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Characterization and Optimization of a Simple, Repeatable System for the Long Term In Vitro Culture of Aligned Myotubes in 3D

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ABSTRACT

Increased recent research activity in exercise physiology has dramatically improved our understanding of skeletal muscle development and physiology in both health and disease. Advances in bioengineering have enabled the development of biomimetic 3D in vitro models of skeletal muscle which have the potential to further advance our understanding of the fundamental processes that underpin muscle physiology. As the principle structural protein of the extracellular matrix, collagen-based matrices are popular tools for the creation of such 3D models but the custom nature of many reported systems has precluded their more widespread adoption. Here we present a simple, reproducible iteration of an established 3D in vitro model of skeletal muscle, demonstrating both the high levels of reproducibility possible in this system and the improved cellular architecture of such constructs over standard 2D cell culture techniques. We have used primary rat muscle cells to validate this simple model and generate comparable data to conventional established cell culture techniques. We have optimized culture parameters for these cells which should provide a template in this 3D system for using muscle cells derived from other donor species and cell lines. J. Cell. Biochem. 113: 1044–1053, 2012.

KEY WORDS: IN VITRO; CELL CULTURE; SKELETAL MUSCLE

Standard 2D cell culture has been used in research into the understanding of skeletal muscle development for several decades. It has its limitations when translated to testing in vivo however, due to its relative simplicity and the complex and highly variable nature of living systems. Behavior of a wide variety of cell types (including skeletal muscle satellite cells) is known to be dependent on the niche they inhabit and greatly affected by alterations in external cues [Cosgrove et al., 2009; Bhatia, 2010]. To promote the greatest levels of cellular maturation and functionality in an in vitro culture it is therefore necessary to devise models that recapitulate the in vivo environment. 3D biomimetic in vitro test systems which accurately model in vivo physiology therefore represent a possible half way point between 2D cell culture and in vivo testing.

Skeletal muscle consists of bundles of highly differentiated, multinuclear fibers (myotubes) orientated uniaxially in order to facilitate contraction of the tissue in a single plane. This functional architecture therefore dictates the key factors for any 3D biomimetic in vitro model to emulate; namely the ability to promote the differentiation of muscle precursor cells (myoblasts) into myotubes and the orientation of these fibers into densely packed and highly orientated "fascicle-like" structures [Liao & Zhou, 2009].

One method for generating such organized muscle cultures in vitro has been to culture muscle derived cells (MDCs) in collagen

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hydrogels [Okano & Matsuda, 1998; Cheema et al., 2003, 2005; Vandenburgh, 2010; Hinds et al., 2011]. These systems are based on the concept that cells seeded within a collagen matrix will quickly adhere to and begin to contract the surrounding matrix. Setting a cell seeded collagen hydrogel between two fixed points leads to the generation of predictable lines of isometric strain in the construct as the cells pull against these stationary posts [Eastwood et al., 1998]. This cell-mediated tension provides a mechanical stimulus sufficient to promote the reorganization and alignment of the seeded cells along the lines of principle strain [Eastwood et al., 1996, 1998]. Gene expression analysis has demonstrated that muscle cells cultured in this environment exhibit significant upregulation of IGF-1 splice variants [Cheema et al., 2003, 2005] and myosin heavy chain (MYH) [Mudera et al., 2010] mRNA, as well as a shift in MYH isoform expression towards a slow phenotype [Mudera et al., 2010]. This data not only indicates the potential for differentiation that these cells possess but also shows how the 3D culture and mechanical environment appear to have strong effects on the construct's emerging phenotype.

Most published work regarding the 3D culture of skeletal muscle cells involves the use of custom built tissue culture environments [Dennis et al., 2001; Mudera et al., 2010; Vandenburgh, 2010; Hinds et al., 2011; Langelaan et al., 2011] (Table I). The development path to these cultures has been largely driven by the desire to recreate the appropriate anatomical structures. This follows the theory that structure can drive function and therefore if the structure is appropriate then the function will also be. To that end, the ease of construction of the relative culture systems has been of secondary importance, and whilst relevant data has been produced, the "scale out" of these systems has been minimal so far.

An example of this is the culture force monitor (CFM) system, which was designed to allow the real time recording of the contractile force generated by cells seeded within an exogenous collagen matrix [Brady et al., 2008]. As with most 3D models, the custom nature of the CFM, in its current guise, negates its widespread use as a standard culture model. Furthermore, the "semi-open" nature of the system makes the maintenance of cultures difficult in the long term.

This 3D collagen based in vitro model has already been shown to generate force as well as possessing other characteristics of skeletal muscle physiology [Cheema et al., 2005; Mudera et al., 2010]. We have now further developed the model, removing it from the custom CFM system into standardized and commercially available chamber slides. Previous work using the CFM system has focused on the use of human derived muscle cells [Brady et al., 2008; Mudera et al., 2010] and the C2C12 mouse muscle cell line [Cheema et al., 2003]. In order to expand the application of the 3D collagen model as a whole, we have now developed optimum conditions for promoting the development and maturation of primary rat muscle cells.

As well as simplifying the model for use in other labs, this modification of the standard protocol allows for the establishment of reproducible, long term cultures, better suited to the study of cellular maturation and physiology over more significant time periods. Successful generation of 3D cultures using primary rat cells as proof of concept will pave the way for the use of this system with cells derived from established animal disease models, providing a biomimetic 3D in vitro environment in which to investigate the behavior of other aberrant cell types.

Development of a simple, reproducible system for the 3D culture of muscle cells, utilizing as few custom built elements as possible, will increase the ease with which other groups are able to establish such systems in their own labs. The establishment of such a model should also lead to greater confidence in achieved results, by reducing the chance that variation in the culture environment will prevent the reproduction of published data by other groups.

