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Reduction of Myoblast Differentiation Following Multiple Population Doublings in Mouse C_2C_{12} Cells: A Model to Investigate Ageing?

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ABSTRACT

Ageing skeletal muscle displays declines in size, strength, and functional capacity. Given the acknowledged role that the systemic environment plays in reduced regeneration (Conboy et al. [2005] Nature 433: 760–764), the role of resident satellite cells (termed myoblasts upon activation) is relatively dismissed, where, multiple cellular divisions in-vivo throughout the lifespan could also impact on muscular deterioration. Using a model of multiple population doublings (MPD) in-vitro thus provided a system in which to investigate the direct impact of extensive cell duplications on muscle cell behavior. C_2C_{12} mouse skeletal myoblasts (CON) were used fresh or following 58 population doublings (MPD). As a result of multiple divisions, reduced morphological and biochemical (creatine kinase, CK) differentiation were observed. Furthermore, MPD cells had significantly increased cells in the S and decreased cells in the G1 phases of the cell cycle versus CON, following serum withdrawal. These results suggest continued cycling rather than G1 exit and thus reduced differentiation (myotube atrophy) occurs in MPD muscle cells. These changes were underpinned by significant reductions in transcript expression of: IGF-I and myogenic regulatory factors (myoD and myogenin) together with elevated IGFBP5. Signaling studies showed that decreased differentiation in MPD was associated with decreased phosphorylation of Akt, and with later increased phosphorylation of JNK1/2. Chemical inhibition of JNK1/2 (SP600125) in MPD cells increased IGF-I expression (non-significantly), however, did not enhance differentiation. This study provides a potential model and molecular mechanisms for deterioration in differentiation capacity in skeletal muscle cells as a consequence of multiple population doublings that would potentially contribute to the ageing process. J. Cell. Biochem. 112: 3773–3785, 2011.

KEY WORDS: SATELLITE CELL; AGEING; IGF-I; IGFBP5; AKT; JNK

Natural muscle wasting with age (sarcopenia) [Rosenberg, 1997] is a significant contributor to the loss of physiological and functional capacity and is strongly correlated with morbidity and mortality [Carmeli et al., 2002; Rantanen et al., 2003]. After the age of 50, humans lose muscle mass at a rate of 1–2% per year [Hughes et al., 2002] which impacts on muscle strength, size, activation [Morse et al., 2005], and metabolic performance [Renault et al., 2002; Rossi et al., 2008]. Indeed more than 50% of the population over 80 years suffer from sarcopenia, which is becoming recognized as a serious geriatric clinical disorder [Cruz-Jentoft et al., 2010]. Thus, preventing muscle wasting with age is of high clinical relevance, not only to reduce the economic burden of ill health associated with ageing, but also to improve the quality of life of increasing numbers of ageing individuals. Despite an overwhelming influx of information regarding the pathophysiological characteristics of muscular ageing, relatively little is known about molecular and cellular events that underpin it, with very few adequate cell-based models available to investigate the mechanisms of skeletal muscle ageing.

Adult skeletal muscle fiber numbers are set in-utero and adult fibers are post-mitotic or terminally differentiated. Despite these phenomena, adult skeletal muscle displays astounding plasticity,

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responding accurately to the soluble and biomechanical cues that it encounters on a daily basis. Skeletal muscle undergoes rapid growth (hypertrophy) during development, exercise, stretch, and mechanical loading, yet severe loss (atrophy) with ageing, disuse, and disease [Sharples and Stewart, 2011]. Changes in protein synthesis and degradation as well as resident adult stem cells (satellite cells) underpin this adaptability. These mono-nucleated cells do have mitotic potential and thus can respond to both positive and negative stimuli essential to successful skeletal muscle plasticity and regeneration. Upon relevant cues, satellite cells are activated and begin to proliferate with a subset of the satellite cells returning to quiescence [Schultz et al., 1978] while the remainder differentiate into myoblasts, which migrate to the damaged site and differentiate further into multinucleated myotubes (or myofibers), repairing the muscle. We recently reported a myoblast model to investigate lack of regeneration with age by understanding the cellular and molecular differences between parental C_2 ("older") and daughter C_2C_{12} ("younger") myoblasts. This enabled the investigation of muscle cell adaptation in the absence or presence of the cytokine tumor necrosis factor-alpha (TNF-α) [Sharples et al., 2010] which is chronically elevated in the circulation of aged populations [Bruunsgaard et al., 2003]. Data revealed reduced differentiation potential (ability to form myotubes) and increased susceptibility of older C_2 cells to TNF-α versus younger C_2C_{12} cells, phenotypes also reported in human muscle cells isolated from biopsies [Grohmann et al., 2005; Pietrangelo et al., 2009]), and previously observed in in-vivo muscular ageing [Lees et al., 2009].

The main focus in the literature on the contributing factors of skeletal muscle ageing investigates the roles of environment and stem cell niche (Conboy and Rando, 2002; Conboy et al., 2003, 2005; Shefer et al., 2006; Gopinath and Rando, 2008), at the expense of the contribution of the myoblasts themselves. Indeed, parabiotic murine pairing studies have illustrated that a young systemic environment can fully rejuvenate the muscular phenotype of old mice [Conboy et al., 2005]. However, in light of recent evidence indicating increased expression of S100B (a negative regulator of differentiation) and reduced expression of RAGE (enhances differentiation) is important in the degenerative phenotypes observed in cells isolated from old versus young human muscle biopsies [Beccafico et al., 2010], and together with data illustrating that serum taken from young humans is unable to change the capacity of muscle cells isolated from elderly individuals to proliferate or differentiate [George et al., 2010], raises the question of the potential role of myoblast contribution to muscle regeneration across the lifespan.

Previous research has investigated the effect of multiple divisions on the proliferative capacity of human skeletal muscle satellite cells [Decary et al., 1997; Di Donna et al., 2000] and focused mainly on division potential and telomere shortening. These studies demonstrated division time to senescence was impaired in muscle cells isolated from elderly versus young and infant muscle [Decary et al., 1997]. Importantly compelling evidence also suggests reduced differentiation capacity with reduced Mrf5, myoD, and myogenin in senescent human myoblasts after multiple divisions [Bigot et al., 2008]. A multiple population doubling (MPD) model, therefore, presents the opportunity to investigate in-vitro the multiple divisions that occur in-vivo with ageing (albeit potentially at an accelerated rate in the former) and which contribute to turnover and regeneration of muscle tissue across the lifespan. This model also guarantees that culture conditions are uniform thus ensuring that myoblasts can be studied in the absence of confounding effects from the stem cell niche or systemic environment that would influence in-vivo studies as well as in-vitro primary cell culture studies where cells are isolated from biopsies donated by older or younger individuals.

Recent evidence has shown that old and young muscle stem cells (satellite cells) isolated from mice (using negative CD45 magnetic sorting and flow cytometry) have robust telomerase activity and it is only their progeny (myoblasts) once grown and induced to differentiate into myotubes in culture that display deterioration of telomerase activity [O'Connor et al., 2009]. Furthermore, it has been established that long-term cultured primary myoblasts, that have little or no telomerase activity, are different to isolated satellite cells that have high telomerase activity [O'Connor et al., 2009]. These, primary cell lines do not regain their ability to maintain telomeres, unlike the C_2C_{12} mouse cell line, which retains robust telomerase activity equivalent to freshly isolated muscle stem cells (satellite cells) [Yaffe and Saxel, 1977; Holt et al., 1996; O'Connor et al., 2009]. As a consequence of this retained, in-vivo-like activity, C_2C_{12} cells provide a good model to investigate the impact of cellular divisions on subsequent myoblast behavior, thus mimicking satellite cell division and renewal.

