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Technique Article

Dissociated Flexor Digitorum Brevis Myofiber Culture System—A More Mature Muscle Culture System

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Considerable knowledge regarding skeletal muscle physiology and disease has been gleaned from cultured myoblastic cell lines or isolated primary myoblasts. Such muscle cultures can be induced to differentiate into multinucleated myotubes that become striated. However they in general do not fully mature and therefore do not model mature muscle. Contrastingly, fresh and cultured dissociated adult mouse flexor digitorum brevis (FDB) myofibers have been studied for many years. We aimed to investigate the possibility of using the FDB myofiber culture system for drug screening and thus long-term cultures of enzymatically dissociated FDB myofibers were established in 96-well plates. Ca^{2+} handling experiments were used to investigate the functional state of the myofibers. Imaging of intracellular Ca^{2+} during electric field stimulation revealed that calcium handling was maintained throughout the culture period of at least 8 days. Western blot and immunostaining analysis showed that the FDB cultures maintained expression of mature proteins throughout the culture period, including α-sarcoglycan, dystrophin, fast myosin heavy chain and skeletal muscle α-actin. The high levels of the fetal proteins cardiac α-actin and utrophin, seen in cultured C2C12 myotubes, were absent in the FDB cultures. The expression of developmentally mature proteins and the absence of foetal proteins, in addition

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to the maintenance of normal calcium handling, highlights the FDB culture system as a more mature and perhaps more relevant culture system for the study of adult skeletal muscle function. Moreover, it may be a useful system for screening therapeutic agents for the treatment of skeletal muscle disorders. Cell Motil. Cytoskeleton 2007.

Key words: muscle; disease; drug screening; foetal isoforms

INTRODUCTION

Skeletal muscle is the principal tissue of the vertebrate body and its main function is to generate movement through the environment. Over the last 20 years the genetic basis of many skeletal muscle diseases has been established (http://www.musclegenetable.org), however treatment has remained elusive even for Duchenne muscular dystrophy (DMD) [Nowak and Davies, 2004] the first major muscle disease for which a gene was identified [Koenig et al., 1987]. Experimental approaches including myoblast transfer, stem cell treatments, upregulation of alternative genes, viral therapy, siRNA and antisense oligonucleotides are currently all being investigated as possible treatments for skeletal muscle diseases [reviewed in Odom et al., 2007; Price et al., 2007; Rando, 2007].

Considerable understanding of skeletal muscle physiology and pathology has been obtained from research using in vitro culture and differentiation of primary or cell-line derived myoblasts [reviewed in Meola, 1991] and these cultures are used to test possible therapies for skeletal muscle diseases [Ashizawa et al., 1982; Quinn et al., 2002; Zhang et al., 2004]. Multinucleated myotubes can be formed through differentiating myoblasts in mitogen-poor media. They express a cohort of myofibrillar proteins and gradually assemble an ordered array of sarcomeres. However converting these cultures into completely mature muscle fibers has remained largely intangible, despite numerous attempts, including co-culturing with neural explants [Kobayashi et al., 1987; Wagner et al., 2003; Larkin et al., 2006], a fibroblastic layer [Cooper et al., 2003] or by exposing the cultures to low frequency electrical stimulation [Naumann and Pette, 1994].

Cross-striated patterns are often only seen in myotubes after multiple weeks of culture [Harvey et al., 1979], even when they are co-cultured with spinal cord [Kobayashi et al., 1987; Wagner et al., 2003]. Most systems result in the predominant expression of immature myosin heavy chain (MHC) isoforms, such as primary rat myotube cultures (even after chronic electrostimulation of myotube cultures for 20 days) [Naumann and Pette, 1994], C2C12 myotubes [Morgan and Madgwick, 1999; Cooper et al., 2004] and 4-week-old three-dimensional myotube constructs co-cultured with fetal nerve explants [Larkin et al., 2006]. However, rabbit primary myotubes cultured on gelatin beads were shown to express solely adult MHC and myosin light chain isoforms after 16 days in culture [Kubis et al., 1997], but this is a time-consuming system that results in the myofibers having an unusual structure bent round the beads, which may be difficult to visualise.

A number of research groups have utilised enzymatically dissociated mouse flexor digitorum brevis (FDB) myofibers for the study of aspects of muscle cell physiology, mostly recently [Cifelli et al., 2007]. Anderson and co-workers have extensively studied the mechanism of action of satellite cell quiescence and activation [reviewed in Wozniak et al., 2005], using short-term cultures of single FDB muscle myofibers, as described initially by Bischoff [1986]. Studies of FDB cultures have included the effect of culture conditions upon fiber survival [Brown and Schneider, 2002], calcium homeostasis [Liu et al., 1997], dedifferentiation [Brown et al., 2006] and the effect of chronic electrical stimulation on fiber type-specific gene expression [Liu and Schneider, 1998]. Both long-term cultured [De Backer et al., 2002] and freshly isolated [Han et al., 2006] FDB myofibers have been employed to study the pathophysiology of dystrophic muscle. Thus the unique properties of the FDB myofibers have been exploited by physiologists for the past 20 years for the study of basic skeletal muscle physiology and the pathophysiology of disease. This article investigates the FDB culture method as a more mature model of skeletal muscle in vitro.

One concept in the search for therapies for various genetic skeletal muscle disorders is the up-regulation of an alternate gene to produce a functional substitute for the absent or defective protein. This has been extensively investigated in animal models of DMD for example, whereby up-regulation of utrophin (the fetal form of dystrophin) has been shown to alleviate the disease phenotype associated with the absence of dystrophin, [reviewed in Hirst et al., 2005]. The up-regulation of fetal isoforms of skeletal muscle proteins as a therapy for muscle diseases may be applicable to a number of skeletal muscle disorders. Conducting small molecule screens for the up-regulation of these fetal proteins in

myotube cultures would be confounded by endogenous expression of these fetal isoforms and drug screening for activating expression of other "rescue" proteins might also benefit from a more mature myofiber culture system. We show that the FDB myofibre culture system results in a more mature muscle fiber phenotype and that it can be adapted as a platform for semi-high throughput screening of therapeutic agents for the treatment of various skeletal muscle diseases.