MATERIALS AND METHODS

MDC ISOLATION

Primary rat MDCs were isolated from skeletal muscle samples from day old, postnatal (P1) rat pups. P1 Sprague Dawley rat pups were sacrificed by cervical dislocation in accordance with the code of practice for the humane killing of animals under schedule 1 of the Animals (Scientific Procedures) Act 1986. Hind limb muscle tissue was excised from the bones and cartilage, minced and incubated in 0.1% type II collagenase (GIBCO/Invitrogen, Paisley, UK) for 50 min in a shaking incubator at 37°C. The cell suspension was subsequently passed through a 100 μm and then a 40 μm mesh, to remove any undigested tissue fragments, before being spun down at 450*g* for 10 min. The cells were resuspended in a standard growth

TABLE I. Comparison of the Chamber Slide System used in this Study With Previously Published Methods for Establishing In Vitro, Three Dimensional Skeletal Muscle Cultures Using Collagen Matrices

System	Mould	Anchor points	References
Chamber slide model	Single well chamber slide (Commercially available)	Polyethylene meshwork (Custom built)	This paper
Culture force monitor	Custom built PTFE mould (Custom built)	Polyethylene meshwork (Custom built)	Cheema et al. (2003, 2005), Brady et al. (2008), Mudera et al. (2010)
Engineered muscle bundles	Longitudinally split silicon tubing (Custom built)	Velcro tabs (Custom built)	Rhim et al. (2007), Hinds et al. (2011)
High-throughput drug screening miniature bioartificial muscles	Vacuum moulded PDMS μ-wells (Custom built)	Vacuum moulded PDMS elastic μ-posts (Custom built)	Vandenburgh et al. (2008)
Murine bio-artificial muscle	Sylgard coated Petri dish (Custom built)	Velcro tabs (Custom built)	Gawlitta et al. (2008), Boonen et al. (2010), Langelaan et al. (2011)
Oriented hybrid muscular tissue	0.9 mm capillary tube mould (Custom built)	Glass rods (Custom built)	Okano & Matsuda (1998)
Skeletal muscle "organoids"	0.4 ml rubber tubing (Custom built)	Velcro tabs/stainless steel screening (Custom built)	Vandenburgh et al. (1996)

media (GM) consisting of 20% fetal calf serum (FCS) (PAA, Somerset, UK), 1% penicillin (100 U/ml) and streptomycin (100 mg/ml) (P/S) (GIBCO/Invitrogen) in high glucose DMEM (GIBCO/Invitrogen). The cell number was calculated using a hemocytometer and the cells were then ready for seeding.

ESTABLISHMENT AND MAINTENANCE OF 2D MDC CONTROL CULTURES

Sterile glass coverslips of 13 mm were placed into 24 well plates and coated with 0.2% gelatin (Sigma–Aldrich, Dorset, UK) for 30 min prior to plating. MDCs were isolated using the protocol described above and seeded at 25,000 or 50,000 cells/cm^2; plates were stored at 37°C in a humidified incubator. When the seeded cells reached confluency, GM was replaced with a differentiation media (DM) to promote myoblast fusion. DM consisted of 2% FCS, 1% P/S and IGF-1 (10 ng/ml) (Sigma–Aldrich) in high glucose DMEM and was used for the remainder of the culture period.

ESTABLISHMENT AND MAINTENANCE OF MDC SEEDED 3D CONSTRUCTS

The protocol for the construction of MDC populated collagen constructs was adapted from those detailed elsewhere [Brady et al., 2008; Mudera et al., 2010]. Briefly, 300 μl 10× MEM (GIBCO/Invitrogen) solution was added to 2.6 ml type 1 rat-tail collagen (First Link, Birmingham, UK) (in 0.1 M acetic acid, protein concentration n = 2.035 mg/ml) and mixed thoroughly. The solution was neutralized using 5 M NaOH (VWR, Leicestershire, UK) until a color change (yellow to pink) was observed and was then mixed with a suspension of MDCs in 300 μl GM. The complete solution was pipetted into a routinely available, single well chamber slide (NUNC, New York) which had a flotation bar fixed to either end (Fig. 1). The flotation bar consisted of three rectangles of polyethylene meshwork (Darice Inc., Strongsville, OH) bound together using 0.3 mm stainless steel orthodontic wire (UCL Eastman Dental Institute, London, UK) (Fig. 1A). Orthodontic wire of 0.7 mm was used to make a loop that could be driven into the flotation bar and bent over to hook onto the rim of the chamber slide, thereby holding the structure in place (Fig. 1B).

The chambers were placed in a humidified incubator at 37°C for 30 min to allow the collagen mixture time to set. Once set, the collagen construct was physically detached from the base of the mould using a sterile needle and floated in GM. The construct was incubated at 37°C and media was changed every day. When 2D control cultures reached confluency, the GM was removed from the constructs and replaced with DM for the remainder of the culture period. Constructs were photographed under sterile conditions at regular intervals during culture using a Canon PowerShot A460 5.0MP digital camera and construct areas calculated using ImageJ software.

qPCR

Levels of differentiation in 2D and 3D cultures were determined by qPCR analysis of myogenin expression. RNA from both 2D and 3D cultures was extracted using the TRIzol® reagent and following the manufacturer's protocol. The total mRNA was then reverse-transcribed into cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, CA). A reaction mix was prepared using the following ratios: 2 μl 10× RT buffer, 0.8 μl dNTPs (100 mM), 2 μl 10× RT random primers, 1 μl multiscribe reverse transcriptase (50 U/μl) and 4.2 μl nuclease-free water. This solution was mixed 1:1 with the RNA dissolved in nuclease-free water to a concentration sufficient to provide cDNA at 4 ng/μl. The reaction mix was then run in a thermo-cycler programmed according to the sequence: 25°C for 10 min, 37°C for 2 h and 85°C for 5 s.

qPCR was performed using Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Warrington, UK). The PCR reaction mixture (12.5 μl TaqMan universal PCR Master Mix, 2.5 μl cDNA, 1.25 μl probe, and 8.75 μl nuclease-free water) was made up for each gene to be analyzed in quadruplicate over a 96 well plate (Applied Biosystems). The plate was incubated at 50°C for 2 min and 95°C for 10 min before being cycled 40 times at 95°C for 15 s and then 60°C for 60 s. This protocol was specifically designed by Applied Biosystems for use with any assays designed according to Applied Biosystems assay design guidelines, therefore no optimization of the thermal cycling parameters was necessary for the probes used; myogenin (assay ID, Rn00567418_m1) and β-actin (assay ID, Rn00667869_m1).