The present study, therefore, adopts a multiple population doublings model to investigate the impact of serial divisions on muscle cell behavior, focusing on the transition from proliferation to differentiation. We here report that C_2C_{12} cells that have undergone 58 population doublings show inappropriate cell cycle progression, with reduced differentiation and associated changes in key molecular pathways (IGF-I/Akt, IGFBP5, and JNK) versus parental cells that have undergone no population doublings. The role of JNK signaling is further investigated using chemical inhibition, deciphering the role of this protein in the reduced differentiation observed. Overall, indicating that despite a retained proliferative capacity, multiple replicative cycles do impact on the capacity of myoblasts to undergo appropriate fusion in-vitro, providing a good model to investigate the impact of a requirement for lifelong survival and division of stem cells at the potential expense of hypertrophy in older age.

MATERIALS AND METHODS

GENERAL MATERIALS

Sterile cell and tissue culture media and supplements were purchased as follows: Dulbecco's modified eagle's medium (DMEM), penicillin/streptomycin solution, and trypsin/EDTA (Bio Whittaker, Wokingham, England, UK); heat-inactivated (hi) new born calf serum (NCS) and hi fetal bovine serum (FBS; Gibco, Paisley, Scotland, UK) and hi horse serum (HS; Southern Group Laboratory, Corby, England, UK). L-Glutamine (BDH, Poole, England, UK). Gelatin type A (porcine skin; Sigma–Aldrich Chemie, Steinheim, Germany) and phosphate buffered saline (PBS; Oxoid Ltd., Basingstoke, England, UK). Gelatin and PBS were autoclaved prior to use (Prestige Medical, Birmingham, UK).

Tissue culture flasks (T75s), 6-well plates and 96-well plates were purchased sterile from Nunc Life Sciences, Thermo Fisher Scientific (Rosklide, Denmark). Clear 96-well UV plates for creatine kinase (CK) assays from BD Biosciences (San Diego, CA) and 96-well plates for real-time PCR from Bio-Rad Laboratories, Inc. (Hercules, CA). Unless otherwise stated, Sigma (Poole, England, UK) and BDH were used to purchase general chemicals and solvents that were of molecular biology/tissue culture or analytical (AnalaR) grade.

CELL CULTURE

C_2C_{12} murine skeletal myoblasts [Yaffe and Saxel, 1977; Blau et al., 1985] were grown in T75 flasks in a humidified 5% CO_2 atmosphere at 37°C in growth medium (GM), composed of: DMEM plus 10% hi FBS, 10% hi NCS, 1% L-glutamine (2 mM final), and 1% penicillin–streptomycin solution, until 80% confluence was attained. Experiments were initiated by washing with PBS, and transferring into low serum differentiation media (DM) composed of: DMEM plus 2% hi HS, 1% L-glutamine, and 1% penicillin–streptomycin solution. C_2C_{12} myoblasts undergo spontaneous differentiation into myotubes on serum withdrawal, and do not require growth factor addition to stimulate the process [Blau et al., 1985].

POPULATION DOUBLINGS OF C_2C_{12} CELLS

Original stock C_2C_{12} mouse skeletal muscle cells (purchased from ATCC, Rockville, MD) were seeded at 1×10^6 cells in T75 flasks in 15 ml of GM and incubated for 48 h until ~80% confluent. Cells were trypsinized and seeded onto new T75s at 1×10^6 for 48 h or at 5×10^5 for 72 h, with doubling time and cell numbers being recorded throughout. This cycle was repeated 20 times over 49 days, creating a stock of cells that had undergone 58 population doublings (MPD; Fig. 1), compared with the original stock that were retained in liquid nitrogen and had undergone no doublings (CON) relative to the MPD cells and were used as the controls in all experiments reported here. Thus, the same "parental cells" that had produced their progeny were used as control cells. There was no observable cell death between cell expansions (determined upon counting by typan blue exclusion).

CELL TREATMENTS AND EXTRACTIONS

Pre-gelatinized (0.2%) 6-well plates were seeded at 8×10^4 cells/ml in 2 ml GM and incubated for 24 h. Cells were washed twice with PBS and transferred to 2 ml DM for up to 72 h. Time point zero (0 h) was defined as 30 min subsequent to transferring into DM. If experiments were taken to 7 days (as with JNK inhibition studies for fusion efficiency measures-see below), then 1 ml of DM was added to wells at 72 h.

Cells were extracted for total protein and CK (biochemical differentiation) assays at 0, 48, and 72 h. Cells were washed twice in PBS and lysed in 200 μl/well 0.05 M Tris/MES Triton lysis buffer (TMT) containing: 50 mM Tris–MES, pH 7.8, 1% Triton X-100. Samples were assayed using commercially available BCA™ (Pierce, Rockford, IL) and CK (Catachem Inc., Connecticut, NE) assay kits according to manufacturer's instructions. Enzymatic activity for CK was normalized to total protein content.

For RT-PCR experiments cells were washed twice with PBS and lysed in 250 μl TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, CA) for subsequent RNA extraction (see below) at 0, 48, and 72 h. For cell cycle analyses, cells were fixed at 0, 24, and 48 h (see flow cytometry below) and for Cytometric Bead Arrays (CBA; see below) investigating phosphorylated intracellular signaling proteins, cells were extracted at 0 min, 5 min, 15 min, 2 h, and 24 h following transfer into DM. Cell culture experiments were performed 3–4 times in total, with each individual experiment performed in duplicate and all samples subsequently assayed in duplicate.

For JNK inhibition studies, JNK inhibitor (SP600125, Merck, Hertfordshire, UK) at 15 μM that has been successfully used in

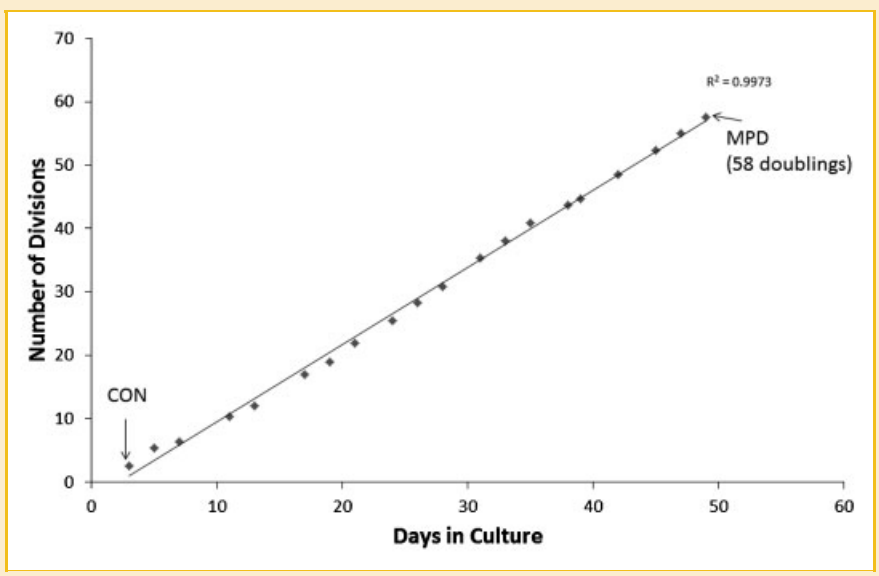

Fig. 1. Population doublings of C_2C_{12} cells. Graphic depiction of days in culture compared with number of divisions. Arrows indicate how many population doublings MPD cells (58) underwent versus CON cells.

C_2C_{12} cells at this does [Frost et al., 2003] was added to both CON and MPD cells at 24 h after transfer into DM. Cells were then fixed (50% methanol/50% acetone), permeabilized, blocked (0.2% Triton X-100, 2% Goat Serum, diluted in TBS solution), and stained for monoclonal anti-desmin (diluted 1 in 200) produced in mouse (Sigma Poole, England, UK). Secondary antibody, anti-rabbit IgG TRITC (Sigma Poole, England, UK) produced in goat (diluted 1 in 200) was then used to bind to the primary desmin antibody to allow detection (see below). Sytox nuclear stain (Molecular Probes, Paisley, UK) diluted 1 in 2,000 was used to detect nuclei for measures of fusion efficiency (see microscopy below) analysed after 7 days after transfer into DM. Cell were lysed with TRIZOL at 72 h and RNA extracted as detailed below.