REAGENTS AND INSTRUMENTS

Myoblasts of the mouse C2C12 myoblast cell line, originally derived from C57BL/6J mice [Yaffe and Saxel, 1977], were a generous gift from Dr Edna Hardeman (Children's Institute for Medical Research, Westmead, NSW, Australia). C57BL/6J and C57BL/10ScSn-Dmd^{mdx}/J (dystrophin negative, [Bulfield et al., 1984]) were purchased from the Animal Resources Centre (Western Australia, Australia).

The collagenase (type I), controlled serum replacement-2 (CSR), laminin and the protease inhibitor cocktail for mammalian cells were all from Sigma-Aldrich (Sydney, NSW, Australia), as were all other chemicals not otherwise mentioned.

The monoclonal antibodies that recognise fast myosin (MY32), α-actinin (EA-53), tropomyosin (CH1) and troponin-T (JLT-12) were all also obtained from Sigma-Aldrich (Sydney, NSW, Australia), as were the fluorescein isothiocyanate (FITC)-conjugated phalloidin, α-bungarotoxin-FITC and horse-radish peroxidase (HRP)-conjugated anti-mouse and HRP-conjugated anti-rabbit IgG antibodies. Monoclonal antibodies recognising fast myosin (NCL-MHCf), dystrophin (NCL-DYS2) and utrophin (NCL-DRP2) were obtained from Novocastra Laboratories (Newcastle upon Tyne, UK). An antibody specific for α-sarcoglycan (IVD3$_1$A9) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, IA) while the cardiac α-actin specific antibody (clone Ac1-20.4.2) was obtained from Research Diagnostics (Concord, MA). The polyclonal skeletal muscle α-actin specific antibody has been previously described [Clement et al., 1999].

Matrigel™ was purchased from Becton Dickinson (North Ryde, NSW, Australia). All the other tissue culture reagents, namely fetal calf serum (FCS), Dulbecco's Modified Eagle Medium (DMEM), penicillin/streptomycin, L-glutamine, heat-inactivated horse serum, phosphate buffered saline (PBS) and trypsin, were obtained from Invitrogen (Mount Waverley, Victoria, Australia). Similarly, the antibody labeling kits (Zenon® Alexa Fluor® mouse IgG$_1$, Zenon® Biotin-XX mouse IgG$_1$) and the strepavidin-Alexa Fluor® 594 secondary antibody were from Invitrogen.

The Criterion SDS-PAGE system and the UV laser scanning confocal microscope (model MRC1000/1024) were from Biorad Laboratories (Regents Park, NSW, Australia). The ECL Plus Western Blotting Detection System™ was from Amersham (GE Healthcare, Buckinghamshire, UK). The BCA™ protein assay kit and polyvinylidene fluoride (PVDF) membrane were purchased from Pierce Biotechnology (Rockford, IL) and the Hydromount from National Diagnostics (Atlanta, GA). The Nanodrop ND1000 spectrophotometer was from Biolab (Victoria, Australia).

The fluorescent microscope (inverted, model IX-71)) and the digital camera (model DP-71) were both from Olympus (Mount Waverley, Victoria, Australia), whilst the IN-Cell Analyser 1000 was from GE Healthcare (Piscataway, NJ).

Fura-2 was obtained from Teflabs (Austin, TX) and pluronic F-127 was obtained from Invitrogen. The isolated pulse stimulator (model 2100) was from A-M Systems (Carlsborg, WA, USA), the dual channel power amplifier (EP500B) from Audio Assemblers (Campbellfield, Victoria, Australia) and the dual monochromator system was from Cairn Research (Kent, UK).

The adenovirus (human adenovirus, serotype 5) was kindly provided by Sherif Boulos [Boulos et al., 2006] and has two promoters to drive both enhanced green fluorescent protein (EGFP) and DsRed protein expression.

METHODS

C2C12 Cultures

C2C12 myoblasts were grown in 10% FCS in DMEM supplemented with 4 mM L-glutamine and 1% penicillin/streptomycin (proliferation medium). Trypsinised suspensions of C2C12 myoblasts were plated into 6-well tissue culture plates or onto glass coverslips coated with 0.1 mg/ml Matrigel™ and maintained in proliferation medium until the cultures reached a confluency of 50%. Cells were then grown in DMEM supplemented with 5% heat-inactivated horse serum, 4 mM L-glutamine and 1% penicillin/streptomycin (differentiation medium). The day of incubation in differentiation medium was denoted as day 0 (D0). Day 1 (D1), day 2 (D2) etc., denotes the number of days following incubation in differentiation medium. Myotubes were cultured at 37°C in 5% CO_2, and differentiation medium was replaced every other day.

Isolation and Culturing of Mouse FDB Myofibers

All the animal procedures were performed in accordance with the guidelines and approved protocols of the University of Western Australia Animal Ethics

Committee. Six to eight-week-old C57BL/6J or C57BL/10ScSn-Dmd^{mdx}/J male mice were killed by sodium pentabarbitone overdose before the FDB muscles were dissected and then incubated in physiological rodent saline (PRS: 138 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.06 mM $MgCl_2$, 12.4 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES) and 5.6 mM glucose, pH 7.3). After removing all connective tissue, blood vessels and other non-muscle tissues, FDB muscles were incubated in PRS supplemented with 0.2% collagenase and 10% FCS at 37°C in 5% CO_2 in air for 90 min. Myofiber bundles were transferred to proliferation medium and incubated for 30 min, prior to trituration with Pasteur pipettes of decreasing diameters to disperse single myofibers. Free single myofibers were separated by gravity sedimentation from broken fibers and single cells by passing through three columns of proliferation medium, followed by seeding into 96-well tissue culture plates coated with 20 μg/ml laminin-DMEM, as detailed in [Shefer and Yablonka-Reuveni, 2005]. Approximately 12 h following seeding of FDB myofibers, the medium was replaced with maintenance medium (DMEM containing 20% controlled serum replacement-2, 4 mM L-glutamine and 1% penicillin/streptomycin). Myofibers were cultured at 37°C in 5% CO_2.