The threshold cycle (C_T) was defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe exceeded a fixed threshold above the baseline. To quantitate the amount of each target gene present, the comparative C_T method was used, as outlined in Applied Biosystems *User Bulletin No. 2: ABI PRISM 7700 Sequence Detection System* (Applied Biosystems). The mean C_T values of quadruplicate samples from each group were determined and normalized to the endogenous housekeeping gene (β-actin). The amount of target amplification relative to the experimental control was calculated by the formula $2^{-\Delta\Delta Ct}$.

IMMUNOCYTOCHEMISTRY

Myoblast numbers and levels of fusion in 2D and 3D constructs were determined through immunocytochemical staining for the muscle specific intermediary filament protein desmin. 3D constructs and 2D controls were fixed using an ice-cold methanol/acetone solution. The 3D constructs were then cut into 2 mm^2 sections using a scalpel blade, collected on poly-lysine coated slides (VWR) and air-dried for 1 h. 3D and 2D samples were permeabilized using 1× TRIS (0.5 M) buffered saline solution (TBS) (+5% normal goat serum (NGS) and 0.2% Triton X-100) for 1 h. They were then incubated overnight at room temperature with a mouse monoclonal anti-human desmin primary antibody (clone D33; Dako, Glostrup, Denmark) diluted 1 in 200 in TBS (+2% NGS and 0.2% Triton X-100). After overnight incubation, the samples were washed thoroughly in TBS before being treated with a goat anti-mouse FITC secondary antibody (Stratech, Suffolk, UK) diluted 1 in 200 in TBS (+2% NGS and 0.2% Triton X-100). Nuclei were identified using the fluorescent minor-groove DNA binding probe DAPI (4,6-diamidino-2-phenylindole; 1.0 ng/ml; Sigma–Aldrich) which was incorporated into the secondary antibody incubation stage. The samples were incubated for 3 h at room temperature in a darkened chamber before being dried and mounted with glass cover slips (VWR) using a drop of MOWIOL mounting medium. 2D controls were dried and affixed to glass microscope slides, again using a drop of MOWIOL mounting

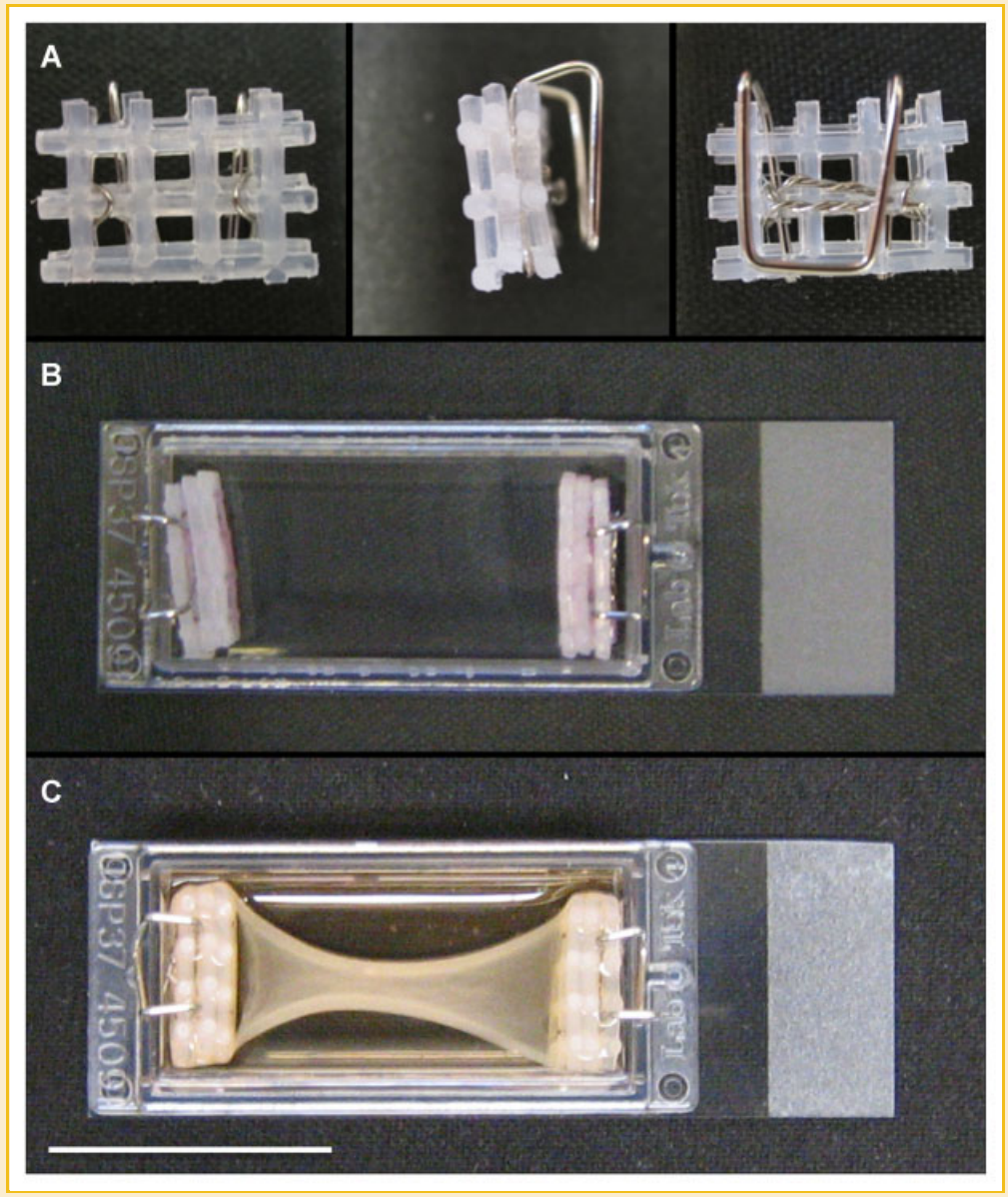

Fig. 1. The chamber slide model. A: Details of the flotation bars used to provide isometric tension to the collagen construct. B: Chamber slide prepared with flotation bars at either end. C: Collagen construct seeded with 5×10^6 MDCs/ml demonstrating the "bowing" caused by contraction of the seeded cells; a phenomenon characteristic of this model. Scale bar = 25 mm. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

medium. Cells were visualized using a Zeiss LSM510 Meta Confocal microscope and accompanying software.