PHENOTYPIC DIFFERENTIATION, MORPHOLOGY, AND FLUORESCENT MICROSCOPY

Morphological parameters of differentiation: Alignment, elongation, and fusion were assessed using a cell imaging system at ×10 magnification (Leica, DMI 6000B). Cells were also processed in the presence of Phalloidin-FITC Stain (see section below) and viewed under a fluorescent microscope (Leica, DMI 6000B) to examine changes in the actin–myosin cytoskeleton. All images were taken at 0, 48, and 72 h following transfer into DM. For JNK inhibitor investigations and measures of fusion index, cells were viewed at 40× using a confocal fluorescent microscope (Zeiss, LMS 510).

PHALLODIN–FITC STAINING

Cells were fixed in 3.7% formaldehyde solution and incubated at room temperature for 10 min. The formaldehyde was removed and the cells were permeabilized in 0.1% Triton X-100 for 3 min. Following Triton aspiration, Phalloidin-FITC (30 μg/ml) was added and incubated for 30 min/RT before visualization using a fluorescent microscope (above).

RNA EXTRACTION AND ANALYSIS

Following TRIZOL extraction RNA concentration and purity were determined using a Biotech Photometer (WPA UV1101, Biochrom, Cambridge, UK). Seventy nanograms of RNA/RT-PCR reaction (10 ng of RNA in 7 μl/reaction) was used.

PRIMERS

Primers (Table I) were identified using a genome browser at www.genome.ucsc.edu and designed using web-based OligoPerfect™ Designer (Invitrogen Life Technologies). Sequence homology searches against the Genbank database ensured specificity. The primers were designed to yield products spanning exon–intron boundaries to prevent any amplification of genomic DNA. Three or more GC bases in the last five bases at the 3′-end of the primer were avoided. Searches for secondary structure interactions (hairpins, self-dimer, and cross dimer) within the primer were also performed and avoided. All primers were between 18 and 22 bp and amplified a product of between 115 and 193 bp. GC content was between 43.5 and 66.7% (Table I). Primers were purchased from Sigma–Genosys (Suffolk, UK) without the requirement of further purification.

QUANTITATIVE RT-PCR AND DATA ANALYSES

RT-PCR amplifications were carried out using Power SYBR Green RNA-to-C_t 1 step kit (Applied Biosystems, Carlsbad, CA) on a Chromo4™ DNA engine that was supported by Opticon Monitor version 3.1.32, MJ Geneworks Inc., Bio-Rad Laboratories, Inc. RT-PCR was performed as follows: 48°C, 30 min (cDNA synthesis), 95°C, 10 min (transcriptase inactivation), followed by: 95°C, 15 s (denaturation), and 60°C, 1 min (annealing/extension) for 40 cycles. Upon completion, dissociation curve analyses were performed to reveal and exclude non-specific amplification or primer–dimer issues. The relative gene expression levels were calculated using the comparative C_t ($^{\Delta\Delta}C_t$) equation otherwise known as the Livak method [Schmittgen and Livak, 2008] for determining normalized expression ratios, where the relative expression is calculated as $2^{-\Delta\Delta C_t}$ and where C_t represents the threshold cycle. Following screening of GAPDH, RPII-α, and RPII-β; RPII-β showed the most stable C_t values and was selected as the reference gene in all RT-PCR assays. A pooled C_t value (22.7 ± 0.75) for the housekeeping gene, RPII-β, was derived from all RT-PCR runs ($n = 20$ in duplicate). To compare MPD C_2C_{12} with CON C_2C_{12} cells, the 0 h time point for CON C_2C_{12} cells was routinely used as the calibrator condition in the C_t ($^{\Delta\Delta}C_t$) equation. RT-PCR data presented in results and all figures are relative gene expression levels determined by the $^{\Delta\Delta}C_t$ equation.

FLOW CYTOMETRY: CELL CYCLE ANALYSES

Following trypsinization (200 μl, 0.5% trypsin/0.02% EDTA solution/well) C_2C_{12} cells were harvested, re-suspended in PBS, and pooled with detached cells. Cells were pelleted (500*g* for 10 min) and

TABLE I. Real-Time qPCR Primer Sequences

Target gene	Primer sequence (5′–3′)	Ref. seq. number	Amplicon length (bp)
IGF-I	F: ATCAGCAGCCTTCCAACTC R: AAGGTGAGCAAGCAGAGC	NM_010512	116
IGF-IR	F: CTACCTCCCTCTCTGGGAATG R: GCCCAACCTGCTGTTATTTCT	NM_010513	185
IGFBP5	F: GACCCAGTCCAAGTTTGTGG R: GAATCCTTTGCGGTCACAGT	NM_010518	193
IGFBP2	F: GAGGAGCCCAAGAAGTTG R: CGCCATCCAGTACATTGAGC	NM_008342	133
Myogenin	F: GGGACCGAACTCCAGTGC R: GCTCCAACTGCTCTGATG	NM_031189	172
MyoD	F: TAGTAGGCGGTGTCGTAG R: GGCTGTCTGTGGAGATGC	NM_010866	85
RP-IIß	F: GGTCAGAAGGGAACTTGTGGTAT R: GCATCATTAAATGGAGTAGCGTC	NM_153798.1	197

washed in PBS prior to fixing in 3 ml (−20°C) 75% ethanol. After a minimum of 24 h, cells were pelleted (300*g* for 5 min), washed in PBS (2 × 500*g*, 10 min), and re-suspended in 0.5 ml of PBS. Ribonuclease A (50 μl) was added at a concentration of 20 μg/ml, cells were gently vortexed and left at room temperature for 30 min. Ten microliters of propidium iodide labeling buffer (50 μg/ml propidium iodide, 0.1% sodium citrate, and 0.3% Nonidet P-40, pH 8.3) was added to each sample. Cells were stored in the dark at 4°C for 24 h, prior to assaying at room temperature, using a FACSCalibur™ (Becton Dickinson, Franklin Lakes, NJ). Data were collected using Cell Quest (Becton Dickinson, Oxford, England) where 10,000 cells were acquired per sample and analyzed using Modfit™ software (Verity Software House, Topsham, ME).

FLOW CYTOMETRY: CYTOMETRIC BEAD ARRAY (CBA) FOR QUANTIFICATION OF PHOSPHORYLATED PROTEINS

BD™ CBA is a flow cytometry application for simultaneous quantification of multiple proteins, including intracellular phosphorylated signaling proteins [Schubert et al., 2009; Manjavachi et al., 2010]. The BD™ CBA uses flow cytometry and fluorescence together with phycoerythrin (PE) antibody-coated beads that capture the proteins of interest; samples are loaded according to protein concentration (15 μg/sample). Cells were washed at 4°C in PBS and lysed using 1× lysis buffer provided in the Cell Signaling Master Buffer Kit (BD™ CBA). The cell lysates were denatured at 100°C and dispersed using a 26-gauge needle. A protein assay was performed (already described) to determine protein concentrations of individual samples. Cell lysates were stored at −70°C until required for the CBA. Samples were thawed and added to the assay diluent provided in the Cell Signaling Master Buffer Kit (15 μg/sample). Standards were prepared using a stock of recombinant protein (50,000 U/ml) contained in each BD™ CBA Cell Signaling Flex Set (separate flex set for combination of total and phosphorylated protein of interest: Akt, ERK, p38 MAPK, and JNK). Serial dilutions of the top standard (1000 U/ml) were performed.

Four phosphorylated proteins per sample were analyzed simultaneously in the presence of test capture beads. All samples were incubated in the dark for 2 h prior to further investigation. PE detection reagent was added to each sample and incubated at RT (protected from light) for a further 1 h. The samples were washed in wash buffer (provided in CBA kits) and centrifuged at 300*g* for 5 min. Excess liquid was removed and 300 μl of fresh wash buffer was added to each pelleted sample, vortexed gently, and then analyzed using Cell Quest Pro (Becton Dickinson) on a BD™ FACSCalibur. Three hundred events were captured per analyte per sample according to manufacturer's instructions. Data were uploaded from Cell Quest Pro, filtered using FCS Filter™ and analyzed using FCAP array software (Hungary Software Ltd., for BD Biosciences).

