue is more common, due to its more *in vivo*-like representation; however, indirect cultures are also reported (Ou et al. 2011). In some cases, direct co-cultures in 3D do not allow for direct cell interaction due to the extra-cellular matrix acting as a barrier between cell types. Due to the considerable number of interlinked tissues related to the musculoskeletal system, this review will concentrate on those *in vitro* co-culture models linked to the locomotive system; i.e. bone, tendon/ligament and skeletal muscle. Reviews of individual tissue engineered skeletal muscle and bone models can be found in Chapters 6 and 7 respectively.

2.6.2.1 Bone – Tendon/Ligament (Enthesis) Models

As discussed in Section 2.4.2, two enthesis types exist depending on the site of attachment. However, as the “quintessential” tendon to bone attachment, the Achilles fibrocartilaginous enthesis has been the choice of study for tissue engineering. The four zoned interface is a complex graded interaction and, as a result, has produced many varied solutions. This interface is almost identical in composition to the ligament-bone interface, therefore comparisons can be drawn from both interface solutions. Bone is characterised as a stiff tissue responsible for forming a frame that the other tissues and organs in the body are organised around. This stiffness also provides a platform to pull against to provide movement. As such, multiple tissue-engineered approaches have been used to create biomimetic structures which allow osteogenic cultures to differentiate and create a bone model *in vitro* (Bose et al. 2013; Jones et al. 2010; Neßler et al. 2016). Contrastingly, tendons and ligaments, although predominantly a material tissue, are strong but flexible and are observed as having a crimped structure to allow extension (Maffulli et al. 2005; Calve et al. 2004).

Tissue engineered approaches tend to favour hydrogel-based 3D models (Calve et al. 2004; Yang et al. 2010), although many wholly material-based solutions have been developed to enhance repair of the enthesis. These material-based models have either been tested using a single cell type or directly in animal models (Yang & Temenoff 2009; Lui et al. 2010; Hashimoto et al. 2007; Phillips et al. 2008;
Font Tellado et al. (2015), though could have potential for use as an *in vitro* model of the musculoskeletal junction. Examples of interface solutions are summarised in Table 2-3.

Spalazzi et al. (2006 and 2008) developed a three-tier stratified polymer-based 3D scaffold for use in bone interface repair. Each section of the scaffold sought to mimic the ligament, interface and bone extra-cellular matrix (ECM) and was manufactured separately before affixing together. Due to the specific nature of this scaffold, co-cultures of fibroblasts, chondrocytes and osteoblasts could attach to the material in multiple directions and deposit suitable replacement matrix on top of the degrading material. This work was initially assessed using an *in vitro* co-culture of first bovine and then human osteoblasts and fibroblasts and reported markers for both ligament/tendon and bone located to specific zones within the construct (Spalazzi et al. 2006). This scaffold was then assessed using a rat model showing the feasibility of the model to translate to *in vivo* studies (Spalazzi et al. 2008). However, the complex nature of manufacturing would render this model unsuitable in pre-clinical modelling due to set-up time and cost.

Alternatively, Ma et al. (2009) utilised an organic matrix model, where rat bone marrow stem cells (BMSC) were seeded onto a laminin-coated 35mm plate and left to self-organise into a bone construct using the addition of osteogenic components such as dexamethasone and ascorbic acid-2-phosphate. A rat BMSC ligament cell suspension was also seeded onto an additional laminin-coated plate. Once the ligament cells were confluent, the bone construct was split into two and pinned opposite each other in the centre of the ligament culture to provide anchor points. The ligament culture then rolled up around the bone constructs. Some constructs were subsequently placed *in vivo* and compared to those continued *in vitro* (Ma et al. 2009). Subsequent analysis of the *in vitro* constructs showed that the BMSC bone and ligament derived sections of the co-culture had developed the matrix deposition of bone and crimped morphology of ligament, both *in vitro* and *in vivo* (Ma et al. 2009). Wang et al. (2014) also used organic components, a decellularised tendon infused with rabbit-derived osteogenic, chondrogenic and fibrogenic cell suspensions in collagen, to create the transitional interface of bone and tendon/ligament. Organic components offer flexibility in terms of volumetric shape and are highly biocompatible and in doing so, represent a more desirable matrix. However, in bone constructs, a purely hydrogel matrix lacks the mechanical properties of bone. Ma et al. (2009) first formed the bone-like component in this system through medium-induced differentiation, as follows development *in vivo*, before forming the ligament tissue around it. However, skeletal muscle and bone are formed prior to tendon (Schweitzer et al. 2010) and all tissues are present in injury, so a full musculoskeletal system should seek to produce these tissues before the addition of tendon solutions to observe effects on tissue formation. Reported media for differentiation of each tissue varies greatly and can induce undesirable effects, for
example, the calcification of other tissues, and so an alternative means of differentiation which affects single cell populations is necessary (Brown et al. 2013).

Phillips et al. (2008) used a single cell source (primary rat fibroblasts from digest culture) but attempted to create an interface-like culture using a graded RUNX2-mediated osteogenesic approach. In this, fibroblasts were seeded onto a collagen scaffold which was modified to deliver different concentrations of RUNX to affect a lineage change along a gradient within the scaffold. This method produced a zonal effect where the presence of RUNX2-expressing cells or mineral deposition decreased along the length of the construct. Similarly, Min et al. (2014) used gradients of platelet-derived growth factor-β (PDGF-BB) and bone morphogenic protein-2 (BMP-2) within a Polycaprolactone/Pluronic F127 (PCL/F127) membrane to influence the commitment and differentiation of adipose-derived stem cells to a tenogenic or osteogenic lineage. Whilst material enhanced solutions do offer a way of independently influencing differentiation, the use of proteins or hormones can be costly and requires additional quality-control measures to determine whether the proteins remain active after the manufacturing process.

Paxton et al. (2009 and 2010) used a bone substitute material (hydroxyapatite incorporated polyethylene and brushite cement) to anchor a fibrin/fibroblast ligament model similar to Ma et al. (2009). This demonstrates an alternative solution to interface tissue engineering and one that could be incorporated into a future tissue engineered musculoskeletal junction platform. Additionally, the use of hydroxyapatite and brushite has been shown to enhance osteogenisis in bone models (Yoshikawa & Myoui 2005; Liu & Williams 2010; Boskey 2013)

Each of these enthesis models utilise an extra-cellular matrix to provide/enhance the individual characteristics of each component, thereby better representing *in vivo* tissue than monolayer cultures. Additionally, these models have been deemed successful in a regenerative capacity; however, none of these models have been used for investigating inter-tissue interactions. Furthermore, each of these models forms each component sequentially rather than simultaneously, substantially increasing the time of manufacture which is undesirable in high-throughput studies.
### Table 2-3: Examples of multi-cellular interface solutions detailing the proposed tissue interface, study, material/construct structure and manufacture technique, and culture conditions.

<table>
<thead>
<tr>
<th>Interface</th>
<th>Study</th>
<th>Structure/manufacture</th>
<th>Culture conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior cruciate ligament</td>
<td>(Spalazzi et al. 2006)</td>
<td>Triphasic material:&lt;br&gt;A - Sintered polyglactin knitted mesh&lt;br&gt;B - Sintered emulsion formed PLGA microspheres&lt;br&gt;C - Sintered 4:1 composite microspheres (PLGA: 45S5 bioactive glass)</td>
<td>• <em>In vitro</em> culture of explant-derived bovine and human fibroblasts and osteoblasts&lt;br&gt;• <em>In vitro</em> culture of explant-derived bovine fibroblasts and osteoblasts, and digest culture isolated chondrocytes&lt;br&gt;• <em>In vivo</em> transplantation of scaffold (cellular and acellular) into athymic rats</td>
</tr>
<tr>
<td>Bone-Ligament-Bone (BLB)</td>
<td>(Spalazzi et al. 2008)</td>
<td>Tissue engineered triphasic constructs (BLB):&lt;br&gt;Bone (B) - Self-organised Bone Marrow Stem Cell (BMSC) anchors&lt;br&gt;Ligament (L) - Self-organised Ligament cells</td>
<td>• <em>In vitro</em> BLB constructs created from rat ligament cell construct formation around pre-cultured rat BMSC bone construct.&lt;br&gt;• <em>In vivo</em> transplantation of BLB construct to replace rat medial collateral ligament.</td>
</tr>
<tr>
<td>Tendon/Ligament-Bone interface</td>
<td>(Ma et al. 2009)</td>
<td>Stratified (tri-region) scaffold from decellularised Achillies tendon</td>
<td>• <em>In vitro</em> culture of rabbit-derived fibroblasts (F), chondrocytes (C) and osteoblasts (O) on the scaffold in three distinct regions (F-C-O).</td>
</tr>
<tr>
<td></td>
<td>(Wang et al. 2014)</td>
<td>Stratified (tri-region) scaffold from decellularised Achillies tendon</td>
<td>• Primary rat fibroblasts were cultured <em>in vitro</em> onto the scaffolds and maintained in osteogenic medium.</td>
</tr>
<tr>
<td></td>
<td>(Phillips et al. 2008)</td>
<td>Stratified (tri-region) scaffold from decellularised Achillies tendon</td>
<td>• Primary rat fibroblasts were cultured <em>in vitro</em> onto the scaffolds and maintained in osteogenic medium.</td>
</tr>
<tr>
<td></td>
<td>(Min et al. 2014)</td>
<td>Stratified (tri-region) scaffold from decellularised Achillies tendon</td>
<td>• <em>In vitro</em> culture of primary human adipogenic stem cells (ASC) to create osteogenic and tenogenic regions</td>
</tr>
<tr>
<td>Bone-Ligament-Bone (BLB)</td>
<td>(Min et al. 2009)</td>
<td>Hydroxyapatite incorporated polyethylene glycol hydrogel anchors with a cell-embedded fibrin ligament construct</td>
<td>• <em>In vitro</em> culture of primary rat tendon fibroblasts embedded within an anchored fibrin hydrogel</td>
</tr>
<tr>
<td></td>
<td>(Paxton et al. 2010)</td>
<td>Hydroxyapatite incorporated polyethylene glycol hydrogel anchors with a cell-embedded fibrin ligament construct</td>
<td>• <em>In vitro</em> culture of primary rat tendon fibroblasts embedded within an anchored fibrin hydrogel</td>
</tr>
</tbody>
</table>
2.6.2.2 Muscle-Tendon Models

Individually, both skeletal muscle and tendon models have been heavily studied in the last 30 years by a number of different groups (Calve et al. 2004; Yang et al. 2010; Martin et al. 2013; Player et al. 2014; Vandenburgh 2010; Lin et al. 2014; Neidlinger-Wilke et al. 2001; Dennis & Kosnik 2000; Powell et al. 2002; Mudera et al. 2010; Boonen et al. 2010; Hinds et al. 2013). However, less focus has been placed on in vitro recreation of the myotendinous junction.

Skeletal muscle 3D tissue engineering generally follows two approaches: A developmental biology approach, which mimics the delamination process observed in embryonic limb generation (Martin et al. 2013; Vandenburgh et al. 2008), or a method involving the inherent regenerative capabilities of the tissue. In the developmental biology model, skeletal muscle precursor cells are seeded onto a flat material, most commonly a hydrogel, and cultured until confluent. At this point, differentiation is induced through a reduction in serum concentration or additional supplementation. During the culture period, the material upon which the cells were placed is remodelled and forms a cylindrical, fascicle-like structure. This “self-assembly” results in compact elongated myotubes. The alternative method of skeletal muscle engineering involves the inherent regeneration capabilities of the tissue (Smith et al. 2012; Player et al. 2014). In vivo, skeletal muscle regenerates through the propagation of precursor cells from satellite cell activation. These migrate to the site of damage and fuse to existing structure to reform the myofibre architecture (Grefte et al. 2007). After seeding muscle precursors within a volumetric scaffold and allowing for a period of attachment and remodelling, cellular fusion is again induced by a reduction in serum or additional supplementation. As an isolated tissue, a more in depth review of skeletal muscle constructs can be found in chapter 6.

Both of these techniques have a common mechanical property. Each model is characterised by the formation of cell-mediated lines of isometric tension between two stationary points. As discussed above (Section 2.6.2.1), tendon models are formed using similar techniques due to the reliance of both tissues on longitudinal tension (Kapacee et al. 2008). However, tendon is characterised by the material matrix providing the strong functionality through dense collagen fibres and the cellular population adjusting the material to obtain the necessary properties (Maffulli et al. 2005; Calve et al. 2004). Contrastingly, skeletal muscle function is provided by the cellular fibres, with the surrounding highly flexible matrix providing a frame upon which the fibres sit (Gillies & Lieber 2011). As a result, the interface between these two tissues is complex; the cellular body of the muscle interlinks directly with the material matrix of the tendon (Ovalle 1987).

To form a system that meets the requirements of both tissue types, a variety of techniques have been developed. Depending on the purpose of the system a topological or biologically enabling
scaffold has been chosen. Initially, work in this area stemmed from the individual studies of tissue engineered skeletal muscle and tendon/ligament constructs. Larkin et al. (2006) and Kostrominova et al. (2009) utilised a delamination model for both tendon and skeletal muscle constructs. In their study, the tendon model was cultured first, prior to cutting in half and pinning at the stationary points of a two week skeletal muscle setup. After a further week culture time, the skeletal muscle construct had rolled the matrix around the anchor points, including the tendon construct pieces. In this system, tendon and skeletal muscle were successfully co-cultured and began to form markers present at the in vivo interface and a structure similar under ultrasound analysis to a foetal/neonatal junction. Each of the tendon and skeletal muscle models that were finally co-cultured used a laminin-based scaffold onto which the cells were seeded. This allowed for the continuity of extracellular matrix between the two components and is one of the constituent components present in the skeletal muscle niche. Its hydrogel property also allows for remodelling (Weist et al. 2013).

Alternatively, the advent of 3D printing in tissue engineering has hinted at a new approach. Merceron et al. (2015) used a bespoke system that printed alternative layers of polymers (polyurethane (PU) and polycaprolactone (PCL)) and cells. The myoblasts and fibroblasts were each mixed with a hyaluronic acid, gelatin and fibrinogen solution (components found within native skeletal muscle extra cellular matrix) and high-glucose Dulbecco’s Modified Eagle Medium (DMEM). The addition of polymers and the design of the 3D deposition were placed to try and recreate the individual properties of each tissue. Whilst, this technique produced the cellular morphological characteristics of fused skeletal muscle and tendon individually, the limited culture period was not long enough to allow enough collagen deposition to create focal adhesion between the two tissues.

2.7 Conclusions

Co-culture strategies have clearly demonstrated their capability to provide a more relevant model for in vivo representation compared to single cell type cultures. Monolayer cultures can allow for multiple cell type co-culture, mimicking pathways and physical interactions without the variability caused by introducing an extra-cellular matrix. Through the use of surface modification or physical separation, degrees of control can be asserted on these cultures to enhance a particular area of study or inform later more complex studies, such as medium composition.

Whilst monolayer co-cultures offer many advantages, including simplicity and a relatively short culture period, they lack the complex interactions available in 3D models. For pre-clinical study platforms, 3D co-cultures offer a more relevant model for toxicity testing. Currently, no models of the full musculoskeletal junction exist, which limits the understanding of novel material-tissue/tissue-tissue interactions in vitro. This should be considered especially important given the
effect that skeletal muscle, tendon and bone have on each other in vivo. The models discussed here show tentative steps towards an enthesis or myotendinous junction. However, their development is limited to one or two research groups. Hence, this thesis uses the fundamental aspects of this review, utilising both monolayer and 3D techniques, to develop a 3D co-culture of skeletal muscle and bone, as a step towards the creation of a biomimetic musculoskeletal junction and a platform to be used for novel material pre-clinical testing. Co-culture of skeletal muscle and bone has not yet been reported in monolayer or 3D tissue engineered cultures and so this thesis describes the steps taken to produce a completely novel system.
3 Thesis Aims

This thesis seeks to determine whether three-dimensional (3D) tissue engineered skeletal muscle and bone constructs can be simultaneously manufactured and differentiated in a single culture vessel. Therefore, this study describes the steps needed to develop the necessary processes and platforms to enable this co-culture. It is envisioned that this model will act as an additional toxicity screening step before animal pre-clinical studies. This will reduce the use of animal testing, which is costly, ethically questionable and based on potentially misleading results from previous monolayer cellular preclinical studies. The cell line based system explored in this thesis should also act as a step towards a primary human cell 3D tissue engineered model, which could replace the need for animal studies in future pre-clinical settings, and become a platform for the formation of an integrated human musculoskeletal system.

Chapter 5 – Medium Composition Analysis Towards 3D Tissue Engineered Musculoskeletal Co-culture.

The study detailed in this chapter seeks to identify a single growth and differentiation medium for C2C12 and TE85 cell lines to enable simultaneous culture of 3D tissue engineered models of skeletal muscle and bone. The growth medium must be capable of sustaining the proliferation of both C2C12 and TE85 cell lines, as assessed through a timeline of DNA and protein concentration. This will be followed by a study into the relative effects of myogenic (reduced serum) and osteogenic (reduced serum with ascorbic acid, dexamethasone and β-glycerol-phosphate) media on the differentiation of each cell type. Differentiation will be assessed through the expression of myogenin and RUNX2/CBFa1. Confirmation of C2C12 fusion will be assessed through immunocytochemistry (ICC) for the intermediate filament protein desmin. Similarly, any lineage altering effects within the TE85 cell line will be assessed through desmin positivity in ICC.

Chapter 6 – Development and Optimisation of a 3D Tissue Engineered Skeletal Muscle Model

This study seeks to develop and validate a scale-down model of an optimised skeletal muscle construct. Initially, a brief comparison will be made of differentiated populations in both monolayer and 3D cultures to demonstrate the relative advantages and disadvantages of each system in pre-clinical studies. To enhance the cost-effectiveness of this in vitro model, a previously reported 3D model will be assessed for the dimensions which produce the most uniform cultures and a scaling model developed based upon this investigation. To further enhance the stability of the 3D model, the cell density and width extension past the anchor points, which increases surface area of attachment, will be optimised according to a factorial design of experiment analysis and then the scaling model validated against this optimised construct. Finally, the initial cellular attachment force
will be compared to previous models and long term culture (up to 34 days) will be assessed with a view to co-culture with a bone model which may require longer culture periods.

Chapter 7 – Development and Optimisation of a 3D Tissue Engineered Bone Model

This section of research explores the development of a 3D bone construct. Initially, the effect of precipitated hydroxyapatite (HA) on TE85 maturation differentiation will be assessed, followed by the observation of TE85 morphology in a 3D collagen matrix. HA is soluble in acidic conditions and therefore, the capacity for the HA to survive the addition to the collagen (dissolved in acetic acid) will be assessed using Alizarin Red S calcium staining. Finally, the initial cell density and hydroxyapatite-to-collagen ratio will be investigated to produce an optimised construct according to molecular and biochemical markers, the first time this process has been used in this model.

Chapter 8 – Development of a 3D Tissue Engineered Skeletal Muscle and Bone Co-Culture System

This chapter seeks to investigate the influences of each cell line, C2C12s and TE85s, on the differentiation profile of both cell populations when in indirect contact culture. Firstly, the effect of any HA degradation particles on C2C12 differentiation will be investigated by conditioning HA in myogenic medium and then culturing the C2C12s in this medium. To recreate the envisaged 3D tissue engineered co-culture system in monolayer, C2C12s and TE85s will be cultured together in a single well, separated by low barrier of Sylgard, to enable both cell populations to be exposed to secreted factors. This culture will be assessed using immunocytochemistry for C2C12 fusion and the production of Alkaline Phosphatase in TE85 populations for adverse effects. Finally, a platform suitable for the co-culture of optimised 3D tissue engineered skeletal muscle and bone shall be developed and optimised constructs cultured simultaneously. Each model shall be assessed for differentiation characteristics as a proof-of-concept design.

Chapter 9 – General Discussion

The implications of the results reported in Chapters 5, 6, 7 and 8 will be discussed in relation to the initial aims laid out within this text and individual findings within the literature. Effects from other co-culture systems will be analysed and compared for observations that are consistent between systems.

Chapter 10 – General Conclusions, Limitations and Future Perspective

This chapter will set out the limitations of the investigations and models described within this thesis, with a view to enabling adaptations and amendments for further study in this area. Also discussed will be the placement of this work within the wider timeline of an animal replacement platform for preclinical studies and the direction that should be taken to progress towards this goal.
4 General Materials and Methods

This chapter states the general materials and procedures used within this body of research. Unless otherwise stated, all cell culture work was conducted in a Class II biological safety cabinet (BSC) under sterile conditions.

4.1 Cell Culture

C2C12 murine myoblasts (ECACC, UK) were cultured under humidified atmospheric O₂ and 5% CO₂ conditions at 37°C (5% CO₂ in air). Cell populations were expanded in Growth Medium (GM) which consisted of High Glucose Dulbecco’s Modified Eagle’s Medium (HG-DMEM) supplemented with 4mM L-Glutamine (Hyclone), 20% Foetal Bovine Serum (FBS) (PAN Biotech, UK) and 1% Penicillin/Streptomycin (PSF) (10,000U/mL and 10,000µg/mL, Gibco). All experiments were conducted prior to passage 8 (P8, relative to passage received from supplier, >P14).

TE85 human osteosarcoma (hOS) cells (ATCC, UK) were initially expanded at the Centre for Biological Engineering, Loughborough University. TE85s were then obtained at passage 54 and cultured under humidified atmospheric O₂ and 5% CO₂ conditions at 37°C (5% CO₂ in air). Cell populations were expanded in Growth Medium (GM) which consisted of either Eagle’s Modified Essential Medium with Earle’s Bicarbonate Soda Salts (Sigma) with 10% FBS (PAN Biotech), 1% L-Glutamine (200mM, Sigma), 1% non-essential amino acids (NEAA) (100x, Sigma) and 1% PS; or growth medium as detailed for C2C12s. All experiments were conducted prior to passage 60.

When cell coverage reached 80% confluency they were routinely passaged using trypsin/EDTA (ethylenediaminetetraacetic acid) and counted (as detailed in Section 4.2: Cell Counting). Subsequently, cells were either re-plated into culture vessels in order to generate adequate cell numbers for experimentation, or seeded onto plates or into collagen constructs for experimentation.

4.2 Cell Counting

Specific seeding densities were required in the experiments for this study. As such, the number of cells within a solution was calculated utilising the Trypan Blue exclusion method and a haemocytometer. Trypan blue is excluded by cells with intact membranes, therefore indicating living and dead cells. 10µL of 0.4% Trypan Blue (Fisher) was added to either 10µL or 90µL of cell suspension from a sample to create a 1:1 or 1:10 dilution depending on predicted cell density. This was to ensure accuracy of counts under brightfield microscopy. From these dilutions 10µL of suspension/Trypan Blue was placed in both chambers of the prepared haemocytometer, created by placement of a coverslip. Cell number was counted using the four corner boxes, then divided by four.
to obtain the mean. The mean number of cells was then applied to the following formula to find the cell density of the original sample:

\[ C = \bar{n} \times d \times 10^4 \]

- \( C \) = Cell concentration (cells/ml)
- \( \bar{n} \) = Average cell count of corner square
- \( d \) = Dilution correction
- \( 10^4 \) = Conversion factor for counting chamber (0.0001 of a ml)

Cell counts were conducted under a x10 magnification using a Leica DMIL-LED microscope.

### 4.3 Tissue Engineered 3D Collagen Constructs

#### 4.3.1 Skeletal Muscle Model

Collagen-based skeletal muscle constructs were prepared according to a protocol adapted from previous descriptions (Smith et al. 2012; Player et al. 2014). 10x Minimal Essential Medium (MEM) (Gibco) was added to 2.0mg/mL type-1 rat tail collagen in 0.1M acetic acid (First Link, UK) and mixed thoroughly. This solution was then neutralised using 5M and 1M NaOH until a colour change from yellow to pink was observed. Myoblasts suspended in GM were then added to the collagen solution and pipetted into a defined setting area with bespoke anchor points, termed “A-frames” at either end (Figure 4.1). The final solution comprised 85% Collagen, 10% 10x MEM and 5% cell suspension. Each A-frame consists of poly(ethylene-co-1-octene) plastic 10 count canvas mesh (Darice, US) bound together with 0.3mm stainless-steel wire (Scientific Wire Company, UK) to form a floatation bar. 0.7mm or 0.3mm wire was used to create a hook which could be pushed into the floatation bar and hung over the side of the setting chamber to keep the construct stable (Figure 4.2 Top). A-frames were kept in 70% industrial methylated spirits (IMS) for a minimum of 24 hours and allowed to dry fully within a class II Bio-Safety Cabinet prior to use.

Once the collagen/cell solution was pipetted into the setting area, the chamber was placed into a 37°C humidified incubator and left until fully polymerised. Once set, the construct was detached from the walls of the setting area and GM added to the well, allowing the construct to float (Figure 4.2 Bottom). Constructs were cultured for four days in GM, ensuring complete replacement of the medium twice daily. To induce cellular fusion, constructs were then cultured in differentiation medium (DM) consisting of HG-DMEM with 4.0mM L-Glutamine (Hyclone) supplemented with 2% Horse Serum (Sigma-Aldritch) and 1% PS for a further 10 days ensuring complete replacement of the DM every 24 hours.
Figure 4.1: Tissue engineered 3D skeletal muscle constructs 8 well plate setup schematic.
A bespoke Sylgard boundary was placed so to define the fourth edge of the setting area. Bespoke A-frames were placed at opposite ends of the setting area. Collagen/cell suspension was pipetted into the setting area. Once polymerised, the Sylgard boundary was removed and growth medium placed into the well ensuring that the collagen construct was suspended.

Figure 4.2: 1.5mL construct A-Frames and constructs in culture. Scale bar – 5.0mm. (Top) Bespoke linear low-density polyethylene (LLDPE) mesh A-Frames. (Bottom) 1.5mL construct in growth medium in Nunc 8-well plate.
4.3.2 Bone Model

4.3.2.1 Hydroxyapatite Solution Preparation

Hydroxyapatite (HA) solution was precipitated as previously described by Liu and Williams (Liu et al 2010). 500mL of 130mM analytical grade ammonium phosphate tribasic trihydrate (NBS Biologicals Ltd) was dripped into a continuously stirred 500mL of 210mM calcium acetate solution maintained at pH12 and 3°C, by adding concentrated ammonium hydroxide and housing in a bath of iced water (Figure 4.3). The precipitate solution was then placed into a refrigerator at 4°C and left to age overnight. Calcium acetate was chosen as a calcium source rather than a calcium chloride or nitrate because the acetate ions are not incorporated into the apatite (Hearn 2013).

![Conc. Ammonium](image)

![Triammonium Orthophosphate](image)

![pH meter and thermometer](image)

![Calcium Acetate (HA solution)](image)

![Iced water](image)

Figure 4.3: Hydroxyapatite precipitation setup

During the aging process, the precipitate settles creating a clear phase separation (Figure 4.4). The HA was washed periodically in double distilled H₂O whilst in storage to retain phase separation.
Samples of the HA suspension were then aliquoted into 50mL tubes (FisherBrand) and centrifuged at 4000rpm for 5 minutes. The clear phase was removed and replaced with dH₂O. After resuspension in dH₂O, dilute hydrochloric acid (HCl) was added to adjust the pH to 7.5 followed by a final wash in double distilled H₂O. Once neutralised, the centrifugation process was repeated until unbound water was negligible creating a concentrated HA paste.