SDS-PAGE

Cultures were rinsed once, then scraped off the plastic or glass surfaces in PBS using pipette tips. Cells were pelleted by centrifugation at 1000 rpm for 2 min. Cell pellets were snap frozen on dry ice and stored at −80°C. Cell pellets were resuspended in 150 μL Laemmli sample buffer, containing 1% protease inhibitor cocktail for mammalian cells. Samples were heated to 94°C for 3 min, vortexed and centrifuged (14,500 rpm for 3 min) prior to protein quantification. Protein quantification was performed using the BCA™ protein assay kit and absorbance of the standards and samples were measured by spectrophotometry. For all Western blots shown, samples were loaded to give an equal quantity of total protein (in the range of 5–10 μg, depending on the antibody to be used).

Samples containing 5% β-mercaptoethanol were electrophoresed on 4–15% gradient gels at ∼150 mV, in 25 mM Tris, 192 mM glycine and 0.1% sodium dodecyl sulfate (SDS), with 10 mM β-mercaptoethanol added to the upper chamber, until the bromophenol dye-front ran off the gels. Samples were electroblotted onto PVDF membrane in Towbin's transfer buffer (TTB: 25 mM Tris, 192 mM glycine and 20% methanol) at 300 mA for 2 h at room temperature (RT) for transfer of small molecular weight proteins (less than 100 kDa). For transfer of larger molecular weight proteins, electroblotting was performed in TTB containing 0.1% SDS and in the absence of methanol, overnight at 16°C.

Membranes were blocked for at least 1 h at RT in PBS containing 0.1% Tween-20 and 5% skim milk powder (blocking solution). Membranes were incubated in blocking solution containing primary antibodies for at least 1 h at RT (see Table I for antibody concentrations used), with the exception of the utrophin antibody that was instead incubated overnight at 4°C. After washing in PBS containing 0.1% Tween-20 for three × 5 min washes, the membranes were incubated in blocking solution containing the appropriate HRP-conjugated secondary antibody at 1:15,000 for 1 h at RT. Following another three × 5 min washes with PBS containing 0.1% Tween-20, detection was performed using ECL Plus.

Immunostaining

FDB cultures seeded onto 13-mm round glass coverslips were fixed in chilled PBS containing 2% paraformaldehyde and 10% FCS for 5 min, on ice. Samples were washed three times in PBS, and then incubated in blocking buffer (PBS plus 10% FCS and 1% BSA) con-

TABLE I. Primary Antibodies Used for Immunohistochemistry or Western Blot Analysis

Antibody	Clone	Species	Concentration for immunohistochemistry	Concentration for Western blot
α-Skeletal muscle actin	Clement et al. [1999]	Rabbit polyclonal	N/A	1:5000
α-Cardiac actin	Ac1-20.4.2	Mouse monoclonal	N/A	1:4000
α-Actinin	EA-53	Mouse monoclonal	1:20	1:100,000
α-Sarcoglycan	IVD3$_1$A9	Mouse monoclonal	1:20	N/A
Dystrophin	NCL-DYS2	Mouse monoclonal	1:10	N/A
Fast MHC	MY32	Mouse monoclonal	N/A	1:200,000
Fast MHC	NCL/MHCf	Mouse monoclonal	1:20	N/A
Sarcomeric troponin-T	JLT-12	Mouse monoclonal	1:10	1:200,000
Sarcomeric tropomyosin	CH1	Mouse monoclonal	1:10	1:200,000
Utrophin	NCL-DRP2	Mouse monoclonal	1:5	1:200

N/A, not applicable.

taining 0.25% saponin for 30 min at RT. Fibers were incubated with the primary monoclonal antibodies overnight at 4°C. All antibodies were labelled with the Zenon® Alexa Fluor® mouse IgG_1 labelling kits except for the utrophin antibody that was conjugated with the Zenon® Biotin-XX mouse IgG_1 labelling kit. In the case of the utrophin antibody, following washing in PBS, coverslips were incubated with the strepavidin-Alexa Fluor® 594 secondary antibody (1:1000 dilution) for 1 h at RT. All myofibers were incubated with DAPI for 5 min to stain the nuclei, washed three times in PBS and mounted on glass coverslips using Hydromount and visualised using a fluorescent microscope and digital camera, a confocal microscope or an IN-Cell Analyser 1000.

Intracellular Ca^{2+} Measurements

Intracellular Ca^{2+} levels were measured using the fluorescent Ca^{2+} indicator Fura-2. FDB fibers were incubated in PRS containing 3 μM Fura-2 (acetoxymethyl ester form) and 0.0125% pluronic F-127 for 45 min in the dark, and then washed three times in PRS and left for 30 min to allow time for dissociation of the dye into the Ca^{2+}-sensitive form. The coverslips were placed into a cell bath and mounted on the stage of an inverted Nikon microscope, equipped for epifluorescence. The fibers were illuminated alternatively at excitation wavelengths of 340 and 380 nm using a Xenon light source in conjunction with a dual monochromator system and the fluorescence emission at 510 nm was acquired with a photomultiplier tube. The fluorescence data were collected using a Cairn data acquisition software and analysis package.

Electrical stimulation of the fibers was delivered via a pair of platinum electrodes situated within the cell bath. An isolated pulse stimulator connected serially to a pulse amplifier was used to provide the stimulus pulse. Cultured fibers were stimulated supra-maximally, with a pulse duration of 0.2 ms.