STATISTICAL ANALYSES

Statistical analyses and significance were determined using Minitab version 15.0. Results are presented as mean ± standard deviation (SD). Statistical significance for interactions between cell type (CON and MPD) and time (0, 24, and 72 h) were determined using (2 × 3) mixed two-way factorial ANOVA. Results for factorial ANOVA's post hoc analyses (with Bonferroni correction) were conducted where main effects for cell type or time occurred, without a significant interaction between time and cell type. If there were significant interactions present, independent *t*-tests were conducted to confirm statistical significance between variable of interest, e.g., between cell types, and paired-sample *t*-tests undertaken for variable of interest within cell type and time. For all statistical analyses, significance was accepted at $P \leq 0.05$.

RESULTS

MULTIPLE DIVISIONS INDUCE REDUCED MORPHOLOGICAL AND BIOCHEMICAL DIFFERENTIATION

Morphologically, MPD cells exhibit reduced differentiation and appear to continue to proliferate (see cell cycle analyses below) at 48 and 72 h compared with the differentiating CON cells (Fig. 2A). The myotubes produced by the MPD cells were markedly smaller and thinner and were reduced in number compared with CON cells (Fig. 2A and B). Biochemical differentiation (studied by CK analyses), however, revealed a significant increase in CK activity over time in both cell types: In CON cells; 25.6 ± 12.9, 340.5 ± 46.3, and 490.4 ± 35.8 mU mg ml^{-1} and in MPD cells; 38.9 ± 14.9, 227 ± 65, and 339.4 ± 37.3 mU mg ml^{-1} at 0, 48, and 72 h, respectively; $P \leq 0.001$ for all comparisons and cell types (0 h vs. 48 h, 48 h vs. 72 h, and 0 h vs. 72 h). Biochemical data do, however, also support the morphological images showing significantly reduced biochemical differentiation in MPD versus CON cells at 48 h (227 ± 65 mU mg ml^{-1} vs. 340.5 ± 46.3 mU mg ml^{-1}; $P \leq 0.001$, Fig. 2C) and 72 h (339.4 ± 37.3 mU mg ml^{-1} vs. 490.4 ± 35.8 mU mg ml^{-1}; $P \leq 0.001$, Fig. 2C). Overall, there was a significant interaction between cell type and time ($P \leq 0.001$), suggesting that differentiation had decreased following multiple divisions.

CELLS THAT HAVE UNDERGONE MULTIPLE DIVISIONS HAVE GREATER TOTAL PROTEIN CONCENTRATIONS AT EARLY TIME POINTS

A significant main effect for time was observed ($P \leq 0.001$) as a result of increased levels of total protein content with time (48 h) in both cell types when compared with 0 h (CON; 0.75 ± 0.13 vs. 1.07 ± 0.13 mg/ml, $P \leq 0.001$ and MPD; 0.73 ± 0.08 vs. 1.22 ± 0.16 mg/ml, $P \leq 0.001$ at 0 h vs. 48 h, respectively; Fig. 2D). There was also a significant interaction between cell type and time ($P \leq 0.001$) suggesting a significant increase in protein in MPD versus CON cells at 48 h (Fig. 2D; 1.22 ± 0.16 mg/ml vs. 1.07 ± 0.13 mg/ml; $P = 0.003$). Protein levels plateaued between 48 and 72 h in MPD cells (48 h; 1.22 ± 0.16 vs. 72 h; 1.24 ± 0.15 mg/ml, $P = 0.646$), however, significantly increased in CON cells (48 h; 1.07 ± 0.13 vs. 72 h; 1.27 ± 0.18 mg/ml, $P \leq 0.001$) although the absolute levels in CON were not significantly different to MPD at 72 h (1.27 ± 0.18 and 1.24 ± 0.15 mg/ml, respectively, P = NS). These data suggest the MPD cells are continuing to proliferate at earlier time points (48 h) relative to CON (confirmed by apparent increased cell density depicted via phalloidin staining; Fig. 2B), but that ultimately similar absolute protein levels exist at later time points (72 h; Fig. 2D). MPD cells do appear morphologically denser