4.3.2.2 Hydroxyapatite Concentration Determination

The concentration of hydroxyapatite (HA) in the paste was measured by calculating the dry mass as a percentage of the initial sample mass. The dry mass was obtained by measuring a sample after baking at 80°C for 30 minutes. The HA concentration was then calculated using the following formula:

\[
\text{Final dry mass (mg)} / \text{Initial paste mass (mg)} \times 100 = \text{Concentration HA in water (\%)}.
\]

4.3.2.3 Bone Collagen/Hydroxyapatite Model

Collagen/hydroxyapatite-based constructs were prepared according to a protocol adapted from Liu & Williams (2010). Hydroxyapatite was added to 2.0mg/mL type-1 rat tail collagen in 0.1M acetic acid (First Link, UK) to the required concentration and mixed thoroughly. 10x Minimal Essential Medium (MEM) (Gibco) was added to this mixture and then neutralised using 5M and 1M NaOH until a colour change from yellow to pink was observed. TE85 hOS cells suspended in GM (HG-DMEM, 20% FBS, 1% PS) were then added to the collagen solution and pipetted into a defined setting area. The final solution comprised 85% Collagen/HA solution, 10% 10x MEM and 5% cell suspension.
Following this, constructs were treated as described in Section 4.3.1 (Skeletal Muscle Model). Briefly, once the collagen/cell solution was pipetted into the setting area, the chamber was placed into a 37°C humidified incubator and left until fully polymerised. Once set, the construct was detached from the walls of the setting area and GM added to the well, allowing the construct to float. Constructs were cultured for four days in GM, ensuring complete replacement of the GM twice daily. To induce osteogenic differentiation, constructs were cultured in differentiation medium (DM), as detailed in Section 4.3.1, for 10 days ensuring complete replacement of the DM every 24 hours.

4.4 Brightfield Imaging
Brightfield images were captured using a Leica DMIL-LED microscope with a monochrome camera. These observations were used to qualitatively assess cell population confluency and health.

4.5 Immunofluorescence
After the following fixing and staining procedures, all samples were mounted on glass microscope slides using Fluormount Aqueous Mounting Medium (Sigma) and imaged using a Leica DM2500 fluorescence microscope. Negative controls for desmin are shown in Appendix 1.

4.5.1 Monolayer Procedures
4.5.1.1 Fixing Procedure
Following culture, the cells were washed twice with phosphate-buffered saline (PBS) and fixed by the drop wise addition of Methanol and Acetone (1:1 v/v) to PBS (50% v/v). After 15 minutes incubation, Methanol and Acetone (1:1 v/v) was added for a further 15 minutes prior to immunostaining.

4.5.1.2 Desmin Antibody Stain
Once fixed, constructs were immunostained for the muscle specific cytoskeletal intermediate filament desmin. Initially constructs were placed into a blocking solution consisting of 5% goat serum and 0.2% Triton X-100 in Tris-buffered saline (TBS) for 30 minutes and then incubated with rabbit anti-desmin antibody (Abcam, UK) for two hours diluted 1:200 in TBS. The cells were then incubated in the dark for a further two hours in goat anti-rabbit Immunoglobulin G (IgG) Rhodamine-derived TRITC (Tetramethylrhodamineisothiocyanate) or Chromeo 488 conjugated secondary antibody (Abcam) diluted 1:200 in TBS.

4.5.1.3 F-actin (Rhodamine Phalloidin)
Phalloidin conjugated with the fluorescent tag Rhodamine was used to visualise the cytoskeletal filament F-actin (Life Technologies). Samples were incubated in a 1:200 solution of Rhodamine Phalloidin with 0.1% Triton-X in PBS for 30 minutes.
4.5.1.4 DAPI (4',6-diamidino-2-phenylindole)
DAPI was used as a counterstain to observe cell nuclei. DAPI binds to the A-T regions in DNA. Samples were incubated in a 1:10,000 dilution (1mg/mL, Thermo Scientific Pierce) in dH₂O for 15 minutes in the dark before being washed up to five times in distilled H₂O.

4.5.2 3D Procedures
Due to the collagen matrix, timings for each step were adjusted to enable liquid penetration through the porous scaffold and interaction with all the cells.

4.5.2.1 Fixing Procedure
Following 14 days culture, the constructs were washed twice in PBS and fixed in Methanol and Acetone (1:1 v/v) in PBS (50% v/v) for 30 minutes. Following this constructs were incubated in just Methanol and Acetone (1:1 v/v) for a further 30 minutes prior to immunostaining.

4.5.2.2 Desmin Antibody Stain
Once fixed, constructs were treated as described in Section 4.5.1.2 with adjusted timings. Constructs were placed into a blocking solution for two hours and then incubated with the rabbit anti-desmin antibody overnight. Following this, the constructs were then incubated in the dark for a further two hours in goat anti-rabbit Rhodamine-derived TRITC secondary antibody.

4.5.2.3 F-actin (Rhodamine Phalloidin)
Constructs were treated as described in Section 4.5.1.3 with adjusted timings. Samples were incubated Rhodamine Phalloidin solution with 0.1% Triton-X in PBS for 2 hours.

4.5.2.4 DAPI (4',6-diamidino-2-phenylindole)
Constructs were treated as described in Section 4.5.1.4 with adjusted timings. Samples were incubated in DAPI solution for 30 minutes in the dark prior to being washed up to five times in distilled H₂O.

4.6 Calcium Content
Alizarin Red S (Sigma) was used to confirm the presence of calcium in the culture. Samples were fixed in 4% formaldehyde after washing twice in Tris-buffered saline (TBS). A 40mM solution of Alizarin Red S (pH 4.2) was placed onto the culture for 20 minutes and washed in dH₂O until any residue was negligible. Staining was observed on a Leica DMIIL-LED microscope. Cameras available for culture imaging were monochrome; therefore, colour images were obtained using a Nokia 800 mobile phone camera positioned over an eyepiece. As a result, the scale of the image is difficult to determine and magnification has not been reported.
To positively identify the capacity for Alizarin Red S to correctly mark bone and bone-like substances; Zebrafish bone tissue was used as a positive control (Figure 4.5).

**Figure 4.5: Alizarin Red S zebrafish calcium stain confirmation.**
Tissue containing ribs (left) and vertebrae (right). Alizarin Red S staining presents as a deep red hue. Circled are the stained bones. Lighter shades of red are non-specific staining of tissue.

4.7 **Gene Expression Analysis of RNA Using Quantitative Real-time Polymerase Chain Reaction**

All instruments and surfaces used in RNA processing and quantitative real-time Polymerase Chain Reaction (qRT-PCR) were cleaned thoroughly using RNase Zap (Invitrogen) prior to use.

4.7.1 **RNA Extraction and Isolation**

Monolayer samples taken for later qRT-PCR analysis were first lysed in 500µL TRI-reagent (Fisher). A P1000 pipette tip was used to scrape the culture well surface to ensure full cellular detachment. 3D cultures were placed into 500µL TRI reagent and snap frozen in liquid nitrogen. All samples were stored at -80°C in 1.5mL Eppendorf tubes for later RNA extraction.

Prior to RNA extraction, samples were defrosted at room temperature. A steel ball bearing was added to 3D tissue engineered construct samples and then agitated on a TissueLyser II (Qiagen) for 4 minutes at 10,000rpm to release the cellular components.

RNA was extracted according to manufacturer’s protocols as follows: 100µL of chloroform was added per 500µL of TRI reagent in the original sample and mixed thoroughly by hand for 15 seconds. After incubation for 2-3 minutes at room temperature, samples were centrifuged at 12,000g for 15 minutes at 4°C. This causes a phase separation in the mixture (a lower red-coloured phenol phase, an interphase, and an upper, colourless, aqueous phase). RNA is contained within the aqueous phase. The aqueous phase was removed and placed into a separate 1.5mL Eppendorf tube. The remaining phases were discarded.
RNA was isolated through a precipitation process. 250µL of 100% isopropanol per 500µL of TRI reagent used for lysis was added to the aqueous phase extraction. The sample was then mixed by inversion and incubated for 10 minutes at room temperature. This was followed by centrifugation at 12,000g for 10 minutes at 4°C. This formed an RNA pellet. The supernatant was aspirated and the pellet washed with 500µL of 75% absolute ethanol by inversion mixing for 5 minutes. The sample was centrifuged for a final time at 7,500g for 5 minutes at 4°C. The absolute ethanol was removed and the RNA pellet left to air dry for 10 minutes. The sample was then re-suspended in RNA storage solution (Invitrogen) and stored at -80°C for later quantification and qRT-PCR analysis.

RNA concentration was measured using a Thermo Scientific NanoDrop 2000 spectrophotometer at 280nm and purity levels measured as a 260/280nm ratio. Only samples with a purity ratio between 1.8 and 2.0 were taken for qRT-PCR.

4.7.2 Quantitative Reverse Transcription Polymerised Chain Reaction (qRT-PCR)

A one-step reaction chemistry using QuantiFast SYBR Green RT-PCR kit (Qiagen) was performed on a ViiA 7 (Applied Biosystems) thermal cycler. To enable this reaction, a mastermix for each gene was prepared in the following ratios: 94% SYBR Green, 2% QuantiFast RT mix, 2% forward primer, 2% reverse primer. For each gene of interest, primer set validation was conducted by creating a standard curve of known RNA concentrations to ensure that reaction efficiency was between 90 and 110%, and to determine an appropriate starting RNA concentration for experimental samples (Appendix 2). Each reaction was found to require 20ng of RNA. All RNA samples were then re-suspended in nuclease-free water such that the concentration for each reaction was 4ng/µL. This was then mixed with the mastermix solution for each gene in a 1:1 ratio (5µL:5µL per reaction). All qRT-PCR preparation was conducted in RNase-free 1.5mL Eppendorf tubes on ice. All 1:1 sample reactions were prepared in 384 well plates (Applied Biosystems).

qRT-PCR reactions were performed as follows: 50°C for 10 min (reverse transcription/cDNA synthesis), 95°C for 5 min (transcriptase inactivation and initial denaturation step) followed by PCR steps for 40 cycles; 95°C for 10 sec (denaturation), 60°C for 30 sec (annealing and elongation) (Figure 4.6 and Figure 4.7). Finally a dissociation/melting curve analysis was performed to allow exclusion of non-specific amplification or primer-dimer interference. Relative gene expression was calculated using ΔΔCT equation (Livak & Schmittgen 2001) where relative expression is calculated as $2^{-\Delta\Delta CT}$. Each individual sample was assessed in triplicate and normalised to a single designated control value.
Figure 4.6: qRT-PCR experimental run including a holding step (reverse transcription/cDNA synthesis and reverse transcription and initial denaturing) followed by the PCR cycling step (denaturing and annealing and elongation). This was followed by a melt/disassociation curve stage.
Figure 4.7: Quantitative reverse transcription polymerised chain reaction schematic from cDNA.

1. **Denaturation** – the DNA is heated to separate the two strands.
2. **Annealing** – cooling step enables primers targeted at your gene of interest to form a hydrogen bond with the end of the target sequence.
3. **Elongation** – DNA polymerase adds nucleotides to the 3' end of each primer. This process is repeated at each cycle causing the DNA to increase exponentially.

Fluorescent tags are bound to the DNA during the annealing and elongation stages and the released during subsequent denaturation steps. Once bound the SYBR green emits fluorescence which is measured.

(Adapted from http://openwetware.org/images/b/b2/JCATutorial_PCRDiagram.jpg)
4.7.3 Primers

Primer sequences used for these studies were previously designed according to a number of guidelines (Sharples et al. 2012; Martin et al. 2013) or acquired as pre-prepared probes (KiCqStart Primers, Sigma Aldrich). Briefly, these guidelines were as follows; Primer length – 18-23bp; GC content – 40-60%; annealing temperature - ~60°C; non-complementary 3’ primer pairs; product length between 76-200bp.

Primers were checked for specificity of the target gene using the gene Blast search available at http://blast.ncbi.nlm.nih.gov/Blast.cgi. POLR2B (mouse) and Myogenin (mouse) had previously been validated for the C2C12 cell line using melt curve analysis. POLR2B (human), RUNX2/CBFa1 (human) and osteocalcin/BGLAP (human) were additionally validated against the TE85 hOS cell line through melt curve analysis and samples from each experimental run by performing a melt curve analysis at the end of each qRT-PCR run (Appendix 3). RUNX2/CBFa1 (human) was found to also be suitable for mouse cell line reactions through Blast searching and was therefore also validated against the C2C12 cell line.

Table 4-1: Quantitative reverse transcription polymerised chain reaction (qRT-PCR) Primers

<table>
<thead>
<tr>
<th>Target mRNA (M-mouse; H- human)</th>
<th>Primer sequences 5’-3’</th>
<th>Reference Sequence Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>POLR2B (M)</td>
<td>GGTCAGAAAGGAACCTGTGGAT</td>
<td>NM_153798.2</td>
</tr>
<tr>
<td>POLR2B (H)</td>
<td>AAGGCTTGTTAGACAACAG</td>
<td>NM_000938.1</td>
</tr>
<tr>
<td>Myogenin (M)</td>
<td>CCAACTGAGATTGTCTGTC</td>
<td>NM_031189.2</td>
</tr>
<tr>
<td>RUNX2/CBFa1 (H/M)</td>
<td>GCAGTATTTACAACAGAGGG</td>
<td>NM_001145920</td>
</tr>
<tr>
<td>Osteocalcin/BGLAP (H)</td>
<td>CTCACACTCTCGCCCTATT</td>
<td>NM_199173</td>
</tr>
</tbody>
</table>

4.8 Alkaline Phosphatase Activity, DNA and Protein Quantification

Samples were lysed in 500µL of dH2O. For monolayer samples, a P1000 pipette tip was used to scrape the culture well surface to ensure full cellular detachment. 3D constructs were placed into a 1.5mL Eppendorf with the dH2O and snap frozen in liquid nitrogen. All samples were stored at -80°C until used. A steel ball bearing was added to 3D tissue engineered samples and then agitated on a TissueLyser II (Qiagen) for 4 minutes at 10,000rpm. Monolayer samples were taken through three freeze-thaw cycles.

Alkaline phosphatase (ALP) activity was determined using a 4-Methylumbelliferyl phosphate (4-MUP, Sigma) reaction. 50µl of the cell/lysis buffer solution was placed into the well with 50µl of dH2O. To this 50µl of 4-MUP was added and left for 30 minutes. To stop the reaction, 50µl of 100mM EDTA buffer was added and then absorbance readings using a Varioskan Flash (ThermoScientific) taken at 440nm.
For studies not involving direct contact with hydroxyapatite, DNA concentrations were measured using Thermo Scientific NanoDrop 2000 spectrophotometer at 260nm. Hydroxyapatite crystals were found to interfere with the spectrophotometer readings of the Nanodrop and so, for studies involving direct contact with hydroxyapatite, commercially available Quant-iT PicoGreen dsDNA Kits (Invitrogen) were used with the following protocol. 50μl of each cell/lysis buffer solution was placed into a 96 well plate with 50μl of dH2O to make a 1:1 dilution. The PicoGreen solution was prepared as a 1:200 dilution in 1x Tris-EDTA (TE) buffer and 100μl added to each sample well. This was placed in the dark for 5 minutes before fluorescence readings were taken at 480:520nm (excitation/emission). ALP was normalised to DNA for later construct configuration comparison.

Protein concentrations were assessed using Thermo Scientific NanoDrop 2000 spectrophotometer at 280nm (absorbance of 1.0 equates to 1.0mg/mL of protein).

### 4.9 Factorial Design of Experiment Optimisation

Within this thesis, factorial design of experiment has been utilised to enable the effects of multiple variable to be analysis simultaneously, including interactions of these variables, rather than conventional single factor testing (Thomas et al. 2008). As part of this analysis, Pareto charts were used to assess whether investigated variables had a significant effect on a specific output (Figure 4.8) and a table of optimisation was produced which assessed each configuration parameter according to the desired output (Figure 4.9).

![Pareto Chart of the Standardized Effects](image)

**Figure 4.8:** Example pareto chart of standardised effects detailing significant effects. This details the magnitude that a tested factor (variable) has on a desired response. A reference line, detailing the significance boundary, is drawn onto the chart. Any factor whose effect passes this line can be deemed to be significant. In the above chart, HA/collagen is the only factor with a significant effect on BGLAP although cell density has a greater effect than the interaction of both factors.
**Figure 4.9: Factorial design of experiment optimisation table.**
The results for each configuration are displayed according to the mean output from each construct and optimised according to desired outcomes.

<table>
<thead>
<tr>
<th>Name of output (i.e. Alignment Angle)</th>
<th>Desired characteristic outcome: High or Low (maximum or minimum configuration)</th>
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<td>Optimum configuration based on desired characteristic outcome</td>
</tr>
<tr>
<td></td>
<td>(i.e. smallest alignment angle was produced in configurations with a cell density of 4 million/mL with no width extension)</td>
</tr>
</tbody>
</table>

<table>
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<th>Variable</th>
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<table>
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<table>
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<th>Cell Des Overhang</th>
<th></th>
</tr>
</thead>
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<td></td>
</tr>
<tr>
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<td></td>
</tr>
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<td>d = 0.92354</td>
<td></td>
</tr>
<tr>
<td>y = 23.5469</td>
<td>d = 0.92230</td>
<td></td>
</tr>
</tbody>
</table>
5 Medium Composition Analysis Towards 3D Musculoskeletal Co-culture

5.1 Introduction

Increases in musculoskeletal injuries, due to increased intensity of recreational sports participation and an aging population, as well as current methods of repair not resulting in complete healing (high reoccurrence of injury), mean that new materials and methods for healing are required (Maffulli et al. 2005; Laurencin et al. 1999; Butler et al. 2008; Font Tellado et al. 2015). Prior to any novel material or medicine's regulatory approval, a series of pre-clinical in vitro trials and animal studies are required to determine their safety and efficacy (Chuang-Stein 2004).

Currently, in vitro bio-toxicity testing is performed on in vitro monolayer cell culture models (ISO10993). Monolayer models are capable of identifying cytotoxic effects through biochemical assays, or assessing changes in gene expression (Nam et al. 2014). However, these results do not effectively translate across to in vivo tissue systems (Nam et al. 2014; Vandenburgh et al. 2008). This is due to a general failure to accurately reproduce the complex nature of in vivo tissue structures and the biochemical pathways that accompany such architecture and necessitates the use of animal models (Eschenhagen & Zimmermann 2005; Smalley et al. 2008; Smith et al. 2012; Mulhall et al. 2013). Animal models clearly demonstrate complex tissue structures and pathways, however, these models have accompanying high costs, contentious ethical considerations and results that do not always translate cross-species (Mobasheri & Lewis 2013; Langley 2009). Therefore, more complex system models are required, which can provide a greater insight in the human in vivo response. Recent work in 3D tissue engineering has indicated the potential of increasing the relevancy of the models for in vitro preclinical toxicological testing (Elliott & Yuan 2011; Vandenburgh et al. 2008; Juhas et al. 2014; Sharples et al. 2012).

Engineered constructs require a cell source capable of forming the structures and processes associated with in vivo tissues, in addition to environmental conditions, such as substrate/scaffold and nutrients, which enable the establishment of these characteristics. The basic conditions to facilitate proliferative and differentiative stages of skeletal muscle and bone have been extensively studied in monolayer and the results translated across to facilitate the creation of 3D engineered models. Myogenic differentiation in both monolayer and 3D requires muscle progenitor cells, or myoblasts (MPCs), to exit the cell cycle. In vitro, this is generally met through a reduction in serum content to induce cellular fusion (Choi et al. 2013; Fujita et al. 2010; Martin et al. 2013; Hinds et al. 2013; Player et al. 2014). Similarly, the addition of ascorbic acid, β-glycerophosphate and dexamethasone has been shown to induce/enhance osteogensis in osteoblast/osteoblasts-like cells.
in the form of increased RUNX2/CBFa1 expression and matrix production and formation of hydroxyapatite (Langenbach & Handschel 2013). Comparing the effects of these media combinations on each cell type allows for a suitable protocol for musculoskeletal co-culture.

Although these medium supplements have been shown to induce desirable characteristics in both myogenic and osteogenic cultures, their relative effects on each other has not yet been reported. To produce a co-culture of 3D musculoskeletal tissues, an individual medium which allows for proliferation and differentiation of both cell types must first be identified. Three commonly reported proliferative/maintenance medium compositions were investigated to observe effects on proliferation rates and cell size (DNA, protein and protein/DNA ratios) on both C2C12 murine MPCs and TE85 human osteosarcoma (hOS) cell lines. Subsequently, after determination of a single proliferative medium composition, the effects of myogenic and osteogenic medium were assessed according to effects on DNA, protein and gene expression. Immunocytochemistry was used to confirm differentiation within C2C12 cultures and indicate potential lineage changes within TE85 populations.
5.2 Materials and Methods

5.2.1 Cell Culture
C2C12 murine myoblasts and TE85 human osteosarcoma (hOS) cells were expanded and routinely cultured as described in Section 4.1 unless otherwise stated.

5.2.2 Medium Composition Culture Comparisons

5.2.2.1 Medium Compositions

Medium compositions were chosen according to local SOPs and as detailed in literature (Table 5-1 and Table 5-2) (Player et al. 2014; Wimpenny et al. 2010; Bölgen et al. 2008; Heinemann et al. 2011; Cheng et al. 2003):

Table 5-1: Proliferation phase medium composition

<table>
<thead>
<tr>
<th>Medium Composition</th>
<th>Composition Details</th>
</tr>
</thead>
</table>
| M1 – Skeletal Muscle Precursor maintenance medium: |  • DMEM - High-Glucose (Hyclone)  
  • 20% Foetal Bovine Serum (FBS) (PAN Biotech)  
  • 1% Penicillin/Streptomycin (PS) (10,000U/mL and 10,000µg/mL, Gibco) |
| M2 - Human Osteosarcoma maintenance medium: |  • EMEM - EBSS (Sigma)  
  • 10% FBS  
  • 1% L-Glutamine (200mM, Sigma)  
  • 1% Non-Essential Amino Acids (NEAA) (100x, Sigma)  
  • 1% PS |
| M3 - Osteoblast-like cell maintenance medium: |  • DMEM - Low-Glucose (Sigma)  
  • 10% FBS  
  • 1% PS |

Table 5-2: Differentiation phase medium composition

<table>
<thead>
<tr>
<th>Medium Composition</th>
<th>Composition Details</th>
</tr>
</thead>
</table>
| Myogenic Medium: |  • DMEM (High Glucose)  
  • 2% Horse Serum  
  • 1% PS |
| Osteogenic Medium: |  • DMEM (Low Glucose)  
  • 10% FBS  
  • 0.1µM Dexamethasone (Sigma)  
  • 0.05mM Ascorbic acid (Sigma)  
  • 10mM β-glycerol-phosphate (Sigma)  
  • 1% L-Glutamine  
  • 1% PS |
5.2.2.2 Seeding Density and Timeline Determination

Prior to culture medium composition assessment, a timeline of growth rates (observed through time to full confluency) and differentiation was assessed to ensure comparative assessment between cell lines.

Proliferation Phase

C2C12 murine MPCs were seeded at 1,500 cells/cm² in 6-well tissue-culture plastic (TCP) plates and cultured for 7 days in M1. TE85 hOS cells were seeded at 1500 cells/cm² in 6 well tissue-culture plastic (TCP) plates and cultured for 7 days in M2.

TE85 Minimum Seeding Density Determination

To ensure full uniform confluency before differentiation, a minimum seeding density needed to be obtained to prevent colony formation. As such, TE85 hOS cells were seeded at 1500, 3000, 4500, 6000, 7500, 9000, 10500 and 12000 /cm² on glass cover slips in a 6-well TCP plate (Nunc).

Differentiation Phase

Due to the morphological changes and culture limitations observed during C2C12 differentiation a timeline of differentiation for both cell lines was based on C2C12 culture. Once confluent, C2C12s were cultured in differentiation medium (DM) (high-glucose DMEM supplemented with 2% horse serum and 1% PS) for 4 days.

5.2.2.3 Medium Composition Comparison Cell Culture¹

Proliferation Phase

To understand the effects of medium composition of proliferation in monolayer, both cell lines were seeded at 4500 cells /cm² in 6 well tissue-culture plastic (TCP) plates (n=3) and cultured for 4 days. Cells were taken for DNA and protein analysis at 24hrs, 48hrs and 96 hours post seeding. Brightfield images were taken prior to each time-point analysis.

Differentiation Phase

Based on results of the proliferation phase cell culture (Section 0), a single maintenance medium (M1) was chosen to culture the cells prior to differentiating. Cells were cultured as detailed during the proliferation phase. Once confluent, cells were cultured in either Osteogenic or Myogenic medium for 3 days. M2 was used as a control for the TE85s. Cells were taken for analysis 24hrs, 48hrs and 72 hours post seeding.

¹ Medium Composition cell culture, DNA and protein quantification and ICC conducted with the assistance of Diogo Mosqueira
5.2.3 **DNA and Protein Quantification**
Samples taken for DNA and protein analysis were lysed and quantified as described in Section 4.8. Statistical analysis was run in GraphPad Prism. An ANOVA with a Bonferroni post-hoc analysis was used to determine sources of significant variation.

5.2.4 **qRT-PCR**
RNA extraction and quantification, and qRT-PCR were run as described in Section 4.7.

5.2.5 **Brightfield Microscopy**
Brightfield Microscopy was taken at each time point to qualitatively assess culture progression and proliferation rates as described in Section 4.4.

5.2.6 **Immunofluorescence Imaging**
Immunocytochemistry was used to assess differentiation and potential lineage changes. Cultures to be imaged were fixed, stained and imaged as described in Section 4.5.
5.3 Results

5.3.1 Medium Composition Culture Comparisons

5.3.1.1 Seeding Density and Timeline Determination

Before assessment of medium composition effects on each cell line, a timeline of the proliferation and differentiation phases of the cultures in their standard medium compositions was conducted.

Proliferation

C2C12s were seeded at 1500 cells/cm² onto gelatine-coated glass coverslips in a 6-well plate. Observation of the C2C12s proliferative phase shows full confluency is reached after approximately 96 hours after which cell layering occurs (Figure 5.1). During this period, no evidence of colony formation was observed. At high levels of confluency, upregulation of the sarcomeric skeletal muscle proteins, desmin and actin, can be observed in spontaneously differentiating myoblasts (Figure 5.2).

![Figure 5.1: C2C12 murine myoblast proliferative timeline. Cultured in growth medium for A) 24 hours B) 72 hours C) 96 hours D) 168 hours. Initial seeding at 1500/cm². Increasing levels of surface coverage and cell layering. n=3 + 3 replicates per well. Scale bar - 50µm](image)
Figure 5.2: Immunofluorescent image of spontaneously differentiating C2C12s (scale bar - 50µm).

Green - Desmin, Red - F-Actin filaments, Blue - DAPI nuclei stain. During differentiation, production of desmin and actin increases in line with the production of contractile apparatus, sarcomeres. This is highlighted by the increased desmin intensity and evidence of red within the elongated cell body. As a cell of myogenic potential, all C2C12s within the cell population are desmin positive.

At an initial seeding density of 1500 cells/cm², the TE85 hOS cell line reached full confluency after approximately 1 week (Figure 5.3). This cell density results in areas of high cell density and colony formation, hence non-uniform coverage. This can potentially lead to areas of necrosis and therefore is unsuitable for experimentation.

To avoid necrotic cell cluster formation and to encourage uniform cell coverage, a minimum cell seeding density was investigated. After three days of culture, TE85 hOS with an initial seeding density of 1500 /cm² and 3000 /cm² display colony formation (Figure 5.4). An initial seeding density of 4500 /cm² and greater displays more uniform proliferation and coverage. The higher cell densities of 10500 /cm² and 12000 /cm² are confluent by the third day of culture. 7500 /cm² and 9000 /cm² are above 90% confluency (qualitative determination based on observations). When testing for the effects of external influences in monolayer, two-three days may not be long enough to observe effects on proliferation phase gene expression and morphology become apparent and therefore 4500 /cm² or 6000 /cm² is more appropriate. Due to a balance of resources and uniform confluency potential, 4500 cells/ cm² was chosen for subsequent monolayer experimentation. At this seeding density, uniform confluency was achieved after 4 days culture allowing direct comparison to C2C12 cultures.
Figure 5.3: TE85 hOS cultured in M2 for A) 24 hours B) 72 hours C) 120 hours D) 168 hours showing cell layer detachment from surface. Attached cell layer on right of image. Initial seeding at 1500/cm². n=3 + 3 replicates per well. Scale bar – 50µm
TE85 hOS Minimum seeding density

Figure 5.4: TE85 hOS seeding densities after 3 days culture.

