Development of Tissue Engineered Skeletal Muscle Manufacturing Variables

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Abstract

Three-dimensional (3D) tissue engineered structures enable more representative determination of novel drug or material effects on tissue than traditional monolayer cell cultures. This work sought to better understand how key manufacturing variables affect the myotube characteristics of a skeletal muscle model toward reducing resource use and to develop an understanding of scaling on model consistency.

C2C12 murine myoblasts were seeded in a tethered collagen scaffold from which directional myotubes form in response to lines of tension and a change in medium. Collagen polymerising area length-to-width ratios greater than one were found to reduced cell-matrix attachment and remodelling forces significantly ($p<0.05$) correlating to a reduction in cell-fusion potential. Following this, utilising a factorial design-of-experiment, 4 million C2C12s/mL, with a polymerising area width 150% of the anchor point, produced the most favourable myotube characteristics and dramatically reduced the incidence of rupture. Scaled constructs showed no significant differences when compared to larger models. Approximately 20 myotubes with a variation in alignment of less than 25 degrees in the central region were consistently observed in the final models. This demonstrates the influence of initial manufacturing variables on tissue formation and has produced a benchmark model for consistent production across scaled constructs for future optimisation and as a potential cost-effective pre-clinical testbed.

Keywords

Biomanufacturing, *In Vitro*, Scale down, Skeletal Muscle, Tissue Engineering.

Introduction

In vivo skeletal muscle is a complex structure of uniformly aligned, terminally differentiated, multinucleated fibres arranged as fascicles within a collagen extra-cellular matrix (Baechle & Earle, 2008). 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 (MPCs) subsequently fuse to the existing muscle structure. This mechanism of MPC 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; Smith, Passey, Greensmith, Mudera, & Lewis, 2012; Tang et al., 2017; Zhao, Zeng, Nam, & Agarwal, 2009). In contrast, three dimensional (3D) skeletal muscle models have been shown to closely replicate the observed *in vivo* architecture (Khodabukus & Baar, 2009; N.R.W Martin, Turner, Farrington, Player, & Lewis, 2017; Smith et al., 2012). These models typically utilise two anchor points that act as pseudo-myotendinous junctions, allowing cells to align according to isometric lines of tension (Boonen et al., 2010; Dennis & Kosnik, 2000; Hinds, Bian, Dennis, & Bursac, 2011; Huang, Dennis, Larkin, & Baar, 2005; Neil R W Martin et al., 2013; Mudera, Smith, Brady, & Lewis, 2010; Neidlinger-Wilke, Grood, Wang JH-C, Brand, & Claes, 2001; Powell, Smiley, Mills, & Vandenburgh, 2002).

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 in high aspect ratio constructs. 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 modified for skeletal muscle, demonstrating cellular attachment and fusion (Cheema, Yang, Mudera, Goldspink, & Brown, 2003); the effect of cell density on 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 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 and includes many of the key stimuli for a skeletal muscle models as put forward by Maleiner et al. (2018).

High-throughput screening (HTS) is defined as the simultaneous analysis of multiple samples and, in the drug reporting industry, predominantly takes place in plates upwards of 384-wells (Fox, Wang, Sopchak, & Farr-Jones, 2002). 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, Brey, & Burdick, 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 enabling greater access to nutrients. To progress towards the use of a tissue engineered skeletal muscle model in pre-clinical safety 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 (Dennis & Kosnik, 2000; Garvin, Qi, Maloney, & Banes, 2003; Martin et al., 2013). Following this, utilising a factorial design of experiment (Hussein, Williams, & Liu, 2015), conditions to enable consistent production of skeletal muscle cultures were selected 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, Dennis, Larkin, & Baar, 2005). Utilising aspects of this process to enhance cell-cell interactions, the effect of hydrogel width extension (an area of cell/collagen suspension set wider than the anchor points) and the 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; hence, initial 24 hour force generation was measured,

comparing adjusted and non-adjusted models to observe possible effects of width extension cell-matrix interactions. Due to the variable nature of primary skeletal muscle precursor cells in producing myotubes of consistent number and characteristics (Nikolić et al., 2017), the murine myoblast cell line C2C12, a well characterised and commonly utilised cell source (Li et al., 2012; Sharples et al., 2012), was chosen to eliminate as much cell variation as possible. This enabled a focus on the influences of the manufacturing parameters on cellular architecture. Similarly, all serum (foetal bovine and horse) and collagen used were from a consistent batch to maintain continuity throughout the study.

Materials and Methods

Cell Culture

C2C12 murine MPCs (ECACC, UK) were cultured under humidified atmospheric O_2 and 5% CO_2 conditions at 37^{o}C (5% CO_2 in air). Cell populations were cultured in 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 (PS) (10,000U/mL and 10,000µg/mL, Gibco).