Collected images from 3D constructs were analyzed using Metamorph software (Universal Imaging Corporation, Downington, PA); an image was taken every 5 μm through the 3D construct at five random points in the gel. For each image, the number of nuclei incorporated into myotubes, as well as the total number of desmin positive nuclei present was counted. In this study, all nuclei incorporated into myotubes were counted as desmin positive nuclei. The numbers from each image were totalled and the fusion efficiency for each construct was then calculated as the number of nuclei incorporated into myotubes expressed as a percentage of the total number of desmin positive nuclei counted.

Myoblast number and fusion efficiency was likewise calculated for 2D control cultures, counting desmin positive nuclei from 10 random images per 13 mm coverslip and using 3 coverslips per culture. The number of images taken in both 2D and 3D was demonstrated to be sufficient to provide a true indication of the mean as determined by cumulative frequency analysis (data not shown). Across all experiments, a myoblast was defined as any mono-nuclear desmin positive cell, while a myotube was defined as a desmin positive cell possessing three or more nuclei.

STATISTICAL ANALYSIS

Statistical significance between mean values for 2D and 3D cultures were assessed using *t*-tests performed in Sigma Stat (version 2.03,

Erkath, Germany). Significant differences in fusion efficiency at multiple seeding densities in 3D, as well as myogenin expression levels over 2D and 3D time-courses were each assessed using one way analysis of variance (ANOVA), again in Sigma Stat. Values are expressed as mean (±standard error of the mean) and a minimum of $n = 3$ was used in all experiments. Significance was set at $P < 0.05$.

RESULTS

MDC ISOLATION

2D cultures of primary rat MDCs, isolated using the described method and immunostained for desmin, possessed a consistent population of myogenic cells. Myoblasts were found to make up 28.80% (±2.69, $n = 9$) of all cells in culture, indicating a substantial and consistent population of cells committed to the myogenic lineage in these cultures.

DIFFERENTIATION OF MDCs IN 2D AND 3D CULTURE MODELS

Myogenin expression levels from MDCs cultured in 2D and 3D were measured over a 3 week culture period using qPCR. Myogenin has been used previously as an indicator of the potential for differentiation in such cultures where live imaging is problematic due to construct thickness [Mudera et al., 2010]. Determination of whether or not differentiation abilities are equivalent in 2D and 3D systems was achieved through comparison of 2D cultures established at 25,000 MDCs/cm^2 and 3D constructs set up at 1 million MDCs/ml. Levels of differentiation were inferred from expression levels of myogenin mRNA.

Analysis of the myogenin qPCR data (Fig. 2) indicated a decrease in myogenin expression over time as a general trend in both 2D and 3D. In 2D culture, there was a significant drop in myogenin expression levels between days 3 and 9 ($P = 0.003$) and between days 6 and 13 ($P = 0.006$). From day 13 until the end of the culture period myogenin expression remained at a constant low level, with no further significant differences seen at any subsequent time point.

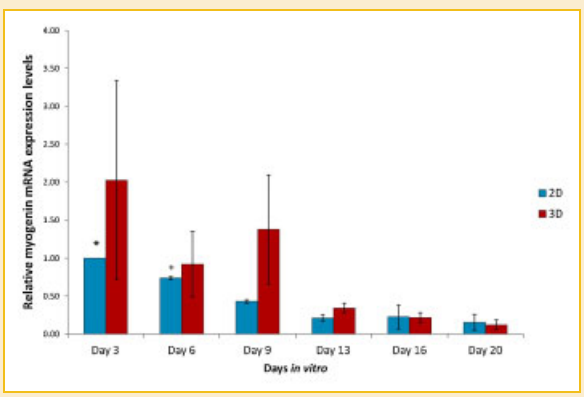

Fig. 2. Myogenin expression in MDCs from 2D and 3D cultures, over 3 weeks in vitro, as determined by qPCR. CT values were normalized to an internal housekeeping gene (β-actin) and expressed relative to levels recorded for 2D culture at day 3. $n = 3$, error bars = standard error of the mean. In 2D, myogenin expression at day 3 was found to be significantly greater than expression at days 9, 13, 16, and 20 ($^*P < 0.005$). Expression at day 6 was also found to be significantly greater than expression at days 13, 16, and 20 ($+P < 0.01$). No significant differences were observed between 2D and 3D cultures at equivalent time points ($P > 0.05$). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

By comparison, myogenin expression in the 3D model was considerably more variable and remained at a consistently high level over the first 9 days in culture. By day 13, expression patterns more closely resemble those in 2D culture, with a substantial drop both in expression and in variance between cultures. While the mean myogenin expression at day 3 was twice that seen in 2D culture, the substantial variability between cultures removed any significance from this difference ($P = 0.478$). Likewise, there was no significant difference in expression between the 3D cultures and their 2D counterparts at days 6 ($P = 0.696$) and 9 ($P = 0.258$), despite substantially greater mean expression levels in 3D at the latter time point. From day 13 onwards, expression levels were broadly equal between 2D and 3D cultures, with no significant differences found at any time point ($P > 0.05$). This data suggests that fusion events in 2D cultures occur early on and quickly tail off. While myogenin levels in 3D are not significantly different from 2D cultures, and likewise tail off over time, the rate at which this occurs is far more variable at early time points, indicating a more changeable rate of fusion in such cultures.

A lack of significant difference between the two culture models at each time-point examined indicates that the potential for differentiation in conventional 2D culture, while not improved, is preserved in 3D. Cells were seeded in 2D at a low density to prevent their detachment from the plastic and ensuring their survival over the entire 3-week culture period. This low seeding density led to low levels of myotube formation in these cultures as indicated by immunostaining of duplicate cultures at equivalent time points (Fig. 3A). Similarly, immunostaining of frozen sections of the 3D constructs revealed very low levels of myotube formation (Fig. 3B).

