

Generation and Use of Cultured Human Primary Myotubes

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1. Introduction

Cell culture is a widely used technique in biomedical research. It permits the analysis of cell specific functions which can relate to changes in certain disease states at the single cell level. A number of cell culture models have been established. These include immortalised cell lines which replicate indefinitely in culture and retain the ability to differentiate and primary cell lines which can be isolated directly from the host tissue and grown in culture. However, primary cell lines have limited replicative potential and become senescent in culture. In the case of muscle cells, cell lines can provide a valuable means of investigating physiology in the absence of confounding factors (such as circulating hormones, adipokines and other bioactive molecules) which arise when dealing with the body as a whole.

Human primary cell lines provide additional benefits in research, when compared to immortalised cell lines, as primary cultures have been shown to retain the metabolic characteristics of the tissue donor and thus reflect alterations in metabolism as seen in specific disease states such as obesity and type 2 diabetes mellitus (Gaster et al., 2004; Steinberg et al., 2006). Of particular interest to the treatment of myopathies is the use of human primary myotube cultures which can be isolated from muscle extracts in normal and diseased states. We (Chen et al., 2005; McAinch et al., 2006b; McAinch et al., 2007; Steinberg et al., 2006) and others (Bell et al., 2010; Mott et al., 2000; Thompson et al., 1996) have shown that the resultant myotube cultures retain phenotypic traits of the donor related to defects in fatty acid oxidation, impairment of insulin stimulated glucose uptake, leptin and/or adiponectin resistance. Thus, there is increased clinical relevance associated with the use of primary cell cultures in researching disease pathogenesis, making human primary cell cultures an invaluable tool in assessing aberrant cellular metabolism and how this may be targeted experimentally to alleviate metabolic dysregulation.

In this chapter we aim to provide the reader with a detailed explanation of the methodological considerations in isolating and culturing of human primary myotubes and their subsequent experimental uses.

2. Current applications of human primary myotubes

2.1 Skeletal muscle cell culture

The skeletal muscle is a highly metabolically active tissue and has a profound influence over systemic metabolic function through the regulation of carbohydrate and fatty acid metabolism (Baron et al., 1988; Zurlo et al., 1990). Historically, the study of cellular mechanisms regulating skeletal muscle physiology was limited by difficulties in maintaining metabolically viable isolated muscle extracts and the ability to manipulate specific variables *in vivo*. The development of cell culture models has provided an invaluable tool by which cellular physiology can be studied. Over the last several decades cell culture techniques have evolved and the resultant studies have contributed greatly to the current understanding of cellular metabolism. A vast number of cell types are cultured to investigate pertinent research questions. Typically the cells used in these models will either be an immortalised cell line or a primary cell line, each of which has distinct advantages. Herein, we provide a brief discussion of the development of both immortalised and primary skeletal muscle cell culture models.

2.1.1 Immortalised cell lines

Undifferentiated immortalised skeletal muscle cell lines have an indefinite myogenic potential, with cells continuing to undergo mitotic divisions when maintained in the appropriate culture conditions. Accordingly, these cell lines expand rapidly in culture to provide a readily repeatable experimental model and can provide an alternative to isolating primary cell lines. A key consideration with the use of immortalised cell lines is the retention of physiological functions similar to that of the tissue source (Obinata, 2001). Therefore it is important that immortalised cell lines are adequately characterised to demonstrate that differentiated cell lines recapitulate the physiology of the original tissue.

Clonal cell lines were initially derived from transgenic animal models, tumour cells, treatment of cells with carcinogens or arose through mutations of specific cells within primary cultures (Efrat et al., 1988; Obinata, 2001; Richler & Yaffe, 1970; Todaro & Green, 1963; Todaro et al., 1963). These cells do not readily respond to the normal apoptotic signals and continue to replicate and divide beyond the life span of un-mutated cells. Isolation and cloning of these myogenic cells enables clonal cell lines to be formed. Despite their benefits in experimental molecular and cellular biology, the relative lack of clonal cell lines and the difficulty in generating new cell lines has inhibited research capacity. More recent techniques utilised to produce immortalised cell lines involve cellular transfection with immortalising genes or oncogenes (Condon et al., 2002; Douillard-Guilloux et al., 2009; Jat et al., 1991). Such methods utilise transfection of genes including telomerase, SV40 Large T-antigen, cyclin-dependent kinase 4 and Bmi to induce immortalisation of a number of cell types (Condon et al., 2002; Di Donna et al., 2003; Douillard-Guilloux et al., 2009). These methods are limited initially by the number of cells stably expressing the transfected DNA needed to generate a viable population of the immortalised cell line. Additionally, cellular incorporation of the immortalising gene can occur at different sites in theoretically identical cells which can lead to variable gene expression and cellular behaviours. The use of clonal cell lines is further confounded by the potential for the introduced DNA to alter the cellular

phenotype and therefore cellular physiology (Ridley et al., 1988). This issue may also be apparent in certain cell lines derived from transgenic animal models and tumour cells which reflect the host's expression of certain genes and proteins (Efrat et al., 1988). This may be partially overcome by the generation of conditionally immortalised cell lines in which the effect of the immortalising agent is abrogated under certain culture conditions, such as changes in serum concentrations or temperature (Macpherson et al., 2004; Obinata, 2001).

Immortalised cell lines that are commonly used for investigating skeletal muscle physiology are L6 and C₂C₁₂ skeletal muscle myotubes which are derived from rat and mouse origin, respectively (Blau et al., 1985; Yaffe, 1968; Yaffe & Saxel, 1977). These cell lines can be terminally differentiated into skeletal muscle myotubes by altering the culture environment to low serum conditions and thus provide a relevant method of investigating muscle physiology.

The L6 cell line was initially described by Yaffe (1968). This cell line was established from newborn rat thigh muscle treated with the carcinogen 20(3) methylcholanthrene in the growth medium during the first two growth passages (Yaffe, 1968). In initial experiments, the L6 cell line was maintained *in vitro* for more than 18 months without losing the ability to fuse to form differentiated myotubes (Yaffe, 1968). In subsequent studies, Richler & Yaffe (1970) observed the L6 cell line to develop into multinucleate, cross-striated myotubes which retain contractile properties. The clonal mouse C₂C₁₂ cell line is a diploid sub-clone generated by Blau et al., (1985) as a derivative of the cell line described by Yaffe & Saxel (1977). This cell line was developed by Yaffe & Saxel (1977) from thigh muscle of CH3 mice and was shown to retain the capacity to proliferate and differentiate in culture. The C₂C₁₂ cell line has subsequently been used in muscle physiology studies due to its rapid proliferation and ability to differentiate to form contractile myotubes which express myogenic proteins (Blau et al., 1985; Yaffe & Saxel, 1977). The initial characterisation of both the L6 and C₂C₁₂ cell lines indicating pronounced differentiation and retention of contractile properties suggest that these cell lines are appropriate models for studying muscle physiology.

2.1.2 Primary cell lines

Adult myotubes are not capable of mitotic divisions and therefore primary skeletal muscle culture systems rely on the ability to induce activation and subsequent myogenic differentiation of quiescent satellite cells from within the muscle fibre (Berggren et al., 2007; Blau & Webster, 1981). Unlike immortalised cell lines, once isolated, primary skeletal muscle cultures are fated for senescence and cease to proliferate after a relatively short period in culture. Consistent with this Machida et al., (2004) showed that with passaging the myogenic potential (as measured by myogenic markers, proliferation and differentiation potential) of rat primary cells to decrease. However primary cells do retain phenotypic traits of the donor cells, and are able to be cultured from small muscle samples of approximately 50 mg to counter this limitation (Berggren et al., 2007; McAinch et al., 2007). For example, studies investigating the phenotype of human primary skeletal muscle myotubes have shown the retention of aberrant glucose metabolism in primary myotubes cultured from insulin resistant Pima Indians (Thompson et al., 1996). Further studies have demonstrated that phenotypic traits of impaired insulin signalling (Bell et al., 2010), fatty acid uptake and oxidation (Bell et al., 2010; Mott et al., 2000), action of the anti-obesogenic and -diabetic adipokines, adiponectin and leptin and aberrant expression of genes which regulate

substrate metabolism within skeletal muscle (McAinch & Cameron-Smith, 2009; McAinch et al., 2006b; McAinch et al., 2007) are also retained. Thus, cultured primary myotubes enable the study of muscle cell structure and function in any number of physiological and pathophysiological states. Moreover, cultured myotubes enable the investigation of many metabolic abnormalities that exist *in vivo*, while eliminating confounding environmental influences on the muscle (such as circulating hormones, adipokines and other bioactive factors). Myogenic satellite cells can be isolated from a number of tissues including skeletal muscle (Blau & Webster, 1981; Chen et al., 2005; Gaster et al., 2001a; McAinch & Cameron-Smith, 2009). When cultured in specific conditions, quiescent satellite cells isolated from donor skeletal muscle can be stimulated to re-enter the cell cycle and proliferate before being terminally differentiated to recapitulate the phenotype of skeletal muscle from the donor.