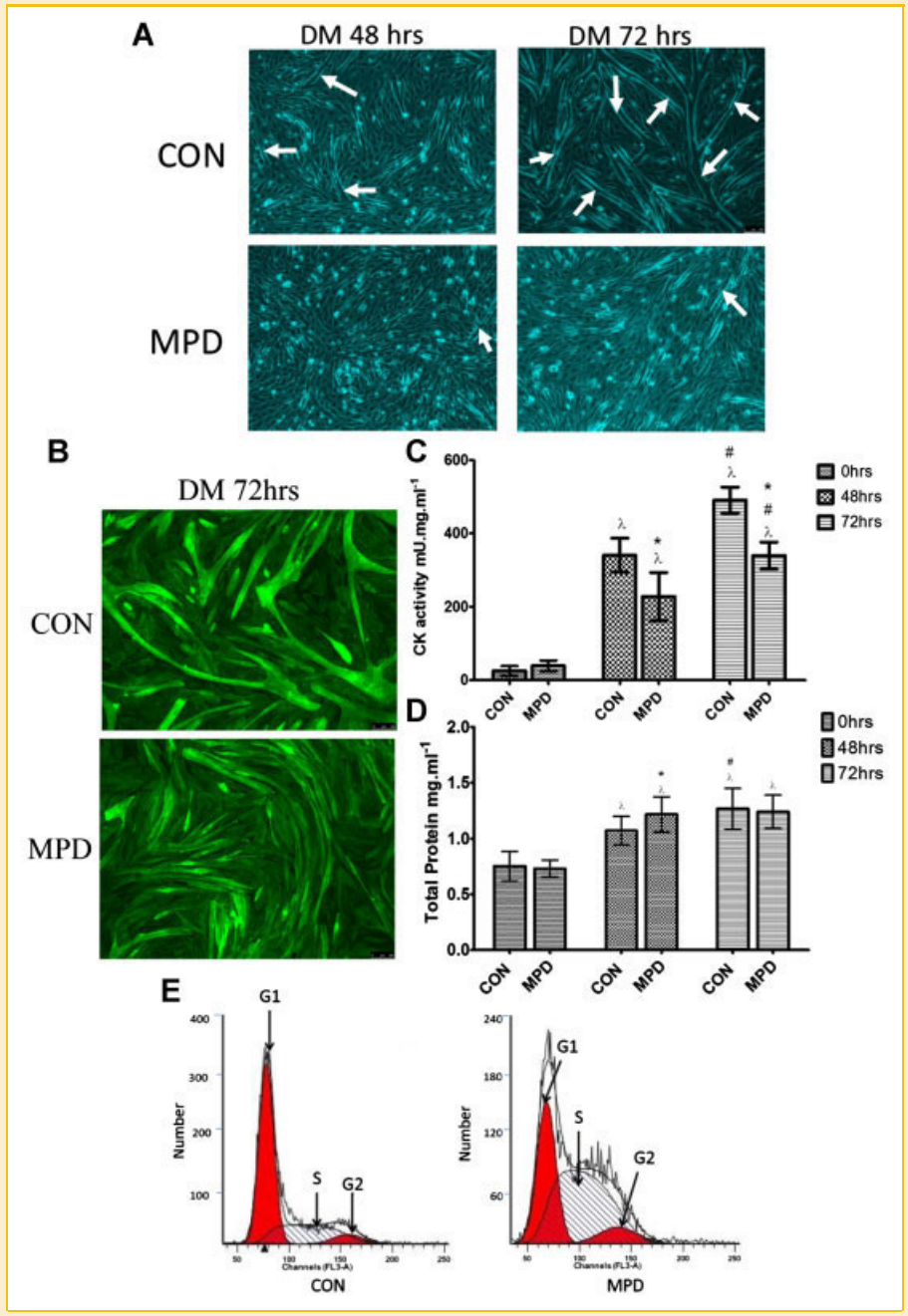

Fig. 2. A: Morphological images of C_2C_{12} cells that have undergone multiple population doublings (MPD) versus control (CON) cells in differentiation medium at 48 and 72 h. There was increased myotube formation in CON versus MPD cells. Early cell alignment and myotube formation at 48 h appears greater in CON versus MPD with clear reductions in myotube formation at 72 h in MPD cells indicated by white arrows (not all myotubes indicated). There also appears to be continued proliferation at the expense of differentiation MPD conditions versus CON, best observed in Figure 2B. Phase contrast images are representative of a minimum of four experiments performed in duplicate. Magnification 10×. B: Phallodin cytoskeleton staining of replicative cells MPD versus CON. Morphology suggests that MPD cells are more densely packed suggesting continued cycling (see Fig. 4E), with individual cells appearing thinner and elongated versus CON cells that have a more hypertrophic appearance demonstrated by huge myotube formation. Data are representative of three experiments in duplicate. Magnification 10×. C: Creatine kinase activity of myoblasts that have undergone multiple divisions compared with control cells. There was a significant increase in CK activity over time in both cells types where significant differences versus 0 h are indicated by λ ($P \leq 0.001$) and significant differences versus 48 h are indicated by # ($P \leq 0.001$). Despite a similar temporal trend between cell types, importantly there was a significant reduction in CK for MPD myoblasts at both 48 and 72 h in compared with CON indicated by * (all $P \leq 0.001$). Results are taken from four separate experiments with the assays performed in duplicate. Graph depicts mean ± SD. D: Total protein concentrations in basal conditions between MPD cells and CON myoblasts. Significant temporal increases in total protein were observed between 0 and 48 h and between 0 and 72 h in both cell types (all $P \leq 0.001$ indicated by λ). Significant increases in total protein were observed in MPD at 48 h versus CON ($P = 0.003$) indicated by *. CON cells significantly increased their temporal expression between 48 and 72 h indicated by # ($P \leq 0.001$) although the absolute levels in CON were not significantly different to MPD at 72 h. Data representative of three experiments in duplicate. Graph depicts mean ± SD. E: Example of CON versus cells that have undergone multiple divisions (MPD) at 0 h [30 min after switching to low serum media (DM)]. There are a larger proportion of cells in S/G2 phases in MPD with a smaller proportion of cells in G1 phase versus CON. Suggesting an continued proliferation and inability to exit the cell cycle in G1 and differentiate. Data are representative of three experiments in duplicate. 10,000 events were recorded per sample. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

than CON cells at 48 h, however, this also remains the case at 72 h (Fig. 2A and B) without apparent differences in protein content, perhaps indicating together with the cell cycle data directly below, is a consequence of continued cycling (MPD) versus differentiation (CON).

INCREASED CELL CYCLE PROGRESSION AT EARLY TIME POINTS IN MPD VERSUS CON CELLS

Due to the apparent increase in cell density and reduced ability for cell fusion in MPD cells, flow cytometry was undertaken to assess the impact of multiple population doublings on cell cycle exit. Following transfer to DM, the MPD cells continued to progress through the cell cycle, with a greater percentage of cells in the S ($60.67 \pm 2.83\%$ vs. $34.01 \pm 9.8\%$ in MPD vs. CON, $P = 0.004$; Fig. 2E), and G2 phases compared with CON ($12.05 \pm 8.47\%$ vs. $1.66 \pm 3.72\%$ $P = 0.05$) and reductions in the G1 phase when comparing MPD vs. CON cells ($37.67 \pm 0.94\%$ vs. $53.94 \pm 9.82\%$; $P = 0.021$; Fig. 2E). Collectively, these findings suggest that cells that have undergone 58 doublings were more likely to continue cycling and not exit the cell cycle in G1 to differentiate compared with CON.

DECREASED DIFFERENTIATION OF MPD CELLS IS ASSOCIATED WITH A REDUCTION IN PHOSPHORYLATION OF AKT

Together with reduced differentiation in MPD versus CON cells, levels of phosphorylated Akt were also reduced (Fig. 3A) at 0 h (24.7 ± 2.54 U/ml vs. 37.98 ± 9.06 U/ml, $P = 0.007$), 5 min (25.83 ± 0.52 U/ml vs. 37.33 ± 8.96 U/ml, $P = 0.005$), 15 min (28.95 ± 0.63 U/ml vs. 43 ± 10.4 U/ml, $P = 0.004$), and 2 h (29.28 ± 0.29 U/ml vs.