Seeding densities (cells/cm²) A) 1500 B) 3000 C) 4500 D) 6000 E) 7500 F) 9000 G) 10500 H) 12000.

Confluency – surface coverage increases with each initial seeding density. 1500 and 3000 cells/mL shows evidence of colony formation (ellipse). n=3 + 3 replicates per well. Scale bar - 50µm
Differentiation

Once confluent, GM was replaced with DM, resulting in the C2C12 cultures showing near total fusion of cellular material into distinct myotubes after two days (Figure 5.5). Alignment of the cells was regular in several areas, although the culture as a whole demonstrated characteristic random alignment. Longer cultures of up to 5 days in differentiation medium increased the number of cells committed to fusion. However, without a distinct direction of tension or topography to align to, the cytoskeletal filaments were multidirectional. As skeletal muscle cells mature, they exert a contractile force on the surface to which they are attached. In this study, this caused their detachment in prolonged cultures, hence the spaces observed in Figure 5.6. Therefore, due to this limitation, a three day differentiation phase assessment protocol was chosen for future monolayer cultures.

**Figure 5.5:** Monolayer C2C12 culture on glass coverslips after two days in differentiation medium (Left) Brightfield image. (Right) Immunofluorescent image showing random alignment. Red - Desmin. Blue - DAPI nuclei stain. Scale bar – 100 µm.

**Figure 5.6:** Immunofluorescent images of monolayer culture after four days in DM. Scale bar - 50 µm Green - Desmin, Red - F-Actin filaments, Blue - DAPI nuclei stain. Large multinucleated myotubes evident. Desmin positive across entire cell body. Actin filaments are multidirectional.
Due to the result in Proliferation and Differentiation a culture period of 4 days was chosen to assess effects on proliferation and a period of 3 days to assess effects on differentiation. In addition, C2C12s were shown to be able to proliferate to a uniform confluency at an initial seeding density of 1500 cells/cm². However, TE85s at this density formed non-uniform surface coverage. After assessment of different seeding densities 4500 cells/cm² was chosen as the initial seeding density for future experiments in monolayer.

### 5.3.1.2 Medium composition analysis

Current tissue engineered systems rely on a number of conditions in order to allow for the successful culture of various tissues. The defining factor of successful culture is the nutrient medium in which the cells grow. In a multi-lineage system (muscle-tendon, tendon-bone), each cell type has different specific requirement for proliferation and maintenance. Differentiation from precursor to functional cell type can require a different stimulus in the form of scaffold characteristic or supplement changes within the medium. To create a system in which the major musculoskeletal components of skeletal muscle and bone can be co-cultured in 3D, a medium must first be identified that can sustain the growth and differentiation of both cell types. Three proliferation phase medium were tested; M1 (HG-DMEM, 20% FBS, 1% PS), M2 (EMEM with EBSS, 10% FBS, 1%NEAA, 1% PS), and M3 (LG-DMEM, 10% FBS, 1% PS). This was followed by differentiation phase analysis using a myogenic medium (HG-DMEM, 2% horse serum, 1% PS) and an osteogenic medium (LG-DMEM, 10% FBS, 0.1µM Dexamethasone, 0.05mM Ascorbic acid, 10mM β-glycerol-phosphate, 1% L-Glutamine, 1% PS).

**Proliferation Phase**

To assess the effects of each medium composition on each cell population during attachment and proliferation, each cell type was initially seeded at 4500 cells/cm² and analysis of each culture taken at periodic time-points. As a qualitative measure of cell growth, brightfield images of each culture condition were taken at 24, 48 and 96 hours post seeding. After 48 hours, little observable difference was seen between each of the culture conditions in both C2C12 (Figure 5.7) and TE85 (Figure 5.8) cultures, although TE85 M3 (DMEM-low glucose with 10% FBS/1% PS) cultures demonstrate a slightly reduced coverage. This trend was observed in the DNA concentrations for each culture (Figure 5.9), in which there were no significant differences between culture configurations. TE85 M3 cultures do show lower concentrations of DNA matching the qualitative observations but this difference is not statistically significant. At 96 hours, C2C12s (Figure 5.7 G, H and I) are fully confluent, however, M1 (DMEM-high glucose with 20% FBS/1% PS) and M2 (EMEM-EBSS with 10% FBS/1% L-glutamine/1% NEAA/1% PS) cultures appear more “cramped”. 
Despite comparative levels of confluency under observation, the DNA concentrations at 96 hours within M1 (C2C12 control medium) cultures are significantly greater than M2 or M3 (p≤0.001). Similarly, TE85 M2 cultures (TE85 control medium) have significantly greater DNA concentrations at 96 hours than M1 or M2. TE85s cultured in M3 medium shows significantly reduced concentrations of DNA compared to both C2C12 and TE85 control cultures (M1 and M2 respectively). Therefore, to achieve a consistent proliferation timeline comparative to previous cultures and models, M3 would be unsuitable for co-culture. This lack of growth could be due to the low glucose and glutamine concentrations compared to M1 and M2, potentially negatively impacting metabolic activity. Protein levels (Figure 5.10) follow a similar trend in both C2C12 and TE85 cultures. Protein/DNA (Figure 5.11) ratios indicate changes in cellular population morphology. During the culture period, the cultures go through periods of hypertrophy and hyperplasia when compared to measurements at 24 hours. After 96 hours no significant differences occur between M1, M2 or M3 conditions in both C2C12 and TE85 cultures. This indicates only a negative proliferative effect from different medium conditions and no morphological changes.

Each cell type was pre-conditioned in each medium prior to experimentation. However, a bias towards control medium (C2C12 - M1 and TE85 M2) was observed. Cultures conducted without preconditioning (Appendix 4) exhibited a greater bias toward control medium conditions, with a change in basal medium (DMEM - EMEM) resulting in significantly reduced DNA concentrations after 4 days and necessitating a pre-conditioning step. From the pre-conditioned culture results, although M1 and M2 demonstrated a capacity to sustain both C2C12 and TE85 cultures, M2 negatively affected C2C12 populations to a greater extent than M1 influenced TE85 populations. Therefore, M1 was chosen as a proliferation medium for subsequent medium composition and co-culture experiments.
Figure 5.7: C2C12 medium composition analysis surface coverage brightfield images. Confluency was observed in all cultures after 96 hours however M1 (skeletal muscle precursor maintenance medium) and M2 (human osteosarcoma maintenance medium) appear to have a high cell density than M3 (osteoblast-like cell maintenance medium). n=3 per timepoint per condition + 3 replicates per well.

Figure 5.8: TE85 medium composition analysis surface coverage brightfield images. Confluency was observed in M1 (skeletal muscle precursor maintenance medium) and M2 (human osteosarcoma maintenance medium) after 96 hours. M3 (osteoblast-like cell maintenance medium) has large areas of uncovered surface. n=3 per timepoint per condition + 3 replicates per well.
Figure 5.9: Proliferation phase culture DNA concentration over 4 days.
(A) C2C12 cultures. DNA concentration increases for all conditions over 4 days. M1, DMEM-high glucose with 20% FBS/1% PS, displays significantly greater concentrations of DNA than M2 (EMEM with 10% FBS/1% L-Glutamine/1% NEAA/1% PS) and M3 (DMEM-low glucose with 10% FBS/1% PS) at 4 days. (B) TE85 cultures. M2 show significantly greater concentrations than M1 and M3 at 4 days. n=3 culture replicates + 3 repeat measures for all conditions. *** = P<0.001
Figure 5.10: Proliferation phase culture protein concentration over 4 days
(A) C2C12 cultures. Protein concentration increases for all conditions over 4 days. M1 displays significantly greater concentrations of protein than M2 and M3 at 4 days. (B) TE85 cultures. M2 shows significantly greater concentrations than M1 and M3 at 4 days. n=3 culture replicates + 3 repeat measures for all conditions. *** = P<0.001
Figure 5.11: Proliferation phase culture protein/DNA ratio over 4 days.
(A) C2C12 cultures. No significant differences between different medium conditions after 4 days. (B) TE85 cultures. No significant differences between different medium conditions after 4 days. n=3 replicates for all conditions. * = P<0.05, ** = P<0.01.
**Differentiation Phase**

During the differentiation phase, each cell type undergoes changes for specialisation. Once skeletal muscle precursor cells (MPCs) have exited the cell cycle, they fuse together to form elongated, multinucleated myotubes with an accompanying upregulation of myogenin expression. Similarly, during differentiation, osteoblasts upregulate the osteogenic molecular switch RUNX2/CBFα1. This initiates a transcriptional cascade resulting in bone formation.

To assess the feasibility of co-differentiation of C2C12 muscle precursor cells and TE85 osteosarcoma cells, each cell type was cultured in myogenic (high glucose-DMEM with 2% horse serum, 1% PS) and osteogenic medium (EMEM with 10% FBS, 0.1µM dexamethasone, 0.05mM ascorbic acid, 10mM β-glycerol-phosphate, 1% L-Glutamine, 1% PS). Cultures were first grown to confluency in M1 (high glucose-DMEM with 20% FBS, 1%PS) due to the capability of both cell lines to proliferate successfully (TE85s were affected less by culture in M1 than C2C12s in M2).

Brightfield observations offer a qualitative assessment of morphological changes across the culture population which enables assessment of some cell behaviours, such as proliferation or cell fusion. C2C12s (Figure 5.12) cultured in myogenic medium show a distinct reduction in cellular boundaries with an increase in elongated cellular bodies over 72 hours. C2C12 in osteogenic medium display an initial “cramping” of cellular bodies after 24 hours. However, elongated cellular bodies are apparent after 72 hours although at a comparable level to myogenic cultures at 48 hours. TE85 human osteosarcoma cells (Figure 5.13) show no obvious morphological changes under each condition other than an increase in observable cell density. Although, H and I show smaller cell bodies than G indicating greater cell numbers at these points.
Figure 5.12: Brightfield observations of C2C12 murine muscle precursor cells (MPCs) after culture to confluency in M1 and then exposure to either myogenic (A, C and E) or osteogenic medium (B, D, F).
Cellular fusion is evident within myogenic cultures. However, no fusion is evident within osteogenic cultures. n=3 per timepoint per condition + 3 replicates per well. Scale bar – 50µm.

Figure 5.13: Brightfield images of TE85 human osteosarcoma cells after culture to confluency in M1 and then exposure to either myogenic (A, D and G) or osteogenic medium (B, E, H).
C, F and I show the exposure to osteogenic medium after culture during the proliferative phase in medium composition M2 (control). n=3 per timepoint per condition + 3 replicates per well. Scale bar – 50µm.
Analysis of DNA confirms greater cell densities within the C2C12 osteogenic cultures after 24 hours. The significantly greater DNA concentration (Figure 5.14A) is most likely a delayed response to the reduction in serum (20% to 10% FBS) when compared to myogenic medium (20% FBS to 2% Horse Serum) implying a continuation in proliferation. After 48 hours, osteogenic culture concentrations reduced so that no statistical differences occurred between medium types. Myogenic culture’s DNA concentrations then decreased to be significantly lower than osteogenic cultures. This may be explained by detaching cells observed through brightfield microscopy; caused either through the reduction in serum or an increase in surface coverage resulting in the cell detachment due to a reduction in available attachment area.

Within TE85 cultures (Figure 5.14B), at all time-points, M1+Myogenic has significantly less DNA presence than M2 + Osteogenic (24 hours p<0.05, 48 hours p<0.01, and 3 days p<0.05). At both 24 and 48 hours, M1 + Myogenic is significantly less than M1 + Osteogenic (P<0.05). However, at 3 days, there were no significant differences between the M1+Myogenic and M1+Osteogenic cultures. There were no significant differences between M1+Osteogenic and M2+Osteogenic cultures at any time-point, which also correlates with the brightfield observations. Higher serum levels within the osteogenic medium caused a continuation in TE85 proliferation, which accounts for the discrepancy in the DNA concentrations between conditions. Protein concentrations (Figure 5.15) follow a similar trend, resulting in no significant differences after 3 days between culture conditions. The formation of myotubes represents the hypertrophy of cell bodies. However, this was not seen to translate into an increase in protein concentration relative to DNA (Figure 5.16). Once cell coverage reached 100% (full confluency) followed by cellular fusion, multiple nuclei were seen to be contained within a single membrane (no DNA change) and cellular coverage remained complete (no protein change), hence protein/DNA ratios remained stable.
Figure 5.14: Differentiation phase culture DNA concentrations

(A) C2C12 cultures. Concentrations are significantly different after 24 and 72 hours with a period of no significance differences at 48 hours. (B) TE85 cultures. Osteogenic cultures show similar arrested proliferation to myogenic cultures, however, DNA concentrations are greater. n=3 culture replicates + 3 repeat measures for all conditions, * = P<0.05, ** = P<0.01, *** = P<0.001.
Figure 5.15: Differentiation culture protein concentrations

(A) C2C12 cultures. Cultures subjected to osteogenic medium have significantly greater concentrations of protein after 3 days culture. (B) TE85 cultures. Cultures subjected to myogenic medium had significantly less protein than control cultures (M2+ostegenic) however there were no significant differences to cultures exposed to M1 followed by osteogenic medium. n=3 culture replicates + 3 repeat measures for all conditions, * = P<0.05, ** = P<0.01, *** = P<0.001
Figure 5.16: Differentiation cultures protein to DNA culture ratios at 24 hours, 48 hours and 3 days post seeding.
(A) C2C12 cultures. (B) TE85 cultures. No significant differences between cultures after 3 days. n=3 culture replicates + 3 repeat measures for all conditions, ** = P<0.01, *** = P<0.001
Myogenin is a muscle specific transcription factor that regulates skeletal muscle differentiation and repair. The qRT-PCR results of myogenin expression (Figure 5.17A) showed an increase over 72 hours in myogenic cultures, with no changes evident within osteogenic medium. To confirm no osteogenic effects within the C2C12 population, RUNX2 expression was also assessed (Figure 5.17B) and no significant changes were observed. RUNX2 expression within TE85 populations increased significantly in all cultures after 72 hours (Figure 5.18).

Immunocytochemical (ICC) staining for desmin, an intermediate filament protein present within the contractile apparatus of skeletal muscle, was used to confirm differentiation within the C2C12 cultures (Figure 5.19). Desmin positive C2C12s were found in abundance after 24 hours in both differentiation media. After 48 hours, C2C12s in myogenic medium displayed clear multinucleated myotubes. In contrast, C2C12s in osteogenic medium showed a reduction in desmin positive cells. After 72 hours, myogenic cultures displayed numerous highly-multinucleated elongated cellular bodies, although some nuclei appear unassociated. Osteogenic medium cultures revealed further reductions in desmin positive cells. Immunocytochemistry of TE85 cultures revealed no desmin presence across all cultures indicating no lineage altering effects (Figure 5.20).

This demonstrates that M1 (HG-DMEM supplemented with 20% FBS and 1% PS) followed by myogenic medium (HG-DMEM supplemented with 2% horse serum and 1% PS) is a suitable protocol to enable the development of a skeletal muscle and bone co-culture system.
Figure 5.17: qRT-PCR of C2C12 differentiation cultures.
(A) Myogenin expression and (B) RUNX2 expression in myogenic and osteogenic medium. Myogenin is upregulated over 72 hrs in myogenic cultures. No significant changes in osteogenic cultures. No significant changes in RUNX2 expression across all configurations at all timepoints. All conditions cultured to confluency in M1. n=3 culture replicates + 3 repeat measures for all conditions.
Figure 5.18: qRT-PCR of RUNX2/CBFα1 expression of TE85 differentiation cultures. RUNX2 expression in myogenic and osteogenic medium. Cultures subjected to osteogenic medium had significantly greater expression of RUNX2/CBFα1 than myogenic cultures. Each conditions was cultured to confluency in M1 (C2C12 GM) with M2 (TE85 GM) + osteogenic medium as control. n=3 culture replicates + 3 repeat measures for all conditions.
Figure 5.19: Monolayer C2C12 morphological observation. Immunofluorescence images after culture in myogenic (A, C and E) and osteogenic (B, D and F) medium. The intermediate filament protein desmin is shown in red. DAPI nuclei stain is represented by blue. C2C12 myogenic cultures show clear presence of cellular fusion however osteogenic medium composition inhibits myotube formation and reduces desmin presence. n=3 per timepoint per condition + 3 replicates per well. Un-fused nuclei mostly show little or no presence of Desmin (denoted by →). Scale bar – 50µm
Figure 5.20: Monolayer TE85 morphological observations. Immunofluorescence images after culture in myogenic and osteogenic medium. The muscle specific intermediate filament protein desmin was used to assess any lineage changes and is shown in red. DAPI nuclear stain is represented by blue. No evidence of desmin presence therefore no potential lineage changes. n=3 per timepoint per condition + 3 replicates per well.
5.4 Discussion

In order to successfully create a platform capable of supporting the co-culture of skeletal muscle and bone, it is important to create culture conditions amenable for both cell types in a single system. As one of the major factors affecting myoblast fusion and bone matrix secretion, investigating the effects of different medium composition on proliferation and differentiation is of high importance. This work has sought to evaluate common proliferative, myogenic and osteogenic medium for C2C12 murine muscle precursor (MPC) and TE85 human osteosarcoma (hOS) cell lines as a pre-requisite to co-culture development. DNA, protein and protein/DNA ratios were taken as a measure of cellular population growth and to indicate potential effects of hypertrophy and hyperplasia in the proliferative and differentiative phases. These results were coupled with brightfield and immunofluorescent images along with qRT-PCR to assess and confirm cell population growth and differentiation. Monolayer cultures were chosen to assess the different medium compositions due to their relatively short growth and differentiation periods, compared to 3D cultures, and a lack of matrix to interfere with analysis methods.

In co-cultures, each cell type may have a standard medium which has previous been determined as acceptable for the individual requirements of each cell population. Co-culture systems described within the literature report the success or failure of a particular cell in influencing the other cell type (Mikos, Herring, Ochareon, Lu, et al. 2006; Eckle et al. 2014; Kino-oka et al. 2013). These papers have yet to describe the conditions which allow for the successful culture and differentiation of both cell types in a single medium system through experimental comparisons.

Myogenic cell lineages are typically cultured in a high glucose (4500 mg/L) basal medium with at least 10% foetal bovine serum (FBS) (Katagiri et al. 1994; Cooper et al. 2004; Park et al. 2008), although, 20% FBS supplementation has been shown to produce more favourable results (Sharp et al. 1995). As a basal medium, DMEM (Dulbecco's Modified Eagle Medium) contains four times the vitamins and amino acids of Eagle's Minimal Essential Medium (EMEM) and is most commonly reported for use with a skeletal muscle cell line (Katagiri et al. 1994; Morimoto et al. 2013; Martin et al. 2013; Player et al. 2014). Osteogenic cell lineages are typically cultured in low glucose (1000 mg/L) medium with 10% FBS and an addition of non-essential amino acids (San Martin et al. 2009; Struewer et al. 2014; Jones et al. 2010; Gomes et al. 2001). The specific differences of each medium have varying effects on the cell populations: FBS contains proteins and growth promoting factors pertinent to cellular growth; glucose is involved in the maintenance of efficient energy metabolism within the cell and is the primary source of energy (Behjousiar et al. 2012; Lu et al. 2013); and glutamine acts a secondary source of energy and assists in the creation of proteins and nucleic acids (Yuneva et al. 2007).
Within this thesis, a restriction in the concentrations of glucose between medium significantly affected the C2C12 line and was not compensated by the glutamine concentrations. A deficit in the FBS supplement concentration, and therefore the growth factor concentrations, was also implicated in the restriction of population growth of the myogenic cells. Conversely, the TE85 cell line showed greater proliferation with a lower glutamine, glucose and FBS concentrations. This suggests that the basal medium is a key influencer in cellular growth dynamics as well as supplementation in these cell lines. Both skeletal muscle and osteoblast-like cell types show a capacity for proliferation in all medium with a bias towards each cell type’s long term culture medium. However, C2C12s were affected to a greater extent than TE85s resulting in high-glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (PS) to be chosen as a suitable proliferation medium for both cell lines. Whilst a common medium of choice for osteogenic cell lineages, low-glucose DMEM with 10% FBS has been shown to inhibit growth rates in this work. This may not affect the desired characteristics for the cell, however, for a preclinical testbed in which results can be required in a relatively short period, this is not desirable.

Myogenic cells have shown the ability to differentiate along an osteoblastic lineage (Sinanan et al. 2006; Mulhall et al. 2011; Katagiri et al. 1994; Starkey et al. 2011; Davies et al. 2015). Conditions promoted through blast and crush injury demonstrate this capacity in vivo (Davies et al. 2015). Katagiri et al. (1994) and Nakashima et al. (2002) utilised bone morphogenic proteins (BMPs) to affect the osteogenic differentiation of unfused C2C12 MPCs. As a marker for osteoblast progression, RUNX2 has been established as the initial trigger of the osteogenic gene cascade resulting in alkaline phosphatase production and an upregulation of BGLAP/Osteocalcin. Under culture with common osteogenic supplement’s (β-glycerol-phosphate, ascorbic acid and dexamethasone), C2C12s showed no change in the MPC fusion associated gene, myogenin, across three days and no significant differences in RUNX2 expression. This is in comparison to the classic upregulation followed by subsequent decrease in myogenin expression observed in the myogenic medium serum-starved cultures (Langenbach & Handschel 2013; Maffulli et al. 2005; Maegawa et al. 2007; Khatiwala et al. 2009).

An immunofluorescent marker for the muscle specific protein, desmin, allowed for observation and qualitative analysis of population phenotypic changes. Under serum starved conditions (myogenic medium), C2C12s fused to form small myotubes dispersed between desmin positive cells, which later progressed to form multiple hypertrophic myotubes, in line with classic in vitro skeletal muscle culture. However, C2C12s cultured in osteogenic medium (low-glucose, 10% FBS and osteogenic supplement’s) demonstrated a decrease in desmin presence with an increase in non-desmin associated nuclei and punctate staining. The low glucose aspect of the osteogenic medium
potentially caused a reduction in metabolic activity, accompanied by the presence of dexamethasone, a known inducer of atrophy (Qin et al. 2013; Krug et al. 2012; Girón et al. 2015) and other osteogenic factors, may have caused a reversion to a more progenitor-like state, alluded to in skeletal muscle calcification conditions (Davies et al. 2015). A lack of RUNX2/Cbfa1 upregulation supports a reversion theory to a less committed cell type, rather than a direct lineage change as in the case of BMP-2 stimulated culture (Katagiri et al. 1994).

As shown from the DNA and protein differentiation profiles, proliferation of the TE85s is arrested once placed in a differentiation medium. Much like the skeletal MPCs, osteoblasts exit the cell cycle prior to differentiating (Funato et al. 2001; Gaur et al. 2005). Once the TE85 cultures growth profiles stabilised, they displayed an upregulation of RUNX2/Cbfa1 in all cultures after 3 days and no desmin associated nuclei with no evidence of cellular fusion. The concentration or the type of serum in the differentiation medium seemingly does not affect the final expression of RUNX2/CBFA1 without significant difference occurring after 3 days. However, the osteogenic supplements may reduce the latent time before expression is upregulated. Therefore, low non-foetal serum (2% horse serum) could be used in subsequent studies involving osteoblasts-like cells, which would reduce the costs involved in cultures of this type.

Previous work has conserved the medium composition between monolayer work and later 3D models (Martin et al. 2013; Mulhall et al. 2013). Despite the addition of an extra-cellular matrix (ECM) impeding nutrient delivery through porous material barriers, cells are able to undergo similar transformations as observed in the preceding monolayer cultures. The addition of an ECM within the culture does not change basic cellular nutrient requirements, except perhaps for concentration of supplements due to a potential increase metabolism through remodelling or differentiation. In comparison to previous skeletal muscle and bone culture, both within this thesis (Chapter 6 and 7) and the literature, cells cultured within an ECM do not require a modified medium composition. They do, however, exhibit longer culture periods before definitive differentiation (Martin et al. 2013; Burattini et al. 2004; Player et al. 2014). Hence, the medium compositions described within this chapter will be continued into 3D investigations.
5.5 Conclusions

It is important to create favourable conditions for both muscle and bone cultures to develop in a single system. Therefore, prior to the co-culture of skeletal muscle and bone, a single proliferative medium and a single differentiative medium must first be ascertained to enable the development of desired characteristics.

By comparing different medium compositions during the proliferation phase of cell culture, it is possible to gain an understanding of the specific nutrient base that will allow for surface attachment and successful maintenance of the culture. This informed subsequent 3D cultures and co-cultures in which cellular attachment to the matrix is of utmost importance. In this study high-glucose (4500mg/mL) DMEM + 20% FBS + 1% P/S (M1) proved successful for the culture of both C2C12s murine skeletal muscle progenitor cells (MPCs) and TE85s human osteosarcoma cells. As an alternative medium for TE85 osteosarcoma cells and a widely used osteoblast-like proliferation medium, low-glucose (1000mg/mL) DMEM + 10% FBS + 1% P/S (M3) was found to inhibit proliferation in comparison to EMEM (EBSS) + 10% FBS + 1% L-Glutamine + 1% Non-Essential Amino Acids + 1% P/S (M2) or C2C12 MPC medium (M1). Myogenic medium (high glucose DMEM + 2% horse serum) enabled the fusion of MPCs to form myotubes and the upregulation of the osteogenic cascade trigger Runx2/Cbfa1 in TE85s. In contrast osteogenic medium seemingly inhibits differentiation of myogenic cultures and therefore is unsuitable for co-culture.

Skeletal muscle cultures can be simply influenced to differentiate by inducing the progenitor cells to exit the cell cycle, causing an up-regulation of certain transcription factors (e.g. myogenin). This can be affected by simply decreasing the serum content of the medium. Inducing osteogenesis is considered more complex, commonly requiring additional supplements to medium such as dexamethasone, ascorbic acid and β-glycerol-phosphate. High-glucose DMEM + 20% FBS followed by high-glucose DMEM + 2% horse serum was found to enable the desired characteristics of both cell types to be obtained in similar time frames and is therefore a suitable combination for skeletal muscle and bone culture.
6 Development and Optimisation of a 3D Tissue Engineered Skeletal Muscle Model

6.1 Introduction

Chapter 5 investigated different medium compositions effects on C2C12 murine myoblast and TE85 human osteosarcoma in both proliferative and differentiative cultures. The high-glucose DMEM (HG-DMEM) supplemented with 20% FBS and 1% PS followed by HG-DMEM supplemented with 2% horse serum and 1% PS was found to be capable of supporting proliferation and differentiation in both cell lines. This chapter utilises these media as a basis for the development and optimisation of a 3D tissue engineered skeletal muscle model.