EFFECT OF INCREASING MDC SEEDING DENSITY ON MYOBLAST FUSION EFFICIENCY IN 3D CULTURE

Since differentiation of muscle precursor cells is known to be highly dependent on cell–cell contact [Krauss et al., 2005], the effect of seeding density on the ability of MDCs to fuse in a 3D environment was investigated. Constructs were established at 3, 4, 5, and 6 million MDCs/ml and cultured for 2 weeks. They were then fixed, stained, and analyzed by confocal microscopy for desmin positivity.

At 5 million MDCs/ml the fusion efficiency was calculated as 73.16% (±2.05) of all myogenic nuclei present (Fig. 4). At this density, only 30% of the desmin positive cells in 3D culture failed to fuse into myotubes, indicating the strong potential for differentiation in cells seeded at this density. By comparison, at 3 million MDCs/ml the fusion efficiency was just 15.37% (±5.66) demonstrating that the majority of cells at this density were unable to differentiate into multinuclear myotubes. The collected data indicates that MDC seeding density is a critical parameter in defining the degree of construct maturation possible in 3D. It should therefore be carefully considered when beginning to use cells from different sources within this model.

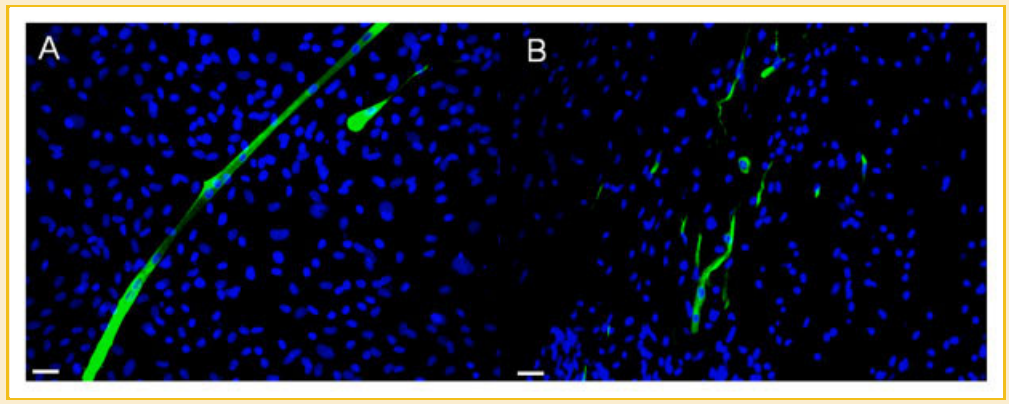

Fig. 3. Primary rat MDCs in culture immunostained for desmin (green) with a DAPI nuclear counter stain (blue). Note the low levels of fusion in (A) 2D culture at 25,000 MDCs/cm^2 and (B) in 3D at 1 million MDCs/ml (B). Scale bars = 20 μm.

Significant increases in fusion efficiency were found at each seeding density examined up to 5 million MDCs/ml (Fig. 4). At 6 million MDCs/ml it was not possible to reliably culture the cells for the full 2 week period since the constructs generated sufficient force to pull themselves off of the frames, thereby destroying the uniaxial tension developed. From this data it is clear that there exists a critical balance between cell seeding density and the level of force that these cells generate. This balance defines the upper and lower limits of the 3D system; critical parameters which should be kept in mind when adapting the model for using different cell sources.

As a comparison, 2D culture seeding density was increased to 50,000 MDCs/cm^2; in our hands, the highest density to promote reliable cell attachment, survival, and differentiation. At this density, the seeded MDCs demonstrated a fusion efficiency of 61.46% (±1.16) which is significantly lower than its equivalent in 3D culture (Fig. 5). A weakness of conventional culture techniques for maintaining differentiated MDCs is that such cultures do not commonly survive at high density for more than a few days in vitro [Vandenburgh et al., 1988]. To ensure cells were analyzed when still alive, 2D cultures were examined following 4 days in DM. Although this time point is not equivalent to the 2 week time point for 3D cultures, it was the longest the cultures could be reliably maintained. Both models can therefore be said to have been analyzed at time points which demonstrate the most mature phenotype attainable given the culture limitations.

The level of differentiation of high density primary MDCs using conventional 2D tissue culture methods is not only significantly lower than in 3D culture, but the maintenance of such cultures for any substantial length of time is problematic due to the cell's spontaneous contractions pulling them off of the substrate. By comparison, differentiated cells at high density in 3D were maintained for 2 weeks in this instance and if necessary can be maintained for significantly longer periods (up to 8 weeks in our hands).

MACROSCOPIC OBSERVATION OF THE DEVELOPING 3D CULTURE MODEL

Macroscopic observations of collagen constructs seeded with 5 million MDCs/ml revealed significant contraction of the gel area

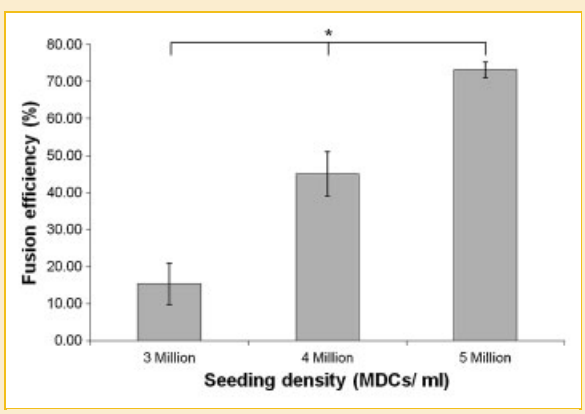

Fig. 4. Effect of increasing seeding density on the fusion efficiency (percentage of myogenic nuclei incorporated into myotubes) of primary rat MDCs in 3D collagen culture. n = 3, $^*P < 0.001$.

