Large-scale isolation of human skeletal muscle satellite cells from post-mortem tissue and development of quantitative assays to evaluate modulators of myogenesis

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Abstract

During aging, there is a decreased ability to maintain skeletal muscle mass and function (sarcopenia). Such changes in skeletal muscle are also co-morbidities of diseases including cancer, congestive heart failure and chronic obstructive pulmonary disease. The loss of muscle mass results in decreased strength and exercise tolerance and reduced ability to perform daily activities. Pharmacological agents addressing these pathologies could have significant clinical impact, but their identification requires understanding of mechanisms driving myotube formation (myogenesis) and atrophy and provision of relevant assays. The aim of this study was to develop robust in vitro methods to study human myogenesis.

Methods

Satellite cells were isolated by digestion of post-mortem skeletal muscle and selection using anti-CD56 MicroBeads. CD56+ cell-derived myotubes were quantified by high content imaging of myosin heavy chains. TaqMan-polymerase chain reaction arrays were used to quantify expression of 41 selected genes during differentiation. The effects of activin receptor agonists and tumour necrosis factor alpha (TNFα) on myogenesis and gene expression were characterised.

Results

Large-scale isolation of CD56+ cells enabled development of a quantitative myogenesis assay with maximal myotube formation 3 days after initiating differentiation. Gene expression analysis demonstrated expression of 19 genes changed substantially during myogenesis. TNFα and activin receptor agonists inhibited myogenesis and...
downregulated gene expression of muscle transcription factors, structural components and markers of oxidative phenotype, but only TNFα increased expression of pro-inflammatory markers.

Conclusions We have developed methods for large-scale isolation of satellite cells from muscle and quantitative assays for studying human myogenesis. These systems may prove useful as part of a screening cascade designed to identify therapeutic agents for improving muscle function.

Keywords Myogenesis · Satellite cells · CD56 · Myostatin · TNFα · Image analysis

Abbreviations
AIDS Acquired immune deficiency syndrome
BSA Bovine serum albumin
COPD Chronic obstructive pulmonary disease
ddH₂O Double-distilled water
DMEM Dulbecco’s modified Eagles medium
FC buffer Flow cytometry buffer
IL-6 Interleukin-6
NCAM Neural cell adhesion molecule
PBS Phosphate buffered saline
SEM Standard error of the mean
TNFα Tumour necrosis factor alpha

1 Background

Skeletal muscle mass in humans declines markedly in old age, a process that has been termed sarcopenia [1]. This age-related loss of muscle mass and strength is associated with mortality and disability and is an important aetiological factor in falls in older people. As life expectancy continues to grow, there is an increasing need to address this issue. Loss of skeletal muscle mass and function is also characteristic of muscular dystrophies and is a common co-morbidity of a number of other diseases including cancer, AIDS, congestive heart failure, COPD, sepsis and renal failure [2]. Although disuse atrophy probably explains the majority of the muscle mass loss observed in some of these conditions, other mechanisms such as inflammation and impaired myogenesis may also play a role.

Myogenesis is the process by which new myofibres are formed and therefore is important in muscle growth, remodelling and repair. Satellite cells constitute the main myogenic activity in adult muscle and in response to stimuli such as myotrauma become activated, proliferate and express myogenic markers [3]. Ultimately, these cells fuse to existing muscle fibres or fuse together to form new myofibres. Changes in the number and function of satellite cells have been reported in elderly patients [4, 5] and in those suffering from certain muscular dystrophies [6], although similar studies in other diseases have, to our knowledge, not been undertaken. The relative abundance and ease of accessibility of satellite cells has encouraged significant pre-clinical research into this stem cell population and their potential for therapeutic applications in disorders of skeletal muscle. In many instances, such studies have employed satellite cells or myogenic cell lines derived from rodents, although a number of investigators have successfully isolated satellite cells from human skeletal muscle [7–11]. These latter research efforts have largely used small biopsy samples from patients undergoing surgery and focused on the characterisation of the isolated cells. Much less effort has been applied to developing assay systems using these cells to allow investigation of the effects of pharmacological agents on myogenesis and the molecular events underlying this process. Such research will nevertheless be instrumental in identifying new targets and mechanisms that modulate myogenesis and may ultimately lead to new therapeutic treatments for muscle disorders.

The aim of this study was to establish a robust method for isolating sufficiently large numbers of satellite cells from adult post-mortem human skeletal muscle to allow the development of myogenesis assays suitable for the study of pharmacological agents in a quantitative and relatively high-throughput manner. As part of the assay validation procedure, we investigated the effects of activin receptor ligands and tumour necrosis factor alpha (TNFα) both of which have been reported to inhibit the myogenesis process [12] and may be relevant to a number of diseases characterised by loss of muscle mass and function.