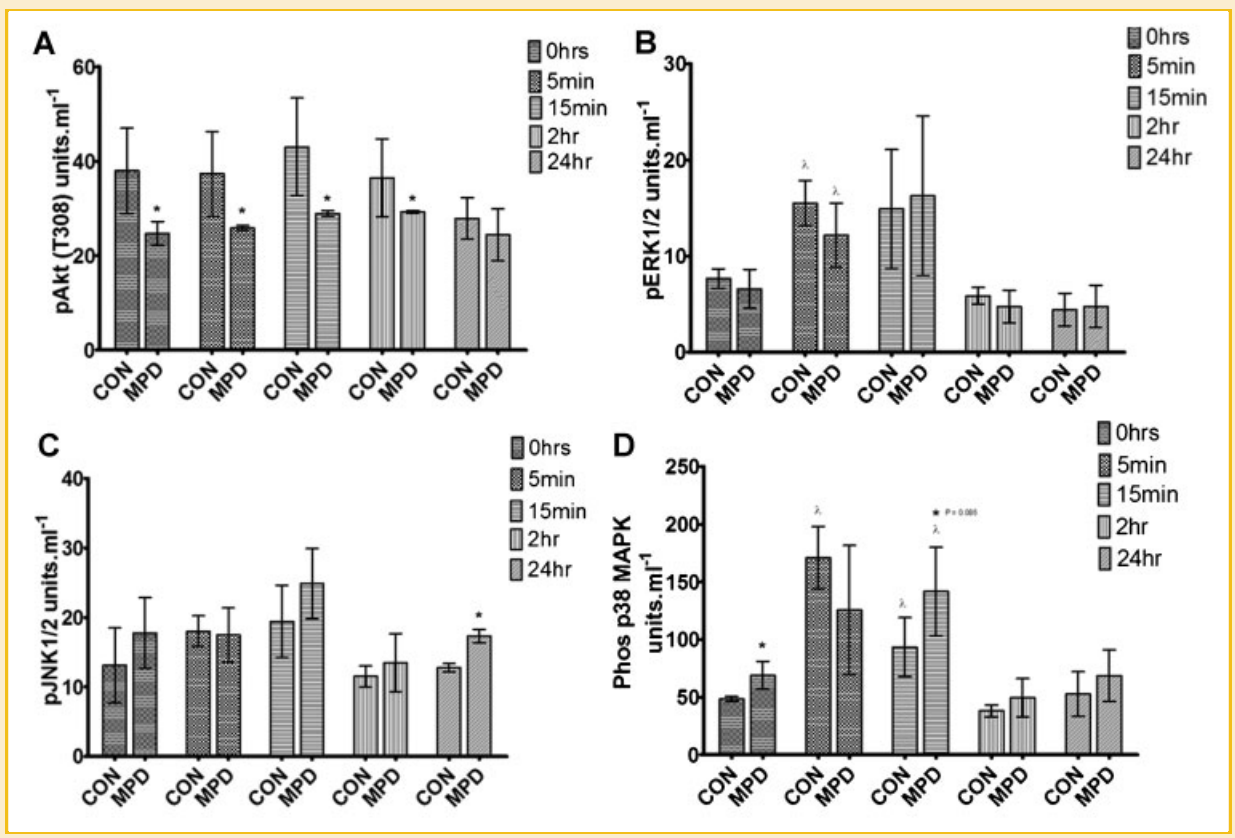

Fig. 3. A: Phosphorlated Akt (T308) (pAkt) concentrations in basal conditions between control (CON) and cells that have undergone multiple population doublings (MPD). There were statistically significant reductions in phosphorylated Akt between CON and MPD myoblasts at 0 h ($P = 0.007$), 5 min ($P = 0.005$), 15 min ($P = 0.004$), and 2 h ($P = 0.03$). Statistically significant bars compared with CON of the same time point are indicated using *. Data represent three experiments performed in duplicate. Graph depicts mean ± SD. B: Phosphorlated ERK1/2 (pERK1/2) concentrations in basal conditions between CON versus MPD myoblasts. There were significant temporal increases in expression of pERK1/2 between basal (0 h) and 5 min time points in all cell types (CON, $P = 0.004$, MPD; $P = 0.048$ indicated by λ). However, no significant changes were observed between CON and MPD cells at any time point. Data are representative of three experiments in duplicate. Graph depicts mean ± SD. C: Phosphorlated JNK1/2 (pJNK1/2) concentrations in basal conditions between CON and MPD myoblasts. An increase in temporal expression in both cell types was observed between basal (0 h) and 15 min, however, this trend was not significant. An increase in JNK1/2 was observed at 24 h between MPD and CON ($P = 0.006$) indicated by *. Data are representative of three experiments in duplicate. Graph depicts mean ± SD. D: Phosphorylated p38 MAPK (Phos p38 MAPK) concentrations in basal conditions between CON and MPD myoblasts. Basal levels of p38 MAPK (0 h) were significantly higher in MPD cells versus CON myoblasts ($P = 0.044$). This increased phosphorylation was also shown at 15 min in MPD versus CON (almost significant; $P = 0.086$). At 5 min, however, CON cell had higher levels of p38 MAPK versus MPD cells but this was not significant ($P = 0.219$). All significant differences between cell types within same time points are indicated by * where they are significantly different versus CON cells of the same time point. Temporal increases within cell type compared with baseline (0 h) are depicted by λ at 5 min in CON ($P = 0.003$) and at 15 min in CON and MPD cells (CON, $P = 0.039$; MPD, $P = 0.035$). Data are representative of three experiments performed in duplicate. Graph depicts mean ± SD.

36.49 ± 8.32 U/ml, $P = 0.03$) after transfer into DM. No differences were seen in Akt phosphorylation at 24 h.

ALTERATIONS IN JNK1/2 AND P38 MAPK PHOSPHORYLATION IN THE ABSENCE OF CHANGES IN ERK1/2 FOLLOWING MULTIPLE DIVISIONS

Despite the morphological differences, there were no significant increases in ERK1/2 phosphorylation at any time point in the MPDs versus CONs (Fig. 3B). There were, however, significant temporal increases in ERK phosphorylation in both cell types at 5 and 15 min, compared with baseline that resulted in a significant main effect for time ($P \leq 0.001$). Indeed, the temporal increases were confirmed between basal (0 h) and 5 min for both cell types (CON; 7.67 ± 1.01 U/ml vs. 15.49 ± 2.36 U/ml, $P = 0.004$, in MPD; 6.59 ± 2.01 U/ml vs. 12.17 ± 3.34 U/ml, $P = 0.046$). Increases in phosphorylation returned to baseline by 2 h and remained at this level at 24 h (Fig. 3B).

Although phosphorylation of JNK1/2 increased in both cell types over 15 min (Fig. 3C) significance was not achieved. At 24 h, importantly, there were higher levels of phosphorylated JNK1/2 in MPD versus CON cells (17.33 ± 0.98 U/ml vs. 12.76 ± 0.61 U/ml; MPD versus CON, respectively, $P = 0.021$). Overall, this resulted in a significant main effect for cell type for JNK1/2 phosphorylation ($P = 0.003$).

Temporal expression of p38 MAPK was increased over 15 min and returned to baseline at 2 and 24 h in both cell types (Fig. 3D) shown by increases (not all significant) between basal (0 h), 5 and 15 min in CON [48.3 ± 2.2 U/ml vs. 171 ± 27.2 U/ml, $P = 0.003$ (5 min) vs. 93.5 ± 25.6 U/ml, $P = 0.039$ (15 min)] and between basal and 15 min in MPD (69 ± 12.1 U/ml vs. 141.9 ± 38.1 U/ml, $P = 0.035$) with non-significant changes at baseline versus 2 and 24 h for both cell types. This resulted in a significant main effect for time ($P \leq 0.001$). Most interestingly, phosphorylated p38 MAPK levels were higher in MPD versus CON cells under basal conditions (0 h) (69 ± 12.1 U/ml vs. 48.34 ± 2.18 U/ml, MPD vs. CON, $P = 0.044$; Fig. 3D). This trend was evident throughout the time course in MPD versus CON cells, and almost achieved statistical significance at 15 min (141.9 ± 38.1 U/ml vs. 93.5 ± 25.6 U/ml at 15 min; $P = 0.086$). At 2 and 24 h no difference was evident between MPD versus CON cells.

INHIBITION OF JNK LEADS IN TO A NON-SIGNIFICANT INCREASE IN IGF-I mRNA AT 72 H MPD CELLS WITHOUT AN INCREASE IN DIFFERENTIATION

With the increase in JNK1/2 at 24 h in MPD cells observed (Fig. 3C), next we wished to inhibit JNK1/2 using the chemical JNK inhibitor SP600125. At 72 h JNK inhibitor increased IGF-I mRNA expression in the MPD cells (1.98 ± 2 DM vs. 3.45 ± 0.42 JNK 15 μM) although this was not significant (Fig. 4A). Differentiation was also not improved at this time point (data not shown). Therefore, an extension of the time course up to 7 days was performed as this initial increase in IGF-I may have suggested a delayed temporal response with differentiation resulting at a later time point. However, at 7 days differentiation was not increased in the MPD cells versus MPD in control DM, shown by no changes in fusion index (9.8 ± 9.8 MPD in DM vs. 16.2 ± 16.2%, P = NS Fig. 4B).

CHANGES IN MYOD, MYOGENIN, AND IGF-I, BUT NOT IGF-IR ARE ASSOCIATED WITH REDUCED DIFFERENTIATION IN MPD VERSUS CONTROL CELLS

In order to ascertain the molecular mechanisms associated with reduced differentiation, myogenic regulatory factors and IGF system components were assessed. A significant increase in myoD mRNA expression with time was observed in both CON and MPD cells (significant main effect for time ($P \leq 0.001$; Fig. 5A) with significant increases observed between 0 and 48 h (CON, 1.3 ± 1 vs. 61.2 ± 20.5, $P = 0.009$; MPD, 3.67 ± 2.08 vs. 39.8 ± 3.99, $P = 0.009$) and between 0 and 72 h (CON, 1.3 ± 1 vs. 99.5 ± 44.7, $P = 0.023$; MPD, 3.67 ± 2.08 vs. 44.45 ± 7.81, $P = 0.019$). Although on average temporal increases in expression were seen between 48 and 72 h in both cell types there was no significant increase at these time points.