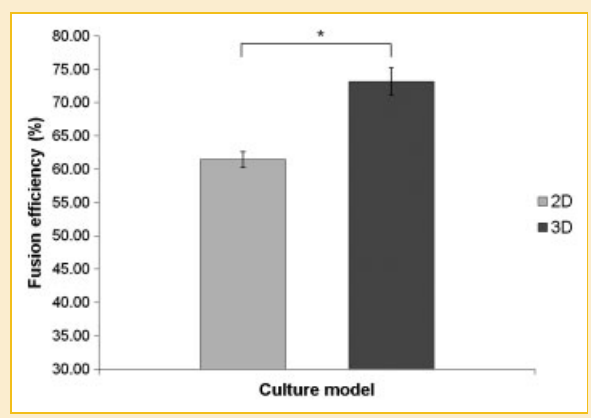

Fig. 5. Difference in the fusion efficiencies of primary rat MDCs seeded at high density in 2D and 3D culture models. Fusion efficiency was measured after 2 weeks for 3D cultures and after 4 days in DM for 2D cultures. n = 3, $^*P = 0.008$.

over a 3-week culture period. The fixed flotation bars within these constructs prevent matrix contraction across the long axis and means that the only mechanical give in the system is across the construct's short axis. Contraction of the seeded cells therefore leads to a characteristic bowing across the short axis which increases incrementally over time (Fig. 6A). This trait is also seen when observing constructs established within the CFM system [Eastwood et al., 1996].

An attempt to quantify the degree of matrix contraction in these constructs was made through measurement of collagen matrix surface area at regular intervals throughout the culture. Constructs were established with an initial surface area of 550 mm^2 which reduced to 176.99 mm^2 ($\pm$41.68) after 21 days in vitro. This represents a decrease in construct area of approximately 70% over 3 weeks (Fig. 6B). Decrease in construct area was found to be rapid during the first week in culture and slower at later time points. A reduction in construct area by roughly 40% was seen in the first week; over 7 days in vitro, the gel area reduced from 550 to 334.94 mm^2 ($\pm$37.69). Contraction in the second week of culture was by a further 20%, from 334.94 to 225.03 mm^2 ($\pm$45.08) and by just 10% in the third week, from 225.03 to 176.99 mm^2 ($\pm$41.68). Matrix remodeling of this type is not thought to be indicative of direct contraction of stimulated muscle fibres in culture but rather the result of slow passive remodeling of the matrix by supporting cell types. This phenomenon is characteristic of this culture type and indicative of the strength of the directional mechanical signal delivered to the seeded cells.

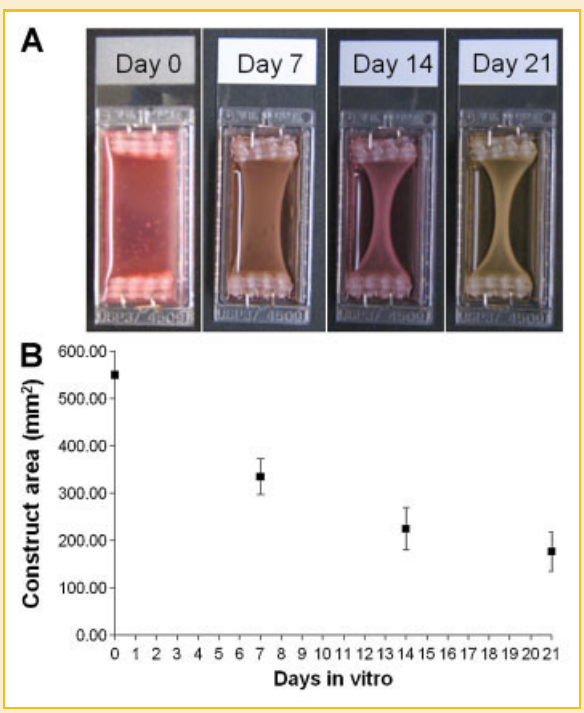

Fig. 6. Contraction of a collagen construct seeded with rat primary MDCs over 21 days in culture. A: Macroscopic appearance of a collagen construct seeded with 5×10^6 ml^{-1}. The construct was photographed at intervals over a 21 day culture period, images from day 0, 7, 14, and 21 are shown. B: Surface area of collagen constructs seeded with 5×10^6 primary rat MDCs/ml over 21 days in culture, n = 5. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

CELLULAR ARCHITECTURE OF MDCs SEEDED IN 3D CULTURE

Phase contrast microscopy of the cells seeded within the 3D collagen constructs revealed that the MDCs showed alignment along the lines of principle strain in a longitudinal direction between the anchor points at each end of the construct as early as 48 h post-seeding. This behavior has been described previously in 3D collagen constructs using the established CFM system and is not observed in collagen constructs in the absence of the uniaxial tension provided by fixed floatation bars [Vandenburgh et al., 1988].

Immunohistochemical analysis of the 3D constructs demonstrated the alignment of differentiated myotubes in parallel with the long plane of the gel (Fig. 7A). 2D control cultures (Fig. 7B) showed no such organization, indicating the improved cellular architecture of MDCs cultured in 3D. Comparison of 2D and 3D culture images to sections of in vivo skeletal muscle tissue (Fig. 7C) further demonstrates the improved biomimicity of MDCs cultured under uniaxial tension. In 3D culture, differentiated myotubes were found densely packed and lying in a parallel conformation, mirroring that seen in vivo, while 2D cultures demonstrated no organized structure or notable orientation. Furthermore, and again similar to in vivo muscle, the myotubes in 3D constructs were entirely unbranched in all cultures examined (n = 9). In 2D culture however, branching was seen in 8.59% ($\pm$1.38) of the myotubes present (n = 3).

DISCUSSION

The requirement to study the behavior of cells in a biologically relevant in vitro environment necessitates the advent of culture systems which accurately mimic in vivo conditions. In the case of skeletal muscle, this means the alignment of densely packed, differentiated myotubes into a parallel array of fibres, capable of performing directed work. Comparison of immunostained 2D and 3D cultures to sections of in vivo muscle tissue clearly demonstrates that the cellular architecture of skeletal muscle is much more closely recapitulated by the 3D collagen construct then by standard 2D tissue culture models.