Two-way analysis of variance (ANOVA) was conducted on data sets and the follow-up post hoc tests took the form of the Newman–Keuls multiple comparison test, using GraphPad Prism 3 analysis software. Statistical significance is defined as $P \leq 0.05$.

Adenoviral Transduction

The FDB myofiber cultures were exposed to the EGFP/DsRed adenovirus at a multiplicity of infection (MOI) of 100, 200 and 500 for 24 h. The myofibers were visualised on a fluorescent microscope at 48 h post-transduction.

RESULTS

FDB Myofiber Cultures Are Amenable to 96-well Plates

Each FDB muscle contains ~1500 myofibers and roughly 50 myofibers were plated into each well of a 96-well plate, therefore two mice were required to achieve sufficient myofibers to fill a plate. Myofibers could be maintained in culture for up to 10 days in the absence of sera and under these conditions the proliferation of fibroblasts and satellite cells was minimal, with little sprouting of the myofibres. Approximately 85% of the fibers comprising the FDB muscle expressed fast MHC isoforms and only 15% expressed the slow isoform (data not shown), thus the FDB culture system is a suitable model for the study of fast skeletal muscle.

Protein Expression in Cultured C2C12 Myotubes and FDB Myofibers

The expression profiles for a range of sarcomeric and sarcolemmal proteins in cultured C2C12 myotubes and FDB myofibers maintained for up to 10 days, as detected by western blotting, are presented in Fig. 1. While the FDB myofibers displayed no detectable levels of cardiac α-actin, detectable levels of cardiac α-actin occurred in C2C12 myotubes at D2 and expression was maintained at all further time-points. Similarly, utrophin was undetectable in FDB myofibers, while expression was evident in C2C12 myoblasts (D0) and throughout the course of myotube formation.

Furthermore, FDB cultures displayed high levels of skeletal muscle α-actin, fast MHC, tropomyosin and troponin-T compared to developing C2C12 myotubes. Samples were loaded to give equal amounts of total protein, hence the relative contribution of mature skeletal muscle proteins to the total protein pool was greatly elevated in cultured FDB fibers compared to developing C2C12 myotubes.

FDB Myofiber Light Microscopy

Representative bright-field images of FDB myofibers maintained in culture for various lengths of time are shown in Fig. 2. The characteristic striated pattern (see insets) of skeletal muscle and the peripheral location of the nuclei can be seen throughout the culture period and are indicative of the myofibers remaining structurally mature, compared to cultured myotubes and de-differentiating myofibers, which lacked the striated appearance and contained numerous centrally-located nuclei (data not shown).

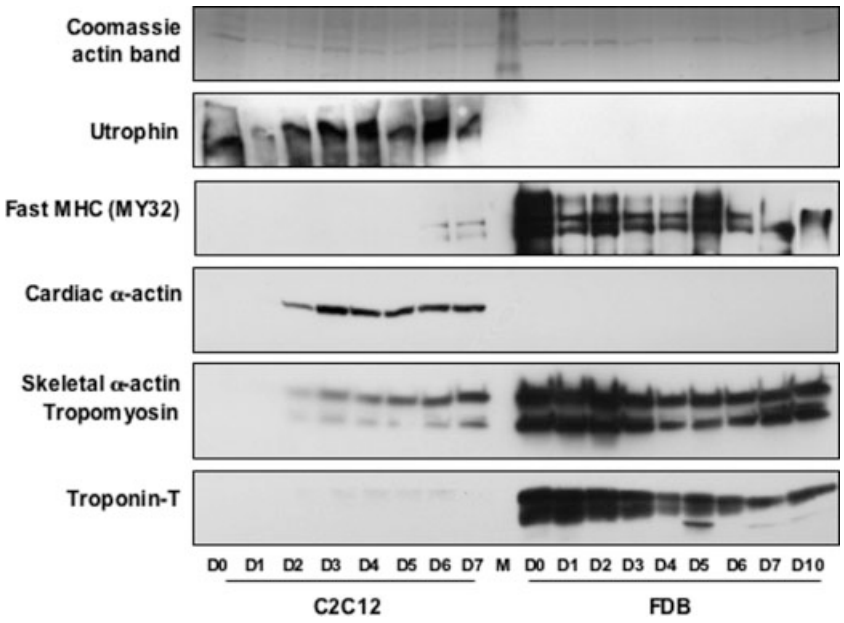


Fig. 1. A time course study of the expression of various muscle proteins in C2C12 myotube and FDB myofiber cultures, where D1, D2...D10 denotes the time point in days at which the cells were harvested after the differentiation medium was added (C2C12s) or the myofibers were dissociated (FDBs). Results shown are for the pooled protein lysates generated from three independent time course studies of both the C2C12 and FDB myofiber cultures. M = Benchmark™ prestained protein ladder (Invitrogen, Mount Waverley, Victoria, Australia).

Imaging of Protein Localisation in Cultured FDB Myofibers

Labelling of the neuromuscular junction (NMJ) of cultured FDB myofibers was conducted via staining with α-bungarotoxin and the DRP-2 antibody to detect α-acetylcholine receptors (α-AChRs) and utrophin, respectively. Immunostaining conducted on D1, D4 and D8 myofibers revealed the characteristic aggregation of the α-AChRs and the associated expression of utrophin (Fig. 3), a feature of developmentally mature skeletal muscle [Bewick et al., 1996]. However, FDB myofibers maintained for 8 days in culture displayed a more diffuse staining for utrophin than those cultured for up to 4 days, indicative of utrophin spread away from the NMJ.