In vivo skeletal muscle displays a complex structure in which uniformly aligned, terminally differentiated, multinucleated fibres are arranged as fascicles within a collagen extra-cellular matrix (Baechle & Earle 2008). As terminally differentiated units, individual fibres cannot regenerate themselves (Grounds 1998). Therefore, during muscle growth (hypertrophy) and regeneration, fibre-adjacent satellite cells (Mauro 1961) undergo asymmetrical mitosis, resulting in a population of cells committed to the myogenic lineage (Grounds 1998). These muscle precursor cells (myoblasts) subsequently fuse to the existing muscle structure. This mechanism of myoblast fusion is recreated in vitro through the formation of immature muscle fibres or myotubes. Within monolayer cultures, myotubes are formed in randomly orientated, branched structures (Burattini et al. 2004; Zhao et al. 2009; Smith et al. 2012). In contrast, 3D skeletal muscle models have been shown to closely replicate the observed uniaxially aligned in vivo architecture (Smith et al. 2012; Khodabukus & Baar 2009). These models typically utilise two anchor points that act as pseudo-myotendinous junctions, allowing cells to align according to isometric lines of tension (Huang et al. 2005; Neidlinger-Wilke et al. 2001; Dennis & Kosnik 2000; Powell et al. 2002; Mudera et al. 2010; Martin et al. 2013; Boonen et al. 2010; Hinds et al. 2011).

Work by Eastwood, Mudera, et al. (1998) demonstrated the influences of length and width ratios (high and low) on fibroblast alignment within a 3D collagen-based in vitro model. They found that fibroblasts align parallel to lines of isometric tension with greater alignment occurring high aspect ratio gels. In particular, fibroblasts were most aligned in the central region and towards the edges of the constructs with random alignment occurring around the anchor point. This model was later developed for skeletal muscle tissue engineering, demonstrating the effectiveness of cellular attachment and fusion (Cheema et al. 2003); the effect of cell density on the production of internal construct forces using primary human muscle derived cells (Mudera et al. 2010); for modelling atrophy and ageing (Sharples et al. 2012; Smith et al. 2012) and investigating the effects of acute
mechanical stimulation on atrophic/hypertrophic gene transcription (Player et al. 2014). Each of these investigations point towards the physiological relevance of this 3D model in low throughput, basic cellular and mechanistic studies.

High-throughput is defined as the simultaneous analysis of multiple samples. In the drug reporting industry, high-throughput screening predominantly takes place in plates upwards of 384-well plates (Fox et al. 2002). For high-throughput testing, as required in bio-toxicity studies, each test must be both resource efficient and highly reproducible in both manufacture and output, in order to remain cost effective and to increase speed of development (Peters et al. 2009). The proportional scale down of previous models has existed for many years in the world of electronics, specifically in computers (Whitesides 2003) and this has been accompanied by a decrease in cost and an increase in efficiency. Within tissue engineering, the direct scaling down of established models will result in a reduction in the required cell number and reduction in raw materials and consumables and therefore an overall reduction in cost. In addition, a reduction in the construct dimensions decreases the distance between those cells in the centre and the medium surrounding, thereby allowing greater access to nutrients. In order to make use of a tissue engineered skeletal muscle model in pre-clinical screening, it must be validated as a repeatable, scalable system without compromising the representative skeletal muscle architecture observed in previous models.

By analysing previous iterations of the collagen model presented here, hydrogel dimensions and calculated volume to surface area ratios were used to develop a basic scaling model for further investigation (Eastwood et al. 1998; Cheema et al. 2003; Sharples et al. 2012). The effects of dimension variation on construct attachment and remodelling forces, previously demonstrated through construct size reduction, were also compared (Martin et al. 2013; Dennis & Kosnik 2000; Garvin et al. 2003). Following this, utilising a factorial design of experiment (Hussein et al. 2015), conditions most amenable to the consistent production of skeletal muscle cultures with desirable morphological traits were optimised and validated. Within other reported designs, areas of hydrogel outside the boundaries of the anchor points have allowed for self-organisation of myotube populations through remodelling of the surrounding matrix (Huang et al. 2005). Utilising aspects of this process to enhance cell-cell interactions, the effect of hydrogel width extension and initial seeding density on myotube characteristics and interactions between the two variables were investigated. Increasing the width increases the surface area of adherence to the anchor point and changes the mechanics of the system by varying the superposition of forces generated through cell attachment and remodelling. Furthermore, detachment of the collagen construct from the anchor points results in complete failure and as such, initial attachment forces comparing optimised and non-adjusted models were conducted to observe possible effects of width extension cell-matrix
interactions. Finally, long-term (33 days) cultures were attempted to increase the flexibility of the skeletal muscle in vitro system in preparation for co-culture with 3D tissue engineered bone. Tissue engineered bone is normally reported in cultures lasting in excess of the 14 day protocol of this skeletal muscle construct to attain greater maturation.
6.2 Materials and Methods

6.2.1 Cell Culture
Cells were expanded and routinely cultured as described in Section 4.1.

6.2.2 Monolayer and 3D Comparison
C2C12s were cultured on glass coverslips or in 3D tissue engineered skeletal muscle constructs. Monolayer cultures were seeded at 1500 cells/cm². 3D constructs were manufactured as described in Section 4.3 and below (Section 6.2.3). Cells were seeded at 4 million cells/mL in a 1.5mL collagen hydrogel (a density previously determined to be optimal for this cell type by Player et al. (2014) and Sharples et al. (2012). Setting areas were approximately 15x28mm with an anchor point approximately 10x3mm (length and width measurements).

Alignment measurements were taken from n=4 3D constructs (739 myotubes – A-frame, 761 myotubes – Centre) and n=3 monolayer well culture (526 myotubes). Myotubes were measured using ImageJ (NIH) using an end-to-end method to obtain mean direction.

6.2.3 3D Tissue Engineered Collagen Skeletal Muscle Constructs
Collagen constructs (Figure 6.1) were prepared as described in Section 4.3. Constructs were cultured for four days in 4.0mL of GM, ensuring complete replacement of the GM twice daily (equating to between 1.0mL and 2.0mL per million cells in 24 hours, initial construct configuration dependant). To induce cellular fusion, constructs were cultured in 4.0mL of DM for 10 days ensuring complete replacement of the DM every 24 hours.

![Figure 6.1: Macroscopic images of collagen-based skeletal muscle construct set-ups. C2C12 myoblasts suspended within a collagen hydrogel, fixed at either end by A-frames. Construct volumes: A) 2.0mL. B) 0.4mL. C) 1.0mL. D) 0.75mL. E) 0.28mL. Scale bar - 5mm](image-url)
6.2.4 Scaling Model and Dimension Variation

To ensure continuity of a model for scaling, image analysis and physical measurements were used to analyse the width and length of constructs described previously (Eastwood, Mudera, et al. 1998; Smith et al. 2012; Sharples et al. 2012). Depth was calculated from the initial pipetted construct volume and known dimensions of each construct. The scaling model was then devised using calculated surface area and known volume ratios.

Width reduction was assessed in constructs with total volumes of 0.28, 0.4, 0.75, 1.0, 2.0 and 3.0mL (Figure 6.1), in order to better understand how cell mediated contraction through initial attachment and remodelling was affected by dimension variation and scaling (Figure 6.2). These volumes were based upon a scaling model (Figure 6.3) and the vessels available to culture in (configurations shown in Table 6-1). The 3.0mL and 2.0mL constructs were cast along the length of bespoke glass chambers of 45 x 20 x 10mm (Soham Scientific, Ely, UK). The 0.75mL and 0.28mL constructs were set across the width of the chambers with a Sylgard boundary as the fourth wall. Nunc rectangular 8 well plates were used as setting areas for the 0.4mL and 1.0mL constructs with a Sylgard boundary to define the setting area. Constructs were set using a seeding density of four million cells/mL of total construct volume.

Figure 6.2: Dimension variation construct schematics showing individual construct relationships. 2.0mL and 0.28mL constructs are scaled. 3.0mL, 0.75mL and 0.4mL represented changes in depth, width relative to constant length and length relative to constant width respectively.
Figure 6.3: Scaling Model based on the width of the anchor point, and the length and volume of the construct, in conjunction with the V/SA ratio (1.3). For a given volume, dimensions of the A-frame and setting area can be obtained.

Table 6-1: A-frame and volume characteristics of collagen constructs.
Configurations based on previously published models (3.0mL), scaled constructs from 2.0mL model (1.0mL and 0.28mL) or dimension changes (Depth change - 3.0mL to 2.0mL; length increase relative to width - 0.28 to 0.4mL; width increase relative to length - 0.28mL to 0.75mL)

<table>
<thead>
<tr>
<th>Cell density (x10^6/mL)</th>
<th>Volume (mL)</th>
<th>Anchor Point Width (mm)</th>
<th>Distance Between Anchor Points (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>3</td>
<td>16</td>
<td>38</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>16</td>
<td>38</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>0.75</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>0.4</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>0.28</td>
<td>6</td>
<td>14</td>
</tr>
</tbody>
</table>
6.2.5 **Manufacturing Variables Optimisation Experimental Design**

Utilising elements of Quality by Design (Thomas et al. 2008) through factorial design of experiment (DOE) software (Minitab® Statistical Software, Minitab Inc., USA), the influences of variation in cell number/mL construct and size of width extension of collagen (Figure 6.4) on myotube characteristics were assessed. Individual construct configurations are shown in Table 6-2.

![Diagram of construct configurations](image)

**Figure 6.4: Manufacturing Variables Optimisation: Construct width extension schematics.**

<table>
<thead>
<tr>
<th>Cell density (x10^6/mL)</th>
<th>Width Extension (%)</th>
<th>Volume (mL)</th>
<th>Setting area (mm)</th>
<th>Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>50</td>
<td>2.0</td>
<td>Width 20  Length 28</td>
<td>4L</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>1.0</td>
<td>Width 10  Length 28</td>
<td>4N</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>1.5</td>
<td>Width 15  Length 28</td>
<td>3S</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>1.0</td>
<td>Width 10  Length 28</td>
<td>2N</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>2.0</td>
<td>Width 15  Length 28</td>
<td>2L</td>
</tr>
</tbody>
</table>

![Table of construct configurations](image)

**Table 6-2: Optimisation of manufacturing variables: Construct configuration. Setting area, volume, cell density and hydrogel width extension (measured as a % of the width of the A-frame. i.e. 25% refers to an addition of 25% to either side of the A-frame)**

6.2.6 **Long Term Culture**

3D tissue engineered skeletal muscle constructs were manufactured according to optimised configurations as described above. Construct were then cultured for four days in GM followed by 10 days in DM. At this point, cultures were either kept in DM for a further 20 days (medium replenished every two days) or switched to maintenance medium consisting (MM) of HG-DMEM + 7% FBS + 1% PS as described previously by Khodabukus & Baar (2009); Vandenburgh et al. (1996).
6.2.7 **Immunofluorescence**
Cultures were stained and imaged as described in Section 4.5

6.2.8 **Construct Width and Myotube Characteristics**
Macroscopic images of collagen constructs were analysed using ImageJ software [National Institutes of Health (NIH)] in order to determine construct remodelling, over the culture period. Each construct width was measured three times at the centre point where remodelling is typically most easily observed. Images were captured using an Epson Perfection V330 Photo flatbed scanner.

Fluorescent images of each construct, separated into regions (central half - n=9 images, two A-frame quarters - n=10 images) were analysed (ImageJ, NIH) for mean length and width (measured at respective maximums) and alignment of myotubes per image (Figure 6.5). Mean individual myotube area (an analogue of individual myotube volume) was calculated per image from the mean length and width measurements. The number of images taken was assessed as generating a representative mean as indicated by stable mean analysis (Appendix 5).

![3D skeletal muscle construct example myotube length/alignment measurement.](image)

*Figure 6.5: 3D skeletal muscle construct example myotube length/alignment measurement. Long axis of myotube denoted by white arrow and bars. Each myotube characteristic was measured in triplicate. Red – Desmin; Blue- DAPI.*
6.2.9 Initial Attachment Force Measurement (Culture Force Monitor)
To assess the initial force generation by cellular attachment in cultures with and without width extension, tissue engineered constructs were set according to Section 6.2.3 and then transferred onto the culture force monitor (CFM) (Mudera et al. 2010; Player et al. 2014; Cheema et al. 2003). Construct force generation was measured for the first 24 hours of culture by a force transducer (Figure 6.6). The force transducer (Measurement Group) utilises a strain gauge to measure deflection of the cantilevered beam. The strain was captured at a rate of one data point every minute.

![Force Transducer, Stationary point, Culture Chamber](image)

Figure 6.6: Culture Force Monitor (CFM) schematic detailing PTFE construct culture chamber, force transducer and stationary point. Constructs placed with frames hooked onto the transducer and stationary point. As the cellular population attaches, the construct reduces in length causing the force transducer to flex and record this force as micro-strain.

6.2.10 Statistical analysis
Construct width reduction/myotube width correlation analysis was conducted using Pearson’s product moment correlation coefficient (PMCC) in Minitab® Statistical Software, (Minitab Inc., USA). DOE pareto and optimisation analysis was also conducted in Minitab. All other statistical analysis was performed using GraphPad Prism (GraphPad Software, USA). Significant differences were assessed using a two-way ANOVA with a Bonferroni post-hoc correction for data with significant F-ratio values. Alpha was set at \( p<0.05 \).
6.3 Results

6.3.1 Monolayer and 3D Culture Comparison

Currently, standard toxicity testing involves monolayer cell culture and so to better understand the advantages and limitations of monolayer and 3D cultures, a direct comparison is needed. Brightfield microscopy and immunofluorescence imaging in conjunction with image analysis was used to highlight distinguishing features and assess characteristics of each culture type.

6.3.1.1 Pre- and Post-Differentiation

Pre-differentiation (day of transition to differentiation medium, DM) monolayer cultures showed areas of spontaneous alignment within mononuclear populations (Figure 6.7 Left). However, for the majority of the population, no distinct orientation was found. Within the 3D collagen constructs, the biomimetic collagen matrix provides a visco-elastic matrix to which the cells can attach and remodel. These myoblasts align along lines of uniaxial tension (Figure 6.8), although two distinct regions were evident. In the central half of the construct, cells within the collagen were seen to be the least directionally restricted, and could therefore be described as highly aligned and elongated (Figure 6.7 Right-A). Due to the superposition of forces applied by the attachment of the cells, the central edges should experience the most deflection. However, due to the increased matrix and cellular density, brightfield imagery is unable to resolve individual cells in this area. Adjacent to the anchor point, multiple boundary conditions cause multi-directional cell attachment, which projects away from the anchor point for approximately a quarter of the length of the hydrogel (Figure 6.7 Right-B).

Figure 6.7: Light micrographs of pre-differentiated culture (day of transition to DM). Scale bar - 100 µm. (Left) Monolayer culture on glass coverslips. 100% confluency after 5 Days culture. (Right) Collagen gel construct. Four days culture depicting (A) the centre and (B) the A-frame region gel. Arrow represents alignment of cells within the gel.
Figure 6.8: Schematic of skeletal muscle collagen construct. Cells align along lines of uniaxial tension (green), created by cellular attachment and remodelling (red arrows).

After the addition of DM at confluency, transitioning monolayer cultures (Figure 6.9 Left) displayed large numbers of elongated cell bodies. These elongated cell bodies formed both aligned and randomly aligned areas. This could be due to cells that fuse first, forming a topographical ridge against which adjacent cells can align. However, randomly aligned areas are more common and are most likely an effect of cell layering and a lack of directional stimulus through tension or topography. In comparison, brightfield imagery of 3D constructs shows highly aligned cultures within the central region (Figure 6.9 Right-A), although individual cellular bodies are difficult to distinguish. In the A-frame region, cells continue to appear attached in multiple directions, although the general shape is more elongated longitudinally along the construct (Figure 6.9 Right-B).

Immunofluorescent imaging confirms the presence of myotube formation through nuclei association with the intermediate filament protein, desmin (Figure 6.10, Left). Within the monolayer culture, alignment of the myotubes is predominantly random with areas of parallel alignment. Within the collagen constructs, immunofluorescence allows for greater determination of cell morphology although position of the nuclei within the myotubes is difficult to discern (Figure 6.10, Right). High cell density at the edges form due to a reduction in the width of the collagen scaffold. Compared to fusion within the monolayer cultures, the filaments appear thinner and were less uniformly distributed. This is most likely because of greater cell proximity in monolayer. Full differentiation was considered to have occurred after three days in DM in monolayer, after which detachment was observed. In 3D cultures, a 10 day differentiation period was used, so despite a more in vivo-like alignment within 3D cultures, fusion within monolayer cultures happens at a much reduced timescale.
Figure 6.9: Brightfield images of monolayer and 3D models of C2C12 differentiation cultures. (Left) Aligned area of monolayer culture on glass coverslips. Two days in differentiation medium (DM). (Right) Collagen gel construct at four days in DM. (A) Centre region (B) A-frame region. Scale bars - 100 µm.

Figure 6.10: Fluorescence imaging of monolayer and 3D differentiated cultures. Scale bars - 100 µm Red - Desmin. Blue - DAPI. (Left) Monolayer culture after two days in DM. (Right) Centre of collagen constructs after 10 days in culture.

6.3.1.2 Differentiated Culture Cellular Alignment

Analysis of myotube alignment from desmin staining (Figure 6.11) further indicates the random alignment of myotubes, within the monolayer culture, where a large distribution of the mean direction of the long axis exists. The 3D culture had a much smaller distribution with a prominent peak, indicating high alignment within the structures at both the Central and A-frame regions.
Figure 6.11: Monolayer and 3D culture myotube alignment. Monolayer culture alignment 0° taken as horizontal axis of each image. 3D culture alignment 0° taken as average alignment for all construct images. 3D constructs n=2 constructs x 2 passages, monolayer cultures n=3 wells + 3 replicates per well.

6.3.2 3D Tissue Engineered Skeletal Muscle Model

6.3.2.1 Dimension Variation and Scaling Model

The majority of recent work in this tissue engineered skeletal muscle model has focussed on using 3.0mL of collagen (Table 6-3). This model is predicated on using a simple collagen matrix with a seeding density of 4 million cells/mL with an approximate length to width ratio of 3:1 (~2.5:1 plus A-frame length); a ratio that was previously defined in a larger 5.0mL model. Analysis of the physical characteristics of these previously published biomimetic constructs (5.0mL, 3.0mL and 2.0mL constructs) showed a volume to surface area (V/SA) ratio of ~1.3 (Table 6-3) in both the 5.0mL and 2.0mL constructs. The 3.0mL construct had a V/SA ratio of 1.72. Larger V/SA ratios imply a greater distance between cells at the centre of the constructs and the surrounding media, indicating less access to nutrients. Therefore, based on the width of the anchor point and the length and volume of the 5.0mL and 2.0mL models, in conjunction with a V/SA ratio of 1.3, a scaling model was developed to enhance the flexibility of this skeletal muscle model for use in pre-clinical testing (Figure 6.3). This enabled a degree of continuity between previous publications and the work reported here (Eastwood, Mudera, et al. 1998; Smith et al. 2012; Sharples et al. 2012).
Table 6-3: Dimension analysis of previously published constructs.

Analysis conducted using image analysis and physical measurement of culture areas. Depth, Surface Area (SA) and Volume (V) to SA ratio calculated from known dimensions and known volume quantities. 5.0mL and 2.0mL models demonstrate similar V/SA ratios indicating similar cellular nutrient access. 3.0mL has a greater V/SA ratio indicating potentially lower cellular nutrient access.

<table>
<thead>
<tr>
<th>Volume (V) / mL (mm³)</th>
<th>Length / mm</th>
<th>Width of A-frame / mm</th>
<th>Depth / mm</th>
<th>Surface Area (SA) / mm²</th>
<th>V/SA (mm³/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 (5000)</td>
<td>65</td>
<td>25</td>
<td>3.08</td>
<td>3803.85</td>
<td>1.31</td>
</tr>
<tr>
<td>3 (3000)</td>
<td>38</td>
<td>16</td>
<td>4.93</td>
<td>1748.89</td>
<td>1.72</td>
</tr>
<tr>
<td>2 (2000)</td>
<td>38</td>
<td>16</td>
<td>3.29</td>
<td>1571.26</td>
<td>1.27</td>
</tr>
</tbody>
</table>

To confirm consistent cell-matrix interaction responses in scaled models and to better understand the effects of length, width and depth change, the constructs defined in Table 6-1 were analysed for hydrogel width reduction, an indicator of cell attachment and remodelling. Width at the centre of each construct was analysed through repeated measures of macroscopic images of each construct using image analysis (Figure 6.12). After 14 days in culture, different length to width ratios produced significant differences in final width reduction. Both the 0.75mL (p<0.001) and 0.4mL (p=0.06) construct width reduced to a lesser extent than the 0.28mL constructs although 0.28mL exhibited greater variability, possibly due to the measurement resolution. However, a change in hydrogel depth (3.0mL to 2.0mL) or a scaled decrease (2.0mL, 1.0mL and 0.28mL) in size did not have a significant effect after 14 days (p>0.05). A reduction in width implies a decrease in the distances between individual cells allowing for greater fusion through increased contact. Although, there were no significant differences between the 3.0mL and 2.0mL models, the 2.0mL construct is a more advantageous design on which to base a scaling model. This is due to a reduction in material and cellular resource use over the deeper 3mL construct and the potential for greater cellular access to nutrients from the lower V/SA ratio.
Figure 6.12: 3D collagen construct dimension variation: construct centre final width (% of initial width). Width reduction at centre of construct. For construct dimensions see Table 6-1. Comparisons between 3.0mL and 2.0mL demonstrate effects of depth change; 0.28mL and 0.4mL show effects of a high length to width ratio; 0.28mL and 0.75mL show effects of a high width to length ratio. 2.0mL, 1.0mL and 0.28mL are scaled constructs. (n=2 constructs x 2 passages, * p ≤ 0.05, *** p ≤ 0.001, bars represent ±SD). Changing length to width ratios (0.75mL and 0.4mL) relative to scaled constructs (2.0mL, 1.0mL and 0.28mL) show less width reduction. Depth change does not significantly affect width reduction.

6.3.2.2 Width reduction/Myotube characteristic correlation analysis

The relationship between hydrogel width reduction and microscopic structure was also investigated to determine whether attachment and remodelling affected myotube formation. The 1.0mL model (scaled construct) was chosen for subsequent analysis due to the flexibility of the 8-well plate system used for culture, a reduced resource use and similarity in construct width reduction to the 2.0mL. Following quantification of myotube length, width and alignment, a correlation was determined to exist between mean width of myotube for the whole construct and width reduction of the collagen hydrogel as a percentage of the initial width (1.0mL construct). This was described as follows:

\[ y = -14x + 1207.6 \]  

\[ (Pearson \ PMCC = -0.977, \ p=0.023, \ n=4) \]

Where \( y \) = myotube width and \( x \) = % width after 14 days. This correlation suggests that a smaller width after 14 days corresponds to a greater individual myotube width. This indicates a potential non-invasive method of distinguishing construct variation and prediction of myotube characteristics.
6.3.2.3 Optimisation of Manufacturing Variables

Three-dimensional tissue-engineered models are wholly reliant on the interaction effects of the cellular population and the culture environment (media contents, matrix material, topography and mechanical forces). To define the optimum conditions for a model suitable for pre-clinical experimentation that best represented in vivo architecture, the effects of varying the cell density and extending the hydrogel width past the A-frame were investigated. Variation in the cell density changes the number of cells available for fusion and changes the magnitude of cell-mediated tension within the hydrogel scaffold. Width extension increases the surface area of adhesion of the hydrogel around the A-frame and changes the mechanics of the construct by increasing the material available to remodel, thereby changing the superposition of force created by cell attachment. Skeletal muscle and myotendinous junctions are morphologically driven tissues; therefore, the descriptive characteristics of the myotube population (length, width and alignment) and prevalence of detachment from the A-frames were used as determining factors for construct optimisation to best represent in vivo architecture. All constructs for this study were based on the length and anchor point dimensions of the 1.0mL (Table 6-1) model due to no significant differences in cell-matrix interactions (construct width reduction) to the previously established 3.0mL model and the ease of multiple condition experiments in identical vessels.

To understand the effects of hydrogel width extension and cell density on cell-mediated matrix contraction and remodelling, relative width reduction after 14 days was assessed for each configuration (Figure 6.13). This demonstrates that regardless of extension, the final width (as a percentage of the initial measurement) does not vary significantly between configurations, indicating an equilibrium point between cell-mediated contraction forces and the stress-strain resistant properties of the collagen matrix. Based on these reduction figures, the final construct volume, and therefore final cell density, can be calculated (Table 6-4). Two million C2C12s/mL with no extension (2N) had a calculated final volume that was significantly greater (p<0.05) than all other conditions with no other final volume differences between configurations. This implied a lower final cell density in 2N constructs potentially resulting in lower levels of fusion. Significant differences (p<0.05) between calculated final cell densities of 4 million/mL with no extension (4N) and 2 million/mL with a large extension (2L), and between 2L and 3 million/mL with a small extension (3S) were also apparent. No other significant differences occurred. This indicates that the size of width extension influences the calculated effective final cell density, potentially influencing the number of cells available for myotube formation and therefore fusion.
Figure 6.13: Width Reduction at centre of each construct (% of original width).
After 14 days culture, there are no significant differences between constructs. (n=2 constructs x 2 passages, * p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001, bars represent ±SD). This indicates that there are no significant observable macroscopic effects on cellular attachment and remodelling.

Table 6-4: Calculated final cell density (millions/mL) from calculated final volume (assuming constant depth and no net change in cell number).
Initial manufacturing conditions, cell density and width extension affect final cell density and therefore the number of cells available for fusion. 4L has a significantly greater calculated cell density and 2N has a significantly lower final cell density that all other conditions.

<table>
<thead>
<tr>
<th>Construct Configuration</th>
<th>Calculated Final Volume (mL) ± SD</th>
<th>Calculated Final Cell Density (x10^6/mL) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>4L</td>
<td>0.52 ± 0.02</td>
<td>15.52 ± 0.74</td>
</tr>
<tr>
<td>4N</td>
<td>0.65 ± 0.03</td>
<td>6.20 ± 0.27</td>
</tr>
<tr>
<td>3S</td>
<td>0.56 ± 0.02</td>
<td>7.98 ± 0.24</td>
</tr>
<tr>
<td>2N</td>
<td>0.70 ± 0.07</td>
<td>2.85 ± 0.30</td>
</tr>
<tr>
<td>2L</td>
<td>0.56 ± 0.05</td>
<td>7.19 ± 0.63</td>
</tr>
</tbody>
</table>
Alignment within the myotube population is a result of tension within the construct and remodelling caused by cell-matrix interaction, and is an important feature of *in vivo* skeletal muscle architecture. In the central region, the cells are limited from pulling longitudinally by the A-frames but are able to reduce the width. In the area around the A-frame, the width of the anchor point also acts as a limiting factor; so the mechanical forces acting on the cellular population produce differing alignment. The collagen model demonstrates that there is observable variation in myotube orientation within each region (Figure 6.14). Four million/mL with a large extension (4L) shows significantly less myotube alignment (*p*<0.05, Figure 6.14 bar chart) than all other conditions in the A-frame region whilst demonstrating greater alignment in the central half. Between construct sections, significant differences are found in the mean width (*p*<0.05) and alignment (*p*<0.001) of 4L and in the alignment of 2L (*p*<0.05), 3S (*p*<0.001) and 4N (*p*<0.05). Alignment was found to be significantly greater within the central region, compared to the A-frame region (*p*<0.05) with the exception of 2N. This demonstrates how the attributes of the cellular population is heavily defined by their interaction with the surrounding matrix and the mechanics generated from different configurations. The myotubes are highly aligned along the edges of the constructs where they are directed inwards due to cell-mediated width reduction, whilst cells close to the centre of the anchor point are limited from contracting the matrix due to the boundary nature of the A-frames.