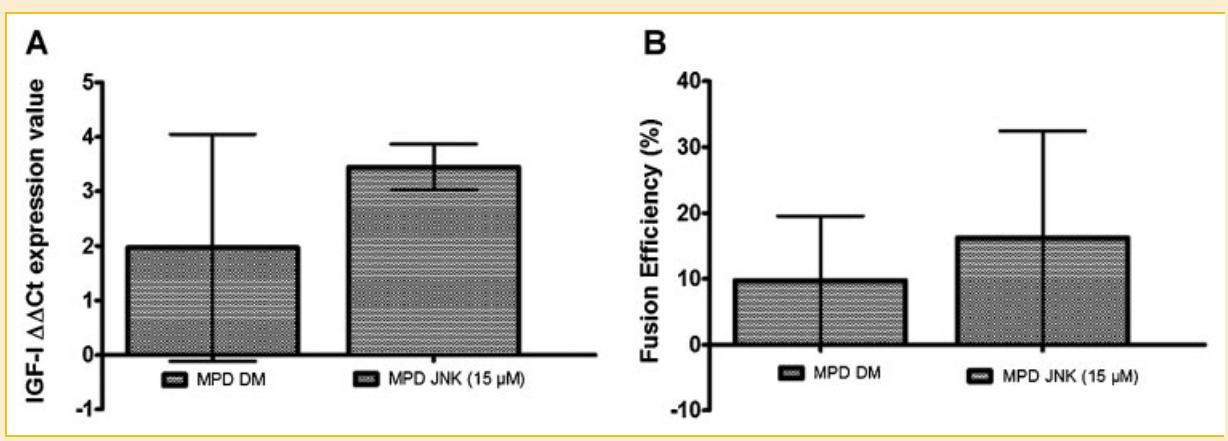

Fig. 4. A: IGF-I expression levels at 72 h ($\Delta\Delta C_t$ expression value as detailed in methods) over time in. Multiple population doubling (MPD) myoblasts in DM control versus JNK inhibitor SP600125 (15 μM). Despite an increased trend there was no significant difference following JNK inhibition with regards to IGF-I mRNA expression. Data are representative of three experiments performed in duplicate. Graph depicts mean ± SD. B: Fusion efficiency after 7 days in DM MPD myoblasts versus JNK inhibitor SP600125 (15 μM). Despite an average increase in fusion efficiency there were no significant differences after JNK inhibition. Data are representative of three experiments performed over four coverslips, with 20 images taken per experiment. Graph depicts mean ± SD.

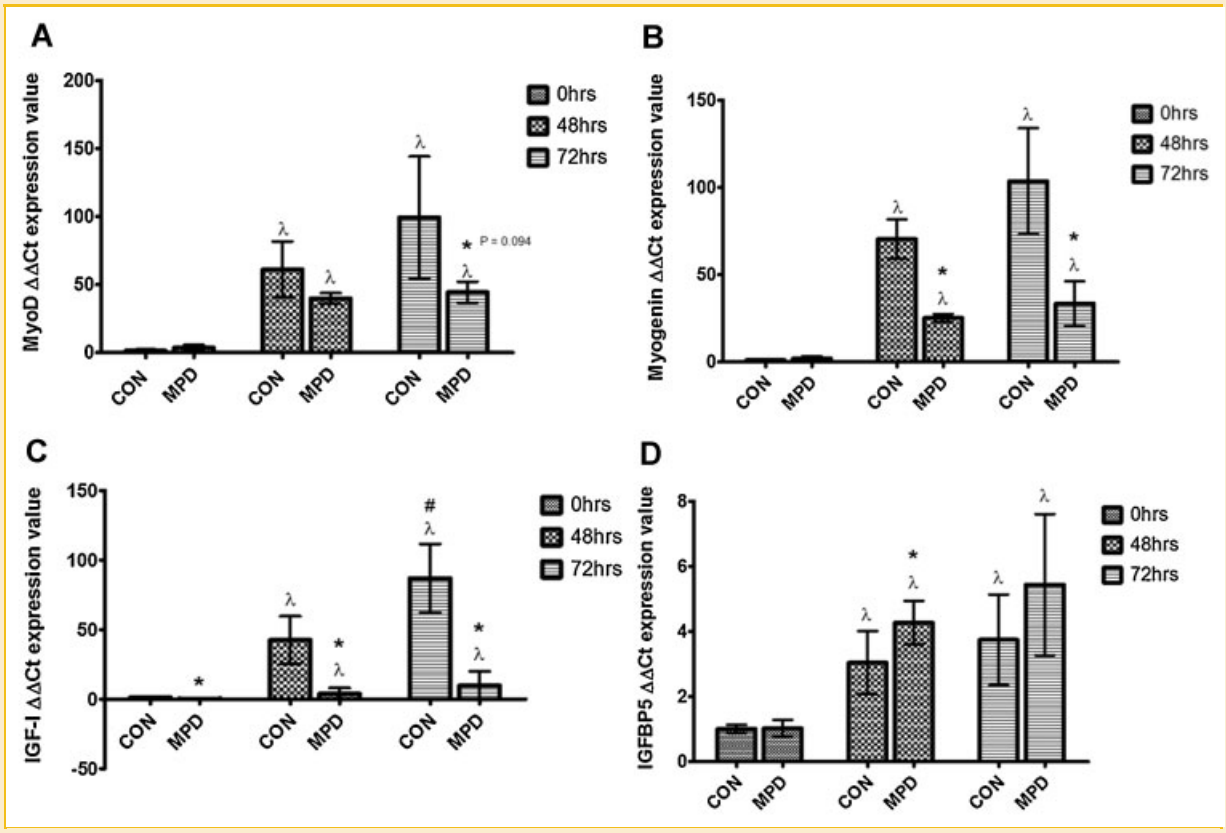

Fig. 5. A: MyoD expression levels ($\Delta\Delta C_t$ expression value as detailed in methods) over time in control (CON) versus multiple population doubling (MPD) myoblasts. Temporal expression increased in both cell types at 48 h (CON, $P = 0.009$; MPD, $P = 0.009$) and 72 h (CON, $P = 0.023$; MPD, $P = 0.019$) versus baseline (0 h) indicated by λ. Despite decreases in myoD expression between CON and MPD cells, significance was not achieved at 48 h, yet approached significance at 72 h indicated by * ($P = 0.094$). Data are representative of three separate experiments in duplicate. Graph depicts mean ± SD. B: Myogenin expression levels ($\Delta\Delta C_t$ expression value as detailed in Methods) over time in CON versus MPD myoblasts. Significant temporal increases were observed in both cell types at 48 h (CON and MPD, $P \leq 0.001$) and 72 h (CON, $P \leq 0.007$; MPD, $P = 0.017$) versus baseline (0 h) indicated by λ. Temporal expression plateaued in both cell types at 48–72 h. Significant decreases in myogenin were observed in MPD versus CON cells at 48 h ($P \leq 0.001$) and 72 h ($P = 0.013$) indicated by *. Data are representative of three separate experiments in duplicate. Graph depicts mean ± SD. C: IGF-I expression levels ($\Delta\Delta C_t$ expression value as detailed in Methods) over time in CON versus MPD myoblasts. There were temporal increases in CON and MPD cells between 0 and 48 h (CON, $P = 0.001$; MPD, $P = 0.048$), and 72 h (CON, $P = 0.001$; MPD, $P = 0.032$), yet between 48 and 72 h IGF-I expression was only increased in CON cells ($P \leq 0.001$ indicated by #) and no increase was observed in MPD cells ($P = 0.156$). Most significantly there were strong significant decreases in IGF-I in MPD cells versus CONs at 0, 48, and 72 h (all $P \leq 0.001$) indicated by *. Data are representative of three separate experiments in duplicate. Graph depicts mean ± SD. D: IGFBP5 expression levels ($\Delta\Delta C_t$ expression value as detailed in Methods) over time in CON versus MPD myoblasts. Significant temporal increases were observed in both cell types regardless of passage at 48 h (CON, $P = 0.004$; MPD, $P = 0.003$) and 72 h (CON, $P = 0.005$; MPD, $P = 0.027$) versus baseline (0 h) indicated by λ. Temporal expression plateaued in both cell types at 48–72 h. Significant increases in IGFBP5 production are reported in cells at 48 h versus CON cells ($P = 0.049$) indicated by *, and although mean increases were observed in MPD cells versus CON at 72 h, significance was not reached. Data are representative of three separate experiments in duplicate. Graph depicts mean ± SD.