2 Methods

The study was approved by the Nottingham Research Ethics Committee 1 (reference number 08/H0403/132), and informed consent was provided by the patients’ next of kin.

2.1 Human skeletal muscle satellite cell isolation and purification

Human skeletal muscle satellite cell lines were derived from vastus lateralis quadriceps muscle of cadaver tissues at post-mortem. Details of the donors and tissue samples used are summarised in Table 1. Skeletal muscle tissue samples (50–100 g) were shipped and maintained at 4 °C for 2–4 days in Dulbecco’s modified Eagles medium (DMEM). They were trimmed of excess connective tissue and minced into pieces of approximately 1 mm³. The minced muscle was digested in 1 % type II collagenase (Worthington, Lakewood, NJ) at 37 °C for 1 h with intermittent agitation (using 1 ml digestion solution per gram tissue). Digested tissue was sieved through a course sieve and the filtrate removed. Incompletely digested tissue was further digested with 10× Trypsin (Invitrogen) at 37 °C for 2 h with intermittent agitation. Digestion was stopped by addition of DMEM containing 10 % foetal calf serum, and the cell suspension was filtered through a 100 μm filter. Cells
were collected and re-suspended in skeletal muscle growth medium (Promocell) and plated into 225 cm$^2$ flasks (Costar) coated with 0.2 % gelatine (using 12 flasks per 100 g of tissue). After 3–5 days incubation, cell debris and non-adherent cells were removed by replacing the skeletal muscle growth medium. Adherent cells were grown to 80 % confluency and recovered from flasks with Accutase (Invitrogen). Skeletal muscle satellite cells were purified using anti-human CD56-conjugated MicroBeads according to standard protocols (Miltenyi Biotec).

2.2 Flow cytometry analysis

Cell preparations were re-suspended in phosphate buffered saline (PBS) containing 0.1 % bovine serum albumin (BSA) (flow cytometry (FC) buffer) at $1 \times 10^6$ cells/ml and incubated with anti-human CD56-PC5 antibodies (clone 2A3) or IgG1 isotype control antibodies (Beckman Coulter) diluted 1:100 in FC buffer at room temperature for 20 min. Cells were washed twice in FC buffer and re-suspended in 0.3 ml of FC buffer. Percentage CD56 expression in cell preparations before and after CD56 microbeads purification were determined by standard methods on a Guava flow cytometer.

2.3 Myogenesis assay

The CD56$^+$ purified cells were stored at passages 0 or 1. Each donor was assessed for its CD56$^+$ expression and ability to form myotubes after storage. Assays were performed in black-walled half-well 96-well plates (Greiner) for immunocytochemical staining or clear 12-well plates (Costar) for gene expression studies. Plates were coated with 100–150 $\mu$g/ml ECM gel in DMEM (Sigma) for 2–4 h prior to addition of cells. Skeletal muscle satellite cells were used at passages 1–2 and plated at 31,250 cells/cm$^2$ (approximately 90 % confluence) in DMEM/F12 medium containing 10 % horse serum (Lonzza) for 24 h. Medium was removed (day 0) and cells incubated in DMEM/F12 (Lonza) containing 2 % horse serum (Lonza) (differentiation medium). Myostatin (Peprotech) and GDF-11 (R&D systems) were reconstituted in sterile double-distilled water (ddH$_2$O) containing 0.1 % BSA and 4 mM HCl; activin A and TNF$\alpha$ (R&D systems) were reconstituted in PBS containing 0.1 % BSA. Ligands were further diluted in differentiation medium prior to addition to cells. Cells were incubated between 1 and 7 days with no replenishment of medium or ligands.

2.4 Immunocytochemical staining for myosin heavy chains

Cells were fixed in PBS containing 4 % paraformaldehyde, permeabilised in PBS containing 0.1 % Triton X-100 and blocked in PBS containing 3 % BSA. Cells were immunostained with anti-myosin slow- (clone Q7.5.4D) and fast-chain (clone MY-32) monoclonal antibodies or isotype control IgG1 (Sigma) followed by secondary labelling with an Alexa 488 conjugated anti-mouse IgG antibody (Invitrogen) and Hoescht 33342 dye (H-1399, Invitrogen). Cells were washed three to five times in PBS between fixing, permeabilisation and staining steps and stored in PBS.