The stability of the sarcomeric organisation was investigated in cultured FDB myofibers at D1, D4 and D9, using immunostaining for a number of sarcomeric proteins. The sarcomeric localisation of filamentous actin (phalloidin), fast MHC, α-actinin, tropomyosin and troponin-T remained unchanged throughout the culture period. Representative confocal microscopy images for FDB fibers maintained for 4 days in culture are shown in Fig. 4. Alternate staining of filamentous actin and fast MHC can be observed by confocal microscopy (Fig. 4a) and the In-Cell Analyzer 1000 (Fig. 4b). The co-localisation of filamentous actin and α-actinin can be seen in Fig. 4c.

Dystrophin and α-sarcoglycan, two important membrane-associated proteins from both a functional and disease perspective, remained localised at the surface and at the myotendinous junctions at the ends of the cultured myofibers at all time points examined. A representative image of this is presented in Fig. 4d. Both dystrophin and α-sarcoglycan staining were markedly absent or diminished respectively in cultured *mdx* FDB myofibers at all time points examined.

Calcium Handling Experiments

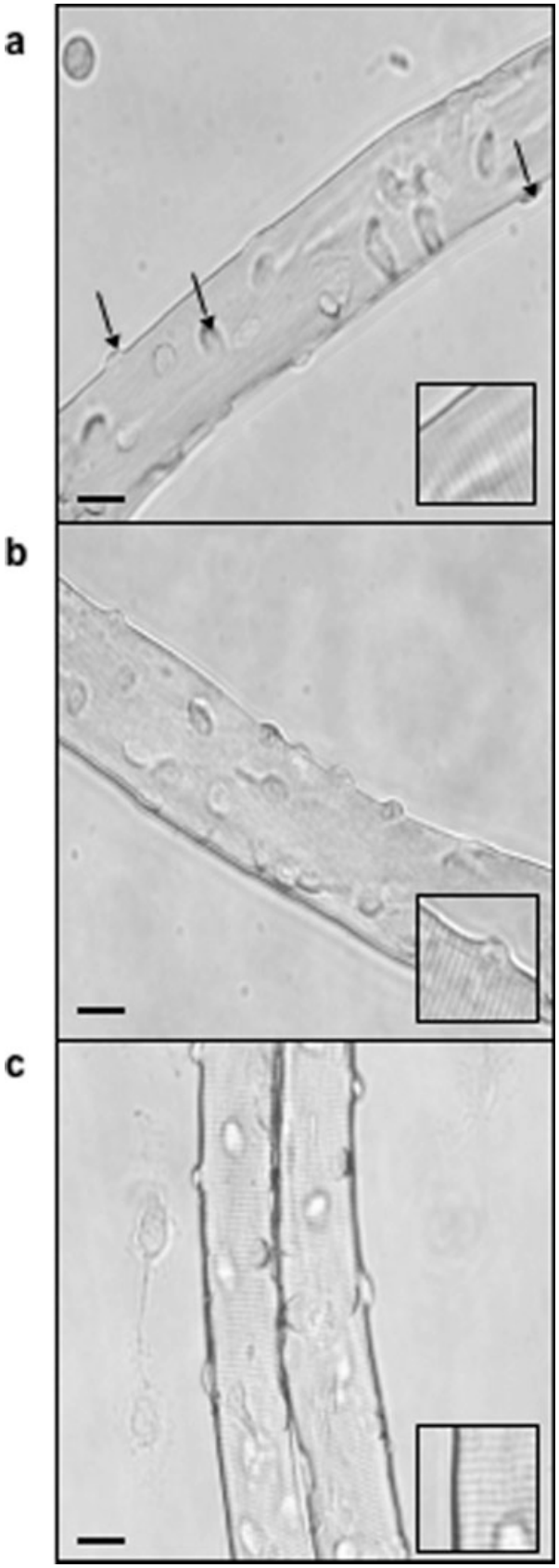
Cultured FDB myofibers loaded with Fura-2 were activated via electric field stimulation (EFS) to produce electrically-evoked Ca^{2+} transients. These responses are representative of Ca^{2+} release and Ca^{2+} reuptake by the sarcoplasmic reticulum triggered by a single action potential. In response to this Ca^{2+} release, the cultured myofibers were observed to contract after delivery of each electrical stimulus. Figure 5a is a representative recording of intracellular Ca^{2+} during EFS in a myofiber maintained in culture for 5 days. The general characteristics of the electrically-evoked Ca^{2+} transients were examined over 8 days in culture (Fig. 5b). At all time points examined the amplitude (i), time to peak (ii) and half relaxation time (iii) of the electrically evoked Ca^{2+} transients remained unchanged.

Transduction by Adenovirus

At 48 h post exposure to the EGFP/DsRed adenovirus, ~40% of the fibers expressed both EGFP and DsRed fluorescent protein (MOI 500) whilst ~20 and 0% of the fibers expressed these proteins with an MOI of 200 and 100, respectively (data not shown).

DISCUSSION

Enzymatically dissociated FDB myofibers can be maintained in 96-well culture plates for at least



10 days and express a cohort of proteins that are typical of mature, terminally-differentiated skeletal muscle. At the same time, the cultured FDB myofibers remained functionally normal showing little change in dynamic Ca^{2+} handling throughout the culture period. We therefore propose that the FDB myofiber cultures are a more relevant and accurate representation of mature skeletal muscle.