As such primary skeletal muscle myotubes have multiple research applications including but not limited to; 1) the study of the effects of myopathies and systemic metabolic diseases on skeletal muscle function, 2) tissue regeneration and renewal for tissue engineering purposes, 3) gene therapy and 4) drug screening (Berggren et al., 2007; Chen et al., 2005; Kessler et al., 1996; Loro et al., 2010; McAinch & Cameron-Smith, 2009; McAinch et al., 2006a; McAinch et al., 2006b; McAinch et al., 2007; Stern-Straeter et al., 2008).

The following sections outline the process of culturing human primary myotubes from their isolation from muscle biopsies to their growth and maintenance in culture and finally a brief look at their current and future applications. At this point we are compelled to mention that methodological variations arise at almost every stage of primary myotube culture, especially in regards to medium composition and the differentiation protocol. Whilst we endeavour to consider the differences in protocols and the implications of these, we primarily report the established methods of our laboratory. Therefore some optimisation may be necessary on behalf of the end user where experimental outcomes differ significantly.

3. The muscle biopsy

A number of techniques can be used to obtain a skeletal muscle biopsy including needle biopsy and surgical excision (Dietrichson et al., 1987; Tarnopolsky et al., 2011). Successful isolation of myogenic satellite cells can be undertaken from small samples of muscle biopsies ranging in size from 50-100 mg (wet weight) (McAinch et al., 2007). This suggests that the typical muscle yield from suction-enhanced needle biopsies is sufficient to culture viable primary myotubes (Melendez et al., 2007; Tarnopolsky et al., 2011). The use of needle biopsy has additional benefits in the relative ease of obtaining muscle biopsies, enabling sampling from a number of accessible skeletal muscles with only the use of local anaesthetic (Dietrichson et al., 1987). The suction-enhanced method described by Tarnopolsky et al., (2011) is described briefly as follows.

The area from which the muscle biopsy is to be obtained is sterilised and the skin and subcutaneous tissue is numbed with a local anaesthetic. A small stab incision (4-5 mm) is made in the skin at the biopsy site. With the aperture closed, the biopsy needle is advanced through this incision to penetrate at least 1 cm beyond the fascia. This motion will be associated with the sensation of deep pressure within the muscle. Once the needle is in position within the muscle, the aperture is opened, and if using a suction enhanced

technique, suction is applied using a sterile 60 ml syringe (approximately 20 ml per sample). It is important to note that the equipment used by these authors is modified to accommodate the suction procedure. However, the biopsy size is significantly increased by using suction compared to no suction (approximately 125 mg compared to 35 mg, respectively. However significant variation in muscle biopsy size exists). The needle is then closed to isolate the muscle biopsy. The needle can then be rotated to obtain another muscle sample as above. The authors describe taking up to 3 samples in quick succession in the one biopsy to obtain adequate muscle sample and is associated with minimum discomfort. The needle is removed using a twisting motion during withdrawal. Pressure is applied to the skin incision and it is closed with a single suture or a tape closure (although a suture may be associated with a lesser degree of scarring) and then pressure is reapplied for 10-15 minutes often with concomitant icing. It is then important the area be kept clean by the subject in the period following the procedure. If closed with a suture, the suture is removed 6 days after the biopsy procedure.

Alternately, skeletal muscle samples can be obtained by open biopsies during surgical procedures. This has the benefits of enabling visualisation of the muscle extract prior to excision and allows for an increase in extract size and/or more controlled sample size in comparison to needle biopsy techniques (Derry et al., 2009; Edwards et al., 1983). In these instances patients are usually under general anaesthetic and a muscle sample is taken from a pre-existing surgical incision, removing the need for an additional surgical incision and superficial trauma. Despite this, it is typically accepted that the needle biopsy procedure presents a more efficient means of obtaining a muscle biopsy (Derry et al., 2009; Edwards et al., 1983).

In our hands, skeletal muscle extracts are obtained from patients undergoing routine bariatric surgery for obesity or obesity and type 2 diabetes mellitus by the attending surgeon. In brief, patients undergo a 12-18 hour fast prior to surgery. They are anaesthetised via short acting propofol and maintained via a volatile anaesthetic mixture of fentanyl and rocuronium. Skeletal muscle extracts are obtained via surgical excision from the *rectus abdominis* muscle by the attending surgeon (McAinch et al., 2007). Skeletal muscle samples extracted from donors to be used for cell culture are trimmed of any visible fat or connective tissue and approximately 50 mg of the muscle biopsy is suspended in serum-free alpha minimum essential media (Gibco, distributed by Invitrogen, Carlsbad, CA). These samples are immediately placed on ice for transportation and undergo no further processing prior to satellite cell isolation. Muscle samples stored in cell culture medium can be maintained at 4 °C for up to 24 hours prior to isolation of the satellite cell population with minimal adverse effects on cellular yield and viability (Blau & Webster, 1981).

4. Tissue culture methods

4.1 Isolation of myogenic satellite cells

The myogenic satellite cell population of skeletal muscle fibres was first characterised by Mauro in 1961. This study correctly identified and proposed satellite cells to be important in muscle regeneration and growth (Mauro, 1961). Degenerative myopathies or muscle injury stimulates quiescent satellite cells to re-enter the cell cycle (Charge & Rudnicki, 2004). Active satellite cells proliferate rapidly promoting muscle regeneration. Therefore controlled

growth of satellite cells is seen to be favourable in the treatment of myopathies and in tissue engineering processes.

Here we describe the process of isolation of these myogenic satellite cells from skeletal muscle extracts which is modified from the methods of Blau & Webster (1981). These methods have been adapted by our laboratory in accordance with optimisation by Gaster et al., (2001a). All processing of skeletal muscle extracts for cell culture should be carried out in a sterile isolated environment, free of biological contaminants. Accordingly, biological safety cabinets should be sterilised under UV light for 20 minutes and cleaned with 70% ethanol prior to use to minimise the risk of contaminating the sample with extraneous cell types. All equipment used must be sterilised.

Satellite cells are isolated from skeletal muscle extracts of 50-100 mg through enzymatic dissociation in 0.05% trypsin-EDTA (Gibco, distributed by Invitrogen, Carlsbad, CA) via a series of incubations. Skeletal muscle extracts are washed twice in approximately 5 ml of alpha minimum essential medium and then washed 3 times in approximately 5 ml of ice-cold 1x Dulbecco's phosphate buffered saline (Gibco, distributed by Invitrogen, Carlsbad, CA). Any remaining connective tissue is removed at this stage. Subsequently, the skeletal muscle extract is manually homogenised in 3 ml of 0.05% trypsin-EDTA in a tissue culture dish using a sterile scalpel. The desired size for muscle fragments is less than 1 mm³ (Blau & Webster, 1981). The resultant homogenate is then combined with an additional 12 ml of 0.05% trypsin-EDTA in a sterile vial, sealed and agitated on an orbital mixer for 20 minutes. Thereafter, the supernatant is aspirated and combined with 5 ml of foetal bovine serum in a 50 ml falcon tube before being placed on ice. Care must be taken at this step to ensure the finely homogenised muscle extract is not aspirated with the supernatant. An additional 15 ml of 0.05% trypsin-EDTA is added to the extract and again agitated as specified above for 20 minutes. The supernatant is combined with the initial yield in the 5 ml of foetal bovine serum. Three repetitions of this process are necessary to fully dissociate the muscle fibres. The supernatant (trypsin and foetal bovine serum mixture) is next filtered through a BD Falcon™ 100-µm nylon cell strainer (BD Biosciences, Bedford, MA). To isolate the cellular fraction of the supernatant, it is transferred to a sterile 50 ml falcon tube and is centrifuged for 7 minutes at 0.5 x g. The resulting pellet of cells will localise at the bottom of the tube and appear reddish-brown in colour. The cellular fraction is gently resuspended in 5 ml of growth medium (alpha minimum essential medium supplemented with 10% foetal bovine serum (v/v), 0.5% penicillin streptomycin (v/v) (Gibco, distributed by Invitrogen, Carlsbad, CA) and 0.5% amphotericin B (v/v) (Sigma-Aldrich, St Louis, MO)) via repeated pipette mixing.