2.5 Quantification of myotube formation

Immunostained myotubes were imaged by digital microscopy on the automated ImageXpress 5000A high-content platform (Molecular Devices). Images were acquired in two channels (Hoescht and Alexa-488) using a ×4 Plan Fluor objective and a single field of view per well representing approximately 45 % of the well area. The percent area of a

| Donor | Age | Gender | Cause(s) of death | Time of cell isolation after death (h) | CD56 % expression pre-purification | CD56 % expression post-purification | Cell yield per gram tissue$^a$ |
|-------|-----|--------|------------------|-------------------------------------|-----------------------------------|-----------------------------------|----------------------------------|
| 1     | 55  | Male   | Pneumonia, lung abscess | 54                                  | 85 %                              | 97 %                              | $2.9 \times 10^6$                  |
| 2     | 64  | Male   | Congestive heart failure, renal failure | 35                                  | 77 %                              | 97 %                              | $2.4 \times 10^6$                  |
| 3     | 67  | Male   | Multi-organ failure | 74                                  | 94 %                              | 99 %                              | $4.0 \times 10^6$                  |
| 4     | 58  | Male   | Pneumonia, renal cancer | 97                                  | 84 %                              | 94 %                              | $4.0 \times 10^6$                  |
| 5     | 72  | Female | Myocardial infarction | 66                                  | 56 %                              | 98 %                              | $0.4 \times 10^6$                  |
| 6     | 62  | Male   | Pneumonia, leukemia | 47                                  | 37 %                              | 95 %                              | $1.2 \times 10^6$                  |
| 7     | 66  | Female | Renal failure, breast cancer | 50                                  | 54 %                              | 96 %                              | $1.8 \times 10^6$                  |
| 8     | 66  | Male   | Pneumonia, non-small cell lung cancer | 66                                  | 72 %                              | 88 %                              | $0.8 \times 10^6$                  |
| 9     | 73  | Male   | Cerebro-vascular haemorrhage | 73                                  | 87 %                              | 98 %                              | $5.4 \times 10^6$                  |
| 10    | 77  | Male   | Heart failure | 77                                  | 92 %                              | 99 %                              | $0.2 \times 10^6$                  |

$^a$ Post-CD56 purification
field of view covered by myotubes was quantified using the MetaXpress Angiogenesis Tube Formation application module (Molecular Devices). Total nuclei counts per field of view were quantified using the MetaXpress Count Nuclei application module (Molecular Devices) as a measure of cell number and viability. The application modules classified myotubes and nuclei based on minimum and maximum width (micrometers) and intensity above background (gray levels). All treatments were performed in quadruplicate, and analysis was performed using data obtained from experiments with cells from three independent donors.

2.6 RNA isolation and cDNA synthesis

RNA was extracted using the RNeasy-96 RNA isolation protocol (Qiagen) that included a DNase incubation step and cDNA synthesised using VILO polymerase (Invitrogen). RNA and cDNA quantity and purity were measured using the Nanodrop ND8000 with sample quality criteria of RNA & cDNA 260/280 ratio >1.9; cDNA 260/230 ratio >1.9.

2.7 Gene expression analysis

Two hundred fifty ng of cDNA was assayed on custom-made TaqMan low-density arrays (TLDA; Applied Biosystems) containing 2× qPCR mastermix RT-QPRT-032X (Eurogentec). The genes analysed in TLDA assays are shown in Table 2. Polymerase chain reaction (PCR) conditions used were 94.5 °C for 10 min, followed by 40 cycles of 97 °C for 30 s and 59.7 °C for 1 min. Gene expression was normalised to a geometric mean of the reference gene Cqs for the sample of interest. DDCq is the difference between the target gene Cq and the geometric mean of the reference gene Cqs for the sample of interest. DDCq is the difference between the DDCq of the sample of interest and the baseline sample DDCq. DDCq (%max) is 100 multiplied by the difference between the DDCq of the sample of interest and the sample with the lowest DDCq (the highest relative expression level) per treatment group. For the treated samples, the lowest DDCq is taken from the control (i.e. non-treated) group. Relative quantification is expressed as $2^{-\Delta\Delta Cq}$; RQ(%max)=$2^{-\Delta\Delta Cq(%max)}$. All treatments were performed in duplicate, and analysis was performed using data obtained from experiments with cells from a minimum of three independent donors.