All experiments were conducted prior to passage 8 (P8, relative to passage received from supplier, >P14).

3D Tissue Engineered Collagen Skeletal Muscle Constructs

Collagen-based skeletal muscle constructs were prepared according to a protocol adapted from previous descriptions (Player et al., 2014; Smith, Passey, Greensmith, Mudera, & Lewis, 2012). 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), mixed thoroughly and then neutralised using 5M and 1M NaOH until a colour change from yellow to pink was observed. MPCs 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 1). The final solution comprised 85% Collagen, 10% 10x MEM and 5% cell suspension. Each A-frame consists of poly(ethelene-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.

Once the collagen/cell suspension was pipetted into the setting area, the chamber was left to polymerise at 37^{o}C for 15 minutes. Following this, the construct was detached from the walls of the setting area

using a needle and growth medium (GM) added to the well. Constructs were cultured for four days in GM. 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-Aldrich) and 1% PS for a further 10 days.

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; Sharples et al., 2012; Smith et al., 2012). Depth was calculated from the initial pipetted volume and known dimensions of each construct. The scaling model was then devised using calculated surface area and known volume ratios.

Construct width reduction was assessed in constructs with total volumes of 0.28, 0.4, 0.75, 1.0, 2.0 and 3.0 mL (Figure 1A-E), to better understand how cell mediated contraction through initial attachment and remodelling was affected by dimension variation and scaling (Figure 1F). These volumes were based upon a scaling model (Figure 1G) and the vessels available to culture in (configurations shown Table 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 polydimethylsiloxane (PDMS, Sylgard® 184 Elastomer) 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 PDMS boundary to define the setting area. Constructs were set using a seeding density of four million cells/mL of total construct volume.

Manufacturing Variables Optimisation Experimental Design

Utilising elements of quality-by-design (Thomas, Hourd, & Williams, 2008) through factorial design of experiment (DOE) software (Minitab Statistical Software, Minitab Inc., USA), the influences of variation in cell number/mL and size of width extension of collagen (Figure 1H) on myotube characteristics were assessed. Individual construct configurations are shown in Table 2. Width extension refers to the additional area added to the width of the collage/cell polymerisation area beyond the width of the A-frames (Figure 1H). This size is expressed as a percentage of the width of the A-frame, i.e. 25% width extension is an addition of a distance of 25% of the width of the A-frame to both sides of the setting area.

Immunofluorescence

Following culture, the constructs were washed twice with phosphate-buffered saline (PBS), fixed with methanol and acetone (1:1 v/v) and subsequently stained for desmin. Once fixed, constructs were placed into a blocking solution consisting of 5% goat serum and 0.2% Triton X-100 in Tris-buffered saline (TBS) for 2 hours and then incubated with rabbit anti-desmin antibody (Abcam, UK) diluted 1:200 in TBS overnight. The constructs were then incubated in the dark for a further two hours in goat anti-rabbit IgG Rhodamine-derived TRITC conjugated secondary antibody (Abcam) diluted 1:200 in TBS. DAPI was used as a counterstain to observe cell nuclei. Samples were incubated in a 1:10,000 dilution (1mg/mL, Thermo Scientific Pierce) in dH_2O for 3 minutes in the dark before being washed at least five times in distilled H_2O.

Construct Width and Myotube Characteristics

Macroscopic images of collagen constructs were analysed using ImageJ software [National Institutes of Health (NIH)] to determine construct remodelling over the culture period. Each construct width was measured three times at the centre point where remodelling effects are typically most observed. Images were captured using an Epson Perfection V330 Photo flatbed scanner. From this, cell densities after cell-mediated width reduction were calculated using the starting cell number and the calculated final volume. This method was chosen over others (MTT or nuclei counts) due to the effects of senescence, cell death and difficulties distinguishing nuclei in a multilayer tissue.

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. Mean individual myotube area (an analogue of individual myotube volume) was calculated per image from the mean length and width measurements.

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 first set and then transferred onto the culture force monitor (CFM) (Cheema et al., 2003; Mudera, Smith, Brady, & Lewis, 2010; Player et al., 2014). Construct force generation was measured for the first 24 hours of culture by a force transducer. 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 per minute.

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 $p<0.05$.

Results

3D Tissue Engineered Skeletal Muscle Model

Dimension Variation and Scaling Model

Much of recent work in this tissue engineered skeletal muscle model has focussed on using 3.0mL of collagen (Supplementary Table 1). This model was established on 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), previously defined in a 5.0mL model. Analysis of the physical characteristics of these published biomimetic constructs (5.0mL, 3.0mL and 2.0mL constructs showed a volume to surface area (V/SA) ratio of ~1.3 (Supplementary Table 1) 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. Based on the width of the anchor point and the length and volume of the 5.0mL and 2.0mL models and a V/SA ratio of 1.3, a scaling model was developed to enhance the flexibility of this skeletal muscle model (Figure 1G).