Following the above protocol should yield approximately 5×10^3 viable, proliferating satellite cells from a 0.1 cm³ muscle sample (Blau & Webster, 1981).

4.2 Myoblast culture and maintenance

As the cellular population isolated does not consist solely of myogenic satellite cells, the cell suspension is initially cultured on an uncoated 25 cm² tissue culture flask (Greiner Bio-One, Monroe, NC) for 20 minutes in a controlled humidified environment of 37 °C and 5% carbon dioxide. This increases the satellite cell content of the cultured sample and minimises contamination by fibroblasts which preferentially adhere to the surface of the tissue culture vessel. Gaster et al., (2001a) provide evidence of increased sample purity after pre-plating

showing a time dependent increase in satellite cell fraction. However, increased duration of pre-plating is associated with a greater total loss of satellite cells and therefore we choose a duration of 20 minutes. After the 20 minute incubation period, gently aspirate the cell suspension taking care not to disturb the base of the flask to which the majority of fibroblasts will have attached. This flask is then discarded. The aspirated cell suspension is then seeded onto a 25 cm² flask coated with an extracellular matrix (Geltrex™ Reduced Growth Factor Basement Membrane Matrix; Invitrogen, Carlsbad, CA; allow extracellular matrix coating to dry in a sterile environment before seeding cells) to simulate the basement membrane under endogenous conditions. Extracellular matrices containing laminin and collagen IV, such as Geltrex™, have been shown to promote preferential satellite cell adhesion over fibroblast adhesion to enhance the satellite cell fractional content (Kuhl et al., 1986). Cells are then incubated in a cell culture CO₂ incubator in the conditions described above. Viable cells will adhere to the coated base of the tissue culture flask over the next 24 hours. These cells are considered to be at passage 1. The isolation and initial culturing of the myogenic satellite cells is depicted in figure 1.

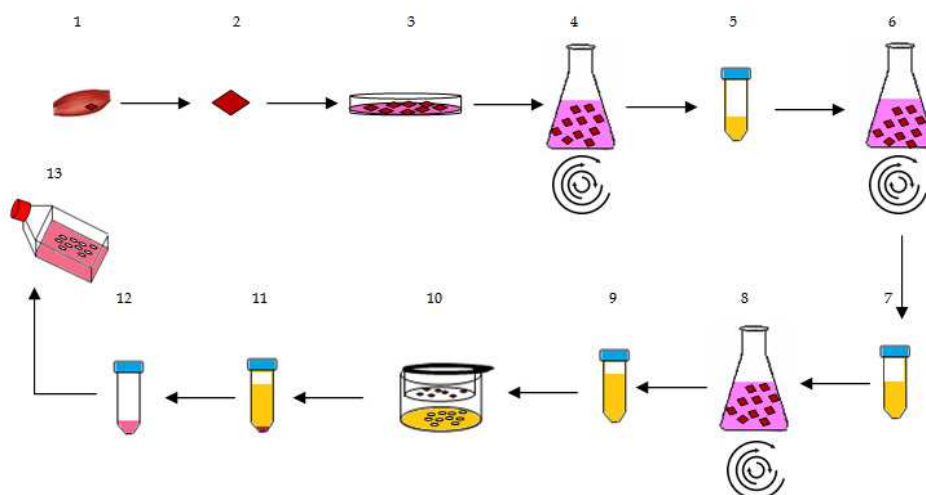


Fig. 1. Schematic representation of the isolation and initial culturing of myogenic satellite cells. 1, site of muscle biopsy is chosen; 2, biopsy extracted; 3, muscle biopsy is manually minced in trypsin-EDTA; 4, first enzymatic dissociated in trypsin under constant agitation; 5, supernatant is combined with foetal bovine serum; 6, second enzymatic dissociated in trypsin under constant agitation; 7, supernatant is combined with foetal bovine serum; 8, third enzymatic dissociated in trypsin under constant agitation; 9, supernatant is combined with foetal bovine serum; 10, supernatant/foetal bovine serum solution is filtered; 11, cellular fraction is pelleted via centrifugation, 12, cellular pellet is resuspended in growth medium; 13, isolated cells are seeded into a 25 cm² tissue culture flask for pre-plating and then transferred to an extracellular matrix coated flask and maintained at 37 °C and 5% carbon dioxide.

24 hours after the initial seeding, the cells are washed twice with 1x phosphate buffered saline to remove cellular debris along with non-adherent and non-viable cells. Thereafter,

growth medium (5 ml) should be refreshed every other day subsequent to a washing step with approximately 5 ml 1x phosphate buffer saline. Continue to maintain the growth of the cells until approximately 70% confluence is reached. This may take 2-3 weeks and therefore it is essential to monitor cell growth to ensure they do not become over-confluent within this period. If myoblastic cells become over-confluent, spontaneous differentiation to mature myotubes will occur and the mitogenic properties of the cells will be lost. At 70% confluency, dissociate the cells from the flask through the addition of 1.5 ml of 0.05% trypsin-EDTA and subsequent incubation at 37 °C, 5% CO₂ for 3 minutes. At this point cells will appear suspended in the media and therefore successful dissociation can be confirmed via microscopic examination. Next, inactivate the trypsin with 3 ml of growth medium, gently ejected over the base of the flask to remove any remaining adherent cells. Isolate the cellular fraction through centrifugation (5 minutes at 0.4 x g) and resuspend the cell pellet in 50 ml growth medium. Gently mix to ensure homogenous cell distribution and then seed onto five extracellular matrix coated 75 cm² cell culture flasks. These cells are designated to be at passage 2. Cells are again grown to approximately 70% confluent following the same procedure as describe for cells at passage 1, before dissociation and isolation via centrifugation. At this point we usually cryopreserve four of the 75 cm² flasks and maintain one for continued growth to passage 3. Cryopreservation enables establishment of a number of stored cell vials for future experimental use to assist in the investigation of your chosen research field. The process of cryopreservation is given in section 4.2.1. The remaining flask is passaged once more as above and seeded onto ten extracellular matrix coated 75 cm² flasks (split ratio 1:10). At 70% confluence cells are trypsinised and isolated via centrifugation (5 minutes at 0.4 x g) and can at this point be cryopreserved.

It is common throughout this process for cell lines isolated from different individuals to exhibit a distinct growth pattern which may be reflected in considerably longer or shorter times taken to reach confluence. Moreover, myoblasts may initially exhibit slow growth patterns, however once maintained in culture for a number of days, growth rates may increase rapidly. Therefore, regularly observing the confluence of the myoblasts is critical to ensure that passaging takes place at the appropriate confluence. Similarly to the dissociable growth patterns of myotubes derived from different individuals, myotubes may also differ in appearance depending on the donor. This can complicate the determination of confluence and therefore each cell line needs to be judged on an individual basis relative to area for growth.

4.2.1 Cryopreservation of myoblastic cells

Cryopreservation of cell stocks is an important practice in any laboratory as it helps to protect against loss of cell lines through bacterial or fungal contamination. Furthermore, as primary cell lines can be passaged finitely before becoming senescent, cryopreservation of cell lines ensures that cell stocks are maintained. It is important that viable cells are chosen for cryopreservation and that the cells are not depleted of myogenic properties due to becoming over-confluent at an earlier passage.

The cryopreservation method used in our laboratory for human primary myoblasts are described herein. Using this method we have established stocks of a large number of viable cell lines derived from muscle extracts. One 75 cm² flask at 70% confluent provides sufficient

cells for distribution to two cryotubes. Thus, the four flasks from passage 2 can be frozen-down in eight cryotubes whilst the ten flasks from cells at passage 3 enable twenty cryotubes to be frozen. This facilitates the rapid expansion of stores of subcultures. In order to ensure consistency between studies, we cryopreserve all cells prior to experimental use. Cells are cryopreserved in a solution containing 25% growth medium (v/v), 25% foetal bovine serum (v/v) and 50% filter sterilised freezing medium. Freezing medium can be made through the addition of 10% dimethyl sulfoxide (v/v; Sigma-Aldrich, St Louis MO) to standard growth medium (as defined in section 4.1). Dimethyl sulfoxide is a freezing agent which acts to reduce the freezing point of the medium to facilitate gradual cryopreservation and prevents ice crystals forming during freezing and lysing the cells. Dimethyl sulfoxide is photosensitive and therefore it is important to protect the freezing medium from light at all stages. Freezing medium is then filter sterilised prior to use through a 0.2- μ m nylon cell filter. Dissociated cells and isolate the cellular fraction via centrifugation as outlined in section 4.2 and then resuspend the pellet in growth medium (25% of total volume). Add to this the foetal bovine serum (25% of total volume). Add the pre-made freezing medium to the cell solution (50% of total volume) and gently mix. Aliquot 1 ml of this solution into each labelled cryotube. The specific volumes to be used depends on the number of flasks to be cryopreserved, however as a guide for two 75 cm² flask use 1 ml of growth medium, 1 ml of foetal bovine serum and 2 ml of freezing medium. To minimise cell death, cells are incrementally frozen at -20 °C for 30 minutes and then enclosed in a foam box for freezing at -80 °C overnight, before finally being stored in liquid nitrogen until required. Alternatively use a cryopreservation freezing container such as "Mr. Frosty" (Nalgene® Labware as part of Thermo Fisher Scientific, New York) which results in a controlled decrease in temperature of 1 °C per minute. Gradually decreasing the temperature during the freezing process aids in preserving cell viability and hence is critical to successful cryopreservation of a viable myoblastic cell population. However, it is also important to note that dimethyl sulfoxide is cytotoxic and therefore once cells are combined with the freezing medium it is important to progress to the freezing stage as rapidly as possible.