FDB Myofiber Cultures Express a Cohort of Mature Skeletal Muscle Proteins

The protein expression profile determined by western blotting for cultured FDB myofibers is markedly different to that obtained for developing C2C12 myotube cultures, regardless of the time point examined (Fig. 1). C2C12 myotubes expressed high levels of utrophin and cardiac α-actin throughout the time course of myotube development compared with FDB myofiber cultures. Despite utrophin being detected by immunohistochemistry at the NMJ (Fig. 3) of the cultured FDB myofibers, these levels were undetectable by western blotting. In contrast, the FDB fibers displayed distinctly elevated levels of the thin filament proteins skeletal muscle α-actin, tropomyosin and troponin-T, compared to developing C2C12 myotubes. Fast MHC expression was present in FDB myofibers at all time points, while expression of this MHC isoform was only initiated at D6 of C2C12 myotube development. C2C12 myotube cultures have previously been shown to initially express fast MHC at D6 and tropomyosin at D2 [Cooper et al., 2004], in agreement with our findings. Given that samples were loaded according to equal total protein, mature sarcomeric proteins account for a noticeably elevated percentage of the total protein pool of the FDB myofibers, compared to the C2C12 myotubes. This is indicative of the tight packing of sarcomeres within mature skeletal muscle compared to that of developing myotubes. The expression of cardiac α-actin and utrophin, the fetal forms of skeletal muscle α-actin [Ilkovski et al., 2005] and dystrophin [Blake et al., 1996], respectively, coupled with the relative lack of expression of mature sarcomeric proteins, including skeletal muscle α-actin, fast MHC, tropomyosin and troponin-T, highlights the developmentally immature state of C2C12 myotubes and the comparative maturity of the FDB fiber culture system.

Fig. 2. Transmitted light images of cultured FDB myofibers on D2 (**a**), D6 (**b**) and D10 (**c**) of the culture period, showing the characteristic striated pattern (see insets, magnification ×1.8) of skeletal muscle and the presence of peripherally-located nuclei (arrows). Scale bars represent 10 μm.

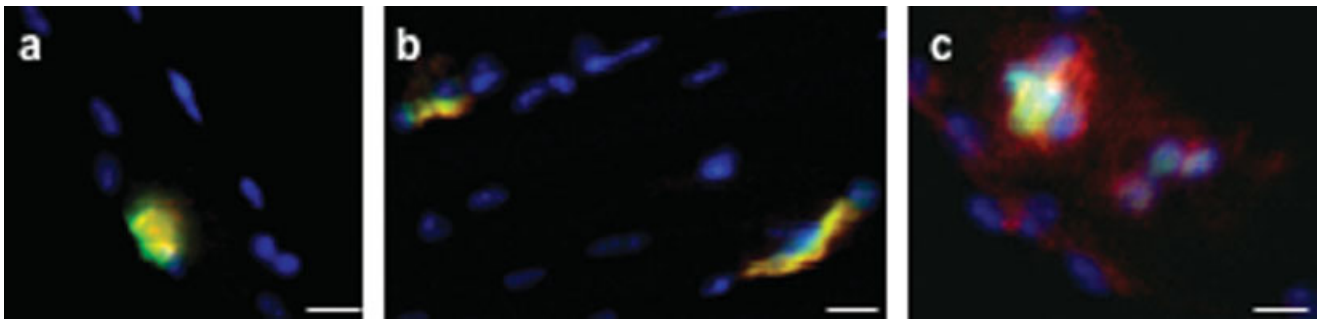


Fig. 3. Fluorescent imaging of the neuromuscular junction using α-bungarotoxin-FITC (green) to label the α-acetylcholine receptors and the NCL-DRP2 antibody (red) against utrophin in a D1 FDB myofiber (**a**), two D4 FDB myofibers (**b**) and a D8 FDB myofiber (**c**). Regions that are yellow represent co-localisation of α-acetylcholine receptors and utrophin expression. Scale bars represent 10 μm.