3 Results

3.1 Skeletal muscle satellite cell isolation and purification

Human skeletal muscle satellite cells were isolated from vastus lateralis quadriceps muscle of cadaver tissues at post-mortem. The donor characteristics are summarised in Table 1. The time of cell isolation after death ranged from 35–97 h across the ten donors with no apparent correlation to downstream cell viability or yield. Following enzymatic digestion of the muscle tissue and removal of cell debris, morphological analysis of the adherent cultured cells by light microscopy suggested the presence of skeletal muscle satellite cells, endothelial cells and fibroblast-like cells (Fig. 1a-c). Cell preparations were further analysed by flow cytometry before and after purification (an example is shown in Fig. 1d-f). The expression of CD56, a marker of skeletal muscle, satellite cells ranged from 37–94 % in crude adherent cell preparations and increased to 88–99 % following purification using CD56-conjugated MicroBeads (Table 1). The cell yield after purification averaged $2.3 \times 10^6$ cells/g tissue across the ten donors. Purified CD56+ cells, but not CD56− cell preparations, formed myotubes expressing myosin heavy chains after 7 days in differentiation medium (Fig. 1g, h). Co-staining of myotubes for myosin heavy chains and cell nuclei demonstrated that myotubes were multi-nucleated (Fig. 1i, j).

3.2 Development of a quantitative myogenesis assay

A 96-well format myogenesis assay was developed to enable characterisation of the myotubes formed from the CD56+ purified cells and to provide a platform for pharmacological screening. Myogenesis was quantified in an automated fashion using image analysis algorithms that classified myotubes based on both skeletal myosin expression (myosin heavy slow and fast chains) and morphology, using intensity and size thresholds, respectively. The percentage area of a field of view covered by myotubes was captured from the image analysis. Only differentiated multinuclear myotubes had significant skeletal myosin expression, and, as such, percentage area covered represented a robust measure of myogenesis (Figs. 1h and 2a). In order to determine the kinetics of myotube formation, a time course was performed. This revealed low-level expression of skeletal myosin at day 1 (24 h after eliciting differentiation) constituting less than 10 % of the area covered. This increased to a maximum coverage of 80 % by day 3 and then plateaued at this level for a further 4 days (Fig. 2b).

3.3 Characterisation of gene expression changes during myogenesis

TLDAs were employed to quantitatively explore the expression of 41 genes during myogenesis. The selected genes,
Table 2 Summary of genes analysed using TaqMan-PCR low-density assays (TLDA)