Conversely, the thawing procedure must take place as rapidly as possible again due to the toxic nature of dimethyl sulfoxide. We recommend immersing the bottom two thirds of the cryotube in a waterbath heated to 37 °C. Take care not to let the water reach the level of the lid as this will increase the risk of contamination of the subculture. When partially thawed the sample should be removed from the waterbath and rapidly combined with approximately 1–2 ml of growth medium to dilute the dimethyl sulfoxide and gently pipette mixed to facilitate thawing. Once fully thawed, combine the cell solution with 9 ml of growth medium and centrifuge at 0.4 x g for 5 minutes to pellet the cellular fraction. Pour off the growth medium to remove the dimethyl sulfoxide and gently resuspend the pellet in growth medium. Myoblasts can then be seeded onto 75 cm² or 175 cm² tissue culture flasks, depending on the volume of cells required for experimentation. As a rough guide one 75 cm² tissue culture flask at 80% confluence provides adequate cells for ten 6-well tissue culture plates or ten 100 mm tissue culture dishes. We recommend that experimentation be conducted at passage 4 or 5 to avoid senescence. For our purposes cells cryopreserved at passage 3 will be at passage 4 when thawed and subcultured. Thus at 80% confluence these cells must be seeded onto the appropriate vessel for differentiation and experimentation at passage 5. Therefore, when thawing cells it is important to be aware of the volume of cells that will be required to complete the study.

4.3 Myotube formation

The process of myoblast fusion is critical to the development of mature multinucleate skeletal muscle myotubes. During the process of myoblast differentiation, the myogenic cells exit the cell cycle and mitotic activity ceases (Linkhart et al., 1981; Yaffe, 1968). Myogenic commitment is under the synergistic regulation of a vast number of myogenic promoters (including MyoD, myogenin, Myf5, myogenic regulatory factor 4, myocyte specific enhancement factor 2) and inhibitors (including ID, Msx1, Twist and BMP-4) (reviewed by Brand-Saber & Christ, 1999; Charge & Rudnicki, 2004). Table 1 further highlights the expression patterns of myogenic markers associated with different stages of myogenic development.

Extracellular factors also mediate myotube differentiation. Such factors include the cell-cell interactions and proximity and cellular interactions with the extracellular matrix. Concordant with this, studies by Osses & Brandan (2002) and Langen et al. (2003) show that the extracellular matrix enhances myogenic differentiation of clonal C₂C₁₂ myoblasts. *In vitro*, protocols used to induce myoblast fusion vary greatly between studies. This has the potential to confound the comparison of results between studies. Predominately, differentiation protocols vary in the duration and the composition of media used to induce myogenic differentiation.

Myogenic stage	Increase expression	Decreased expression	Variable expression
Muscle derived stem cell	Sca-1 Bcl-2 All MRFs		CD34
Quiescent Satellite cell	c-MET Truncated CD34 Pax7 VCAM-1		m-cadherin Myf5 MyoD Desmin
Myogenic precursor cell	m-cadherin c-Met Full length CD34 Myf5 MyoD Desmin		Myogenin
Terminal differentiation	Myogenin MRF4 Other MRFs	CD34 Pax7	

Adapted from review by Deasy et al., (2001)
MRF; Myogenic regulatory factor.

Table 1. Expression of myogenic markers at different stages of myotube formation.

Myoblast fusion and subsequent myotube formation has been reported to occur over a period of 4-8 days (Gaster et al., 2001b). In our hands, a differentiation protocol of 4 days is

sufficient to induce myoblasts elongation and fusion to produce multinucleate myotubes. The results of Gaster et al., (2001b) substantiate this, showing that on the day after culture conditions were changed to induce differentiation, myoblast fusion to form myotubes had begun and there was an increase in creatine kinase activity and fast myosin heavy chain content up until day 4 when these markers began to decline. This study also reported the presence of developing myofibrils and cross-striations at day 8 of differentiation (Gaster et al., 2001b), however no data is reported evaluating these parameters before this time. Therefore, it is hard to compare the presence of the developing contractile apparatus with functional measures such as creatine kinase activity and fast myosin heavy chain content which were seen to decline from day 4 onwards. Contrary to this Stern-Straeter et al., (2008) observed increased expression of myogenic genes from day 4 of differentiation but peaked at day 16. Creatine kinase activity in this study was also seen to peak at day 16 (Stern-Straeter et al., 2008). These results suggest a longer differentiation period may facilitate further maturation of the myotube cultures and therefore provide a more physiological representation of skeletal muscle. However, longer durations of differentiation can lead to myotube detachment and subsequent cellular death. Moreover, it has been shown in rats that the source of skeletal muscle biopsy (that is, soleus compared to extensor digitorum longus) affected the rate at which the isolated cells differentiated (Lagord et al., 1998). This suggests that the source of muscle will alter the myogenic properties of the derived human primary myotubes.

Aside from variations in the duration of culturing, myoblast fusion can be induced under a number of conditions such as a reduction in sera concentration and substitution of foetal bovine serum with horse serum, which contains less growth factors (Gaster et al., 2001a; Gaster et al., 2001b; Linkhart et al., 1981; McAinch et al., 2007). Further, addition of growth factors (vitronectin, B27 supplement, basic fibroblast growth factor, cardiotropin-1, brain-derived neutrophic factor, glial derived neurotrophic factor and neutrophin 3 and 4) (Das et al., 2009) and provision of soluble basement membrane in the absence of additional growth factors and serum (Langen et al., 2003) can also be used to promote differentiation.

In view of these variations, some contention exists as to the optimal protocol for myoblast differentiation. Accordingly, the differentiation process may need to be optimised by the end user for their specific needs. Differentiation can be confirmed via a number of different measures including analysis of sarcomere formation, creatine kinase activity, myosin heavy chain and alpha actin content and expression of myogenic markers MYoD (up-regulated in earlier stages of differentiation), myogenin, desmin, and myogenic regulatory factor 4 (Blau & Webster, 1981; Stern-Straeter et al., 2008; Stern-Straeter et al., 2007). Such analyses enable individual optimisation of the differentiation process to suit the demands of specific studies.

For our purposes the optimised differentiation protocol is as follows. At approximately 70-80% confluent, cells which have previously been seeded onto extracellular matrix coated tissue culture vessels are washed in 1x phosphate buffered saline. A simple substitution of 10% foetal bovine serum for 2% horse serum is made in the replacement medium. All other components remain the same as for the growth medium (that is, differentiation media contains 2% horse serum (v/v), 0.5% penicillin streptomycin (v/v) and 0.5% amphotericin B (v/v)). Cells are washed and differentiation media is changed every other day until day 4 is reached. When visualised microscopically cells will appear elongated and fusion will be evident. At this point myotubes are ready for experimentation.

5. Experimentation

At the conclusion of the optimised differentiation protocol, myotubes are ready for experimental purposes. However, the specific protocol used in the final stages of myotube culturing is dependent on the end use of the myotubes. That is, the way the myotubes are subcultured and subsequently lysed will depend on the experiment to be conducted. Here we will briefly examine some of the key considerations in designing experiments for analysis of different metabolic parameters.