The myoblast population was found to make up 28.80% of the total number of MDCs isolated from P1 rat pups in this study. This result is similar to that observed in work published previously regarding primary rat muscle cells from the hind limbs of newborn rat pups [Kaufman et al., 1991]. While purification methods for myoblasts have been developed [Baroffio et al., 1993; Rouger et al., 2007; Brady et al., 2008], previous work using the CFM system has demonstrated that the nonmyogenic/fibroblast fraction is required in order to promote matrix contraction and thereby promote the tension required to facilitate cellular reorganization [Brady et al., 2008]. It was therefore decided to utilize the mixed myogenic and nonmyogenic MDC population throughout this study.

The bowing of the collagen constructs (Fig. 1C) as they develop is characteristic of this culture model. Recent work involving electrical stimulation of a similar 3D collagen model [Langelaan et al., 2011] has demonstrated that a twitch response is elicited from that

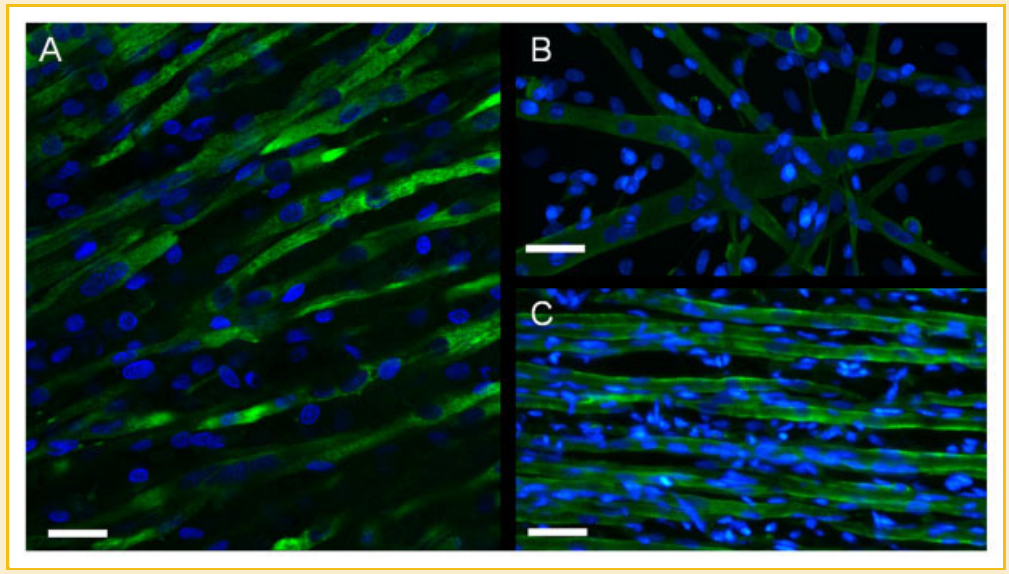

Fig. 7. A: A longitudinal section from a 3D collagen construct seeded with primary rat MDCs at 5 million cells/ml and immunostained for desmin (green) and the nuclear marker DAPI (blue). B: Primary rat MDCs seeded on 2D tissue culture plastic at 50,000 cells/cm^2 and again stained for desmin (green) and DAPI (blue). C: A longitudinal section of in vivo skeletal muscle from the hind limb of a P1 rat pup stained for desmin (green) and DAPI (blue). Scale bars = 20 μm.

construct when stimulated. The rapid twitch observed is believed to be the direct result of active contraction of the differentiated myotubes in culture. This in turn suggests that the slow and incremental bowing seen in these un-stimulated constructs equates to matrix remodeling in response to both active myotube contraction and the passive contraction of supporting cell types within the 3D collagen hydrogel. After seeding, cells (particularly fibroblasts) quickly adhere to the collagen matrix surrounding them. As they attach, cells generate contractile force which in turn begins to draw the surrounding matrix in [Stopak & Harris, 1982]. The floatation bars maintain the length of the construct but not the width, leading to contraction across the short plane of the construct over time. From this it can be deduced that the level of bowing (i.e., the reduction in construct area) provides a strong indication as to the level of matrix remodelling carried out by the cells as they mature and is therefore indicative of the strength of the mechanical signal delivered to the seeded cells. Comparison of levels of matrix remodeling may become important when analyzing the performance of different cell types within such 3D models and should be considered in cases where certain populations fair better than others in terms of alignment or cellular maturation.

In the case of primary rat MDCs, the directional cue provided by the aforementioned matrix contraction was sufficient to promote cellular reorganization in parallel with the lines of tension in all cultures examined ($n = 9$) as indicated by immunohistochemical data and as has been demonstrated previously [Eastwood et al., 1996]. This uniformity in cellular activity provides an indication of the reliability of this culture method for generating organized in vitro muscle cultures along lines of principal cell generated tension. Furthermore, it demonstrates that the cellular alignment seen in constructs utilizing both primary human and C2C12 cells in the CFM system [Cheema et al., 2003, 2005; Mudera et al., 2010], is conserved when using primary animal cells in the chamber slide model. Hence it seems clear that the predominant cues that the cells respond to in these models are predictable and mechanical and not altered depending on donor cell type.

The unbranched conformation of the cultured myotubes indicates the added benefit to MDC differentiation in vitro that provision of a directional signal affords. In conventional 2D culture, adherence of the cells to a flat, rigid surface provides multiple attachment points and directional cues which prevent the development of the cells in an organized manner. This results in the fusion of randomly aligned myoblasts, which in turn leads to the formation of branching myotubes; a feature uncharacteristic of in vivo muscle fibres. The integration of a uniaxial directional signal into the 3D culture model therefore promotes the orientation of cultured fibres into a far more physiologically relevant cellular model for in vitro study.

Comparing myogenin expression levels between 2 dimensional and 3 dimensional constructs as an indicator of muscle differentiation ability reveals no significant difference between the two systems. While 2D cultures exhibit significant drops in myogenin expression levels over the first 2 weeks however, 3D cultures maintain consistent expression levels up to day 9. This observation implies that while differentiation of MDCs in 2D culture is rapid, with most fusion events occurring at the very beginning of the culture period, it is somewhat more varied in 3D culture with consistent expression levels over 9 days. The disparity in myogenin expression trends between culture systems is possibly attributable to more complex cellular interactions in 3D. While cells in 2D culture need only align themselves on 2 axes, cells in 3D must align in 3 planes before fusion is possible. In all probability this will lead to greater variation in the levels of myoblast fusion occurring at any one moment within this system. Furthermore, the contraction of the construct as it develops will bring a greater number of myogenic nuclei into contact with one another over time which will in turn lead to new opportunities for fusion events to take place.