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 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. After 14 days in culture, different length to width ratios produced significant differences in final

width reduction (Figure 2). Both the 0.75mL and 0.4mL construct width reduced to a lesser extent than the 0.28mL constructs ($p<0.001$ and $p<0.05$ respectively). 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.

Width reduction and myotube characteristics

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:

$$y = -14x + 1207.6$$

Where y = myotube width (μm) and x = % width after 14 days (Pearson PMCC = -0.977, ρ=0.023, n=4). 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.

Investigation 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 understand the best observable conditions for a model suitable for pre-clinical experimentation that closest represents *in vivo* architecture, the effects of varying the cell density and extending the hydrogel width past the A-frame were investigated. Varying the cell density changes the number of cells available for fusion and influences 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 forces 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 manufacture. All constructs for this study were based on the length and anchor point dimensions of the 1.0mL model (Table 1) 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 (Supplementary Figure 1). This demonstrates that regardless of extension, the final width (as a percentage of the initial measurement) does not vary significantly between configurations. Based on these reduction figures, the final construct volume, and therefore assumed final cell density, can be calculated (Table 3). 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.

Alignment within the myotube population results from 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 can 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 observable variation in myotube orientation within each region (Figure 3). Four million/mL with a large extension (4L) shows significantly less myotube alignment ($p<0.05$, Figure 3F) 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) except for 2N. This demonstrates how the attributes of the cellular population are 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.

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 4). From observations of myotube characteristics through fluorescent imaging (Supplementary Figure 2), 4L produces myotubes of significantly greater length (Figure 4D, 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 number of myotubes (p<0.05) in both regions (Figure 4E). 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.

The myotube characteristic results were analysed (Table) in Pareto charts 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 3 and Figure 4 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.

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 (Supplementary Figure 3).

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. 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 present under best observed conditions, a small extension of width would be necessary.

Confirmation of Manufacturing Variables and Scaling Model

4 million cells/mL was chosen as the best observed 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 5, no significant differences were seen between the best observed configuration, 4N, and 4S except in favour of the adjusted model. Likewise, no significant differences were observed 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. Myotube formation was extensive in all models (Figure 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 best observed construct configuration (4 million cell/mL + 25% width extension, 1.5mL construct) was compared with 4N constructs (Figure 5). By measuring the initial cellular attachment force, it becomes apparent that despite a total of 6 million cells within the extended constructs, these constructs produced 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.

Discussion

This work sought to define the manufacturing variables required to produce a consistent, scalable skeletal muscle model towards a pre-clinical safety analysis system. 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 HTS.

Currently, bio-toxicity testing for materials and drugs takes place using monolayer models in accordance with ISO10993 for medicinal devices or through HTS in drug discovery (ISO Identification of Medicinal Products). However, in materials particularly, 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. This can result in large numbers of false positive which causes unnecessary animal tests. Tissue engineering shows huge promise in creating representative *in vitro* models of *in vivo* systems which can be used as test-beds for investigative and 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.

Published literature provides 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 dimension ratios on the attachment of C2C12's to the collagen, and their subsequent remodelling of the matrix, construct width reduction was analysed. Once MPCs have been seeded, 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, 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, Dennis & Kosnik 2000, Garvin et al. 2003). After 4-5 days of culture, the C2C12s within the collagen matrix presented here show equilibrium of mechanical forces, demonstrated by no significant reduction after this time point. During this period, 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 implies a greater cell density at the point of greatest reduction, and since MPCs 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, 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 width of 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).

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 vitro* myotubes are much smaller than *in vivo* skeletal muscle fibres and, therefore, larger myotubes better represent *in vivo* structures. The basic properties of the engineered skeletal muscle described here were analysed based on myotube characteristics. Regardless of the size of width extension and cell density, myotube characteristics did not differ significantly. Considering the variation in the number of MPCs available for fusion (total cell number/construct differed depending on volume), this result is seemingly paradoxical. However, within primary cell populations, MPCs 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 and only able to access a localised population for fusion. 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). However, a larger cell density (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 MPC percentage can differ between cultures depending on cell source. Within the 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 HTS. 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).

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 reported 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 4 million cells/mL and a 25% width extension had 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.

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. In its current guise, this model should only be utilised as an early safety indicator for pharmaceutical and material testing, with a particular focus on morphological variables.

Considering the effects of C2C12 MPC 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 dramatically 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.

Future work will seek to understand the manufacturing parameters influence on functional response and investigate the mechanical properties of the model. Furthermore, to truly validate this model and progress toward industry adoption, comparisons to existing accepted model’s biological responses to characterised pharmaceutical and physical interaction stimuli will be undertaken.