| Gene name       | Official symbol | Gene description                        | Functional category | TLDA assay number       |
|-----------------|-----------------|-----------------------------------------|---------------------|-------------------------|
| MYOD1           | MYOD1           | Myogenic differentiation 1              | Differentiation marker | Hs00159528_m1          |
| MYF5            | MYF5            | Myogenin factor 5                       | Differentiation marker | Hs00271574_m1          |
| Myogenin        | MYOG            | Myogenin (myogenic factor 4)            | Differentiation marker | Hs00231167_m1          |
| PAX7            | PAX7            | Paired box 7 isoform 1                  | Differentiation marker | Hs00242962_m1          |
| MEF2A           | MEF2A           | Myocyte enhancer factor 2A              | Differentiation marker | Hs00271535_m1          |
| Creatine kinase | CKM             | Creatine kinase, muscle                 | Differentiation marker | Hs00176490_m1          |
| NCAM1 (CD56)    | NCAM1           | Neural cell adhesion molecule 1         | Satellite cell marker | Hs00941833_m1          |
| Syndecan 3      | SDC3            | Satellite cell marker                   |                     | Hs0006320_m1           |
| Troponin I      | TNN1            | Troponin I type 1 (skeletal, slow)      | Skeletal muscle structure | Hs00268531_m1          |
| Troponin II     | TNN2            | Troponin I type 2 (skeletal, fast)      | Skeletal muscle structure | Hs00268536_g1          |
| MYH1            | MYH1            | Myosin heavy chain-2 (MYH-1) IIX fast   | Skeletal muscle structure | Hs00428600_m1          |
| MYH2            | MYH2            | Myosin heavy chain-2 (MYH-2) IIa fast   | Skeletal muscle structure | Hs00430042_m1          |
| MYH3            | MYH3            | Myosin heavy chain-3 (MYH-3) embryonic  | Skeletal muscle structure | Hs00159463_m1          |
| MYH4            | MYH4            | Myosin heavy chain-2 (MYH-4) IIb fast   | Skeletal muscle structure | Hs00255652_m1          |
| MYH7            | MYH7            | Myosin heavy chain-7 (MYH-7) slow       | Skeletal muscle structure | Hs00165276_m1          |
| IGF1            | IGF1            | Insulin-like growth factor 1 (somatomedin C) | Anabolic | Hs00559804_m1          |
| Calpain 1       | CAPN1           | Calpain 1, (mu/I) large subunit         | Atrophy              | Hs00156280_m1          |
| Calpastatin     | CAST            | Calpastatin                             | Atrophy              | Hs00156280_m1          |
| FBXO32          | FBXO32          | F-box protein 32 (MAFbx)                | Atrophy              | Hs00369714_m1          |
| FOXO1           | FOXO1           | Forkhead box O1                         | Atrophy              | Hs00231106_m1          |
| TRIM63          | TRIM63          | Tripartite motif-containing 63 (MURF-1) | Atrophy              | Hs00261590_m1          |
| Myostatin       | MSTN            | Myostatin                               | Atrophy              | Hs00193363_m1          |
| FRAP1           | FRAP1           | FK506 binding protein 12-rapamycin      | Catabolic            | Hs00234522_m1          |
| GSK3β           | GSK3B           | Glycogen synthase kinase 3 beta         | Catabolic            | Hs00275656_m1          |
| TNFa            | TNFa            | Tumour necrosis factor (TNFa superfamily, member 2) | Inflammation | Hs00174128_m1          |
| TWEAK           | TNSF12;TNSF13   | Tumour necrosis factor (ligand) superfamily, member 12 precursor (TWEAK) | Inflammation | Hs00356411_m1          |
| NOS2A           | NOS2            | Nitric oxide synthase 2A (iNOS)         | Inflammation         | Hs00167257_m1          |
| IL6             | IL6             | Interleukin 6 (interferon, beta 2)      | Inflammation         | Hs00174131_m1          |
| p38             | MAPK1           | Mitogen-activated protein kinase 1 (p38) | Inflammation | Hs00177066_m1          |
| NFkB            | NFKB1           | Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105) | Inflammation | Hs00231653_m1          |
| IL15            | IL15            | Interleukin 15 preproprotein            | Inflammation         | Hs009999039_m1         |
| IL15            | CD36            | CD36 molecule (thrombospondin receptor) | Oxidative metabolism | Hs00354519_m1          |
| Citrate synthase| CS              | Citrate synthase                        | Oxidative metabolism | Hs02574374_s1          |
| PPARδ           | PPARD           | Peroxisome proliferator-activated receptor delta | Oxidative metabolism | Hs00606407_m1          |
| PPARγ1α         | PPARGC1A        | Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha | Oxidative metabolism | Hs00173304_m1          |
| PPARγ1β         | PPARGC1B        | Peroxisome proliferator-activated receptor gamma, coactivator 1 beta | Oxidative metabolism | Hs00370186_m1          |
| PEPCK1          | PCK1            | Phosphoenolpyruvate carboxykinase 1 (soluble) | Oxidative metabolism | Hs00159918_m1          |
| PRKAA1          | PRKAA1          | Protein kinase, AMP-activated, alpha 1 catalytic subunit | Oxidative metabolism | Hs01562315_m1          |
| LDHA            | LDHA            | Lactate dehydrogenase A                 | Oxidative metabolism | Hs00855332_g1          |
| Pyruvate kinase | PKM2            | Pyruvate kinase, muscle isofom M2       | Oxidative metabolism | Hs00987247_g1          |
| β-HADH           | HADH            | Beta hydrox acyl CoA dehy              | Oxidative metabolism | Hs00193428_m1          |
| 18S             | 18S             | Ribosomal 18S                          | Housekeeper         | Hs99999901_s1          |
| RPL13A          | RPL13A          | RPL13A                                  | Housekeeper         | Hs03043885_g1          |
| Ubiquitin C     | UBC             | Ubiquitin C                             | Housekeeper         | Hs00824723_m1          |
encoding markers of myogenic differentiation, satellite cells, muscle atrophy, catabolism, inflammation, oxidative metabolism and structural components (Table 2) were studied over the 7 days post-induction of differentiation. These data are comprehensively tabulated in Online Resource 1. All data presented is the average percent change in gene expression in cells isolated from donors 2, 3 and 4 (Table 1). The expression of genes encoding the myogenic transcription factors MEF2A, MYOD1 and MYOG were increased during the time course of myogenesis. Gene expression of MYOD1 peaked on day 1, MEF2A on day 2 and MYOG on day 6 (Fig. 3a and Online Resource 1). In contrast, gene expression of myogenic transcription factor MYF5 was expressed at highest levels in myoblasts at the onset of culture and decreased over the time course of myogenesis (Fig. 3a). Expression of CKM, a marker of differentiated myotubes, and PPARGC1A, a marker of oxidative metabolism, increased from days 1 to 3 with high expression maintained from days 4–7 (Fig. 3a, b). Gene expression of CD36, another marker of oxidative metabolism, increased over the time course with highest expression at day 7 (Fig. 3b). Gene expression of structural components of both skeletal muscle slow fibres, e.g. MYH7 and fast fibres, e.g. TNNI2, MYH1, MYH2 (Fig. 5h and Online Resource 2) and fast fibres MYH7, TNNI1 (Fig. 5g and Online Resource 2) increased during myogenesis with the highest expression after 3 and 7 days, respectively (see Online Resource file 1).