The variability in cellular interaction over the first 2 weeks also likely explains the substantial deviation in expression levels seen between individual 3D cultures at single time points. This variability between 3D cultures results in large standard errors and removes any significance in the different myogenin expression levels seen between 2D and 3D on days 3, 6, and 9. Based on this data therefore, it cannot be said that MDC differentiation is significantly improved or inhibited by transfer to the 3D model, despite the apparent differences in trends between the culture systems.

While this analysis seems logical based on the myogenin expression data alone, conclusions regarding equal levels of differentiation in 2D and 3D are not upheld when the seeding density is increased. Analysis of immunohistochemical data from high-density cultures demonstrated significantly higher fusion efficiencies in 3D cultures seeded at 5 million MDCs/ml when compared with 2D controls seeded at 50,000 MDCs/cm^2 (Fig. 5). The ability for the 3D model to promote greater levels of fusion is difficult to confirm however, since it cannot be stated for certain that the seeding densities examined in 2D and 3D are in any way equivalent. For each culture model the highest density to produce viable cultures was used, but it seems likely that the compliant nature of the 3D model permits the successful culture of more densely packed cells than is possible in 2D. If this is the case then the significant increase in fusion efficiency observed would rather be a result of a higher density in 3D rather than any inherent differences in the culture environment.

Low density and high density data combined suggests therefore that the capacity for MDC differentiation is broadly equal in 2D and 3D as measured by myogenin data. The compliant nature of the 3D model however permits the establishment of more densely seeded cultures which in turn allows for greater levels of fusion to be achieved.

The varied rate of differentiation over the first 2 weeks in culture holds strong implications for the future use of this model. Between days 9 and 13 there is a substantial drop in myogenin expression in 3D culture and the variance between cultures is greatly diminished. Myogenin is known to be constitutively expressed at low levels in skeletal muscle tissue in vivo [Sinanan et al., 2006; de Almeida et al., 2010]. The shift in expression pattern between these two time points implies that by day 13, myogenin expression has dropped to the low levels seen in mature tissues and, consequentially, that no further fusion events are occurring. This in turn suggests that any future analysis of these constructs should occur after 13 days in vitro, once differentiation of MDCs has subsided and the construct enters a more stable state. Adherence to this experimental protocol should ensure the greatest levels of reproducibility between experimental cultures. Alternative methods for culturing aligned myotubes using a fibrin based model have also stated that construct maturation takes up to 10 days [Khodabukus et al., 2007], indicating that similar lengths of time are required for construct maturation regardless of the model used.

Initial experiments utilized constructs seeded at 1 million MDCs/ml; a density previously optimized using primary human fibroblasts for generating predictable lines of tension within the system [Eastwood et al., 1996, 1998]. However, immunohistochemical data confirms that the ability of seeded MDCs to differentiate into multinuclear myotubes in 3D is highly density dependent. This is likewise known to be the case in conventional 2D culture [Lindon et al., 2001] and follows a simple logic; the greater the seeding density, the greater the number of myoblasts in culture and hence the greater the probability that they will be seeded at a sufficiently close proximity to fuse with their neighbors. In theory then, by continuing to increase the seeding density of MDCs, it should be possible to achieve greater and greater levels of myotube formation and the increased density of the resulting fibres into a more fascicule like arrangement. This ability is limited by the inherent strength of the model tissue however; a seeding density of 6 million MDCs/ml led to the formation of an unreliable construct that sometimes survived and sometimes pulled itself off the frames. The tendency for densely seeded MDCs to detach themselves from their substrate is not completely removed when using the 3D model it seems, but the flexibility of the matrix permits far higher fusion efficiencies to be maintained for weeks in culture rather than days.

An optimum seeding density of 5 million MDCs/ml is suggested for primary rat cells by the data collected here. In comparison, similar work using the C2C12 mouse cell line [Cheema et al., 2003] highlighted 4 million MDCs/ml as an optimum seeding density. Conversely, work with primary human cells in this model suggests an optimum of 8 million MDCs/ml for generating the most physiologically relevant model tissue possible [Mudera et al., 2010]. This disparity is likely attributable to the significant differences in the size of the myogenic fraction in MDC populations from different sources. The C2C12 cell line is a homogeneous population of myogenic cells and as such requires lower seeding densities than primary MDCs which possess a significant number of fibroblasts and other nonmyogenic cell types. The reduced presence of myogenic cells in culture, when compared to the C2C12 cell line, suggests that a larger seeding density would be required in primary cells in order to illicit similar levels of fusion and construct maturation. The human cells from the published study were from adult human donors while the rats used in this study were from P1 pups. Since the fraction of myogenic precursors from tissue of increasing age is known to drop off substantially [Kaufman et al., 1991] it seems likely that this higher optimum seeding density is attributable to a smaller myogenic fraction in cells from these donors.

Although a considerable amount of work has gone into the study of these 3D collagen based muscle constructs at early stages of development (0–7 days in vitro) [Cheema et al., 2003; Brady et al., 2008; Mudera et al., 2010], long term culture has been difficult due to the open nature of the CFM system. By moving this model into a completely sterile environment we have been able to establish and optimize 3D cultures capable of surviving for weeks in culture. This step forward not only reduces the custom nature of the culture model for use elsewhere, but also allows for analysis of the behavior and maturation of the cultured cells over a greater length of time. Greater levels of differentiation, the ability to maintain densely seeded cultures over many weeks and the improved cellular architecture afforded the seeded cells, all act to highlight the significant advancement to standard culture techniques that this model represents. The free availability of the required reagents and relatively minor expertise required to establish and maintain these highly reproducible cultures,

make this model a strong candidate for the continued in vitro study of skeletal muscle development and physiology.

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