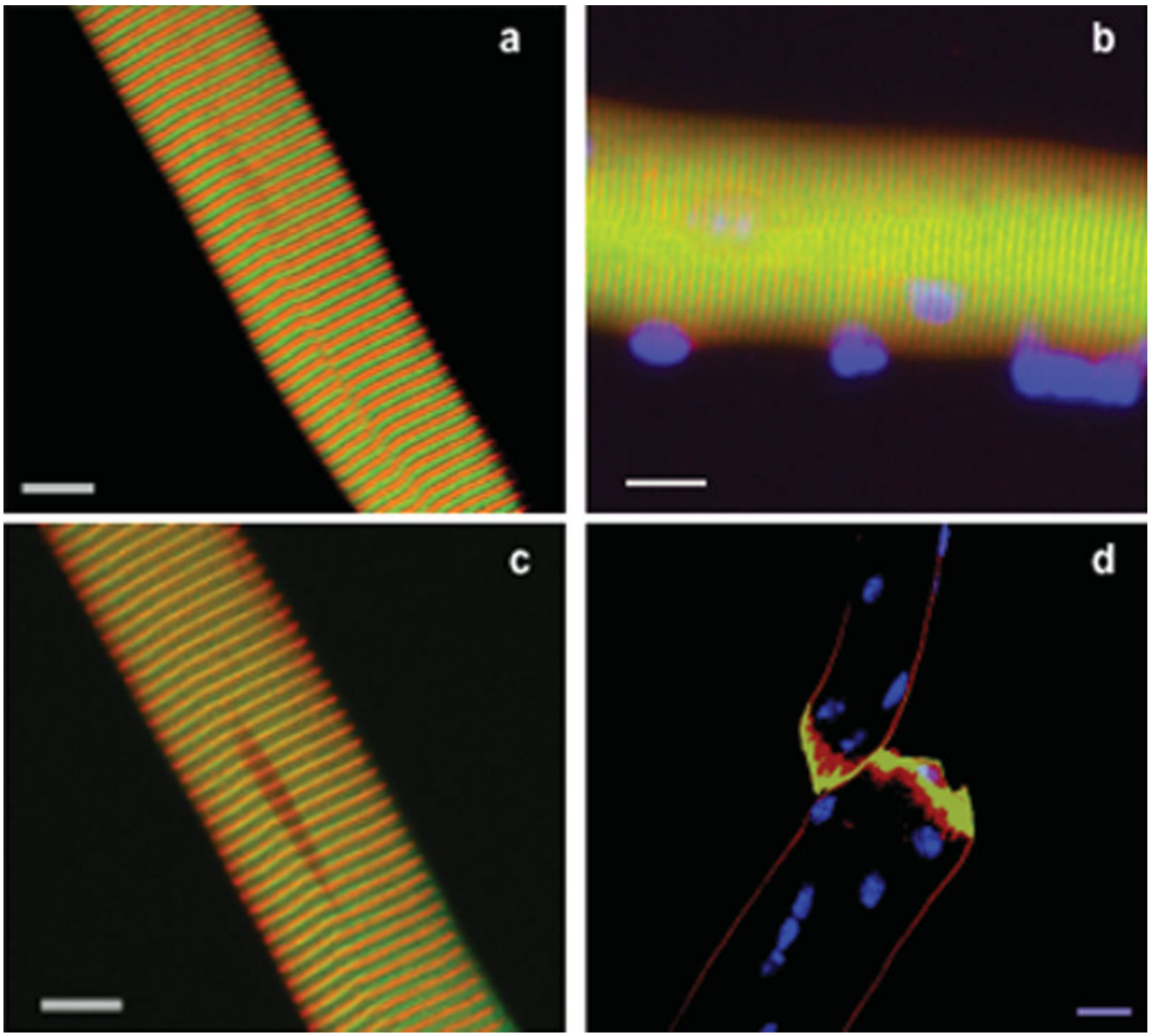


Fig. 4. Cultured D4 FDB myofibers imaged using confocal microscopy (**a**, **c** and **d**) and an IN-Cell Analyzer (**b**). In both illustrations (a) and (b), myofibers are labeled with phalloidin (green) and fast MHC (red). Image (c) shows phalloidin (green) and α-actinin (red) whilst image (d) shows dystrophin (red) and α-sarcoglycan (green). Nuclei are labeled with DAPI (blue) and the scale bars represent 10 μm.

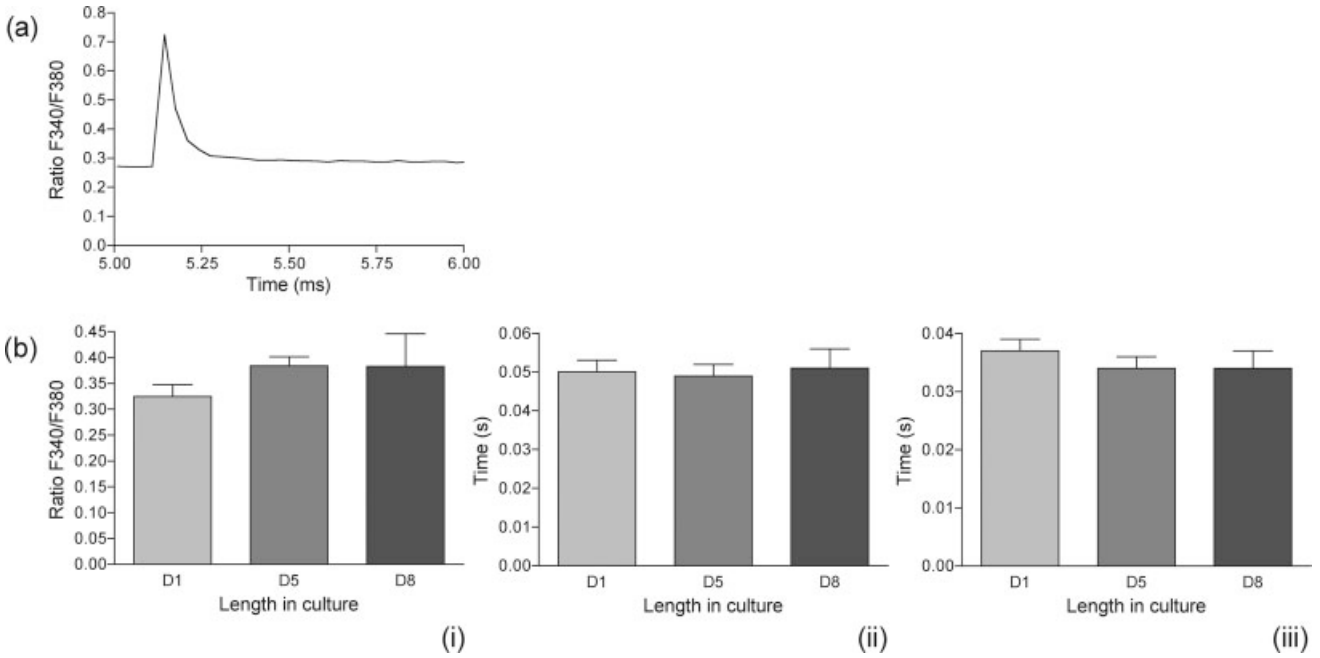


Fig. 5. (**a**) A representative recording of intracellular Ca^{2+} in a FDB myofiber during electrical stimulation, following culturing for 5 days. (**b**) The characteristics of the electrically-evoked Ca^{2+} transients in FDB myofibers maintained in culture for 1, 5 or 8 days, (i) peak amplitude, (ii) time to peak and (iii) half relaxation time.

The Localisation of Sarcomeric and Membrane-Associated Proteins Is Maintained in Cultured FDB Myofibers

Confocal microscopy conducted on cultured FDB myofibers at D1, D4 and D8 revealed that the localisation of filamentous actin, fast MHC and α-actinin remained unaltered at all time points, while the expression of dystrophin and α-sarcoglycan remained localised to the sarcolemma and at the myotendinous junctions (Fig. 4). Thus, in addition to cultured FDB myofibers expressing high levels of skeletal muscle proteins, their spatial localisation within the myofibers remains intact. It has previously been demonstrated that the expression of components of the dystrophin-associated protein complex are initiated relatively late in myotube differentiation, specifically dystrophin and α-sarcoglycan are first detected at only D6 [Cooper et al., 2004]. Thus the FDB myofiber cultures are a more time-efficient and more accurate culture system for the study of various mature skeletal muscle proteins and isoforms.