3.4 Effect of activin receptor ligands and TNFα on myogenesis

To characterise the myogenesis assay further, we quantified the effects of myostatin, growth and differentiation factor-11 (GDF-11), activin A and TNFα on myobute formation. All ligands inhibited myotube formation in a concentration-dependent manner during 4 days of treatment (Fig. 4b). TNFα was the most potent inhibitor of myogenesis. Average pEC_{50} values±SEM from experiments with cells derived from donors 1, 2 and 3 (Table 1) were 11.1±0.2 (EC_{50}=7.9 pM) for TNFα, 9.9±0.2 (EC_{50}=125.9 pM) for GDF-11, 9.8±0.3 (EC_{50}=158.5 pM) for activin A and 9.1±0.1 (EC_{50}=794.3 pM) for myostatin. Total cell nuclei count per well were similar across all samples, suggesting no marked change in cell number or viability (Fig. 4c).

3.5 Effect of myostatin and TNFα on gene expression changes during myogenesis

The gene expression changes elicited by myostatin (10 nM) and TNFα (1 nM) during the inhibition of myogenesis were studied over a 4-day time course. These data are comprehensively tabulated in Online Resource 2. All data presented are the average percent change in gene expression in cells isolated from donors 1, 8, 9 and 10 (Table 1). Myostatin and TNFα inhibited the expression of myogenic transcription factors MEF2A, MYOD1 and MYOG (Fig. 5a, b and Online Resource 2). TNFα but not myostatin partially restored the expression of MYF5 (Fig. 5c). Myostatin and TNFα inhibited the expression of CKM and CD36 (Fig. 5d, e); however, myostatin but not TNFα inhibited the expression of PPARGC1A (Fig. 5f). Myostatin and TNFα also inhibited the expression of markers of both skeletal muscle slow fibres MYH7, TNNI2, MYH1, MYH2 (Fig. 5g and Online Resource 2) and fast fibres TNNI2, MYH1, MYH2 (Fig. 5h and Online Resource 2). TNFα but not myostatin increased gene expression of inflammatory markers including IL-6, IL-15 and NFKB1, but neither myostatin nor TNFα substantially changed the expression of

Table 2 (continued)

| Gene name            | Official symbol | Gene description            | Functional category | TLDA assay number |
|----------------------|----------------|----------------------------|---------------------|------------------|
| β-2-Microglobulin    | B2M            | Beta-2-microglobulin       | Housekeeper         | Hs99999907_m1    |
| GAPDH                | GAPDH          | GAPDH                      | Housekeeper         | Hs99999905_m1    |
| HPRT1                | HPRT1          | HPRT                       | Housekeeper         | Hs99999909_m1    |
| HMBS                 | HMBS           | Hydroxymethyl-bilane synthase | Housekeeper       | Hs00609297_m1    |

Summary of 48 genes included on custom-made TLDA plates showing gene name, official gene symbol, gene description and functional category TLDA assay number. The TLDA included 41 skeletal muscle related genes and seven reference genes for normalisation of gene expression data.

Fig. 1 Representative examples of the morphology of adherent cell types in skeletal muscle cell preparations by light microscopy (×200 magnifications): satellite cells (a), endothelial-like cells (b) and fibroblast-like cells (c). Example of CD56 expression on adherent cell preparations pre- (d) and post-purification in CD56- (e) and CD56+ (f) cell fractions. A comparison of immunostaining with anti-CD56 antibodies (red line) and isotype control antibody (pink line) is shown. A linear region M1 was drawn at 1 % of cells in the isotype control (background), and cells in this region were determined to be CD56+. The percentage of CD56+ cells in each cell population is indicated. Representative images illustrating myotubes in CD56- (g) and CD56+ (h) cell fractions 7 days after treatment. Representative images illustrating myotubes co-stained for cell nuclei (i, j) and multinucleated myotubes (j). White box in i shows region of image enlarged in j. Myotubes were identified by immunostaining for myosin slow and fast chains.
catabolic or atrophy markers including TRIM63 and FBX032 (see Online Resource 2).

4 Discussion

There is a clear unmet medical need for efficacious pharmacological agents to treat sarcopenia and the skeletal muscle dysfunction associated with a range of diseases. One potential therapeutic approach to this end is to identify drugs that promote new myofibre generation either by directly stimulating myogenesis or by blocking the effects of endogenous inhibitors of myogenesis. Such compounds may also help realise the potential therapeutic value of skeletal muscle satellite cells. The identification of such modulators of myogenesis requires the provision of robust and suitably high-throughput assays that can deliver quantitative data. The aim of the present study was therefore to develop methods to isolate large numbers of adult human satellite cells that could subsequently be used in pharmacological studies.