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Tables

Table 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)

Cell density ($x10^6$/mL)	Volume (mL)	Anchor Point Width (mm)	Distance Between Anchor Points (mm)
4	3.0	16	38
4	2.0	16	38
4	1.0	10	20
4	0.75	10	14
4	0.4	6	20
4	0.28	6	14

Table 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)

Cell density ($x10^6$/mL)	Width Extension (%)	Volume (mL)	Setting area (mm)		Designation
			Width	Length	
4	50	2.0	20	28	4L
4	0	1.0	10	28	4N
3	25	1.5	15	28	3S
2	0	1.0	10	28	2N
2	50	2.0	15	28	2L

Table 3: Manufacturing parameter effects: 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. # - cell density, million cells/mL; L – 50% width extension; S – 25% width extension; N – no width extension (e.g. 4L – 4 million cells/mL with a 50% width extension). 4L has a significantly greater calculated cell density and 2N has a significantly lower final cell density that all other conditions.

Construct Configuration	Calculated Final Volume (mL) ± SD	Calculated Final Cell Density ($x10^6$/mL) ± SD
4L	0.52 ± 0.02	15.52 ± 0.74
4N	0.65 ± 0.03	6.20 ± 0.27
3S	0.56 ± 0.02	7.98 ± 0.24
2N	0.70 ± 0.07	2.85 ± 0.30
2L	0.56 ± 0.05	7.19 ± 0.63

Table 4: Standardised effects from Pareto Charts 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. Effects listed left to right in order of magnitude.

	A-frame	Centre	Whole Construct
Mean Length	No Significant Effects	B, A	A
Mean Width	AB	No significant Effects	No Significant Effects
Mean Number of Myotubes	AB	AB	AB, A
Alignment	AB	B, A	

Figure legends

Figure 1: Collagen-based skeletal muscle construct setup: C2C12 MPCs suspended within a collagen hydrogel, anchored by A-frames. Construct volumes (Scale bar - 5mm): A) 2.0mL. B) 0.4mL. C) 1.0mL. D) 0.75mL E) 0.28mL. F) 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. G) Scaling Model based on the width of the anchor point, length and volume of the construct and volume/surface area ratio (1.3). For a given volume, dimensions of the A-frame and setting area can be obtained. H) Manufacturing Variables Optimisation: Construct width extension schematics.

Figure 2: 3D collagen construct dimension variation: construct centre final width (% of initial width). Width reduction at centre of construct. For construct dimensions see Table 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=4, * $p \leq 0.05$, *** $p \leq 0.001$, 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 significantly less width reduction. Depth change does not significantly affect width reduction.

Figure 3: Myotube angular distribution - degrees from horizontal. A) 2 million/mL, No extension, B) 2 million/mL, No extension, C) 3 million/mL, Small extension, D) 4 million/mL, No extension, E) 4 million/mL, Large extension, F) Angle (±) covering 90% of distribution. Lower values represent greater alignment. A-frame region alignment is lower than in the central region. Larger width extensions exhibit more aligned myotube populations within the central region (not significant) but lower alignment within the A-frame region. n=4, * $p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$, bars represent ±SD.

Figure 4: Myotube population characteristics showing variation between regions (Red-Desmin). Scale bar – 50 µm. A) Central region - demonstrates high alignment and myotube formation. B) Edge of A-frame region - Arrows denote direction of cellular alignment. Minimal myotube formation with variable alignment. Single cells observable as thinner, less prominent signal. C) A-frame region - Random alignment with formation of myotubes. Single cells observable as thinner, less prominent signal. (D-E) Construct myotube characteristics per image by region (D) Mean myotube length, (E) Mean number of myotubes. (n=4, * $p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$, bars represent ±SD). 4 Million C2C12s/mL with a large extension produces significantly larger myotubes than all other conditions in the central region. 4 Million C2C12s/mL with no extension produces significantly more myotubes than all other conditions across both regions. Legend applies to D and E.

Figure 5: Skeletal Muscle Scaling Model Validation: myotube characteristics per image. (A) Mean myotube length, (B) Mean calculated myotube area, (C) Mean number of myotubes, (D) Myotube angular distribution (lower values indicate greater alignment). (n=4, * $p \leq 0.05$, **$p \leq 0.01$, *** $p \leq 0.001$,

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. E) 1.5mL construct, best observed 4 million/mL 25% width extension configuration myotubes. Red – Desmin; Blue – DAPI. Scale bar – 100 µm. F) Initial construct mean force production through cell mediated contraction and remodelling (micronewtons, µN) (n=3). Constructs with width extension refers to an addition of 25% of the width of the A-frame to either side.