In order to accurately represent the *in vivo* structure of highly aligned muscle fibres, it is important to understand the variation in myotube formation. Interactions between the mechanical forces generated through cell attachment and remodelling, the effective cell density and the alignment of the myotube population affect the Central and A-frame region’s myotube populations differently (Figure 6.15). From immunohistochemical observations of myotube characteristics, 4L produces myotubes of significantly greater length (Figure 6.16A, *p*<0.05) than other conditions in the central region of the construct. Myotube width remained consistent between conditions in both regions. Configuration 4L also demonstrated the largest variability across repeats. However, 4N produced a significantly greater (*p*<0.05) number of myotubes in both regions (Figure 6.16B). A greater mean length of myotube within 4L but lower numbers of myotubes may be explained by the smaller myotubes having fused to form larger structures and therefore having depleted the total number of myotubes present. This implies that greater levels of remodelling could enhance myotube formation whilst greater tension could enhance the distribution.
Figure 6.14: Construct myotube angular distribution.
Angle in which 90% of distribution lies. Graphical representations show alignment distribution as degrees from horizontal. Bar chart shows significant relationships. Lower values represent greater alignment. A-frame region alignment is lower than in the central region. n=2 constructs x 2 passages, * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, bars represent ±SD. Larger width extensions exhibit more aligned myotube populations within the central region (not significant) but lower alignment within the A-frame region 4 million/mL with large width (4L) extension significantly lower alignment than all other conditions. Higher cell densities also increased alignment in the central region (not significant except 2 million/mL with no extension (2N) significantly less aligned than 2 million/mL with large extension (2L) and 4 million/mL with large extension (4L)). Full alignment graphs shown in Appendix 5.
Figure 6.15: Myotube population characteristics showing variation between regions (Red-Desmin).

Figure 6.16: Construct myotube characteristics per image by region.
(A) Mean myotube length, (B) Mean number of myotubes. (n=2 constructs x 2 passages, * p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001, bars represent ±SD). 4 Million C2C12s/mL with a large extension produces significantly larger myotubes than all other conditions in the central region. Legend applies to A and B.
Table 6-5: Pareto charts of standardised effects detailing significant effects on each myotube characterisation parameter by each manufacturing variable (A - seeding density, B - Width extension) or an interaction (AB) effect in each region.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>A-frame</th>
<th>Centre</th>
<th>Whole Construct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Length</td>
<td><img src="chart1.png" alt="Chart" /></td>
<td><img src="chart2.png" alt="Chart" /></td>
<td><img src="chart3.png" alt="Chart" /></td>
</tr>
<tr>
<td>Mean Width</td>
<td><img src="chart4.png" alt="Chart" /></td>
<td><img src="chart5.png" alt="Chart" /></td>
<td><img src="chart6.png" alt="Chart" /></td>
</tr>
<tr>
<td>Mean Number of Myotubes</td>
<td><img src="chart7.png" alt="Chart" /></td>
<td><img src="chart8.png" alt="Chart" /></td>
<td><img src="chart9.png" alt="Chart" /></td>
</tr>
<tr>
<td>Alignment</td>
<td><img src="chart10.png" alt="Chart" /></td>
<td><img src="chart11.png" alt="Chart" /></td>
<td><img src="chart12.png" alt="Chart" /></td>
</tr>
</tbody>
</table>

Significant effects are denoted by the relevant bar passing the red line. Red line denotes significance ($\alpha = 0.05$).
The myotube characteristic results were analysed in Pareto charts (Table 6-5) to determine whether cell density, width extension beyond the A-frame, or an interaction of the two factors influenced myotube population characteristics significantly (n=4, p<0.05). Comparison with Figure 6.14 and Figure 6.16 determined the direction of effect (positive or negative). Increasing cell density was found to significantly increase the mean myotube length (centre and whole construct), mean number of myotubes (whole construct) and alignment in the central region. Increasing width extension resulted in a significant increase in the mean myotube length (centre), mean number of myotubes (centre) and alignment (centre). The interaction of cell density and width extension positively affected the mean number of myotubes present in the A-frame region, mean number of myotubes (whole construct), mean myotube width (A-frame) and alignment in the A-frame region. These results demonstrated that within this model, isolated effects (cell density or width extension) were predominantly limited to the central region, whereas interaction effects (cell density and width extension) are mostly limited to the A-frame region. Myotube width was not as significantly affected, regardless of cell number or width extension. By increasing cell density and material available to remodel through hydrogel width extension, cell-cell proximity may be enhanced, leading to an increase in cell-cell interactions. Therefore, it could be postulated that variations in myotube characteristics are predominantly affected by cell-cell interactions as opposed to cell-matrix interactions due to combination effects only having significance at the A-frame (other than the mean length across the construct).

Using the response optimising feature of the DOE function, mean myotube length, mean number of myotubes, and mean myotube width were set to be maximised and the range of myotube alignment minimised to best represent in vivo architecture. This allows for the configurations of each measured output of each construct to be analysed and assessed according to desired characteristics. Of the tested configurations, a cell density of 4 million/mL with no extension was calculated to produce the most desirable morphological outputs (Figure 6.17).
Figure 6.17: Construct myotube characteristics optimisation plots.
Myotube characteristic responses maximised; myotube population response, (alignment) minimised. Shows optimum conditions based on tested configurations are 4 million C2C12s/ml with no hydrogel width extension.

Along with biological structural validation, the physical success of the construct’s manufacture is an important part of the optimisation process. Constructs with a cell density of 2 and 3 million cells/mL did not rupture from the A-frames during the culture period (n=8 and 4 respectively), whereas constructs of 4 million/mL with no extension detached from the A-frame 33.3% of the time (n=6) after which the construct must be discarded. By increasing cell density, the number of cells interacting with the matrix is increased and in turn, the tension through cell mediated contraction and remodelling is also greater. This places greater stress onto the site of collagen attachment to the A-frames and the collagen is therefore more susceptible to detachment. By adding a width extension, the collagen is able to adhere around the A-frame, increasing the surface area of attachment. Within constructs of 4 million cells/mL density, a width extension reduced the prevalence of detachment despite an increase in the total cell number in the construct and without compromising morphology. Therefore, to keep the incidence of detachment as low as possible whilst maintaining morphological features observed under optimised conditions, a small extension of width would be necessary.
6.3.2.4 Confirmation of Optimisation and Scaling Model

In order to produce myotubes with the greatest alignment and to maintain favourable myotube characteristics (number, length and width), whilst decreasing hydrogel detachment rates, a cell density of 4 million/mL with a minimal width extension has been determined as optimum. Due to the artisan nature of the manufacture of these constructs, in the smallest tested model, 14mm length between A-frames, an extension of less than 25% becomes untenable due to the inability to accurately produce width extensions smaller than this. Therefore, following optimisation, 4 million cells/mL was chosen as the optimum cell density with a modified extension of 25% to increase the total width of collagen by 50% of the width of the A-frame. This configuration was then validated against the “no width extension” model using myotube characteristics in 1.5mL (modified 1.0mL model) and 0.42mL (modified 0.28mL model) constructs to confirm the adjusted parameters and model for scaling.

As evidenced by Figure 6.18, no significant differences were seen between the “optimised” configuration, 4N, and 4S except in favour of the adjusted model. Likewise, between the 1.5mL and 0.42mL constructs, indicating that 4 million cells/mL with a 25% extension either-side of the anchor point is a suitable, scalable model to take forward for future experimental testing. Comparisons with 4L results also show that a 25% extension (S - small) is associated with significantly larger myotube length (central region) as observed in the larger width extension model, thus eliminating the weaknesses of the no extension model. This also validates the scaling model, enhancing the flexibility of the system.
Figure 6.18: Skeletal Muscle Model Validation. 
(A) Mean myotube length, (B) Mean calculated myotube area, (C) Mean number of myotubes, (D) Myotube angular distribution (lower values indicate greater alignment). (n=2 constructs x 2 passages, * p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001, bars represent ±SD). 4 million C2C12s with a small (S) width extension (25% of the width of the A-frame added to either side) demonstrates no significant differences between No extension (N) constructs except in favour of the modified construct. Legend applicable to all graphs.
6.3.2.5  **Effects of Initial Muscle Precursor Cell Attachment Force**

To better understand the cell-generated mechanical forces that result in construct detachment from the A-frame, the initial attachment forces of the optimised construct configuration (4 million cell/mL + 25% width extension) was compared with 4N constructs (Figure 6.19). By measuring the initial cellular attachment force, it becomes apparent that despite a total of 6 million cells within the extended constructs, these constructs produces less than 50% of the total force of those constructs with 4 million cells per construct (no extension). The increase in force witnessed in the 4N compared to the 25% width extension is likely responsible for the detachment of the collagen from the A-frame.

![Graph](image)

**Figure 6.19**: Initial construct mean force production through cell mediated contraction and remodelling (micronewtons, µN) (n=3 constructs per condition across three passages). Constructs with width extension refers to an addition of 25% of the width of the A-frame to either side.
6.3.2.6 Long Term Culture

To successfully create a model of the musculoskeletal system, each component must be capable of culture duration equal to the component with the greatest time to maturity. Three dimensional bone models are described as having a culture period of up to 33 days to attain maturity. The optimised skeletal muscle model was cultured following two protocols; 4 days in GM followed by 30 days in DM or 4 days in GM followed by 10 days in DM then maintained for 20 days MM.

Both culture protocols resulted in a similar timeline (Figure 6.20). Cultures proceeded as normal to 14 days; cells attached to, then remodelled, the collagen matrix followed by skeletal muscle precursor cell fusion to form myotubes. Alignment and density observably increased up to 28 days. After which, abnormal morphological features were observed in both medium protocols. Large circular vesicles appeared within myotubes with large areas of rounded bodies within the collagen matrix, which is indicative of cell death or detachment from the matrix. In addition, myotubes were observed to become detached from the collagen matrix along the majority of the myotubes length (Figure 6.21). This detachment is most likely caused by un-fused C2C12s continuing to remodel the collagen over this time period.

Immunofluorescence for desmin yielded inconclusive results with large amounts of debris and nonspecific staining apparent. Myotube structure was therefore obscured and made any further conclusions unobtainable.
Figure 6.20: Representative morphological timeline of C2C12 cultured within optimised 3D collagen model for 33 days.
(4 days in high glucose DMEM + 20% FBS + 1% PS followed by 30 days high glucose DMEM + 2% HS + 1% PS). Brightfield images of A) 19 days (x10) B) 23 days (x20) C) 31 days (x10) D) 33 Days (x10). Increasing alignment of myotube morphology up to day 28, after which abnormal morphology (circular vesicles within myotubes, indicated by \( \rightarrow \)) and rounded bodies were observed (indicative of cell death). \( n=4 \) constructs from a single passage.

Figure 6.21: Brightfield image of myotube mostly detached from the collagen scaffold (indicated by \( \rightarrow \)). One end of the myotube remained attached to the collagen. 31 days in culture (4 days in high glucose DMEM+20% FBS+1% PS followed by 27 days high glucose DMEM + 2% HS + 1% PS) (x10 magnification). \( n=4 \) constructs from a single passage.
6.4 Discussion
This work sought to define the manufacturing variables required to produce a consistent, scalable skeletal muscle model towards a pre-clinical analysis system and to assess the effects of long term culture in preparation for future 3D tissue engineered bone co-culture. In particular, the effect of dimension variation on cell-mediated width reduction was investigated. Additionally, the effects of cell density and hydrogel width extension on myotube characteristics were assessed using a factorial DOE paradigm. This represents a continuation in the development of the collagen model first reported by Cheema et al. (2003) by demonstrating a model that is both reproducible in inherent myotube population characteristics and capable of being scaled. This enhances the flexibility of the system, so that it can be used for different purposes, for example, mechanical stimulation (Player et al. 2014) or high-throughput assessment. Finally this work sought to assess the long term culture limitations of this model in preparation for future 3D co-culture.

Currently, bio-toxicity testing for materials and drugs takes place using monolayer models (ISO10993). However, these tests are normally conducted using suggested cells that are irrelevant to the target tissues (i.e. Chinese Hamster Ovary (CHO) cells to test bone implant materials) or relevant cells only with specific justification (ISO10993) rather than as standard practice. Tissue engineering shows huge promise in creating representative in vitro models of in vivo systems which can be used as test-beds for pre-clinical material and drug testing (Nam et al. 2014, Ferrec et al. 2001, Reed et al. 1999, Ryan et al. 2001, Osborne et al. 1995). However, due to resource costs and the current artisan nature of production, the majority of studies currently use very low numbers of statistical repeats. To increase these numbers, the ability to miniaturise models is of vital importance.

As a monolayer culture, the C2C12 murine myoblast displays classic muscle precursor cell characteristics and a culture timeline which features proliferation to full confluency and the potential for spontaneous differentiation. The C2C12 cell line also displays proteins, desmin and actin, identified as specific to muscle growth and sarcomeric formation (Paulin & Li 2004; Shah et al. 2004; Sciote & Morris 2000). Monolayer and 3D cultures display many similar characteristics, most importantly cellular fusion. However, in trying to create a representative biomimetic model of skeletal muscle, the 3D design of the collagen model best represents the in vivo alignment of myofibres. The 3D construct also allows for assessment of force generation facilitated by the suspension of the matrix in the culture vessel. This is not possible in a traditional well plate setting. Monolayer cultures do have an advantage in timescale and resource use requiring less medium and a 7 day culture period when compared to a 14 day culture period in 3D.
Published literature provide examples of a tissue engineered model reported within groups with different physical characteristics tending towards smaller scale models with functional output (Powell et al. 2002, Vandenburgh 1998, 2008, 2010). However, the manufacturing processes and resulting effects on such systems have yet to be reported. Previous work with 5.0mL and 3.0mL constructs has characterised the structure and myogenic gene transcription for the system presented here (Cheema et al. 2003, Smith et al. 2012, Player et al. 2014). To create a successful model of scaling, consistent reproduction of the basic characteristics of larger models is necessary.

To determine the effect of changing construct dimension ratios on the attachment of the C2C12’s to the collagen hydrogel, and their subsequent remodelling of the matrix, the width reduction of each construct was analysed. Once myoblasts have been seeded within the construct, they attach and remodel the collagen matrix prior to fusion (Sharples et al. 2012, Mudera et al. 2010). This attachment causes a reduction in width that is most apparent at the centre of the construct. Within soft tissue models (Smith et al. 2012, Dennis & Kosnik 2000, Garvin et al. 2003) a reduction in the size of the construct is used as an observable measure of the effect of cell-matrix interaction (Smith et al. 2012). After 4-5 days of culture, the C2C12s within the collagen matrix presented here show an equilibrium of mechanical forces, demonstrated by no significant reduction after this time point. In the first 4-5 days of culture, a width reduction in excess of 50% occurs, echoing reports within primary cell models. Smith et al. (2012) reported a 60% reduction in construct area (greater than 50% reduction in width) in the first 14 days using primary rat muscle derived cells. In other models based around the delamination of cellular populated matrices (Martin et al. 2013, Dennis & Kosnik 2000, Garvin et al. 2003), matrix contraction and remodelling is also shown to reduce construct size. A smaller width would imply a greater cell density at the point of greatest reduction, and since myoblasts require contact to fuse (Krauss et al. 2005), a greater density would suggest the capacity for larger or more numerous myotubes. This link has been confirmed in this model through correlative data from immunohistochemical analysis of myotube characteristics within a 1.0mL construct (Section 6.3.2.2), which suggests that width reduction may be used as an indicator of myotube formation.

To create a truly biomimetic model, common practice is to use a “function follows form” paradigm (Smith et al. 2012). In the case of tissue engineered skeletal muscle, a randomly aligned myotube population would not be able to contract efficiently in a single direction. Therefore, it is necessary to replicate the aligned structures of in vivo skeletal muscle tissue. Alignment within tethered constructs is inherently created by cell attachment and remodelling, however, alignment is shown here to be further impacted by the cell density and the size of matrix extension past the anchor point. With a larger number of cells initially embedded within the collagen, ensuring that the
collagen matrix is wider than the A-frame may allow for greater remodelling. In the central region of the construct, the limiting effects of the A-frame are least apparent (Eastwood 1998, Smith et al. 2012), consequently, this area is most aligned. In addition, width extension seemingly increases alignment in the central region and decreases alignment within the A-frame regions. Constructs reported within the literature with regions outside the width of the anchor points demonstrate enhanced remodelling and self-organisation of myotubes (Huang et al. 2005). Considering these parameters, 4 million cells/mL with a 25% width extension was found to be optimum.

The functional capabilities of skeletal muscle have also been linked to the number and size of fibres (Hortobagyi et al. 2000, Fitts et al. 2001). In vivo myotubes are much smaller than in vivo skeletal muscle fibres and therefore larger myotubes better represent in vivo structures. Therefore, the basic properties of the engineered skeletal muscle described here were analysed based on myotube size characteristics. Regardless of the size of width extension and cell density, myotube characteristics did not tend to differ significantly. Considering the variation in the number of myoblasts available for fusion (total cell number/construct differed depending on volume), this result is seemingly paradoxical. However, within primary cell populations, myoblasts are shown to have limited remodelling capability (Smith et al. 2012, Martin et al. 2013, Brady et al. 2008). This may mean that the C2C12 population is constrained within the collagen fibril matrix and only able to access a localised population for fusion and another cell type with greater ability to remodel may be required to more accurately represent in vivo tissue.

As previously mentioned, the greater the density of muscle precursor cells, the more cellular contact there is and therefore the greater the potential for fusion to occur (Mudera et al. 2010, Neumann et al. 2003). We investigated three cell densities and found that the four million C2C12s/mL is the optimum seeding density as assessed by factorial DOE analysis of myotube characteristic. Greater cell densities (6 million cells/mL) resulted in hydrogel detachment 100% of the time. A 4 million cells/mL seeding density has previously been reported in collagen-based constructs (Cheema et al. 2003), although the specific nature of the optimisation was not discussed. The smallest construct shown here uses 1.68 million cells per construct, compared to previous iterations which have required far greater cell numbers, (20 million C2C12s total, Cheema et al., 2003; 12 million C2C12s, Player et al. 2014), and thus demonstrates a reduction in cellular resources of over 85%. Within primary muscle-derived models, up to 5 million muscle derived cells per ml were successfully cultured within the 3.0mL model iteration (Smith et al. 2012), equating to 15 million cells per construct, although the myoblast percentage can differ between cultures depending on cell source. Within our 0.42mL construct, this would be reduced to 2.1 million cells, meaning that for the same number of cells present in the larger model, seven 0.42mL constructs could be manufactured. In
other model designs, utilising a delamination process (Khodabukus and Baar 2009, Martin et al. 2013), a smaller number of cells are initially seeded, although a period of proliferation is shown to occur prior to differentiation, making a direct comparison of cells present prior to fusion difficult. Vandenburgh et al. (2008) have also reported a scaled embedded cell model for high-throughput. In this set up, two million cells/mL of matrix has been used, resulting in 200,000 cells per construct (4mm length). In these models, the advantages of a lower seeding density are offset by the initial cost of the reagents used in manufacture and the complex nature of culture (mixing of reagents and inclusion of biochemical stimulants i.e. growth factors).

Optimisation and validation has shown that a 25% extension of the collagen matrix beyond the width of the anchor point, generated myotube characteristics most representative of in vivo structure and the lowest incidence of detachment. An extension of hydrogel width increases the effective final cell density (4L - 15.52 ± 0.74 x10^6 cells/mL, 4N - 6.20 ± 0.27 x10^6 cells/mL) as well as increasing the surface area of adhesion to the anchor points, whilst attempting to maintain the mechanical forces required for myotube formation. It also decreases the likelihood of detachment when compared to a model with no width extension. Physical success of the construct is linked to resource efficiency and cost, therefore it is an important characteristic of any in vitro model. Considering the biological outputs of the majority of tissue engineering literature, failure rates are not often studied and yet, for pre-clinical testing or implantation, the consistency of the model is of paramount importance. Smith et al. (2012) reported that above a certain cell density (six million cells/mL), the collagen hydrogel matrix detached from the anchor points. This was considered to be due to the interaction of the cells and the matrix generating too much tension through remodelling of the collagen. Study of this initial attachment force showed that constructs with a 25% width extension has a peak force approximately half of those constructs without extension (~1200µN compared to 550µN). A previously defined C2C12 collagen model (Cheema et al. 2003) also obtained a force of approximately 1200µN after 24 hours (5mL construct at 4 million per mL). However, in the model presented here with no extension, a peak force was obtained after 8 hours in comparison to a continuing gradient within Cheema et al. 2003. It is possible that the overall magnitude of the force generated within the 25% width extension model is greater than no extension models due to a higher initial cell number; however, due to the excess material present, this force may be dissipated across the construct.

Fibroblast cultures have been shown to produce much greater initial attachment and remodelling forces (Cheema et al. 2003; Eastwood et al. 1998) suggesting that a mixed cell type (biopsy based primary cell cultures) would need further optimisation to avoid detachment from the A-frames, as described in a fibrin model by Martin et al. 2013. Introduction of a 25% width extension either-side
of the A-frame may allow for greater cell densities, and therefore greater potential for cell fusion, within primary cultures, whilst reducing the absolute cell requirements through a reduction in construct size.

To allow for multi-tissue culture, it is important to ensure that a system is as flexible as possible. Three dimensional bone models are often described as being cultured in excess of 20 days to obtain maturity (Du et al. 1999; Liu & Williams 2010; Jones et al. 2010; Lian & Stein 1992; Chatzinikolaidou et al. 2015). Therefore, it was necessary to determine culture period limits for this construct. Brightfield observations of the skeletal muscle construct indicate a threshold culture period of 28 days, regardless of nutrient protocol. Unfused C2C12s can remodel a hydrogel matrix (Sharples et al. 2012); therefore, myotubes that have been detached from the matrix may have had the collagen removed from around them as a result of this remodelling. Within in vivo skeletal muscle, the extracellular matrix, in which the muscle is embedded, is at a much lower ratio of cell/collagen than in this in vitro model (Fratzl 2008). To keep the muscle fibres in place, each fibre is attached to the collagen present within the tendon. Consequently, to enable a more biomimetic model of skeletal muscle, a tendinous-like attachment for each fibre may be necessary for cultures longer than 28 days.

6.5 Conclusions
This work demonstrates the effects of dimension change on myotube formation and the use of industrial and engineering design of experiment tools to optimise key variables of the model. It also validates the proposed scaling model proposed towards a reduction in material and cellular resource costs and assesses the long term culture limitations in preparation for later co-culture.

When placed in a 3D culture, C2C12s show a capacity for greater alignment than conventional monolayer cultures and therefore would be a more suitable model for preclinical studies. Considering the effects of C2C12 myoblast seeding density and collagen width extension on the desirable morphological traits of the 3D construct, an optimum configuration was found to be four million cells/mL construct volume with a 25% width extension either side of the A-frame. This configuration resulted in a reduction in longitudinal attachment forces despite a higher initial cell number over constructs without width extension, consequently reducing the incidence of construct rupture. Models using this configuration and the defined scaling model can be scaled down without detrimental effect. The smallest model tested here was a 0.42mL construct which represents a sevenfold decrease in resource cost over previously published iterations. This demonstrates the capability for significant reduction in resources used (cellular and reagent) over previous similar models and those reported within the literature. By characterising the physical properties and
verifying scaling within this model, variations between constructs previously observed in biochemical analyses may also be reduced. Previous model characterisations (Player et al. 2014; Sharples et al. 2012; Cheema et al. 2003; Smith et al. 2012), in addition to the work reported here, represent a complete system suitable for early stage preclinical tests that has been characterised according to both molecular and morphological traits.

The capability of the system to withstand long term culture (up to 33 days) was also assessed to determine culture limitations for potential multi-tissue models. Morphological observations indicated that this model has a culture limit of 28 days, after which abnormalities occur. More study should be undertaken to better understand the cause of these traits and whether they negatively affect the overall culture and how to mitigate or minimise this issue.
7 Development and Optimisation of a 3D Tissue Engineered Bone Model

7.1 Introduction
Chapter 5 investigated different medium compositions to identify a single proliferation medium and differentiation medium for both C2C12 and TE85 cell lines. This medium protocol was utilised in Chapter 6 during the use of factorial design of DOE for optimisation of the collagen-based model. This was done in order to create the most biomimetic muscle model based on myotube characteristics and to reduce resource loss due to failure of the model as a consequence of anchor point detachment. Following the success of this process, as reported in Chapter 6, the following study describes the optimisation of a biomimetic bone model towards a 3D tissue engineered skeletal muscle and bone co-culture pre-clinical testbed.

In vivo bone characteristics differ depending on the location upon the limb bone or within the body. The main body, or diaphysis, of long bones is characterised by dense, compact bone whilst the bone around the joint (the epiphysis) has a large surface area to mass ratio and is defined as a comparatively soft trabecular bone (Clarke 2008; Weiner & Wagner 1998; Bianchi & Martinoli 2007). When this structure is damaged, three phrases of repair occur: Inflammatory, reparative and remodelling. The initial inflammatory phase serves to immobilise the fracture and accumulates a large variety of cells including bone absorbing osteoclasts and undifferentiated cells. This is followed by a reparative phase (Sfeir et al. 2005). During this phase there is revascularisation of the damaged area and the formation of a bony callus, in which both intramembranous and endochondral ossification takes place, with osteoblasts present to deposit new woven bone. The final phase is one of remodelling, in which the normal bone structure is recreated (Harry et al. 2008). The later stages of the reparative phase are recapitulated in in vitro models, in which a population of cells are added to a scaffold to deposit a calcified matrix and mature along the osteogenic pathway (Motamedian et al. 2015). During the differentiation of bone depositing cells (osteoblasts), RUNX2/CBFa1 is implicated as a trigger for the osteogenic cascade of transcription factors that culminates with osteocalcin/BGLAP (Franceschi et al. 2003; Ogasawara 2013; Mohseny et al. 2011). Furthermore, alkaline phosphatase (ALP) is an important feature in the cellular production of bone mineral, during which pyrophosphates are reduced to form phosphate groups for the generation of calcium apatites (Golub et al. 1992).

Due to the different types of bone, a variety of different tissue engineered models exist with a number of different applications (Rose & Oreffo 2002; Bose et al. 2012; Shrivats et al. 2014). The requirements for models which are intended to be placed in vivo differ from those intended as
models to simulate disease or study physiology. Both constructs require an inherent biocompatibility and osteoinductive/osteoc conductive characteristics, although constructs designed for in vivo use also incorporate degradation kinetics so that the construct will be replaced with native tissue over time (Brown et al. 2013; Kamath et al. 2014).

Tissue engineered bone models utilise a scaffold and either an osteoblast-like cell line or a multipotent stem cell, for example a mesenchymal stem cell, to act as a source of matrix deposition and remodelling (Laurencin et al. 1999; Brown et al. 2013; Syed-Picard et al. 2009). Within these models, two main categories of scaffold exist: Synthetic or nature-derived. Natural-derived materials include type-I collagen, coral or demineralised bone matrix (DBM) (Kamath et al. 2014; Cheng et al. 2014; La 2003; Petite et al. 2000). Bioactive glasses, polymers (such as polylactic acid [PLA] or polylactic-co-glycolic acid [PLGA]) and ceramics (such as tricalcium phosphate or hydroxyapatite) are examples of synthetic scaffolds (Boskey 2013; Lou et al. 2015; Gentile et al. 2014; Hench & Best 2013). Traditionally, demineralised scaffolds have been used to create bone models for grafting or general study. However, with the advances in materials science and manufacture, new technologies can produce materials with more specifically designed characteristics, hence the development of synthetic scaffolds that do not need special processing before use (Oryan et al. 2014). Individually, nature-derived and synthetic scaffold have advantages and disadvantages, although in many cases these disadvantages can be reduced or eradicated by combining two or more materials to improve the scaffold characteristics to meet functional requirements. Generally, these improved scaffolds are composites of nature-derived and synthetic materials; such scaffolds are termed hybrid or composite scaffolds (Murugan & Ramakrishna 2006; Kim & Kim 2014; Henson & Getgood 2011; Oryan et al. 2014).