5.1 mRNA analysis of genetic markers

The analysis of gene expression provides an invaluable source of information regarding changes in the expression of key regulatory genes. Gene expression often provides the basis for additional studies aimed at assessing the effects of experimental interventions on cellular signalling. The growth of human primary myotubes for gene expression purposes has several subtle differences to that of other outcomes. Myotubes grown for the analysis of messenger RNA abundance are typically grown in 6-well tissue culture plates (Greiner Bio-One, Monroe, NC) as decreased cell volume is typically needed for successful RNA extraction. Given the lower area for growth (approximately 9.6 cm² per well) confluence is often reached in a shorter period and therefore it is important to regularly check confluence to ensure differentiation is conducted at 70-80% confluence. Being over-confluent will risk the cells detaching during the experimental period. Subsequent to experimentation, the next key consideration is the process by which the cells are to be lysed. The experimental treatments should be ceased by rapidly washing each well 3 times in approximately 2 ml of ice-cold 1x phosphate buffered saline. Myotubes should be placed immediately on ice to inhibit RNase activity after the third wash and then rapidly lysed. We find the lysis of cells with TRIzol® reagent (Invitrogen, Carlsbad, CA) is highly efficient and provide a high yield of quality RNA (as determined spectrophotometrically). RNA is a particularly unstable molecule and it is important to ensure that the sample is not contaminated with RNases during the extraction procedure. TRIzol® reagent acts to solubilise cellular membranes and debris whilst inhibiting RNase activity and maintains RNA integrity (Santella, 2006). However, due to the corrosive TRIzol® (including the vapour phases) cells should be transferred on ice to an RNA designated fumehood prior to the addition of this reagent. Using filtered pipette tips add 800 µl of TRIzol® to each well. Gently agitate the TRIzol® to ensure that the entire surface of the well is covered and then repeatedly eject the TRIzol® over the base of the well to ensure myotubes are lysed. Transfer cellular lysates to a labelled tube. At this point the cellular lysates can be stored at -80 °C until ready to continue with the RNA extraction.

Total cellular RNA is then extracted through the addition of 200 µl of chloroform (Sigma Aldrich, St Louis, MO) to the cellular lysates. Mix via short vortex and then allow to sit on ice for 5 minutes. Centrifuge the TRIzol®/chloroform for 15 minutes at 16,000 x g to separate the phases. Three distinct phases should be visible; a bottom phase containing the TRIzol® and cellular debris, a thin opaque interphase and a clear RNA containing supernatant. If the separation of phases does not occur, re-vortex the tube and allow to sit on ice before centrifuging again as above. The RNA containing supernatant is then aspirated into a fresh tube containing an equal volume of isopropanol to supernatant (approximately

600 μ l; Sigma Aldrich, St Louis, MO) and 10 μ l of 5 M sodium chloride (Ajax Finechem, Seven Hills, Australia). Care must be taken not to contaminate the RNA sample with the interphase and therefore it is recommended that 80% of the supernatant volume be aspirated. At this point incubate the samples at -20°C for a minimum of 2 hours (can be left overnight) to precipitate the RNA pellet. Subsequent to the incubation period, centrifuge the samples at $16,000 \times g$ for 20 minutes to isolate the pellet. Take care to orient all tubes in the same manner to enhance the ease of RNA extraction, as the RNA pellet may be small and difficult to visualise. Aspirate the isopropanol/sodium chloride solution taking care not to aspirate the RNA pellet. Wash the pellet once with 400 μ l of 75% molecular grade ethanol (Sigma Aldrich, St Louis, MO) and centrifuge for 8 minutes at $8,000 \times g$. Again aspirate the ethanol taking care not to aspirate the RNA pellet and then allow the pellet to air-dry for 5-8 minutes on ice. Resuspend the RNA pellet in 5 μ l of sterile diethylpyrocarbonate treated water (Invitrogen, Carlsbad, CA). The resuspension of the pellet can be enhanced by heating the diethylpyrocarbonate treated water to 65°C . Transfer 1 μ l of RNA to a fresh tube containing 19 μ l of diethylpyrocarbonate treated water for spectrophotometric determination of RNA content at 260 nm and 280 nm. RNA purity can be determined from OD260/OD280 ratio. Samples with an OD260:OD280 ratio of 1.9 - 2.1 can be considered of high quality and relatively free of contaminants (Santella, 2006). RNA samples should be stored at -80°C until required.

RNA can then be reverse transcribed to complementary DNA via the use of such kits as the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Resultant complementary DNA can then analysed for messenger RNA expression of genes of interest by such techniques as 'real-time' polymerase chain reaction (Heid et al., 1996). Data from 'real-time' polymerase chain reaction can be analysed by either absolute quantification or relative quantification (Livak & Schmittgen, 2001). Absolute quantification determines the input copy number of the gene of interest usually against known standards, whilst relative quantification enables determination of the expression of the gene of interest relative to a reference gene (Livak & Schmittgen, 2001).

5.2 Protein

The growth of myotubes for analysis of protein markers requires a greater cell density than is required for analysis of gene expression. Accordingly, when growing for analysis of protein expression, myotubes should be subcultured and grown to confluence on 100 mm tissue culture dishes (Greiner Bio-One, Monroe, NC) coated with extracellular matrix prior to differentiation. As for messenger RNA analysis cells destined for protein expression studies need to be differentiated at approximately 70-80% confluence to ensure an adequate yield. To stop experimental treatments cells should be washed twice with ice-cold 1x phosphate buffered saline. To avoid dilution of the protein fraction it is important to remove excess phosphate buffered saline with either suction or transfer pipettes. When lysing cells for protein expression the addition of a protease inhibitor to the lysis buffer is necessary to inhibit proteolytic protein degradation. For muscle cultures we find that an immunoprecipitation lysis buffer (10 mM Tris pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% NP-40) containing complete mini protease inhibitor cocktail tablet per 10 ml working stock (Roche Diagnostics, Indianapolis, IN) is suitable in lysing cells for protein extraction.

Adherent skeletal muscle myotubes are lysed using 100 μ l of immunoprecipitation lysis buffer and complete mini-protease inhibitor cocktail tablet. Mechanically detached adherent cells from the base of the 100 mm cell culture dish with a cell scraper (Greiner Bio-One, Monroe, NC). To proceed with protein extraction, centrifuge the cellular lysate at 17,000 \times g for 4 minutes. Carefully aspirate the supernatant, aliquot and store at -80 $^{\circ}$ C. Freeze-thaw cycles should be avoided.

Protein abundance can then be quantified against known albumin standards with a working range of 20-2000 μ g/ml in accordance with the assay microplate procedure using a Pierce Bicinchoninic Acid kit (Pierce Biotechnology distributed by Thermo Fisher Scientific, Scoresby, Australia) at 562 nm. We have previously found that the protein content from a single 100 mm tissue culture dish is low and in order to have sufficient protein to measure multiple markers it may be necessary to have a number of replicates for each experimental condition which can be combined to increase the overall protein concentration. If this is the case up to four 100 mm dishes can be lysed using 100 μ l of lysis buffer transferred from plate to plate along with the cellular lysates. This helps to reduce sample dilution with the lysis buffer.

5.3 Glucose and fatty acid uptake

Aberrant glucose and fatty acid metabolism is observed in human primary myotubes isolated from individuals with obesity and type 2 diabetes mellitus compared to control (Bell et al., 2010; Mott et al., 2000; Thompson et al., 1996). Therefore, determination of glucose and fatty acid uptake in these cells can be a useful tool in screening new pharmacological agents aimed at ameliorating perturbed substrate metabolism. The process described below outlines the key methodological considerations for growing human primary myotubes for glucose and fatty acid uptake assays.

5.3.1 Glucose uptake

Prior to differentiation, skeletal muscle myotubes should be grown to approximately 70% confluence in 12 well tissue culture plates. At confluence, differentiation should be undertaken for 4 days. Previous results indicate that basal glucose uptake decreases upon myoblast fusion (Klip et al., 1984; Mitsumoto et al., 1991). In contrast insulin-stimulated glucose uptake increases subsequent to myoblast fusion particularly in the serum deprived L6 myotubes (Klip et al., 1984; Mitsumoto et al., 1991). Therefore for this particular assay we recommend a differentiation period of 4 days however this may need to be optimised depending on the specific outcome of the study. The protocol for measuring glucose uptake is adapted from that of Ciaraldi et al., (1995).