A number of methodologies have been described for isolation of satellite cells from human muscle tissue. These include explant outgrowth [10, 13] and enzymatic digestion [7–9, 14] and have mainly employed small biopsies of fresh tissue (typically 50–100 mg). Stewart and colleagues [11] have previously shown that cells derived from larger samples (approximately 4 g) of post-mortem tissue can also be successfully differentiated into myotubes. Here, we have extended these studies by developing a method for cell isolation from post-mortem muscle tissue that allows the use of much larger samples (50–100 g). Use of large post-mortem tissue samples has obvious advantages in terms of cell yield. However, as tissue processing occurred several hours after death, this raises potential concerns over the utility of such material as the basis for cellular assays. A comparison between the myogenic potential of satellite cells isolated from fresh tissue and post-mortem tissue may be one way to address this concern, but we are unaware of any studies where this has been investigated. This may be due to the difficulty of securing appropriate tissue samples. Even if such samples could be obtained, the different isolation procedures and cell propagation times needed for biopsy versus post-mortem tissue and the inability to compare results within donors makes such a study difficult to control. Encouragingly however, although our tissue samples were processed up to 97 h after death, we were able to successfully isolate viable cells and use these for functional and transcriptional analyses. Initially, we tried the digestion methods...
employed by other investigators on small-tissue biopsies but were unable to isolate satellite cells. This failure was most likely a consequence of the larger tissue mass relative to the concentration of digestive enzymes used. This assertion is supported by the fact that the method we ultimately used, although based on that of Dellavalle and colleagues [9], employed higher concentrations of collagenase and trypsin and an extended time of digestion. Adaptation of the method allowed for fewer cell manipulations and provided a good cell yield. The cells obtained from this isolation were a mixed population with the main contaminants (as assessed morphologically) being endothelial cells and fibroblasts (Fig. 1a–c).

Following the initial preparation, cells were further purified using CD56+ selection, a marker that has been used previously to isolate cells with myogenic potential [9, 15]. This procedure yielded very-high-purity cell samples (Table 1). We also confirmed the observation of Stadler and colleagues [15] that CD56+ cells do not form myotubes and will overgrow the CD56+ cells in a mixed culture. In summary, our data suggested that this method can be used to isolate large numbers of cells from post-mortem tissue with robust myogenic potential.

Having isolated CD56+ cell preparations with good myogenic potential, we further developed our assay to allow the investigation of the effects of pharmacological modulators of myogenesis. During this process, we found that cell density was critical and that seeding cells at approximately 90 % confluence and eliciting differentiation within 24 h yielded a robust and reproducible myogenic differentiation in all donor cells (Fig. 2). The extent of myogenic differentiation in cultures was also dependent on the lot of horse serum used, and batch testing of serum was routinely performed. Traditionally, methods to measure myogenesis have involved manual counting of the number of nuclei per tube, to obtain the fusion index of multinucleated myotubes. More recently, automated methods employing immuno- or histological staining [16–18] have been used to measure myogenesis. The multi-parametric imaging algorithms we employed allowed myogenesis (percentage myotube area per field of view) and cell viability/proliferation (total cell nuclei) to be measured simultaneously. These algorithms also have the potential to quantify more sophisticated morphological endpoints such as tube branching or diameter. Measurement of the latter parameter offers the exciting prospect of further developing this system to enable assessment of the atrophying effects of agents on pre-formed myotubes.

In this study, we have also established a robust methodology to study gene expression changes during primary human myogenesis in a high-throughput format. Our gene expression methodology has proven useful as assay sensitivity restricted our development of biochemical assays for skeletal muscle markers in a high-throughput format (unpublished observations). Although TLDA can only analyse 10s–100s of genes, compared with 1,000s by global gene microarrays, they have a particularly high sensitivity and dynamic range [19]. This methodology might ultimately find utility in the diagnosis of skeletal muscle disorders and the evaluation of drug efficacy and/or side effects.