For the most part, nature-derived scaffolds do not have the mechanical properties observed in bone, although in comparison to synthetics, they exhibit significantly superior biocompatibility and regenerative characteristics such as osteoconductivity and induction (Padmanabhan et al. 2014; Brown et al. 2013). Synthetic polymer materials can be designed with specific shapes and porosities with incorporated growth factors. However, the biodegradation of these materials renders them unsuitable for in vitro testing, in which the understanding of a novel material’s specific effects on biological characteristics would be confounded (Liao et al. 2005; Middleton & Tipton 2000; Gentile et al. 2014). To maintain biological compatibility and mimic the material features of bone tissue, a more biomimetic structure that supports and stimulates cellular populations can be manufactured by combining a nature-derived scaffold with a stiffer, synthetic matrix.
Hydroxyapatite (HA), a type of calcium apatite similar to the mineral present in bone ECM, is an inorganic material that occurs naturally but can also be synthesised outside of the body by a variety of methods (Wei et al. 2005). HA has previously been shown to be osteoconductive (allows for bone deposition) and to aid cell attachment (Boskey 2013). It has also been approved by the FDA for use as a bone filler and for use as a ceramic coating on metallic implants (Liu & Williams 2010). However, on its own, HA is brittle and, as a ceramic, is difficult to process. Conversely, collagen, the most often used organic material scaffold for tissue engineering, has mechanical properties that differ greatly from natural bone (Wahl & Czernuszka 2006; Rodrigues et al. 2003). When combined with HA, a model which more closely represents the major organic and mineral components in vivo bone can be prepared. Additionally, a composite of collagen and HA has been shown to accelerate osteogenesis in comparison to type-I collagen or HA in isolation (Xie et al. 2004; Wahl & Czernuszka 2006). This type of hybrid has been extensively studied utilising a variety of different manufacturing techniques, such as precipitation, hydrothermal, hydrolysis and mechanochemical synthesis (Jones et al. 2010; Rodrigues et al. 2003; Padmanabhan et al. 2014; Antebi et al. 2013). Wahl & Czernuszka (2006) offers a comprehensive review of these methods.

Investigations into a collagen-HA model have focussed on the mechanical properties of the construct, characterisation of the HA and evidence for calcification (Kamath et al. 2014; Hutmacher 2000; Chen et al. 2015; Stanishevsky et al. 2008). As the purpose of such investigations was to determine suitability for use in vivo, the models would be exposed to more established cell populations in situ. However, an in vitro model for toxicity testing requires an undifferentiated cell population source to differentiate in an engineered biomimetic microenvironment. Some specific parameters, such as initial seeding and pore size, have been assessed for effects on cellular attachment and calcification (Grayson et al. 2008; Chen et al. 2015). However, the specific effects of manufacturing variables on cell differentiation in vitro has yet to be reported. Grayson et al. (2008) suggests that greater cell densities do not necessarily correspond to greater bone formation when using decellularised bone as a scaffold, implying that the best model of bone may not be a direct replication of in vivo composition. Furthermore, after observations of undesirable changes in pore size and construct shape after in vivo implantation (collagen lattice collapse), Liu & Williams (2010) assessed the effect of different HA to collagen ratios on cell-mediated construct reduction. In this study, it was found that incorporating HA within a collagen hydrogel regulated cell-mediated construct diameter reduction and affected distribution of cells within the construct. Specifically, this was dependant on the ratio of HA/collagen.

As a suitable biomimetic in vitro model, a collagen-HA engineered construct was chosen as a basis for the bone component of a pre-clinical skeletal muscle and bone testbed. Additionally, by utilising
type-1 collagen as a basic structural matrix, this allows for a single contiguous system across the skeletal muscle and bone constructs proposed for co-culture. This removes the need for large scale material tests for compatibility between the two components. Presented here are the results from the investigation and optimisation of the manufacturing variables, cell density and HA/collagen ratio (specifically the isolated and interaction effects) leading to the most mature biologically representative model of bone. This utilised the osteoblastic markers of RUNX2/CBFa1 (early differentiation), alkaline phosphatase activity (mid-late differentiation) and osteocalcin/BGLAP (late differentiation) to characterise the differentiation of each model configuration.
7.2 Materials and Methods

7.2.1 Cell Culture

Cells were expanded and routinely culture as described in Section 4.1.

7.2.1.1 Hydroxyapatite Dose Response

To observe the effects of the HA particles produced (as described in Section 4.3.2.1) on TE85 hOS differentiation, TE85s were seeded at 4500 cells/cm² in a 12 well plate and cultured until confluency (4 days). Prior to cellular exposure, HA was prepared in concentrations of 8, 4, 1.6, 0.8, 0.32 and 0.064mg/well in DM (HG-DMEM, 2% horse serum, 1% PS) and compared to a no exposure control.

At confluency, TE85s were directly exposed to all concentrations of HA in media. After 24 hours, the HA had settled and therefore DM could be replenished daily without removing HA. Samples were taken for DNA and Alkaline Phosphatase (Section 4.8) analysis after 4 days in differentiation (DM) media.

7.2.1.2 TE85 Human Osteosarcoma Cell 3D Culture Morphology

Once placed within a 3D matrix cells are shown to behave differently to monolayer cultures. As such, TE85 hOS cells were cultured within a 1.0mL collagen hydrogel (as described in Section 4.3.2.3 without the addition of hydroxyapatite) set within a 24-well plate (Nunc) and seeded at an initial concentration of 10,000 cells/mL. Constructs were cultured for 4 days in high glucose DMEM (HG-DMEM) with 20% FBS and 1% PS followed by 10 days in HG-DMEM with 2% horse serum and 1% PS. Constructs were then fixed and taken for calcium staining as described in Section 4.6. Brightfield microscopy was used to observe continuous morphological changes throughout the culture period.

7.2.2 3D Tissue Engineered Collagen/Hydroxyapatite Bone Constructs

Collagen/HA constructs were manufactured as described in Section 4.3.2. HA was precipitated and the concentration determined as described in Section 4.3.2.1 and 4.3.2.2. Briefly, once the concentration of HA paste has been obtained, the ratio of paste to collagen was calculated. For example, for a 2:1 ratio of HA to collagen in a 1.0mL construct configuration, 4mg of HA is required for a collagen density of 2mg/mL.

The required mass of HA paste was suspended in type-1 rat tail collagen (2.0mg/mL, First Link UK). 10xMEM was subsequently added to the HA/collagen mixture as 10% of the total final construct volume (i.e. for a 1.0mL construct, 100µL of 10xMEM is added to 850µL of HA/collagen). This acts as a pH indicator for later neutralisation and as immediate nutrients for cells suspended within the collagen. The HA/collagen solutions were then neutralised by adding 5M NaOH followed by 1M NaOH solution in a drop-wise manner until a colour change was observed. A cell suspension of 5% of the total volume was added to each solution to give the required cell density per mL. Each 1.0mL
sample was set in a single well of a 24 well plate under humidified atmospheric O₂ and 5% CO₂ conditions at 37°C (5% CO₂ in air). Each gel was released from the edges of the well in preparation of addition of media.

7.2.3 Optimisation of Manufacturing Variables Experimental Design
Utilising elements of Quality by Design (Thomas et al. 2008) through factorial DOE software (Minitab Statistical Software, Minitab Inc., USA), the influences of variation in cell number per mL construct and HA to collagen ratio on myotube characteristics were assessed. Individual construct configurations are shown in Table 7-1.

<table>
<thead>
<tr>
<th>Cell density (cells/mL)</th>
<th>Hydroxyapatite/Collagen (mg/mL ratio)</th>
<th>Designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 000 000</td>
<td>2:1 (4:2)</td>
<td>2H</td>
</tr>
<tr>
<td>10 000</td>
<td>2:1 (4:2)</td>
<td>2L</td>
</tr>
<tr>
<td>505 000</td>
<td>1:1 (2:2)</td>
<td>1M</td>
</tr>
<tr>
<td>10 000</td>
<td>0:1 (0:2)</td>
<td>0L</td>
</tr>
<tr>
<td>1 000 000</td>
<td>0:1 (0:2)</td>
<td>0H</td>
</tr>
</tbody>
</table>

7.2.4 Immunofluorescence imaging
Samples were stained for DAPI and Phalloidin as described in Section 4.5.

7.2.5 qRT-PCR
Samples taken for qRT-PCR were lysed and stored, then RNA extracted and qRT-PCR run as described in Section 4.7 for RUNX2/CBFα1 and Osteocalcin/BGLAP.

7.2.6 Alkaline Phosphatase Activity and DNA Quantification
Monolayer samples taken for DNA/Protein analysis were lysed in double distilled H₂O and taken through 3 freeze-thaw cycles. 3D tissue engineered samples were snap frozen with 500µl of dH₂O in liquid nitrogen. Samples were stored at -80°C for later use. A steel ball bearing was added to 3D construct samples and then agitated on a TissueLyser II (Qiagen) for 4 minutes at 10,000.

For studies not involving direct contact with hydroxyapatite, DNA content was measured using Thermo Scientific NanoDrop 2000 spectrophotometer at 260nm. For studies involving direct contact with hydroxyapatite, commercially available Quant-iT PicoGreen dsDNA Kits (Invitrogen) were used as described in Section 4.8.

Alkaline Phosphatase (ALP) was measured using a 4-MUP assay as described in Section 4.8. ALP was normalised to DNA for later construct configuration comparison.
7.2.7 Construct reduction analysis

Macroscopic images were taken using an Epson Perfection V330 flatbed scanner to view construct cell mediated contraction over the culture period. Construct diameter was measured using ImageJ (NIH).
7.3 Results

7.3.1 Hydroxyapatite Precipitate Effects in Monolayer

As the major component of bone, HA has been used to help in the osteogenic differentiation of osteoblast-like cells lines. When applied to a culture, large quantities of particles placed on top of the cultures can restrict nutrient access. Therefore, a dose response study was undertaken in direct contact with the TE85s (4 days growth media, 4 days differentiation media) to inform any potential future monolayer experiments and to assess the influence of HA on TE85 cultures, specifically relative growth and alkaline phosphatase production. Analysis of culture DNA concentrations showed that high levels of HA negatively affected TE86 population growth (Figure 7.1). No DNA was found in 8mg/mL HA cultures and 4mg/mL cultures were found to be significantly lower (p≤0.0001) than 0.8, 0.32 and 0.064mg/mL cultures. The absence and reduction of DNA within the 8 and 1.6mg/mL conditions is most likely a result of cell death within the culture due to nutrient restriction. Subsequent gentle washing of the cultures to remove HA particles before analysis, potentially removed evidence of cells within these cultures. The 1.6mg/mL cultures also demonstrated significantly lower levels of DNA than 0.8mg/mL (p≤0.05), and 0.32 and 0.064mg/mL (p≤0.001). As a measure of osteogenic maturation, alkaline phosphatase (ALP) concentrations were normalised to these DNA levels (Figure 7.2). Cultures containing 0.32 and 0.064mg/mL of HA were found to have significantly greater ALP/DNA levels (p≤0.001 and p≤0.0001 respectively) than 0.8mg/mL with no other cultures producing ALP, indicating a greater general cellular population maturity than at greater concentrations. 0.32mg/mL concentrations also exhibited lower variation between cultures. In cultures with significantly less ALP, HA concentrations may be such that TE85s did not upregulate the processes needed to produce ALP for matrix formation.

This demonstrates that the exposure of TE85s to the HA particles produced by the precipitation method described (Section 4.3.2.1) could produce cultures in which osteoblast characteristics were not compromised when the amount of HA present is controlled. This is evidenced by ALP and DNA quantitative analysis and could be used to simulate co-culture systems of skeletal muscle and bone in monolayer and to inform 3D tissue engineered cultures.
Figure 7.1: DNA quantification of TE85s in direct contact with hydroxyapatite (HA).
After 4 days in differentiation medium (8 days total culture), DNA in 8 and 4 mg/mL concentrations were significantly lower (p≤0.0001) than all other conditions. 1.6mg/mL also demonstrated significantly lower concentrations of DNA than 0.8, 0.32 and 0.064 mg/mL and no exposure controls. There were no significant differences between 0.8, 0.32 and 0.064 mg/mL and controls. n=4 culture replicates +3 repeated measures, * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001, bars represent ±SD.

Figure 7.2: Alkaline phosphatase (4MU production) normalised to DNA for TE85s after 4 days in differentiation media (8 Days total culture).
Alkaline phosphatase was only found present in 0.8, 0.32 and 0.064mg/mL concentrations and no exposure controls. 0.8mg/mL concentration levels were significantly lower than 0.32, 0.064 and 0.0 mg/mL. There were no significant differences between 0.32 and 0.064mg/mL and controls; however, 0.32mg/mL demonstrated less variation between samples. n=4 culture replicates +3 repeated measures, ** p ≤ 0.01, **** p ≤ 0.0001, bars represent ±SD.
7.3.2 TE85 Human Osteosarcoma Cell 3D Culture Morphology

As discussed in Chapter 2, a 3D biomimetic model would provide results that better reflect cellular responses in vivo. To assess TE85 morphology changes under 3D conditions, brightfield images were used to observed constructs without HA allowing for comparisons to in vivo cellular architecture without stimulation from HA. The TE85 hOS cells were seeded within a collagen gel set in a 24 well plate. Constructs were cultured in HG-DMEM with 20% FBS and 1% PS for 4 days followed by 10 days in HG-DMEM with 2% horse serum with an initial seeding density of 10,000 cells/mL.

TE85 hOS cells cultured within a 3D matrix exhibited an elongated profile during the early stages of culture, with some evidence of extended cell process (Figure 7.3). During the later stages of the 3D culture period, brightfield imagery showed clusters of cells with multiple tendrils extending in all directions (Figure 7.4). This suggests a potential osteocyte-like morphology, indicating a highly differentiated cell population. However, the limitations of brightfield microscopy do not allow individual cells to be identified from a cluster and therefore the nature of the clusters could not be determined. To qualitatively assess the maturation of the cell population, the construct was stained for calcium using Alizarin Red S after 14 days culture (Figure 7.5).

Observing the construct as a whole (Figure 7.5 Left), the Alizarin Red S distribution is heterogeneous with some regular circle patterns evident. At higher magnification (Figure 7.5 Right), a more rounded individual osteoblast-like morphology was observed.

Figure 7.3: TE85 hOS cells within a collagen model set in a 24 well plate after 3 days total culture. Cells exhibit some evidence of extended cell processes (shown by →) indicating attachment to the surrounding matrix. Scale bar - 50µm. Representative images of 6 replicate cultures.
Figure 7.4: TE85 hOS cells within in a collagen model set in a 24 well plate after 10 days total culture. Cells exhibit large numbers of cell processes (examples shown by →) and distinct grouping of cells (examples denoted by ellipse). Individual cells are indistinct. Scale bar - 50µm. Representative images of 6 replicate cultures.

Figure 7.5: TE85 hOS cells cultured for 14 days total culture in a 1.0mL collagen construct set in a 24 well plate, stained for calcium (Alizarin Red S). (Left) Non-homogeneous distribution of Alizarin Red S with circular patterns evident. x5 magnification (Right) TE85 hOS present as circular osteoblast-like morphology (image taken near edge of construct). x10 magnification. (Inset) expanded area showing circular morphology (denoted by→). Representative images of 3 replicate cultures.
7.3.3 Hydroxyapatite Storage
After the wash and centrifugation steps, described in Section 4.3.2.1, the HA was neutralised with dilute hydrochloric acid (HCl), separated into 50mL tubes (FisherBrand), centrifuged to remove unbound water, autoclaved and stored at 4°C. After prolonged storage (> 1 week), evaporation caused the HA to form a conglomerate. When the HA was re-suspended in dH₂O as a particle suspension (an autoclaved spatula was used to break up the HA mass) and placed into culture with TE85s, the HA formed large non-uniform particles. As a by-product of this process, sterility was lost in many of the HA samples. As a result, an alternative storage protocol was tested.

After HA was precipitated, the HA was left to settle within the host liquid. Samples from the HA phase were aliquoted into 50mL tubes and centrifuged. After resuspension, the HA was adjusted to ca. pH7.5 followed by a final wash and centrifuge before use. The original HA precipitate suspension was kept at 4°C and as a high pH solution (>pH10). The high pH of the solution ensured that the HA was kept separate in the solution and prevented contamination.

7.3.4 Hydroxyapatite in collagen
Microscopic imagery of Alizarin Red S stained hydroxyapatite (HA) demonstrates the capability of the HA to withstand the initial acidic conditions of the collagen (2.0mg/mL in acetic acid) during the preparation of the collagen-based bone model (Figure 7.6). The HA remains embedded within the collagen after 14 days culture (four days in growth medium (GM) and 10 days in differentiation media (DM)). Macroscopically, the HA embedded within the collagen has a white, bone-like appearance that is clearly distinguishable from the collagen (Figure 7.7). The use of HA within the collagen as a stimulant to osteogenic differentiation renders the use of Alizarin Red S as a marker of bone formation redundant. Therefore, mRNA expression will be used as a main indicator of maturation of the bone model.
Figure 7.6: Hydroxyapatite within 1.0mL a-cellular collagen gel stained with Alizarin Red S after 14 days culture (four days in growth media followed by 10 days in differentiation media).

Figure 7.7: Macroscopic image of HA embedded within a collagen disc with TE85 human osteosarcoma cells (14 days culture). HA appears as a white, bone-like component within the collagen. Non-uniform distribution of HA to distinguish composite structure from collagen scaffold.
7.3.5  

**Tissue Engineered 3D Bone Constructs: Effects of Cell Density and Hydroxyapatite Concentration**

As a complex tissue, bone is subject to the interaction effects of the cellular population and the constituent parts of the culture environment (mechanical forces, matrix components media contents, etc.). Assessing the interaction effects of the cell density and the scaffold component ratios enables the most favourable conditions for the best representation of *in vivo* biological characteristics to be ascertained.

A change in diameter is linked to the number of cells attaching to the matrix and creating a contractile force on the collagen. However, the addition of HA can disrupt this force due to its stiffness. To understand the interaction effects of these variables (cell density and hydroxyapatite/collagen ratio) on the macroscopic cellular effects (cell attachment and remodelling), the final diameter of the bone constructs was assessed (Figure 7.8). Each construct had an initial diameter of 15.6mm (24 well plate). Figure 7.8 shows significant differences in final diameters between constructs. When assessed with a Pareto analysis (Table 7-2), the HA/collagen ratio can be seen to have the most significant effect on construct diameter reduction, followed by the cell density. There is also a combination effect between the two factors. This implies that the greater the cell density the greater the reduction. Conversely, the greater the HA concentration, the lower the reduction in diameter.
Figure 7.8: Macroscopic images and quantification of diameter reduction of TE85 hydroxyapatite (HA)/collagen constructs 14 days culture in 24 well plates. 
(Top) A) 10,000 cells/mL, 0:1 HA/Collagen B) 1,000,000 cells/mL, 0:1 HA/Collagen C) 505,000 cells/mL, 1:1 HA/Collagen D) 1,000,000 cells/mL, 2:1 HA/Collagen E) 10,000 cells/mL 2:1 HA/Collagen. (Bottom) 3D bone construct configuration final diameters (mm) after 14 days culture. Greater cell densities resulted in greater levels of reduction in diameter. Greater ratios of hydroxyapatite to collagen restricted the reduction in diameter. n=14 replicate constructs, * p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001, bars represent ±SD.
Figure 7.9, Figure 7.10 and Figure 7.11, detail the comparative concentrations and expression of the osteogenic markers of RUNX2/CBFα1, alkaline phosphatase (ALP) and osteocalcin/BGLAP. There were no significant differences (p>0.05) found in the expression of RUNX2/CBFα1 (Figure 7.9), although 1,000,000 cells/mL with a 0:1 ratio (0H) and 10,000 cell/mL with a 2:1 ratio (2L) demonstrated greater expression albeit with greater variability, possibly due to the osteosarcoma nature of the TE85 cell population. Analysis of the mid/late stage marker ALP (Figure 7.10) showed that 0H activity was significantly less (p≤0.05) than that of 10,000 cells/mL with 0:1 HA/Collagen ratio (0L). No other significance was demonstrated, however, 0H and 2L exhibited lower ALP activity, continuing the trend of earlier osteoblastic differentiation where these configurations demonstrated less progression along the osteogenic pathway. Analysis of osteocalcin/BGLAP mRNA (Figure 7.11) showed a significantly greater expression in 1,000,000 cells/mL with a 2:1 ratio (2H) than 0L with no other significance (p≤0.05) indicating a more mature cellular population.

Pareto analysis of these results (Table 7-2) shows that an interaction between the cell density and the HA/collagen ratio had a significant effect on the expression of RUNX2/CBFα1 and ALP activity but only the HA/collagen ratio was significantly implicated in the final expression of Osteocalcin/BGLAP. This implies that the greater the ratio of HA to collagen, the greater the expression of osteocalcin/BGLAP but a combination of these factors (cell density and HA/collagen ratio) has a greater effect on initial osteogenic differentiation.

![Graph showing quantitative real-time PCR (qRT-PCR) results for RUNX2/CBFα1 expression.](image_url)

Figure 7.9: 3D bone construct configuration real time qRT-PCR ($2^{ΔΔCT}$) measurement of RUNX2/CBFα1. No significant differences in expression between constructs. n=4 constructs + 3 repeated measures.
Figure 7.10: 3D bone construct configuration alkaline phosphatase (ALP) activity (4MU production µg/mL) normalised to Total DNA to indicate relative levels of ALP per cell. 10,000 cells/mL with no hydroxyapatite (HA) exhibited significantly greater levels of ALP activity than 1,000,000 cells/mL with no HA with no other significant differences. n=4 replicates + 3 repeated measures, * p ≤ 0.05, bars represent ±SD.

Figure 7.11: 3D bone construct configuration real time qRT-PCR ($2^{ΔΔCT}$) measurement of Osteocalcin/BGLAP. 1,000,000 cells/mL with a 2:1 HA/collagen ratio expressed significantly greater levels of Osteocalcin/BGLAP that 10,000 cells/mL with no hydroxyapatite with no other significant differences. n=4 replicates + 3 repeated measures, * p ≤ 0.05, bars represent ±SD.
Table 7-2: Pareto charts of standardised effects detailing significant effects on each maturation parameter by each manufacturing variable (A - seeding density, B – Hydroxyapatite/Collagen ratio) or an interaction (AB).

<table>
<thead>
<tr>
<th>Final Diameter</th>
<th>DNA</th>
<th>RUNX2/CBFa1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pareto Chart of the Standardized Effects</strong>&lt;br&gt;Response is Final Diameter, Alpha = 0.05</td>
<td><strong>Pareto Chart of the Standardized Effects</strong>&lt;br&gt;Response is DNA, Alpha = 0.05</td>
<td><strong>Pareto Chart of the Standardized Effects</strong>&lt;br&gt;Response is RUNX2, Alpha = 0.05</td>
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<td><strong>Term</strong></td>
<td><strong>Term</strong></td>
<td><strong>Term</strong></td>
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<tr>
<td>B</td>
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<td>2.13</td>
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Significant effects are denoted by the relevant bar passing the red line. Red line denotes significance (α = 0.05)

**Categorical Variables**
- A: Cell density
- B: Hydroxyapatite/Collagen ratio
- AB: Interaction

**Continuous Variables**
- Final Diameter
- DNA
- RUNX2/CBFa1

**Quantitative Variables**
- Standardized Effect

**Statistical Significance**
- Alpha = 0.05
Using the response optimising feature of the design of experiment function, RUNX2/CBFα1 was set to be minimised and ALP/DNA and Osteocalcin/BGLAP maximised to best represent a mature *in vivo* bone molecular signature (Figure 7.12). This allows for the configurations of each construct to be analysed for each measured output and assessed according to desired characteristics. Of the tested configurations, a cell density of 1.0 million/mL with a biomimetic 2:1 ratio was calculated to produce the most desirable outputs.

![Figure 7.12: Optimisation of bone constructs, maximising Alkaline Phosphatase/DNA and Osteocalcin/BGLAP and minimising RUNX2/CBF-α1. 1,000,000 cells/mL with a biomimetic 2:1 was found to be the most desirable configuration.](image)
7.4 Discussion
This work sought to define the manufacturing variables required to produce the most mature bone model towards a high throughput analysis system. In particular, the effect of hydroxyapatite (HA) incorporation within collagen and the initial seeding density affecting cell-mediated contraction and cellular differentiation was investigated. These factors were assessed using a factorial design of experiment (DOE) paradigm. This model is based upon the design reported in Liu & Williams (2010) which reported no cytotoxic effects of the HA upon human mesenchymal stem cells.

As discussed previously (Chapter 2), current preclinical biotoxicity tests rely on monolayer models (ISO10993-5 2009; Wolf et al. 2013). One of the key limitations of these models is the absence of a representative matrix, for example the influence of a hydroxyapatite/collagen matrix for bone. Therefore, a 3D tissue engineered construct was envisaged in which a 3D culture of bone forming cells was able to replicate the morphological and molecular characteristic of bone.

When cultured in a 3D collagen matrix, osteocyte-like processes were observed under brightfield observation. However, at greater magnification, the previously mentioned osteocyte-like morphology was no longer observable but the more rounded shape of classic osteoblasts was evident. A similar progression within other such flexible matrices is reported within the literature (Bosetti et al. 2001; Bölgen et al. 2008; Seidi et al. 2011). Conversely, stiffer matrices show a more variable lineage progression (Du et al. 1999; Kim & Kim 2014). For a preclinical model, a mixture of osteoblast-like bone deposition, as well as overall maturation of the model, is desirable in order to assess the effect of novel materials on the biological outputs of the system.

HA has been previously reported to enhance osteoblast differentiation and has also been shown to be advantageous in 3D tissue engineering in composite form with an elastic polymer such as collagen (Harrison et al. 2014; Stanishevsky et al. 2008; Ferraz et al. 2004). Collagen scaffolds have interlocking fibril structure and so the remodelling/contraction of a localised lattice can have an effect on the whole construct (Fratzl 2008). Therefore, increasing the cellular population creates a greater number of attachments to the matrix and contributes to an enhanced reduction in diameter reduction (Eastwood, McGrouther, et al. 1998; Langelaan et al. 2010; Mudera et al. 2010). However, the addition of the hydroxyapatite has been shown to reduce overall model contraction potentially due to the breakup or stiffening of the fibrils by the HA particles (Liu & Williams 2010; Wahl & Czernuszka 2006). Liu & Williams (2010) discovered that a HA/collagen ratio in excess of 1:1, effectively limited reduction over 37 days. The work presented in this chapter corroborates this finding with statistically significant reductions occurring in the lower ratio constructs; however, the
restriction on cell-mediated reduction observed in high HA/collagen ratios was offset by increases in initial seeding density.