At confluence, myotubes are pre-incubated in serum free alpha-minimum essential medium for 4 hours and subsequently washed three times in uptake buffer (150 mM NaCl, 5 mM KCL, 1.2 mM MgSO₄, 2.5 mM NaH₂PO₄, 1.2 mM CaCl₂, 10 mM HEPES, 0.1% bovine serum albumin, pH 7.4). Myotubes should then be incubated with the compound of interest in 1 ml of uptake buffer for the given time period. Initiate the glucose uptake reaction through the addition of radiolabelled glucose (2-[³H]-deoxy-D-glucose) at 1 μ Ci/ml and 2-deoxy-D-glucose (Perkin Elmer, Waltham, MA) at 10 μ M per well and incubate at 37 $^{\circ}$ C and 5% CO₂ for 15 minutes. Subsequently, aspirate the reaction buffer and rinse the myotubes four times

rapidly with ice cold 1x phosphate buffered saline to stop the reaction. Solubilise the myotubes with 500 μ l of 0.1 M sodium hydroxide for 30 minutes at room temperature. This process can be enhanced by gentle agitation. The cellular lysates should then be divided with 400 μ l being combined with 4 ml of scintillation fluid in scintillation vials for determination of incorporated radiolabelled glucose. The remainder of the lysates should be used to determine cellular protein content. Non-specific glucose uptake can be determined in the presence of cytochalasin B (10 μ M/l) to block glucose transporter mediated glucose uptake (Ceddia et al., 2005; Fletcher et al., 2000; Michael et al., 2001). Cytochalasin B has been shown to be a potent inhibitor of cellular glucose uptake (Kletzien et al., 1972). Non-specific glucose uptake is subtracted from the total glucose uptake values and the net glucose uptake value is expressed relative to cellular protein content in picomoles of 2-deoxy-glucose taken up by the myotubes per mg protein per minute.

5.3.2 Fatty acid uptake

The process of determining fatty acid uptake is similar to that of glucose uptake and accordingly, human primary myotubes should be grown and differentiated in the same manner. Differentiated myotubes should then be exposed to the experimental conditions. Fatty acid uptake can then be measured in the presence of [$1-^{14}$ C] palmitate (Perkin Elmer, Waltham, MA) and non-radiolabelled palmitate (Pimenta et al., 2008). In brief, the palmitate uptake is assayed through the addition of 0.2 μ Ci/ml [$1-^{14}$ C] palmitate and 20 μ M non-labelled palmitate (Fediuc et al., 2008) in serum free alpha-minimum essential medium for 4 minutes. Palmitate should be conjugated to fatty acid free bovine serum albumin at a molar ratio of 1. Cells should be lysed in 500 μ l of 0.1 M NaOH for 30 minutes and palmitate uptake is determined relative to cellular protein content as above.

5.4 Fatty acid oxidation

In addition to determining substrate uptake it is also possible to determine the rate of fatty acid oxidation in human primary myotubes. This allows a more thorough assessment of cellular bioenergetics as corresponding results of enhanced uptake and oxidation may be indicative of improved metabolic function, whilst results of enhanced uptake but impaired oxidation may suggest that the intervention has elicited detrimental effects. Palmitate oxidation is determined from the production of radioactive $^{14}\text{CO}_2$ from [$1-^{14}$ C] palmitate. This outcome can be determined through measurements of radiolabelled CO_2 or water with many variations existing in technique (Fediuc et al., 2006; Fediuc et al., 2008; Pathmaperuma et al., 2010; Petersen et al., 2005; Pimenta et al., 2008; Watt et al., 2006). One such method is described herein and has been adapted from previously described work of Petersen et al., (2005) in L6 skeletal muscle myotubes.

Myotubes should be grown in 100 mm tissue culture dishes until approximately 80% confluence at which point growth medium should be substituted for differentiation medium containing 2% horse serum. On day 4 of differentiation, pre-incubate the myotubes in serum free alpha minimum essential medium contain 0.1% bovine serum albumin for 2 hours. Following a washing step with 1x phosphate buffered saline, myotubes can be exposed to experimental conditions in alpha minimum essential medium containing 0.1% foetal bovine serum (v/v), 4% fatty acid free bovine serum albumin (w/v), 0.1 mM palmitate and 2 μ Ci of

[1-¹⁴C] palmitate with the respective experimental conditions. A 2 hour treatment period has been shown to be sufficient in determining fatty acid oxidation however the period of exposure can be modified to suit the experimental design and therefore should be optimised by the end user. Subsequent to experimental exposure 1 ml of the incubation medium is added to a 20 ml scintillation vial containing 1 ml of 1 M H₂SO₄ and a 0.5 ml microcentrifuge tube containing 1 M benzethonium hydroxide to trap liberated ¹⁴CO₂ over a one hour period. The microcentrifuge tube containing the trapped ¹⁴CO₂ is then placed in a scintillation vial and counted to determine the rate of fatty acid oxidation.

6. Future applications

6.1 Three-dimensional skeletal muscle tissue constructs

Bioengineering of skeletal muscle constructs employs the use of myogenic progenitor cells and specialised scaffolding to form three-dimensional tissue constructs. Given the limited regenerative capacity of adult skeletal muscle, bioengineered skeletal muscle provides a therapeutically relevant method of reducing morbidity associated with various myopathies. Congruent with this, the long term goal of three-dimensional tissue culturing predominately centres around the provision of viable tissue transplants for regenerative purposes after acute injury or disease for instance the fabrication of three-dimensional cardiac muscle constructs. Like adult myotubes, mature cardiac myocytes are terminally differentiated cells and thus cannot regenerate subsequent to damage. Therefore the ultimate goal of bioengineered heart muscle supports myocardial regeneration after myocardial infarction, chronic heart failure or in the repair of congenital heart defects (Eschenhagen et al., 2002). This has the potential to reduce morbidity and mortality associated with cardiovascular diseases. Surgical tissue transplants are widely used in muscle regeneration after muscle injury, however muscle transplantation is associated with significant donor site morbidity, and loss of muscle volume and function (Bach et al., 2004). Implantable bioengineered skeletal muscle constructs remove the need for large muscle extracts from healthy donor tissue and thus pre-engineered tissue constructs provide an appealing treatment method for skeletal muscle pathologies. Bian & Bursac (2008) propose distinct advantages associated with the use of bioengineered muscle constructs as being the ability to engineer constructs with architecture specific for the site of damage, the ability to precondition fabricated muscle for the specific mechanical and metabolic demands of the site and the administration of specific growth factors and hormones to support growth after implantation. However, despite these potential benefits, the field of bioengineered tissues is still in its relative infancy, with muscle constructs exhibiting morphological differences to native muscle with respect to the level of differentiation and organisation of the cell microstructure, making direct comparison with native muscle problematic (Baar, 2005).

The architectural, electrical and functional integrity of fabricated muscles must be similar to that of native muscle in order to compensate for structural and functional deficits within the endogenous muscle. Accordingly, the construct needs to be contractile, demonstrate electrophysiological stability, be flexible yet mechanically robust and have angiogenic potential. Currently, bioengineered muscle constructs are small in size with Huang et al., (2005) generating myoids of $177 \pm 10.5 \mu\text{m}$ in diameter with the cross-sectional area of individual myotubes being only $10 \mu\text{m}$. This is similar to results of Powell et al., (2002) who generated

human bioartificial muscles with individual myotubes being less than 10 μM in diameter. Furthermore, the current methods of generating muscle constructs result in myoids which exhibit significant deficits in force production compared to that of native muscle (Dennis et al., 2001). The absence of fibroblasts in constructs generated from C₂C₁₂ cells also resulted in a force deficit manifesting in a 35% reduction in peak twitch force after tetanic stimulation (Khodabakus & Baar, 2009). The authors noted that addition of 3T3 fibroblasts to C₂C₁₂ myotubes (ratio 1:5) prior to seeding attenuated this force deficit but led to a 50% decrease in specific force. Taken together these results imply that further work needs to be done before bioengineered muscle constructs can be used for functional regeneration of human skeletal muscle. Nonetheless, in spite of the functional limitations, much can be gained through investigations of muscle formation using three-dimensional models of myotube growth (Bach et al., 2004).

Three-dimensional skeletal muscle tissue constructs provide a useful model for the study of muscle structure and function in physiological and pathophysiological states, drug screening and gene therapy despite current limitations in their fabrication (Bach et al., 2004; Vandenburg, 2010; Vandenburg et al., 2008). The use of three-dimensional constructs provides a method with enhanced physiological relevance due to the relative absence of cellular monolayers within their endogenous microenvironments. Interestingly, Larkin et al., (2006) describe a method of co-culturing pre-formed self-organising primary rat tendon constructs with isolated myoblasts in place of artificial tendons, to form a functional myotendinous junction in the three-dimensional constructs formed. Functional testing of these constructs revealed the myotendinous junction could withstand tensile forces beyond the physiological range (Larkin et al., 2006). Rivron et al., (2008) further review recent advances in the ability to generate pre-vascularised tissue constructs *in vitro* which has the capacity to readily anastomose to the host vasculature and thus can enhance viability of implanted tissue constructs. These advances within the tissue engineering field represent promising progress in the development of functional muscles for regenerative purposes.