We catalogued quantitative changes in expression of selected genes encoding markers of myogenic differentiation, satellite cells, muscle atrophy, catabolism, inflammation, oxidative metabolism and structural components (Table 2). Consistent with their role as master myogenic transcription factors, MYF5 and MYOD1 were expressed at highest levels in satellite cells and differentiating cells, respectively, before myotube formation [20]. In contrast, expression of MEF2A, another transcription factor controlling muscle-specific gene expression, peaked during myotube formation [21]. Expression of CKM followed the time course of myotube formation consistent with CKM

![Fig. 3 Time course of gene expression during myogenesis 0–7 days after eliciting differentiation. Panel a: MEF2A (purple triangles), MYOD1 (blue circles), MYF5 (red diamonds), CKM (green squares). Panel b: CD36 (light blue diamonds), PPARGC1A (pink circles), MYH7 (orange triangles) and TNNI2 (brown squares). Data values are RQ(% max) for average expression changes with cells from donors 2, 3 and 4 (Table 1). SEM values are omitted for clarity and are shown in Online Resource 1. Gene expression changes for all 41 genes are shown in Online Resource 1](image)
being a marker of differentiated myotubes with an essential role in energy homeostasis [22]. Myotubes expressed structural components of both fast and slow muscle fibres, including MYH7 and TNNI2, respectively, with expression increasing after the peak of myotube formation. In agreement with gene expression data, myotubes immunostained for myosin slow and fast chains individually (unpublished observations) consistent with a previous study [10]. PPARC1A and CD36, markers of oxidative metabolism, also increased expression after myotube formation plateaued [23, 24]. This suggests that metabolic and structural maturation or remodelling of myotubes continues after their initial formation. Gene expression results for MYOD1, MEF2A, MYOG and MYH are consistent with the expression of their associated proteins during myogenesis [9]. FBX032 and TRIM63 that encode E3 ligases involved in skeletal muscle atrophy increased in expression during myogenesis and extend previous observations [25, 26].
Having established robust myogenesis and gene expression assays, we further characterised their utility by studying the effects of myostatin, the related activin receptor ligands GDF-11 and activin A, and TNFα on myogenesis. These ligands are negative regulators of muscle function that reduce the anabolic activities and increase catabolic activities of muscle cells [27–32]. Consistent with previous findings, we found myostatin, GDF-11, activin A and TNFα inhibited human myogenesis and demonstrated TNFα more potently inhibits myogenesis compared with activin receptor ligands [12, 26, 33]. It is worth noting that inhibition of myogenesis by TNFα might involve upregulation of activin levels [12]. We used our gene expression assay to compare the molecular events regulated by TNFα and myostatin during myogenesis. Consistent with previous studies, both TNFα and myostatin inhibited the expression of myogenic transcription factors.
including MYOD1, MEF2A and MYOG [26, 34, 35]. Myostatin and TNF-α also inhibited expression of structural and functional components of skeletal muscle including slow and fast fibres, e.g. MYH1, 2 and 7, TNNI1 and TNNI2 and CKM, in agreement with previous work [26, 33, 35, 36]. Myostatin inhibited the expression of PPARC1A, a marker of oxidative metabolism associated with improved endurance performance [37, 38]. This finding is in agreement with a study in mice where inhibition of myostatin signalling increased PPARC1A expression and exercise performance [39]. TNF-α did not inhibit the expression of PPARC1A contrasting previous studies in mice that showed TNF-α could suppress PPARC1A levels and contribute to impaired muscle performance [40, 41]. TNF-α but not myostatin substantially increased the expression of inflammatory markers NFKB1, IL-15 and IL-6. This supports previous reports suggesting TNF-α can signal via the NFκB pathway to induce inflammation in muscle cells whereas myostatin acts through an NFκB-independent and non-inflammatory mechanism [30, 34, 36]. Neither TNF-α nor myostatin regulated the expression of the atrophy markers TRIM63 and FBX032 [25]. This contrasts other studies that showed myostatin reduced TRIM63 and FBX032 levels during human myogenesis but increased their expression during mouse myogenesis [26, 34]. TNF-α increased TRIM63 and FBX032 expression in rat L6 myotubes and TRIM63 was shown to be required for TNF-α induced loss of muscle function in mice [42, 43]. In summary, TNF-α and myostatin inhibit human myogenesis and regulate an overlapping but distinct set of genes involved in muscle cell differentiation, structural and metabolic functions.

In conclusion, we have developed a robust methodology for isolating human skeletal muscle satellite cells from large samples of post-mortem tissue. The cell yield was sufficient to allow the development of relatively high-throughput and highly quantitative assays for the measurement of myogenesis and the associated changes in gene expression. Such assays should enhance our understanding of myogenesis and could be employed as part of a drug-screening cascade aimed at identifying new therapeutic agents for treating muscle disorders.

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Conflict of interest Jan Scott is an employee of MedImmune, AstraZeneca, Cambridge, UK. Andrew Walding and Beverley Isherwood are employees of AstraZeneca, Alderley Park, UK; Iain Dougall and Wendy Tomlinson are former employees of AstraZeneca, UK.

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