Electrically-Mediated Ca^{2+} Transients Are Maintained in Cultured FDB Fibers up to D8

EFS of cultured FDB myofibers resulted in the generation of intracellular Ca^{2+} transients which remained unaltered at all time points examined (D1, D5 and D8). Thus it is evident that the necessary components of the excitation-contraction pathway remain unchanged with time in culture, including membrane depolarisation, activation of the dihydropyridine receptors, coupling to the ryanodine receptors of the sarcoplasmic reticulum, and Ca^{2+} release and re-uptake via the Ca^{2+}ATPase pumps. Spontaneous Ca^{2+} sparks are a characteristic of dedifferentiating myofibres [Brown et al., 2006] and embryonic skeletal muscle [Chun et al., 2003], in contrast only a small percentage (<5%) of FDB myofibres began to display spontaneous contractions after 5–6 days in culture. The time to peak of electrically-evoked Ca^{2+} transients has been shown to be approximately eight times slower in developing myotubes, compared to adult FDB myofibers [Bakker et al., 1997]. Furthermore, the rate constant of Ca^{2+} decay is significantly reduced in myotubes compared to myofibres, 1.68 and 28.2 s^{-1}, respectively [Bakker et al., 1996], indicative of functional immaturity of the calcium-handling pathway. The ability of the cultured FDB myofibers to maintain the characteristics of adult skeletal muscle is indicative of the functional maturity and viability of these myofibers in culture.

The Denervation in Cultured FDB Myofibers Is Accompanied by Alteration of the Components of the NMJ

It is well documented that muscle denervation is accompanied by changes in muscle morphology and

protein expression [for a review see Midrio, 2006]. To ascertain the effect of denervation on cultured FDB myofibers, the NMJ was visualised by labelling α-AChRs and utrophin, two prominent components of the NMJ [Blake et al., 1996]. At D1 and D4 (Figs. 3a and 3b, respectively), FDB myofibers displayed a discrete localised co-expression of utrophin and α-AChRs at a defined region of the sarcolemma. However, by D8 the expression of utrophin was more diffuse and spread-out from the site of α-AChR expression (Fig. 3c). It has been shown that denervation is accompanied by re-expression of utrophin at the surface membrane of muscle fibers [Takemitsu et al., 1991]. Thus cultured FDB myofibres may prove a useful system for investigating the consequences of muscle denervation, in vitro.

Cultured *mdx* FDB Myofibers Maintain the Hallmarks of Dystrophin-Deficient Muscle

Immunostaining conducted on cultured *mdx* FDB myofibers revealed that these myofibers were dystrophin-negative and expressed diminished levels of α-sarcoglycan (data not shown), as is seen in *mdx* muscle biopsies [Ohlendieck and Campbell, 1991]. Light microscopy revealed the characteristic striated pattern of skeletal muscle and the peripheral location of the nuclei in FDB myofiber cultures at all time points examined (Fig. 2). However, it was observed that a portion of the cultured *mdx* myofibers displayed central nucleation and that these cultures were more susceptible to dedifferentiation (data not shown). These properties have been described previously for cultured *mdx* myotubes and myofibers [Yablonka-Reuveni and Anderson, 2006]. These findings therefore indicate that cultured FDB myofibers are a reliable culture system for the study of various skeletal muscle diseased states, since the hallmarks of *mdx* skeletal muscle are maintained in this culture system.

Cultured FDB Fibers Can Be Imaged and Analysed on a Platform Suitable for Semi-High-Throughput Small Molecule Screens in a 96-Well Plate Format

The IN-Cell Analyzer 1000 was initially designed for cell-based assay development in pharmaceutical hit-to-lead characterisation where imaging and analysis in high throughput and high content are required. The technology platform was applied to the FDB myofiber cultures in 96-well assay format, using multiple fluorescent probes and transmitted light. The images obtained for fluorescently labeled sarcomeric proteins showed clear banded staining patterns with no requirement for optical sectioning (representative image presented as Fig. 4b). The ability of this system to detect the localisation and intensity of staining with various labeled antibodies in a 96-well plate format is validation that the FDB culture technique can be combined with this imaging and analysis platform for high-throughput drug screening for skeletal muscle disorders.

Cultured FDB Myofibers Can Be Transduced With Adenovirus and Express Foreign Proteins

To further confirm the utility of cultured FDB myofibers, we transduced the myofibers with an adenovirus [Boulos et al., 2006]. Various researchers have reported effective transduction of mature skeletal muscle by adenovirus [Larochelle et al., 1997] and adeno-associated virus [reviewed in Herzog, 2004]. Indeed, these viruses have been successful as a therapeutic delivery system for replacing absent or defective genes [Xiao et al., 2000; Sun et al., 2003; Liu et al., 2005]. We showed that the FDB myofibers were transduced in a dose-dependent manner and synthesised the two fluorescent proteins encoded by the virus. This demonstrates that the cultured FDB myofibers are competent in responding to stimuli, which is a crucial requirement if this system is to be used for drug screening. Additionally, being able to transduce the myofibers opens up a wide array of possible future experiments such as investigating the localisation and pathological implications of mutant or novel proteins in a mature skeletal muscle setting.

CONCLUSION

We have clearly shown that the FDB myofiber culture system is a more relevant and accurate culture technique for the study of various aspects of mature skeletal muscle, compared to the widely used myoblastic cell lines. At both a protein and functional level, the cultured FDB myofibers maintain the characteristics typical of mature skeletal muscle. The protein expression profile of C2C12 myotubes has been demonstrated to comprise predominantly fetal isoforms and a much lower contribution of mature sarcomeric proteins, when compared to the cultured FDB myofibers. In addition, the hallmarks of dystrophic muscle are present in cultured *mdx* FDB myofibers, thus the characteristics of diseased states can be maintained and investigated using this culture technique. The success of this culture method in a 96-well plate format, coupled with imaging using the IN-Cell Analyzer, provides an excellent system for future application to small molecule drug screening for treatments for various skeletal muscle diseases.

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