However due to the differences between native muscle and bioengineered muscle constructs, the challenges which still exist in the formation of *de novo* skeletal muscle cannot be ignored. Specific challenges still exist in supporting growth of sufficient differentiated muscle tissue for regenerative purposes, the capacity to supply sufficient oxygen and nutrients to the core of the construct, the need for these tissue constructs to remain viable and be vascularised and innervated *in vivo* (Bach et al., 2004; Birla et al., 2005; Davis et al., 2007; Gawlitta et al., 2007). Overcoming these challenges to achieve maximal therapeutic benefits centres largely around the ability to accurately recapitulate the endogenous environment for growth *in vitro*. Strategies to enhance myoblast proliferation and differentiation to support these needs are currently in great demand. One such method may be the application of mechanical stretch during construct formation. Endogenously, the electrical impulses generated by the central nervous system are critical developmental stimuli prompting the formation of mature muscle fibres with de-innervated muscle showing reduced numbers of secondary myotubes (Ross et al., 1987). In cell culture models electrical stimulation has been shown to enhance activation of quiescent satellite cells, myoblast alignment, protein synthesis, proliferation and differentiation (Donnelly et al., 2010; Tatsumi et al., 2001; Vandenburg & Karlisch, 1989). Sarcomere formation and the development of contractile apparatus have also been shown to be enhanced by electrical

stimulation in C₂C₁₂ myotubes grown in three-dimensional constructs (Langelaan et al., 2010; Park et al., 2008). An 8 day mechanical stretch/relaxation protocol in bioengineered human muscles also augmented the myofibre diameter and area suggesting adaptation to a phenotype more similar to that of native muscle (Powell et al., 2002). In line with enhanced myotube diameter, Huang et al., (2005) showed that treatment with the trophic factor insulin-like growth factor 1 significantly increased force production of myooids. In further research insulin-like growth factor 1 was shown to induce myotube hypertrophy which was associated with a concomitant increase in active force production (Vandenburgh et al., 2008). This further supports the notion that extracellular paracrine factors are also important in enhancing construct formation. Levenberg et al., (2005) also demonstrated that the co-culture of myoblasts with embryonic fibroblasts and endothelial cells promoted the formation of vessel-like structures within the resultant muscle constructs which has the capacity to advance vascularisation *in vivo*. These findings highlight the significant progress being made in relation to the field bioengineering muscle constructs which resemble native muscle.

6.1.1 Growth of bioengineered tissue constructs

The growth of three-dimensional tissue cultures relies on the manipulation of the *in vitro* environment to support self-organisation of myotubes into bioengineered constructs which resemble the basic structure of *in vivo* skeletal muscle. Parallel alignment of the myotubes along a uni-directional axis is important if force is to be generated by the construct. The ability of the muscle constructs to generate adequate force to restore muscle function is likely dependent on the provision of an extracellular matrix that facilitates interactions of myofibres in a manner similar to that of native muscle (Bian & Bursac, 2008). Several forms of scaffold can be used to support organisation including non-biodegradable and biodegradable scaffolds, of which biodegradable scaffolds are preferable as their controlled degradation supports the development of native extracellular matrix and allows for the formation of densely packed myotubes in new muscle tissue (Koning et al., 2009; Rossi et al., 2010). Biodegradable scaffolds which have been employed for myooid growth include polyglycolic acid, alginate and hyaluronic acid hydrogels (Kamelger et al., 2004), fibrin (Huang et al., 2005), collagen (Hinds et al., 2011) and lamina gels (Dennis et al., 2001). Biomimetic extracellular scaffolds have been shown to enhance myotube fusion and markers of differentiation in two-dimensional cell culture models (Osses & Brandan, 2002). These findings can be extrapolated and applied to three-dimensional tissue culture and thus highlights the importance of the extracellular matrix used.

Moreover, the scaffold needs to be biocompatible if the tissue is to be implanted *in vivo* as this prevents the host's immune system initiating an immune response against the foreign substance (Rossi et al., 2010) which otherwise has the potential to increase the muscle damage sustained. Indeed, recent advances in both skeletal and cardiac muscle engineering have seen bioengineered constructs successfully implanted subcutaneously in rodents (Birla et al., 2005; Levenberg et al., 2005). Birla et al., (2005) initially cultured neonatal cardiac myocytes on laminin coated plates before the bioengineered cardiac constructs were implanted subcutaneously. The authors show the implants become vascularised and cell viability is maintained for at least 3 weeks *in vivo* (Birla et al., 2005). Levenberg et al., (2005) report similar findings with the bioengineered muscle continuing to differentiate *in vivo* and

anastomoses of vessels fabricated *in vitro* with the host's vasculature, as evidenced by the presence of erythrocytes within the lumen of these vessels. Importantly, the explanted constructs showed spontaneous contractility and force production was augmented compared to *in vitro* cultured controls (Birla et al., 2005). Moreover, skeletal muscle grafts cultured in collagen/Matrigel™ hydrogel have been used in myocardial repair subsequent to infarct induced by coronary ligation which was associated with cellular differentiation and vascularisation 4 weeks after implantation (Giraud et al., 2008). These findings represent significant progression towards the end goal of developing implantable tissue constructs and are an important intersect between applications for skeletal and cardiac muscle regeneration.

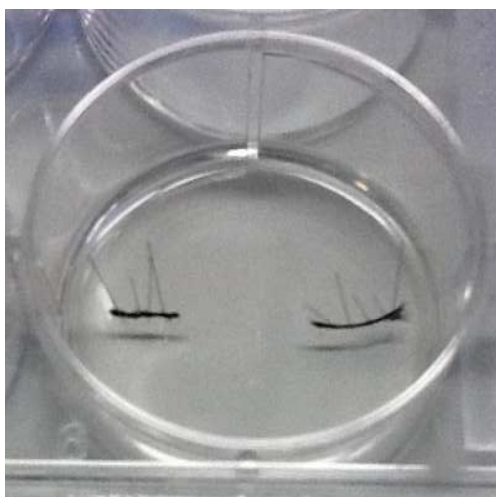
An additional consideration regarding the appropriateness of various scaffolds culture techniques is the provision of sufficient bioactive signals to replicate the endogenous niche to regulate satellite cells quiescence and myoblast proliferation and differentiation. Reproducing a microenvironment which accurately emulates the endogenous niche of muscle cells is complicated by the distinctive combination of cellular, biochemical and biophysical systems which define the biochemical and structural properties of the endogenous microenvironment (Cosgrove et al., 2009). Eberli et al., (2009) support the importance of adequately replicating the microenvironment showing that the addition of fibroblast growth factor, epidermal growth factor, dexamethasone and insulin to collagen coated dishes enhances the formation of differentiated human primary myotubes. However, this issue is confounded due to the extensiveness of the list of factors which regulate myogenesis and myotube formation.

The use of Matrigel™ (BD Biosciences San Jose, CA), a bioactive extracellular matrix derive from the mouse Engelbreth-Holm-Swarm tumour lineage may partially address this issue. Matrigel abundantly produces extracellular matrix factors which support cellular growth and differentiation (reviewed by Kleinman & Martin (2005)). Given these effects, Matrigel™ is an appealing substrate to support the fabrication of three-dimensional tissue constructs. Consistent with this, Hinds et al., (2011) found that the use of a hydrogel matrix consisting of Matrigel and fibrin enhanced myotube formation and density within the construct which was positively correlated with force production. Whilst no single extracellular matrix has been identified conclusively as superior, the use of fibrin gels with aprotinin, a plasminogen inhibitor and the naturally occurring cross-linking agent, genipin, have been shown to enhance force production and time-to-failure of constructs by way of controlled degradation of this matrix (Khodabukus & Baar, 2009). Moreover, fibrin gels provide a more flexible scaffold medium into which cells migrate and proliferate both on top of and within the fibrin gel to increased specific force production (Dennis et al., 2001; Huang et al., 2005). Furthermore, controlled fibrinolysis of the fibrin gel and secretion of extracellular matrix proteins by the muscle cells allows the muscle construct to develop a phenotype more representative of native muscle (Ross & Tranquillo, 2003). When considered in conjunction with the findings of Hinds et al., (2011) this suggests enhanced fibrin gels to be an appealing substrate for fabrication of bioengineered muscle and accordingly is the method we choose to report herein due to these advantages and also the relative inexpensiveness of substrates and ease at which myooids can be formed.

The methods described are based on previous research in skeletal muscle myotubes (Huang et al., 2005). Methods utilising C₂C₁₂ myotubes have since been optimised to support an

extended period for which the constructs can be maintained in culture (Khodabukus & Baar, 2009). The protocol reported below is based on the optimised procedure.