To measure the osteogenic maturity of the culture, RUNX2/CBFa1, alkaline phosphatase (ALP) normalised to DNA and Osteocalcin/BGLAP were measured as markers of initial, mid/late and late stage differentiation respectively (Lian & Stein 1992; Shui et al. 2003; Mohseny et al. 2011; Lumachi et al. 2009; Kapustin & Shanahan 2011; Golub et al. 1992). The TE85 population, as a sarcoma cell line, does not follow the classic maturation profile and is capable of expression proliferative and differentiative markers simultaneously, which can lead to enhanced variability within cultures (Lian & Stein 1992). However, by comparing the relative amounts of these osteogenic markers, the population that has progressed furthest along the differentiative pathway can be determined. After 14 days in culture, no significant differences occurred in RUNX2/CBFa1. However, large variation in 1,000,000 cell/mL with a 0:1 ratio (0H) and 10,000 cell/ml with a 2:1 ratio (2L) occurred, which may be explained by the sarcoma differentiation profile, in which a population demonstrates a mixed maturation profile (Lian & Stein 1992). It is thought that this occurred within the constructs due to a large population (within 0H constructs) in which clusters of the cells may express these markers earlier whilst other areas may not. Variation between constructs may be caused by inter-cell signalling, in which a differentiated/differentiating cell may induce differentiation in nearby cells. This could have occurred on different timescales in different constructs due to the osteosarcoma nature of the cells or inherent variation in the manufacturing process. Within a smaller population (2L), the distribution of cells and HA within the construct may have a gradient effect on stimulating differentiation (i.e. cells more proximal to the HA are stimulate earlier/to a greater extent). Likewise, ALP/DNA ratios showed large variation, with the only significance occurring between 1,000,000 and 10,000 cells/mL with a 0:1 ratio. TE85s have previously been shown to be a poor generator of ALP when compared to alternative cell types (Clover & Gowen 1994) potentially accounting for the general lack of statistical significance. Despite this, a higher HA ratio with a larger initial cell seeding produces a greater amount of ALP/DNA. Measurements of the late stage marker Osteocalcin/BGLAP shows that 1,000,000 cells/mL with 2:1 ratio of HA/collagen produces the most mature cellular population. Once again, statistical significance is limited but a greater HA/collagen ratio appears to produce higher levels of Osteocalcin/BGLAP. This implies that a longer timeline for differentiation may be needed to produce less variable populations.

By analysing these results through Pareto analysis, an interaction effect between cell density and hydroxyapatite/collagen scaffold ratio is shown to significantly influence RUNX2/CFa1 and ALP/DNA. However, the HA/collagen ratio is the sole significant influencing factor on osteocalcin/BGLAP. This data supports previous literature stating the osteogenic enhancing effects
of HA (Harrison et al. 2014; Stanishevsky et al. 2008; Ferraz et al. 2004) whilst specifically investigating the effects that cell density and HA/collagen play in promoting osteogenesis in a 3D tissue engineered model of bone. This demonstrates the multi-interaction effect that manufacturing variables can have and should be taken into account when designing novel systems.

Using the optimising feature of the design of experiment software, setting RUNX2/CBFα1 to be minimised and ALP/DNA and osteocalcin/BGLAP to be maximised, 1,000,000 TE85s/mL with a 2:1 ratio of hydroxyapatite to collagen was found to produce the most mature population over 14 days. This also represents the most biomimetic structure. A 2:1 hydroxyapatite to collagen ratio is most similar to the in vivo ratio of 30% organic (including cellular material and water) to 70% inorganic ratio (Sommerfeldt & Rubin 2001). As the bone model progresses, it would be expected that the osteoblasts would enclose themselves within a mineral shell within the bone and become osteocytes as in vivo (Sommerfeldt & Rubin 2001; Nakamura 2007; Clarke 2008). Literature had reported osteocyte densities between 19,000 and 28,500/mm³ for human long bones (Buenzli & Sims, 2015). In the optimised model, a 1,000,000 cells/mL collagen represents a final density of ~7,713/mm³ (without net change in cell density). Therefore, the higher initial seeding density also better represents in vivo structure. This model does show a deficit in terms of cell density to in vivo bone, potentially indicating that a further increase in initial seeding may be beneficial, Holy et al. (2000) observed no increase in calcification with an increase from 1.0 x10⁶ to 6.0 x10⁶ million cells/mL on a PLGA scaffold. This difference in cell density in producing tissue formation could be attributed to the difference between a maturing and established cell population, and a microenvironment which better represents the bone ECM compared to PLGA.

### 7.5 Conclusions

This work sought to optimise an in vitro 3D tissue engineered bone model by comparing markers of osteogenic progression within a hydroxyapatite (HA)/ type-1 collagen composite model using a factorial design of experiment. This represents a key step in the development of a biomimetic tissue engineered model towards in vitro testing by assessing the individual and interaction effects of cell density and HA/collagen on the expression of RUNX2/CBFα1 and osteocalcin/BGLAP and production of alkaline phosphatase (ALP). An initial seeding density of 1,000,000 cells /mL with a HA/collagen ratio of 2:1 was found to be the optimum of tested configurations in producing a population with the greatest expression of BGLAP and ALP with the lowest expression of RUNX2. This work supports previous literature in the osteogenic enhancing effects of HA and the retardation of cell-mediated diameter reduction and provides a stable platform from which to create a co-culture platform.
8 Development of a 3D Tissue Engineered Skeletal Muscle and Bone Co-culture system

8.1 Introduction

Chapter 5 investigated various medium compositions to determine a suitable protocol for the culture of both skeletal muscle and bone populations. High-glucose DMEM (HG-DMEM) supplemented with 20% FBS and 1% PS followed by HG-DMEM supplemented with 2% horse serum and 1% PS was found to be capable of promoting proliferation and differentiation in both myoblast and osteoblasts-like cell populations. Chapter 6 and 7 used a factorial design of experiment (DOE) to optimise 3D tissue engineered models of skeletal muscle and bone. In this work, C2C12 murine muscle precursor cell (MPC) and TE85 human osteosarcoma (hOS) cell responses were analysed according to gene expression and morphological observations; the formation of immature muscle fibres, or myotubes, and the maturation of bone through presence of RUNX2/CBFa1, alkaline phosphatase and osteocalcin/BGLAP. Optimised models formed systems deemed to be the most stable and biomimetic through DOE analysis. This chapter implements the medium protocol from Chapter 5 to investigate the interactions of secreted factors through segregated monolayer culture and to develop a platform which allows for the co-culture of the 3D tissue engineered biomimetic skeletal muscle and bone models described in Chapter 6 and 7.

Biomaterials have been used to facilitate healing (suturing) or act as a replacement for damaged tissues (bone plates) since before the Christian era, although biocompatibility was not assessed until the 1800s (Ratner 2013). Currently, novel materials must go through a regulatory pathway that includes a series of in vitro pre-clinical tests before human trials and market approval (Lo & Field 2009; Stavropoulos et al. 2015). As a part of these preclinical tests, monolayer cellular investigations into toxicity precede animal studies. However, these monolayer tests often produce results that do not translate to later in vivo systems, due to a lack of physiological relevancy. Animal studies have also shown a lack of translation to human trials (Bhadhiraju & Chen 2002; Huh & Kim 2015; Johnson et al. 2001; Mestas & Hughes 2004).

Tissue engineering shows huge potential in bridging this gap and possibly removing the need for animal studies altogether. Functional tissues have long been the goal of regenerative medicine, with musculoskeletal tissues, particularly bone, being a continuing active area of research (Chatzinikolaidou et al. 2015; Parmar & Day 2015; Chen et al. 2015; Neal et al. 2015; Cleetus et al. 2015). To date, many different models of skeletal muscle, tendon/ligament and bone have been published with both pheno- and genotypic characteristics of in vivo tissues (Martin et al. 2013; Player et al. 2014; Yang et al. 2010; Sah & Ratcliffe 2010; Calve et al. 2004; Dragoo et al. 2003). Each of
these models has been developed according to single tissue optimisation whilst ignoring neighbourhood tissue influences. Recently, investigations into adjacent tissue interfaces, for example: Bone-ligament/tendon; muscle-tendon; muscle-neuron; have become the major progressive research area for 3D tissue engineering (Lu et al. 2010; Seidi et al. 2011; Yang & Temenoff 2009; Charvet et al. 2012; Bitar et al. 2004; Paxton et al. 2012; Kostrominova et al. 2009). Adjacent tissue models account for direct interactions between cell types. However, neighbourhood tissues within complex systems, such as the musculoskeletal system, both directly and indirectly affect other nearby tissues. It has been shown that skeletal muscle can positively affect bone healing (Sartori & Sandri 2015; Davis et al. 2015) and bone can affect muscle mass (Bakker & Jaspers 2015). Therefore, to accurately model the in vivo musculoskeletal system the major tissue influences on differentiation should be investigated.

The work defined in the following chapter details the progression of the skeletal muscle and bone co-culture. Initially, an assessment of C2C12 response to indirect exposure of different concentrations of hydroxyapatite will be made to observe any potential lineage altering effects. This will replicate a 3D tissue engineered bone and skeletal muscle co-culture system in which the skeletal muscle would be subjected to any degradation/leachable products from the hydroxyapatite. In addition, to understand the relative effects of each cell type on the other, indirect contact cultures will be assessed according to differentiation capabilities. Indirect co-cultures are where each cell type is physically separated but any secretions are able to affect each cell type through a shared medium. Finally, a culture system will be developed that allows the successful manufacture and subsequent co-culture of optimised skeletal muscle and bone constructs, which have been previously detailed in Chapters 6 and 7. Each construct was assessed according to markers of fusion; skeletal muscle constructs were assessed for skeletal muscle precursor fusion to form myotubes using immunofluorescence and the bone constructs according to relative expression of alkaline phosphatase, RUNX2/CBFa1 and osteocalcin/BGLAP.
8.2 Materials and Methods

8.2.1 Cell Culture

C2C12 murine myoblasts and TE85 human osteosarcoma (hOS) cells were expanded and routinely cultured as described in Section 4.1 unless otherwise stated.

8.2.2 C2C12 Culture Hydroxyapatite Dose Response

To understand the effects of hydroxyapatite degradation products on C2C12 muscle precursor culture towards a co-culture system, C2C12s were seeded at 4,500 cells/cm$^2$ in a 12 well plate and cultured until confluency (4 days). In preparation for cellular exposure, HA was arranged at a concentration of 0.32 mg/mL in myogenic differentiation medium (DM) based on the results in Section 7.3.1. HA in DM was prepared 24 hours before cellular exposure to C2C12s. At confluency, C2C12s were exposed to medium conditioned for 24 hours. Each culture treatment was replenished from conditioned media stock. Conditioned medium stock was replenished after each well medium change, as detailed in Figure 8.1. Brightfield imagery was used to determine morphological response over 4 days.

![Figure 8.1: Hydroxyapatite media conditioning protocol.](image)

Hydroxyapatite was first quantified and then placed in a differentiation media (myogenic) suspension at the required concentration. Each concentration was conditioned for 24 hours at 37°C in 5% CO2 + air. Condition media was then used on the assigned culture and then the media replaced for conditioning for the next 24 hours.
8.2.3 *Indirect Contact Co-culture of Skeletal Muscle and Bone*

To better understand the effects of secreted factor influence of each cell type’s culture, a system to allow separate culture in a shared medium was devised. Sylgard was pre-set in six well plates (Nunc) and cut to provide a central barrier approximately 0.5x3.5x0.5mm across the wells (Figure 8.2A). C2C12s and TE85s were seeded separately at 4500 cells/cm\(^2\) either side of the barrier. Growth medium (GM) (high glucose DMEM supplemented with 20%FBS and 1% PS) was added to each well until the levels rose above the Sylgard (~6.0mL) and 2.0mL replenished daily. At confluency (72 hours post seeding), GM was aspirated and replaced with 1.0mL myogenic differentiation medium (DM) and HA (0.32mg/mL) in DM to the C2C12s and TE85s respectively. Cultures were left for 2 hours at 37°C/5% CO\(_2\) in air to allow the HA to settle onto the TE85s. DM was then gently added to the well above on top of the boundary to increase levels above the Sylgard (ca. 6.0mL). Controls were as detailed in Figure 8.2B. Cells were taken for analysis after 3 days in DM.

\[^2\] Indirect contact co-culture of skeletal muscle and bone cell culture and ICC conducted with the assistance of Lia Blokpoel-Ferreras
Figure 8.2: Monolayer co-culture systems. Each cell type was separated by a Sylgard boundary with secreted and degradation products shared within the medium. Both cell types exposed to a single medium by filling the well to a volume above the height of the boundary. A) Experimental well layout detailing differentiation conditions. B) Experimental controls.
8.2.4 Co-culture of Tissue Engineered 3D skeletal Muscle and Bone Constructs.

To allow for the co-culture of optimised 3D tissue engineered skeletal muscle and bone models, a novel experimental platform was developed. Co-culture setting areas were created using Nunc Rectangular 8 well plates coated with Sylgard. An area large enough to allow for the setting of the skeletal muscle construct and placement of the boundary was cut away from the Sylgard covering the well. The top threaded section of a 15mL centrifuge tube (FisherBrand) was removed and placed inverted atop the remaining Sylgard covering the well. 2.0mg/mL collagen was used to seal the resultant ring to the Sylgard to create a well to set the bone construct in. A minuten pin was placed at the centre to act as an anchor point for the bone construct. A Sylgard boundary was used to create the setting area for the skeletal muscle construct and sealed used 2.0mg/mL collagen. Experimental layout is detailed Figure 8.3. Skeletal muscle and bone constructs were then cultured as described in Section 4.3.

![Schematic of 3D tissue engineered co-culture system](image)

Figure 8.3: Schematic of 3D tissue engineered co-culture system.

To create the bone construct setting area, the threaded section of a 15 mL centrifuge tube was removed and placed on a Sylgard-coated well. The ring was then sealed to the Sylgard with 2.0mg/mL collagen. A minute pin was placed in the centre of the ring to act as an anchor point for the bone construct. A Sylgard boundary was placed adjacent to the ring and sealed with collagen. Skeletal muscle and bone constructs were then formed as previously described (Chapter 5 and 6)
8.2.5 **Tissue Engineered 3D Hydroxyapatite/Collagen Gel Constructs**
Hydroxyapatite was precipitated and quantified as described in Section 4.3.2. 3D tissue engineered hydroxyapatite/collagen bone constructs were manufactured according to optimised parameters as described in Sections 4.3.2.3 and 7.3.5.

8.2.6 **Tissue Engineered 3D Skeletal Muscle Collagen Constructs**
3D tissue engineered skeletal muscle collagen constructs were prepared according to optimised parameters as described in Sections 4.3.1 and 6.3.2.3.

8.2.7 **Alkaline Phosphatase Activity, DNA and Protein Quantification**
Monolayer samples taken for ALP, DNA and protein analysis were lysed in double distilled H₂O and taken through 3 freeze-thaw cycles. 3D tissue engineered samples were snap frozen with 500µl of dH₂O in liquid nitrogen. Samples were stored at -80°C for later use. A steel ball bearing was added to 3D construct samples and then agitated on a TissueLyser II (Qiagen) for 4 minutes at 10,000. For studies not involving direct contact with hydroxyapatite, DNA content was measured using Thermo Scientific NanoDrop 2000 spectrophotometer at 260nm. For studies involving direct contact with hydroxyapatite, commercially available Quant-iT PicoGreen dsDNA Kits (Invitrogen) were used as described in Section 4.8.

ALP was measured using a 4-MUP assay as described in Section 4.8. ALP was normalised to DNA for later construct configuration comparison. Protein levels were measured using Thermo Scientific NanoDrop 2000 spectrophotometer at 280nm.

8.2.8 **Immunofluorescence imaging**
Cultures to be imaged were fixed and stained as described in Section 4.5.

8.2.9 **qRT-PCR**
RNA extraction and quantification, and qRT-PCR were run as described in Section 4.7.
8.3 Results

8.3.1 Monolayer co-culture

8.3.1.1 Effects of Indirect Contact of Skeletal Muscle and Bone in Monolayer Cultures

Dose response HA on monolayer populations of C2C12s

As the major component of bone, hydroxyapatite (HA) has been used to stimulate the osteogenic differentiation of osteoblast-like cells lines. When applied to a culture, large quantities of particles placed on top can restrict nutrient access and cause harmful secreted factors to remain localised to the cells. *In vivo* and 3D skeletal muscle tissues do not normally come into direct contact with HA, but tissue culture methods may necessitate contact with degradation products. C2C12s exposure to 0.32mg/mL of HA conditioned differentiation medium for 4 days post confluency showed evidence of cellular fusion under brightfield microscopy (Figure 8.4). Large multi-nucleate myotubes were present and therefore, 0.32 mg/well concentration of HA was deemed to not inhibit C2C12 differentiation and is therefore a suitable osteogenic supplement for co-cultures.

![Figure 8.4: Brightfield image showing C2C12s after 4 days in 0.32mg/mL hydroxyapatite conditioned differentiation media (8 days total culture). C2C12s show multi-nucleated myotubes as evidence of cellular fusion (myogenic differentiation) in the presence of potential degradation particles from the hydroxyapatite. White arrows denote areas of multiple nuclei within a myotubes. Representative of 3 replicate cultures.](image-url)
Effects of Indirect Contact of Skeletal Muscle and Bone in Monolayer Cultures

As a monolayer representation of 3D skeletal muscle and bone co-cultures, cultures of C2C12s and TE85/hydroxyapatite (HA) were co-cultured in the same well but separated by a Sylgard boundary. Both TE85 and C2C12 cell lines were cultured to confluency in growth medium (GM) (HG-DMEM, 20% FBS, 1%) and then exposed to differentiation media (DM) (HG-DMEM, 2% horse serum, 1% PS) and HA in DM respectively. Once the HA had settled on the TE85s, DM was added until levels rose above the boundary to allow free-flow of secreted factors.

Analysis of TE85 DNA levels (Figure 8.5) showed that C2C12 and TE85s without the addition of HA had significantly greater levels of DNA that all other conditions. This indicated that the additions of secreted factors from the C2C12s were influencing the growth of the TE85s in co-culture. These factors would not be present in TE85 only cultures and the influence may be reduced by the presence of HA in the TE85/C2C12+HA cultures. ALP levels normalised to DNA (Figure 8.6) showed that the addition of C2C12 and HA does not inhibit production of ALP relative to TE85 only controls and is significantly greater (p≤0.05) the C2C12/TE85 cultures without HA. Immunofluorescence of C2C12 cultures showed evidence of myogenic fusion in all cultures, although C2C12/TE85+HA cultures showed evidence of reduced myotube formation (Figure 8.7). This could be in response to the presence of HA in isolation or an interaction effect of TE85 paracrine and secrete factors and HA.

![Figure 8.5: TE85 cell population DNA quantification after 7 days culture (4 days in growth medium + 3 days in differentiation medium). TE85/C2C12 without hydroxyapatite (HA) cultures had significantly greater concentrations of DNA than all other conditions indicating a positive effect of C2C12s on TE85 population proliferation. n=3 replicate cultures + 3 repeat measures, * p ≤ 0.05, *** p ≤ 0.001, bars represent ±SD](image-url)
Figure 8.6: TE85 cell population alkaline phosphate activity (ALP) normalised to DNA. TE85 only cultures with HA demonstrate significantly greater ALP per cell than co-cultures without HA and TE85 only populations with HA. However, there is no significant difference between TE85 cultures without HA and co-cultures with HA. n=3 + 3 repeat measures, * p ≤ 0.05, *** p ≤ 0.001, bars represent ±SD.

Figure 8.7: Immunofluorescent images of A) C2C12 B) C2C12+HA conditioned media C) C2C12/TE85 –HA D) C2C12/TE85 +HA. All C2C12 cultures show evidence of myogenic fusion although myotube number appears decreased in D. Blue – Nuclei (DAPI), Red – F-actin (Rhodamine Phalloidin), Green – Desmin (Chromeo 488). Scale bar – 20µm. Representative of conditions.
8.3.2 Co-culture of Tissue Engineered 3D Skeletal Muscle and Bone Constructs

Prior to the creation of a musculoskeletal junction, conditions amenable to the successful co-culture of skeletal muscle and bone must be understood. Skeletal muscle and bone cultures were set up according to optimised configurations and cultured in the same well. After 14 days culture (4 days growth media, 10 days differentiation media) samples were taken for markers of differentiation. As an early marker and one required in the cascade of osteogenic differentiation and maturity, RUNX2/CBFα1 from the bone co-cultures was analysed in comparison to bone only constructs (Figure 8.8). Co-culture constructs showed significantly greater (p≤0.05) expression than the controls. Alkaline phosphatase level analysis (mid-late stage marker) was found to have no significant differences between cultures although co-culture levels were lower (Figure 8.9). The late stage marker osteocalcin/BGLAP (Figure 8.10) was found to be expressed significantly more (p≤0.05) in co-cultures. These results indicate that co-culture cell populations exhibit a greater osteogenic genotype than control cultures although calcification profiles were not significant. Additionally, this indicates that C2C12s positively interact with bone cultures.

![Image](image-url)

Figure 8.8: Real time qRT-PCR (2^-ΔΔCT) measurement of RUNX2/CBF-α1 in co-culture and bone model only controls. After 14 days culture, 3D tissue engineered bone constructs exhibit significantly greater expression of RUNX2/CBFα1, an early stage marker of osteogenesis. n=4 repeat conditions + 3 repeat measures, * p ≤ 0.05, bars represent ±SD.
Figure 8.9: Alkaline Phosphatase Activity (ALP) (4MU production µg/mL) normalised to Total DNA to indicate relative levels of ALP per cell in co-culture and bone model only controls. After 14 days culture, 3D tissue engineered bone constructs exhibited a similar concentration of ALP per cell to control construct. n=4 repeat conditions + 3 repeat measures, bars represent ±SD.

Figure 8.10: Real time qRT-PCR ($2^{\Delta\Delta CT}$) measurement of Osteocalcin/BGLAP in co-culture and bone model only controls. After 14 days culture, 3D tissue engineered bone constructs exhibited significantly greater presence of osteocalcin/BGLAP indicating a more mature cell population. n=4 repeat conditions + 3 repeat measures, * p ≤ 0.05, bars represent ±SD.
Skeletal muscle constructs were qualitatively assessed for differentiation through immunofluorescence. Cellular fusion was observed in all constructs (Figure 8.11) although the observable characteristics were not as hypertrophic as controls or previous constructs. Furthermore, large numbers of unfused cells were observed across the co-culture skeletal muscle constructs potentially indicating a population that has not progressed along the differentiative pathway as isolated constructs. This reflects monolayer co-culture systems in which a reduction in differentiation within skeletal muscle cell populations was observed when cultured with TE85 human osteosarcoma populations (Figure 8.7). This could be due to nutrient competition with the bone construct or that the bone model has secreted factors which restricts cellular fusion in the skeletal muscle model, such as bone morphogenic proteins (BMPs). Quantification of the myotube characteristics within these constructs was not possible due to a large number of surviving cells obscuring whole myotubes (Figure 8.12). As was observed within the monolayer direct culture systems, C2C12 populations became the dominant cell type in these co-cultures. This result may be observed in the 3D skeletal muscle constructs through a large number of surviving myogenic cells, indicative of high levels of attachment and remodelling and potential proliferation.

Figure 8.11: Immunofluorescence showing myogenic fusion within a 3D tissue engineered skeletal muscle construct co-cultured with 3D tissue engineered bone construct. Scale bar- 50µm Representative of 4 conditions.
Figure 8.12: Immunofluorescence showing unfused and fused C2C12s within a 3D tissue engineered skeletal muscle construct co-cultured with 3D tissue engineered bone construct. Scale bar-50µm. Representative of 4 conditions.

8.4 Conclusions

This chapter builds on the optimisation of skeletal muscle and bone constructs described in Chapters 6 and 7, utilising the parameters described to create a novel platform and assesses conditions to enable co-culture of these constructs. Considering the wider reaching applications of this chapter, these results will be discussed in Chapter 9 – General Discussion.

In this chapter, investigations were conducted into the interactions of secreted factors interactions in monolayer, as well as the development of a platform to facilitate the co-culture of the 3D tissue engineered biomimetic skeletal muscle and bone models that were previously described in Chapters 5 and 6. This platform enabled the first reported simultaneous differentiation of both skeletal muscle and bone constructs in a single system.
Co-culture of indirect monolayer cultures as a precursor to a final co-culture of optimised 3D tissue engineered skeletal muscle and bone were assessed for any interaction effects. These results show that in indirect contact, TE85 proliferation was positively affected by the addition of C2C12s, although this effect was negated with the addition of hydroxyapatite (HA) to the cultures. Additionally, C2C12 and HA culture with TE85s did not inhibit alkaline phosphatase production (ALP). Within C2C12 cultures, the addition of HA and TE85s did not prevent myogenic fusion, however, in C2C12/TE85+HA cultures displayed qualitative evidence of reduced myotube formation.

To enable simultaneous co-culture of 3D tissue engineered skeletal muscle, a novel platform was developed taking into account the unique requirements of each model. This system enabled the first reported concurrent differentiation of both skeletal muscle and bone cultures. In addition 3D co-culture has demonstrated enhancing effects on gene expression within the bone cultures. However, alkaline phosphatase activity was found not to significantly differ between culture conditions. Although myogenic characteristics within skeletal muscle constructs were not quantified, observation on cellular fusion indicates a less differentiated population compared to both controls and previous model reported within this thesis.
9 General Discussion

The data collected and analysed in this thesis represents the development of a 3D tissue engineered skeletal muscle and bone pre-clinical co-culture platform. Chapter 5 investigated proliferative and differentiative medium compositions to enable the culture of C2C12 and TE85 cell lines. Chapter 6 and 7 utilised the medium compositions determined in Chapter 5, along with a factorial design of experiment to optimise 3D tissue engineered models of skeletal muscle and bone according to specific configurations. High-glucose DMEM (HG-DMEM) supplemented with 20% FBS and 1% PS followed by HG-DMEM supplemented with 2% horse serum and 1% PS was found to be capable of promoting proliferation and differentiation in both myoblast and osteoblasts-like cell populations. Following this, 4 million C2C12 MPCs/mL with a width extension of 25% either side of the anchor point was found to produce the myotubes of greatest length, width and the most highly aligned myotubes in the skeletal muscle construct. Similarly, a biomimetic 2:1 HA/collagen ratio with a 1.0 million hOS/mL initial seeding density, produced the most highly differentiated mRNA expression and alkaline phosphatase production in the bone model.

Chapter 8 implemented the developed 3D tissue engineered constructs in a series of investigations that sought to understand the interaction effects of these tissue types on each other. Initially, segregated monolayer co-culture effects were analysed to study secreted factor interactions. Following this, a platform was developed that enabled the simultaneous manufacture of the 3D tissue engineered skeletal muscle and bone cultures. Finally, this platform was used to manufacture a proof-of-concept 3D co-culture and subsequently analysed for evidence of differentiation and compared to isolated construct controls.

The following text discusses the implications and possible causes for the effects observed in Chapter 8, due to this work building on chapters 5, 6 and 7, as well as comparing these effects to other co-cultures involving skeletal muscle or bone and the placement of this work in the wider body of literature.

9.1 Indirect Monolayer Co-culture

This work sought to investigate the interaction effects of skeletal muscle and bone model co-culture and how each cell type behaves when subjected to secreted factors and paracrine interactions. This is essential in creating a physiologically relevant system. Initial studies within monolayer cultures allow for controlled systems without the variation inherent within the manufacture of 3D systems, which helps to inform observations within later 3D co-cultures.
As a precursor to a biomimetic 3D culture of skeletal muscle and bone, indirect monolayer co-cultures of C2C12s and TE85s were analysed for interaction effects. Prior to this, the effect of hydroxyapatite (HA) on TE85 (direct contact) and C2C12 (indirect contact) was investigated to assess toxicity and the potential to stimulate differentiation. A dose response study was undertaken to optimise the levels of HA added to TE85 cultures (Chapter 7) in order to maximise the osteogenic marker of ALP, which has previous been shown to be influenced by HA addition to culture (Kikuchi et al. 2011; Harrison et al. 2014). The 0.32 mg/well was found to produce the highest ALP/DNA levels with the smallest variation between cultures (n=4). To assess any effects from HA leachables on myogenic populations, both remnant from the manufacturing process and directly from the HA, differentiation media was firstly exposed to HA for 24 hrs for pre-conditioning and then placed upon the C2C12 cultures. At 0.32mg/mL, C2C12 cultures exposed to conditioned medium were capable of forming myotubes and therefore this concentration was taken forward to monolayer co-cultures. This suggests that the precipitation process used in the formation of HA does not produce any factors which inhibit the formation of myotubes and, in conjunction with Liu & Williams (2010), indicates the potential for HA produced in this way to be used in bone therapies without disrupting proximal skeletal muscle regeneration.

In order to model the co-culture of unattached 3D tissue engineered skeletal muscle and bone, indirect co-cultures were set up within a 6-well plate, in which a Sylgard boundary separates each cell population. In these indirect cultures, TE85s within the C2C12/TE85 co-cultures without HA were found to have significantly greater levels of DNA, with no other significances between the other cultures. However, this relationship is reversed when ALP is normalised to DNA. Addition of HA to co-cultures increased ALP/DNA in comparison to those without HA, although no statistical significance was found between TE85-only cultures. TE85 only cultures without HA did produce higher levels of ALP/DNA than both co-cultures without HA and TE85 only cultures with HA. TE85s may have down regulated markers relevant to the production of ALP and are therefore self-regulating responses once exposed to HA. This self-regulation has been reported in reference to organic/inorganic pyrophosphate ratios (Golub et al. 1992). The lack of significant differences between co-cultures with HA and TE85 only cultures is thought to be due to the height of the central boundary not allowing the complete mixing of secreted factors.

Current monolayer toxicity test are generally single cell and conducted according to ISO10993 (fibroblast or CHO cells). This system, by using both skeletal muscle and osteoblast cell lines, and incorporating elements of ECM to induce osteogenesis, creates a more relevant system for testing neighbourhood effects of toxicity can be used. The ease of setup would lend itself to high-throughput techniques in smaller culture wells, although alternatives, such as trans-well inserts...
Additionally, this system could be used to investigate musculoskeletal conditions, such as the exposure of skeletal muscle to bone cells and ECM in blast injuries (Davies et al. 2015) by removal of the central barrier, similar to Wang et al. (2007), in which mature populations were allowed to migrate and mix when observing enthesis healing.

9.2 Co-culture of 3D Tissue Engineered Skeletal Muscle and Bone

A co-culture of tissue engineered skeletal muscle and bone can be utilised for a variety of different purposes. In addition to novel material pre-clinical screening, this system may be used as a developmental step in the production of a tissue engineered full human musculoskeletal junction. This approach was taken following the *in vivo* development of the musculoskeletal junction in which bone and muscle cells are recruited and begin differentiation prior, to co-location of tendon cells and the formation of the interface (Schweitzer et al. 2010).

To enable the co-culture of the skeletal muscle and bone models, a novel platform was developed. This platform allowed for the specific manufacturing requirements of each model, facilitating simultaneous formation of both constructs. This represents the first reported single system culture of tissue engineered bone and skeletal muscle. In this system, co-cultures exhibited significantly higher levels of RUNX2/CBFa1 and Osteocalcin/BGLAP, indicating a more mature cell population, although the population variation in the co-culture RUNX2/CBFa1 was comparatively high. Lian & Stein (1992) showed that osteosarcoma populations do not follow normal osteoblast profiles. The proliferative, early differentiative and mature stages can co-exist in sarcoma populations which are more uniform in classic osteoblast profiles which may explain the population variation observed. ALP/DNA levels were not significantly different between conditions; however, TE85s have been reported as producing alkaline phosphatase in lower concentrations than normal osteoblast cultures. Wang et al. (2007) reported reduced ALP activity and Alizarin Red S staining in the osteoblasts region on a monolayer co-culture of osteo- and fibroblasts although an increase in ALP was observed in the fibroblast cells with no change in Alizarin Red S staining. To recreate the enhanced ALP production observed in the monolayer cultures reported in this thesis, additional direct stimulus may be required (Clover & Gowen 1994). This includes additions of molecular components within the differentiation medium (Langenbach & Handschel 2013) or an applied load, potentially through hydrostatic pressure (Neßler et al. 2016), however this would require further novel system development.

The skeletal muscle constructs exhibited evidence of myotube formation, although distribution and size of these myotubes were qualitatively less extensive than observed in control and previous
constructs indicating some inhibitory effects from the co-culture. This could be due to the interaction of a number of secreted factors. IGF1, TGFβ1 and notch signalling, amongst others, could result in this effect. The GH/IGF1 axis is a fundamental factor of bone health in its essential role in bone growth maturation and maintenance (Locatelli & Bianchi 2014). The GH/IGF1 axis is also an important factor in promoting muscle cell growth and influencing fusion (Perrini et al. 2010). TGFβ1 has been shown to be secreted by osteosarcoma cell lines (Franchi et al. 1998), which acts as an inhibitor of myogenic differentiation, but has been shown to increase proliferation of myogenic cells (Katagiri et al. 1994; Liu et al. 2001; Furutani et al. 2011) and also enhance contractility (Weist et al. 2013). Notch signalling, in particular Notch3, also stimulates the proliferation of myogenic cells (De Salvo et al. 2014).

As a unique 3D tissue engineered system, direct comparisons with similar cultures are impossible, therefore contrasts with the effects of other tissue engineered musculoskeletal co-cultures on bone and skeletal muscle models, as well as the formation methods are discussed. Ma et al. (2009) demonstrated that a co-culture of bone and ligament in direct contact facilitated in the formation of histology indicative of both ligament and bone and therefore differentiation according to desired lineages. No adverse effects were reported on either model and in comparison with a previous single tissue culture reported in the same group, no obvious reported differences in either tissue could be found (Syed-Picard et al. 2009; Calve et al. 2004). However, in this co-culture system, the bone constructs were formed first using a separate osteogenic medium, before formation of the co-culture system in a ligament differentiation medium. The culture period for their study was also longer than the work described in this thesis. The work of Ma et al. represents the repair of a mature enthesis, rather than the developmental approach used here. Larkin et al. (2006) and Kostrominova et al. (2009) used a similar method of construct formation to initially construct a self-organised tendon, which was then split and pinned opposite each other to form anchor points in the developing skeletal muscle construct. Similarly to the bone-ligament-bone construct described by Ma et al. (2009), the formed myotendinous junction construct demonstrated characteristics of both tendon and skeletal muscle with no reported deficiencies when compared to previous single tissue models (Huang et al. 2005), although, again, longer culture periods were used than those presented in this thesis, potentially allowing more mature constructs to form. Despite multiple tissue types in both of these systems, no change in proliferative or differentiative culture times was reported; however Wang et al. (2007) reported a decrease in doubling time within the osteoblast culture in a monolayer co-culture of osteoblasts and fibroblasts. This demonstrated a potential lack of continuity between monolayer and 3D tissue engineered cultures, although culture conditions should be taken into account.
9.3 3D Tissue Engineered Skeletal Muscle and Bone Pre-clinical Co-Culture Platform

Successful development of a skeletal muscle and bone co-culture, which replicates key characteristics of *in vivo* tissue, would be of great benefit to *in vitro* investigations into the musculoskeletal system and could enable the generation of a full *in vitro* musculoskeletal junction. Progression of such a culture with standardised manufacturing parameters could also reduce the reliance on animal models in pre-clinical studies, greatly improving the ethical standing of these practices.

Within the literature, many different medium compositions have been used on both muscle precursor and osteoblast-like cells (Clover & Gowen 1994; Anselme et al. 1999; Ostrovidov, Ahadian, et al. 2014; Burattini et al. 2004; Goto et al. 1999). Each of these compositions has been developed independently without concern of application to other cell types. To develop a co-culture of skeletal muscle progenitor and osteosarcoma cells, a single medium which allows for proliferation, followed by a single differentiation medium, must first be defined if both populations are to be cultured simultaneously. This would also have the secondary effect of reducing resource requirements and therefore decreasing the associated costs. The data obtained here indicates that a previously established medium can initially induce a bias in proliferation rates towards the existing composition, but can be favourably maintained in alternative media after multiple passage exposure. This data also demonstrates the ability to use a simple reduction in serum concentration to affect differentiation in both skeletal muscle and bone models. This represents a significant step in the formation of an *in vitro* musculoskeletal junction by creating a consistent culture environment between cell types.

The skeletal muscle collagen model has previously been developed for use in the culture force monitor (CFM) system (Eastwood et al. 1994; Cheema et al. 2003; Player et al. 2014) and as a component in the formation of a neuromuscular junction (Smith 2012a). This model has also been investigated in comparison to monolayer cultures, which has shown that equivalent formation of myotubes can be observe in both monolayer and 3D cultures, but has demonstrated the advantages of 3D culture in myotube alignment (Smith 2012b). However, this work has limitations when working towards high-throughput, in the form of user manufactured variability and high costs, in comparison with alternative techniques, such as the fibrin delamination model (Martin et al. 2013; Martin et al. 2015). Therefore, this work generated specific manufacturing parameters, using a factorial design of experiment, and a validated a scale-down model to reduce/remove these limitations, most significantly, the cost. This has led to a consistently produced construct which is more representative
of skeletal muscle architecture than monolayer cultures and has increased flexibility of size to suit requirements.

Similarly, a hydroxyapatite (HA)/collagen bone model was developed using a factorial design of experiment to produce a model of greatest differentiation according to gene expression and alkaline phosphatase experimentation. Previously, HA has been used in a number of different studies to generate biomimetic structures, including combination with hydrogels (Murugan & Ramakrishna 2006; Kim & Kim 2014; Henson & Getgood 2011; Oryan et al. 2014). However, analysis of manufacturing variables in a HA/collagen model has not previously been undertaken.

Similar single tissue constructs of skeletal muscle and bone in the literature (discussed in Chapters 6 and 7) may previously have been optimised according to specific characteristics. However, as demonstrated, the medium, mechanical forces and matrix components can significantly change the biological formation of the constructs. Therefore, the assessment of configurations was carried out using a single medium protocol to reduce potential effects when co-cultured.

Considering the different volumetric shapes and mechanical influences to which each of the skeletal muscle and bone construct were developed, a novel co-culture system needed to be created. Previous co-culture systems utilised a well-established delamination model (Ma et al. 2009; Larkin et al. 2006; Kostrominova et al. 2009), creating cylindrical 3D constructs and concentrating on biological outputs without regard to creating individual properties to best support each tissues formation. The platform developed in this thesis sought to enable to formation of each of the constructs as previously described in both shape and cellular differentiation. Subsequent observation of each construct did not demonstrate any obvious deviation from single construct formation and analysis of differentiation showed enhancement of gene expression in the bone construct. Whilst fusion in the skeletal muscle constructs was diminished, an increased culture period length may demonstrate similar myotube characteristics to isolated skeletal muscle constructs.

These observations suggest that a full tissue engineered musculoskeletal junction is possible. As demonstrated in previous studies (Garvin et al. 2003; Sawadkar et al. 2013), tendon formation in a collagen hydrogel is possible and culture of such a model in the same medium as both skeletal muscle and bone is achievable. However, alternative solutions may be possible due to the proposed scaled-down model. This skeletal muscle model has removed the large cell number disadvantage, in comparison to other models, and the use of this with a scaled-down model of bone (not shown due to lack of time for validation) could allow for the tendon fabrication technique described by Ma et al (Ma et al. 2009). Such steps, in addition to the recreation of the studies described in this thesis in
primary human cells, will allow for the formation of a highly relevant system that could eventually replace animal studies in pre-clinical studies.
10 Conclusions, Limitations and Future Perspective

In this chapter, the general conclusions of this thesis will be summarised and the limitations of the models and future work on this topic explored to guide advancement in this area.

10.1 General Conclusions

Utilising the materials and methods outlined in this thesis, the manufacture of a 3D tissue engineered skeletal muscle and bone co-culture platform is possible.

The skeletal muscle model has the potential to form highly aligned, multinucleated myotubes at a greater success rate (non-rupture) than previously seen designs utilising a seeding density of four million C2C12 cells/mL of collagen with a 25% width extension either side of the anchor point. In addition, a scale down model was developed and validated, increasing the cost-effectiveness of the system. Similarly, the bone model was developed to produce the most mature cell population according to gene expression and alkaline phosphatase activity (ALP) using a one million TE85 cells/mL seeding density within a 2:1 hydroxyapatite to collagen ratio.

Co-culture of these in vitro tissues demonstrated enhanced gene expression in the bone model, in comparison to isolated bone controls, with no significant differences in ALP activity. Co-cultured skeletal muscle constructs retained a capacity for myoblast fusion, though this appeared diminished when compared to controls. As such, this represents the first reported instance of simultaneous tissue engineered skeletal muscle and bone differentiation with evidence of an enhancing effect on bone cultures.

This thesis, into the development of a novel skeletal muscle-bone co-culture platform, demonstrates the techniques that should be used in order to implement a tendon component. Considering the already reported success in skeletal muscle-tendon and bone-tendon models (Kostrominova et al. 2009; Ma et al. 2009), the addition of a tendon component to this model should not be considered a prohibitive obstacle to the construction of an in vitro musculoskeletal junction.

10.2 Limitations and Future Perspective

Prior to the creation of a skeletal muscle and bone co-culture platform, both models needed to be optimised according to cellular characteristics and adjustments to best enhance the flexibility of the system. Within the skeletal muscle model, this required production of large numbers of configurations for analysis. As a result, relatively low repeat numbers were used due to the resources needed to manufacture the bespoke materials needed in the manufacture of this model. To produce more robust data, a greater number of repeats may yield further statistical significance although the ultimate outcome should not be modified as a result. The sample sizes required to
determine significance levels can be calculated using the data put forward in this thesis and a statutory power analysis calculations.

Whilst this model produces an absolute measure of differentiation, myotube morphological characteristic measurement is a laborious and time-intensive output measure and would be unsuitable for high throughput screening. Alternatives, such as protein analysis or gene expression through qRT-PCR, have their own limitations. There are no specific skeletal muscle maturation markers in gene expression, although a timeline of expression of markers such as myogenin and MyoD1 could be used (Player et al. 2014; Smith et al. 2012; Mudera et al. 2010). Additionally, protein markers could be used to indicate the progression of the skeletal muscle model, although, the collagen scaffold, as a protein-based matrix, poses a problem in isolation of relevant markers and as a result, a problem in accurate measurement. Greater sensitivity could be achieved by the use of an enzyme-linked immunosorbent assay (ELISA), which assesses the concentration of a protein within a solution (Maegawa et al. 2007; Sun et al. 1997; van der Schaft et al. 2011). However, each of these would require process optimisation and validation to be considered alternatives. All of the above techniques require sacrificial analysis and are indicative rather than absolute. The culture force monitor discussed in Chapter 5, may allow for a non-sacrificial system of assessment which would enable assessment before and after intervention studies. Again, this would just be an indicative assessment rather than absolute but could allow for early detection of constructs that will not be successful. Within the literature, C2C12s have been infected with a green fluorescent protein (GFP) control virus allowing the real-time observation of fusion (Millay et al. 2013). This would allow non-invasive assessment of fusion before usage, but would still require a lengthy quantitative determination. However, if this is then coupled with an in-incubator microscope, such as the Cell-IQ (CM Technologies Oy), an automated protocol to capture and process images of GFP could be implemented to reduce analysis time.

Physical determination of the muscle fibre is the only absolute assessment of skeletal muscle progression, though as the in vivo structure provides a functional output, electrical stimulation and measurement of resultant contraction would be a viable alternative. Such systems exist for delamination style models in which a reduced, highly elastic ECM is present (Larkin et al. 2007; Martin et al. 2015). Collagen is a dense fibrous matrix which undergoes considerable amounts of remodelling during the culture period which makes determination of functional outputs difficult although not impossible (Schutte et al. 2010). During the development of the skeletal muscle, attempts were made to attach small scale models (0.42mL) to the electrical stimulation culture force monitor available to this group. Two barriers to this method were found to exist; attachment to the highly sensitive force monitor and maintaining the shape and tension of the model whilst attaching.
Despite this, a single curve demonstrating the potential for electrical stimulation was observed (not shown n=1). Feasibly, adjustments to both culture force monitors could be made to facilitate determination of both attachment force and functional force under stimulation, although the cost and time required to do so could be prohibitive in a purely research setting.

Additionally, the bespoke manufacture of the anchor points as well as the use of Sylgard barriers to create the setting areas in some designs is an obvious area of variability. In those models that do not require these boundaries, restrictions in the volume of medium available were observed. This can cause negative effects in the culture through reduced nutrients and development of harmful concentrations of waste products, such as ammonium and lactic acid (Glacken et al. 1986). In an ideal system, removal of the waste products and replenishment of the medium in an automated fashion would undoubtedly enhance the culture characteristics, but assessment of optimum flow as well as other process variables (dissolved oxygen, nutrient concentration, pH) would be necessary.

3D printing is a current area of investigation that would allow for reproducible manufacturing as well as rapid adjustment for the creation of parameters conducive to enhancing the anchor points and reducing variability in this area. 3D printing would also allow both scale-up of numbers and scale down of size to fit study requirements. A design that mimics the myotendinous interface or a tendon-like addition to the hydrogel at the A-frame region is also suggested to allow for culture periods in excess of 28 days. This would then secure the myotubes and limit unwanted effects of remodelling.

Similar to the skeletal muscle model, no specific marker of bone maturation exists, hence the use of multiple markers to create a timeline of osteoblast progress. Due to the multi-cellular nature of bone resulting from a single lineage progression, analysis of further differentiation into osteocyte-like cells in tandem with longer-term culture should be undertaken to enhance the relevance of this model. Additionally, the use of hydroxyapatite rendered observations of cellular morphology and matrix deposition difficult to resolve, requiring the use of qRT-PCR and alkaline phosphatase analysis. Sectioning of the models could be used, although an additional process optimisation would need to be carried out.

The co-cultures of skeletal muscle and bone represent a significant step towards an in vitro musculoskeletal junction model. Although, as in the isolated skeletal muscle model, the bespoke nature of the platform poses a large source of variation. The dual nature of the culture also further restricts the nutrient accesses and so a perfused system would be a further enhancement. Quantitative analysis, beyond the proof-of-concept method, needs to be carried out on the skeletal muscle model in co-culture to increase the relevance of the comparison to isolated tissue controls.
As discussed in Chapter 5, long term culture of the skeletal muscle model is limited to 28 days in culture before undesirable characteristics develop. However, fusion in the co-culture appeared limited over the 14 days. Therefore, this timeline should be increased to allow for enhanced differentiation. Whilst the gene expression within the bone model was significantly greater in co-culture, large variability was observed in all markers. Increasing the length of culture could potentially reduce this variation by allowing all cells to progress further down the differentiative pathway. Alternatively, additional supplements could be added to the differentiation medium to enhance bone maturation although further medium composition analysis would need to be undertaken to assess effects on both cell types.

During manufacture, the co-culture is simultaneous, however a lag period occurs between the placement of the bone hydrogel into the setting area and the skeletal muscle hydrogel, which increases the time before the cellular material has access to growth conditions. Whilst this period is not extensive, it is not one that occurs in manufacture of the isolated tissue cultures and therefore could affect the culture in unknown ways. Additional investigations into the possible effects of this lag period should be undertaken, as well as the development of suitable methods to overcome this factor if necessary. Furthermore, small scale models of the skeletal muscle model were validated to reduce costs in anticipation of higher throughput experimentation. Therefore, the bone model should be similarly scaled down and validated to achieve an overall reduction in costs and resources to take this platform forwards.

Finally, to ascertain the relevance of these models, both in co-culture and isolation, simple toxicity tests with known materials or chemicals, both with adverse, neutral and enhancing effects, should be used to compare with monolayer and in vivo studies. As a cell line based model in its current form, this model can be used to act as an additional screening step prior to animal preclinical studies. To take the further step in removing the animal study entirely, this work, as well as the future work to increase the robustness of the system detailed above, should be repeated using human primary cell lines. Using the force, width reduction data and measurement techniques in this thesis, a finite element analysis of these constructs should be pursued and would significantly enhance the predictive nature of this platform and provide a computer simulator for potential biological studies.
A completed platform would ideally be a bone-tendon-skeletal muscle-tendon-bone perfused system in which contractile force can be obtained and can the model manufactured in a highly repeatable, automated fashion. Further functional output of the bone section through compressive loading and tendon section through extension analysis could be included in addition to sacrificial molecular outputs.
References


Bakker, A.D. & Jaspers, R.T., 2015. IL-6 and IGF-1 Signaling Within and Between Muscle and Bone: How Important is the mTOR Pathway for Bone Metabolism? *Current Osteoporosis Reports*. Available at: http://link.springer.com/10.1007/s11914-015-0264-1.


Cummings, P.B., 2009. Axial Skeleton. Available at: https://droualb.faculty.mjc.edu/Course Materials/Elementary Anatomy and Physiology 50/Lecture outlines/skeletal system I with figures.htm


Dept of Health UK, 2012. 2003-04 to 2010-11 programme budgeting data,


European Commission, 2010. *Of mice and men – are mice relevant models for human disease?*,


NC3Rs, 2011. NC3Rs Research review 2011.


Pittenger, M.F., 2008. *Mesenchymal stem cells from adult bone marrow.*, 


Sartori, R. & Sandri, M., 2015. BMPs and the muscle-bone connection. Bone. Available at: http://dx.doi.org/10.1016/j.bone.2015.05.023.


Scottish Government, 2016. Allied Health Professionals Musculoskeletal Pathway Minimum Standards,


Appendix 1 – Negative desmin antibody stain controls

To demonstrate specificity of the primary antibody attachment to the intermediate filament protein desmin and conjugated fluorophore, negative controls were performed by omitting the primary antibody and also using a protein negative cell line. DAPI was used as a counterstain to show the presence of cell bodies.

Figure A1.1: C2C12 cultured for four days in DMEM high glucose +20% FBS + 1% PS. Primary antibody omitted from staining process.
Figure A1.2: C2C12 cultured for four days in DMEM high glucose +20% FBS + 1% PS followed by three days in DMEM high glucose + 2% horse serum +1% PS myogenic medium. Primary antibody omitted from staining process.

Figure A1.3: TE85s cultured for four days in DMEM high glucose +20% FBS + 1% PS followed by three days in DMEM high glucose + 2% horse serum +1% PS myogenic medium. No evidence of desmin presence despite >500ms exposure.
Appendix 2 – qRT-PCR Primer Validation Melt Curves

Once a qRT-PCR has finished, the reaction has produced a saturated product in which all of the SYBR Green dye has been bound to the double-stranded DNA (dsDNA). As the melt curve progresses, the temperature increases, causing the dsDNA to disassociate and become single stranded. As the product disassociates, the fluorescence decreases as the SYBR Green is released. The melt curve plot is the differential of this decrease in fluorescence (Dwight et al. 2011; Huguet et al. 2010; Bruzzone et al. 2013).

Figure A2.1: Validation melt curve for mouse RPIIb (normalising stable gene) using C2C12 murine myoblasts. Single peak indicates a single product production. RNA concentrations per reaction were tested as follows: 50, 10, 2, 0.4, 0.08, 0.016, 0.0032, 0.00064ng/reaction. All reactions produced a single product at all RNA concentrations.
Figure A2.2: Validation melt curve for mouse Myogenin (muscle precursor fusion associated gene) using C2C12 murine myoblasts. Single peak indicates a single product production. RNA concentrations per reaction were tested as follows: 50, 10, 2, 0.4, 0.08, 0.016, 0.0032, 0.00064ng/reaction. Only RNA concentrations above 0.08ng/reaction produced a single peak.

Figure A2.3: Validation melt curve for human RPIIb (left peak, normalising stable gene) and RUNX2/CBFα1 (right peak, early marker for osteogenesis) using TE85 human osteosarcoma cells. Single peak for each product. RNA concentrations per reaction were tested as follows: 500, 100, 20, 4, 0.8, 0.16, 0.032, 0.0064ng/reaction. All RPIIb reactions produced single peak products. Only RUNX2/CBFα1 RNA concentrations above 0.16ng/reaction resulted in a single product. The RUNX2/CBFα1 primer was additionally validated for C2C12 murine myoblasts with the mouse RPIIb primer producing the same melt curve responses.
Figure A2.4: Validation melt curve for human Osteocalcin/BGLAP (late stage maturation marker) using TE85 human osteosarcoma cells.

RNA concentrations per reaction were tested as follows: 200, 50, 12.5, 3.125, 0.781, 0.195, 0.049, 0.012ng/reaction. Only concentrations above 0.195ng/reaction and below 50ng/reaction produced a single peak product.
Appendix 3 – qRT-PCR Experimental Melt Curve Plots

Once a qRT-PCR has finished, the reaction has produced a saturated product in which all of the SYBR Green dye has been bound to the double-stranded DNA (dsDNA). As the melt curve progresses, the temperature increases, causing the dsDNA to disassociate and become single stranded. As the product disassociates, the fluorescence decreases as the SYBR Green is released. The melt curve plot is the differential of this decrease in fluorescence. Small peaks at lower temperature indicate an intermediate stage of disassociation where some products remain in a stage of semi-disassociation (Dwight et al. 2011; Huguet et al. 2010; Bruzzone et al. 2013).

Figure A3.1: Confirmation of single product for each primer for the Medium Composition Culture Comparisons: RPIIb human (H) and mouse (M) (normalising stable gene), Myogenin (M) (myoblast fusion associated gene), RUNX2/CBFa1 (H) (early osteogenic marker).
Figure A3.2: Confirmation of single product for each primer for the Bone Optimisation: RPIIb human (normalising stable gene), RUNX2/CBFa1 (early osteogenic marker), Osteocalcin/BGLAP (late stage osteogenic marker).

Figure A3.3: Confirmation of single product for each primer for the tissue engineered bone model when co-cultured with the tissue engineered skeletal muscle: RPIIb human (normalising stable gene), RUNX2/CBFa1 (early osteogenic marker), Osteocalcin/BGLAP (late stage osteogenic marker).
Appendix 4 – Media Composition without Pre-Conditioning DNA Results

Figure A4.1: Proliferation phase culture DNA concentration of non-pre-conditioned cell lines over 4 days. (A) C2C12 cultures. DNA concentration increases for all conditions over 4 days. M1 (DMEM-high glucose with 20% FBS/1% PS) and M3 (DMEM-low glucose with 10% FBS/1% PS) displays significantly greater concentrations of DNA than M2 (EMEM with 10% FBS/1% L-Glutamine/1% NEAA/1% PS) at 4 days. (B) TE85 cultures. M2 show significantly greater concentrations than M3 and M2 at 4 days. n=3 for all conditions. *** = P<0.001
Appendix 5 – Myotube characteristics representative image analysis

Figure A5.1: Confirmation of a stable mean of the number of myotubes produced by A) 9 images in the Central Region and B) 10 images in the A-Frame Region (5 from each end). Four million C2C12/mL, 25% width extension 1.5mL model.
Figure A5.2: Confirmation of a stable mean myotubes length produced by A) 9 images in the Central Region and B) 10 images in the A-Frame Region (5 from each end). Four million C2C12/mL, 25% width extension 1.5mL model.
Figure A5.3: Confirmation of a stable mean myotubes width produced by A) 9 images in the Central Region and B) 10 images in the A-Frame Region (5 from each end). Four million C2C12/mL, 25% width extension 1.5mL model.

Alignment figures were generated from an accumulation of all alignment measurements for each region. The full alignment graphs are shown below.
Figure A5.4: Four million/mL with a 50% width extension alignment. Each bar represents the number of myotubes within a 5 degree alignment as a percentage of the total number of myotubes.
Figure A5.5: Four million/mL with no width extension alignment. Each bar represents the number of myotubes within a 5 degree alignment as a percentage of the total number of myotubes.
Figure A5.6: Three million/mL with a 25% width extension alignment. Each bar represents the number of myotubes within a 5 degree alignment as a percentage of the total number of myotubes.
Figure A5.7: Two million/mL with no width extension alignment. Each bar represents the number of myotubes within a 5 degree alignment as a percentage of the total number of myotubes.
Figure A5.8: Two million/mL with a 50% extension alignment. Each bar represents the number of myotubes within a 5 degree alignment as a percentage of the total number of myotubes.