Myotubes are cultured on polydimethylsiloxane (SYLGARD 184 Elastomer kit; OBA, Dandenong, Australia) coated 35 mm tissue culture dishes or 6-well tissue culture plates. Prepare the polydimethylsiloxane according to the manufacturer's instructions in a ratio of 10:1 base to curing agent. In brief, using a syringe aspirate 1.5 ml of the base agent and slowly coat the base of each dish, taking care to avoid the formation of air bubbles. Add 150 μ l of the curing agent and gently mix with the pipette tip. Allow the dish to cure for 48 hours at room temperature. This process can be quickened by incubating in a humidity-free incubator at 60 °C. Place two 6 mm long braided silk sutures (Fine Science, North Vancouver, Canada) at either end of the dish with an interposing gap of 12 mm between the end of each suture. Secure the sutures in place with 0.1 mm-diameter stainless steel minuten pins (Fine Sciences, North Vancouver, Canada) as shown in Figure 2. Place multiple pins along the length of the suture to ensure that they are firmly fixed in place.



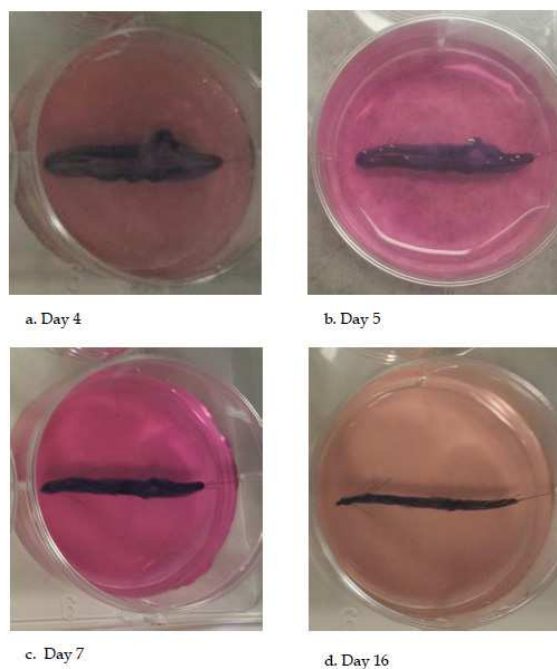
(Cornall et al., Unpublished observation)

Fig. 2. Arrangement of sutures in the polydimethylsiloxane coated well of a 6-well tissue culture plate. As the sutures are to provide the only point of force to which the myotubes align against the suture needs to be firmly affixed with multiple minuten pins along the length of the suture.

At this point it is important to sterilise the dishes to avoid contamination of the myooids constructs. This can be done by soaking the dishes in 70% ethanol for 20 minutes. Following this, aspirate the ethanol and rinse the dish with 1 ml of 1x phosphate buffered saline. The dishes can also be exposed to UV light for 1 hour to assist with sterilisation.

The next step is the preparation of the fibrin gel. Once all components have been diluted to the appropriate concentrations, determine the number of dishes and prepare a master mix of growth medium, genipin (Sigma Aldrich, St Louis, MO), thrombin (Sigma Aldrich, St Louis, MO) and aprotinin (Sigma Aldrich, St Louis, MO). C₂C₁₂ myotubes growth medium is made

with a base of Dulbecco's modified eagle medium supplemented with with 10% foetal bovine serum (v/v), 1% penicillin streptomycin (v/v) and 0.5% amphotericin B. For each dish add 463 μ l of growth medium containing 10 μ l Genipin (stock concentration 10 mg/ml), 25 μ l Thrombin (stock concentration 200 U/ ml) thrombin and 2 μ l Aprotinin (stock concentration 10 mg/ ml). Gently agitate the dish until the entire surface is covered, ensure no air bubbles form around the sutures and pins. Quickly, add 200 μ l of 20 mg/ml fibrinogen (Sigma Aldrich, St Louis, MO) to each dish and gently mix. Allow the gel to polymerise for 1 hour. Seed the cells into each of the dishes (100,000 cell per dish) in growth medium to a final volume of 2 ml. Allow the cells 24 hours to attach prior to changing the growth medium every other day. Myoblasts will migrate through the fibrin gel to proliferate both within and on top of the gel. After 2 days the gel should begin to contract and the myotube will begin to realign themselves between the points of force between the 2 sutures. This process should complete around day 7. The myotubes are then differentiated for 2 days in reduced serum differentiation medium (2% horse serum). Continue to maintain the forming myooids in growth medium containing 7% foetal bovine serum until construct formation is completed.



(Cornall et al., Unpublished observations)

Fig. 3. Formation of three-dimensional C_2C_{12} tissue constructs at different time points after initial seeding of the cells. Note the progressive contraction of the gel and subsequent alignment of the construct between the two sutures resulting in the formation of a densely packed three-dimensional tissue construct. At day 16 the dimensions of the construct were determined as approximately 25 mm length and 1 mm in width and the myooid was deemed to be fully formed.

Once construct formation is complete the myooids can be stimulated to contract using platinum wire electrodes positioned either side of the constructs using a force transducer. Electrical stimulation of constructs has been successfully demonstrated using clonal C₂C₁₂ myotubes in a number of studies (Khodabukus & Baar, 2009; Langelaan et al., 2010; Park et al., 2008). However, in our experience the process of electrically stimulating constructs of human primary myotubes has proven difficult. Whilst we have previously been able to culture the human primary myotubes to form bioengineered skeletal muscle constructs, these myooids have been unresponsive to electrical stimulation (McAinch, Unpublished observations). As primary cells have limited myogenic capacity, this may reflect changes in the electrical potential of the membrane as the cells age in culture. It may be possible to form contractile myooids from human primary myotubes which have been isolated from the muscle sample immediately prior to culturing in this manner, as opposed to being passaged in monolayers initially. However, it remains unclear whether contractile ability is retained. Despite the lack of contractile ability, culturing the myotubes in three-dimensional arrangements provides an interesting model providing a more physiologically relevant arrangement of cells (that is, *in vivo* cells are rarely arranged in monolayers). When cultured in this manner it enables investigations into the importance of cell-cell interactions and spatial arrangements on the maturation and metabolic processes of skeletal muscle myotubes.

7. Conclusion

This chapter has highlighted the diverse nature of research applications associated with the use of human primary myotubes for studies investigating muscle physiology and the implications of this for systemic health. We have identified significant benefits of using human primary myotubes over the use of immortalised cell lines such as the C₂C₁₂ and L6 cell lines due to the fact that these cells retain donor phenotypic traits. This increases the physiological relevance of results obtained in studies utilising such primary myotubes. Moreover, the relative ease at which samples of sufficient size can be obtain through simple, relatively non-invasive biopsy techniques heightens the practicality associated with their use. Whilst we have outlined the practices within our research group of isolating myogenic satellite cells, growing and maintaining myoblasts and the differentiation of myoblasts into myotubes for experimentation, it is apparent that significant variations in methodology do prevail. Therefore, the methods described in this chapter may need optimising by the end user depending on the outcomes and clinical measures of the specific study. Accordingly, we recommend these methods as a highly appropriate starting point for this procedure. We have also provided an indication of methods for a number of experimental techniques associated with the use of human primary myotubes for developing bioengineered skeletal muscle constructs and their application for the treatment of various of myopathies. This list of applications is by no means exhaustive which furthers demonstrates the versatile nature of this experimental model.

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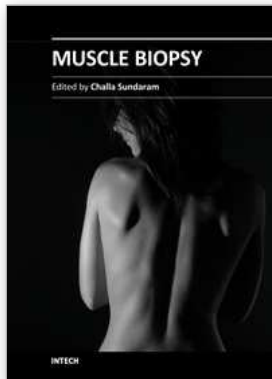
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Muscle Biopsy

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Investigation of muscle diseases has changed dramatically with the understanding of genetic basis of a wide range of muscle diseases. Muscle biopsy has become a powerful tool not only to provide diagnosis but to make tissue available for genetic studies and to basic scientists for biomedical research. Accurate interpretation of muscle biopsy to detect cell dysfunction/ damage/death or absence / abnormality of a protein or genetic defect by the sophisticated technologies is important to guide treatment of various muscle diseases. In this book on muscle biopsy various chapters deal with the procedure and interpretation of muscle biopsy, its use in the culture of myotubes and membrane transport studies. Muscle biopsy is an important technique to investigate mitochondrial dysfunction and the mitochondrial DNA integrity in oxidation. Phosphorylation in various metabolic diseases like obesity, type 2 diabetes mellitus and peripheral vascular disease is explored in the other chapters with detailed descriptions on methodology. This book provides the advances in the basic techniques of muscle biopsy for a neuroscientist.

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