There was, however, a significant interaction between cell type and time between CON and MPD myoblasts ($P = 0.008$) suggesting an overall reduction in MyoD expression levels in MPD versus CON cells over time. Indeed, there was an average reduction of MyoD at 48 h in MPD versus CON cells, yet significance was not attained when individual *t*-tests were performed (61.2 ± 20.5 in CON vs. 39.8 ± 3.99; in MPD, $P = 0.134$). There was also a reduction at 72 h but again, significance was not attained (99.5 ± 44.7 in CON vs. 44.5 ± 7.81 in MPD; $P = 0.094$; Fig. 5A). Similar, but statistically significant trends were observed for myogenin expression, with significant temporal increases in myogenin mRNA resulting in a significant main effect for time ($P \geq 0.001$; Fig. 5B). Importantly, myogenin expression was significantly reduced at 48 h (25.14 ± 2.34 in MPD vs. 70.4 ± 11.2 in CON, $P \leq 0.001$) and 72 h (33.4 ± 12.8 in MPD vs. 103.6 ± 30.4 in CON, $P = 0.013$; Fig. 5B). Overall, this resulted in a significant interaction between cell type and time ($P \leq 0.001$) for myogenin expression (Fig. 5B).

IGF-I mRNA expression increased over time in both CON and MPD cells between 0 and 48 h (CON, 1.17 ± 0.8 vs. 42.4 ± 17.1, $P \leq 0.001$; MPD, 0.13 ± 0.11 vs. 3.72 ± 4.26, $P \leq 0.048$; Fig. 5C) and between 0 and 72 h (CON, 1.17 ± 0.8 vs. 86.9 ± 24.7, $P \leq 0.001$; MPD, 0.13 ± 0.11 vs. 9.8 ± 10.2, $P \leq 0.032$). This trend was maintained in the CON cells between 48 and 72 h (CON, 42.4 ± 17.1 vs. 86.9 ± 24.7, $P \leq 0.001$), however, significance was not attained for MPD cells between 48 and 72 h (MPD, 3.72 ± 4.26 vs. 9.8 ± 10.2, $P =$ NS). Overall, there was a significant main effect

for time ($P \leq 0.001$). Importantly when comparing the two cell types, IGF-I expression was reduced extensively in cells that had undergone MPD versus control cells at all-time points resulting in statistical differences at 0 h (0.13 ± 0.11 in MPD vs. 1.17 ± 0.8 in CON, $P \leq 0.001$), 48 h (3.72 ± 4.26 in MPD vs. 42.4 ± 17.1 in CON, $P \leq 0.001$) and 72 h (9.8 ± 10.2 in MPD vs. 86.9 ± 24.7 in CON, $P \leq 0.001$; Fig. 5C). There was a significant interaction between cell type and time for IGF-I mRNA expression (Fig. 5C; $P \leq 0.001$). Interestingly, and despite the enormous differences in expression levels of IGF-I; IGF-IR expression was not changed between CON and MPD cells (Figure not shown), with no significant interaction between cell type and time. There were also no temporal changes in IGF-IR expression in either cell type. Nevertheless, expression levels were high across both cell types, where CON and MPD cells had average C_t values of 24.7 ± 1.15 and of 24.26 ± 0.76, respectively.

INCREASED IGFBP5 AND IGFBP2 EXPRESSION ARE ASSOCIATED WITH REDUCED DIFFERENTIATION

We have previously shown using transfection technologies that increased IGFBP5 expression is associated with prolonged survival, but reduced differentiation [James et al., 1993, 1996; Meadows et al., 2000]. This led us to speculate that IGFBP5 levels may be elevated in MPD cells as a means of reducing differentiation with MPD. There were significant temporal increases in IGFBP5 mRNA expression resulting in a significant main effect for time ($P \leq 0.001$; Fig. 5D), specifically, between 0 and 48 h, with both cell types increasing IGFBP5 mRNA expression (CON, 1.01 ± 0.13 vs. 3.04 ± 0.97, $P = 0.004$; MPD, 1.03 ± 0.26 vs. 4.27 ± 0.67, $P = 0.003$), similarly between 0 and 72 h (CON, 1.01 ± 0.13 vs. 3.74 ± 1.39, $P = 0.005$; MPD, 1.03 ± 0.26 vs. 5.44 ± 2.17, $P = 0.027$). However, significant increases were not observed in either cell type between 48 and 72 h suggesting a plateau in expression independent of experimental condition. While IGFBP5 levels increased significantly in both cell types over time, IGFBP5 was reduced at both 48 (3.04 ± 0.97 in CON vs. 4.27 ± 0.67 in MPD; $P = 0.049$) and 72 h (3.74 ± 1.39 in CON vs. 5.44 ± 2.17 in MPD; $P =$ NS; Fig. 5D) although significance was not attained at 72 h. Overall, there was a significant main effect for cell type, ($P = 0.002$), and the interaction between cell type and time bordered on significance ($P = 0.079$).

Given its potential role in suppressing differentiation in C_2 myoblasts [Sharples et al., 2010], we also investigated expression levels of IGFBP2 in CON and MPD cells. In both cell groups, IGFBP2 expression was highest at 0 h and declined with time (Figure not shown), confirmed with a significant main effect for time ($P \leq 0.001$). On initial inspection, the decline appeared steeper in MPD versus CON cells; however, significance was not achieved 48 or 72 h, when compared with baseline values. Significance was, however, achieved in CON cells between 0 and 48 h (1.14 ± 0.57 vs. 0.495 ± 0.4, $t = 2.25$, $P = 0.05$) and 0 and 72 h (1.14 ± 0.57 vs. 0.27 ± 0.2, $t = 3.51$, $P = 0.013$). Interestingly, although significance was not achieved, IGFBP2 expression in CON cells was lower than in MPD cells at 0 h (1.14 ± 0.57 in CON vs. 2.24 ± 1.46 in MPD), 48 h (0.495 ± 0.4 in CON vs. 0.7 ± 0.54 in MPD), and 72 h (0.27 ± 0.2 in CON vs. 0.46 ± 0.39 in MPD).

DISCUSSION

The present study used a MPD model to investigate the impact of serial divisions on skeletal muscle growth and regeneration in an attempt to provide a model to investigate the effect of ascending cell doublings throughout the lifespan as a contributor to skeletal muscle ageing. Indeed, differentiation was reduced and associated with a reduction in IGF-I, myoD and myogenin transcripts and Akt signaling with increases in IGFBP5 mRNA and JNK signaling in the MPD C_2C_{12} mouse cells. These data suggest that in the absence of senescence and the presence of retained telomerase activity (O'Connor et al., 2009), "proliferative immortality," a trait of many "true" stem cells, does not compromise the survival of MPD cells, but does appear to do so at the expense of efficient cell differentiation/fusion. These cells could, therefore, be used to potentially investigate the impact of muscle stem cell ageing in-vitro.

The loss of differentiation capacity observed in the present study has also been observed in cells isolated from human biopsies of aged individuals [Pietrangelo et al., 2009; Beccafico et al., 2010], as has a reduction in IGF-I expression and decreased phosphorylation of Akt [Benbassat et al., 1997; Leger et al., 2008], all of which will contribute to the reduction in differentiation observed in the present model, with IGF-I/IGF-IR/PI3K/Akt pathways having been shown to be fundamental to myoblast differentiation [Coolican et al., 1997; Al-Shanti and Stewart, 2008]. Therefore, this model potentially mimics the molecular regulation of reduce protein synthesis seen in elderly skeletal muscle. Indeed, it has been reported that depressed expression of protein initiation and translation targets such as mTOR, p70S6K1, 4E-BP1, and EIF2B, all of which function downstream of IGF-I/Akt (reduced in the present study) is evident in older compared with younger counterparts [Terada et al., 1994; Welsh et al., 1997; Pallafacchina et al., 2002; Cuthbertson et al., 2005; Leger et al., 2008].

Inappropriate cell cycle regulation in MPD cells that continue through the S/G2 phases rather than exiting the cell cycle in G1, a requirement of skeletal muscle cells in order to differentiate, is also observed in human muscle cells, which display altered control of cell cycle regulation as a consequence of increased cyclin and reduced cyclin dependant kinase inhibitors (cdks) being expressed with age [Kim et al., 2005; Bigot et al., 2008]. Specifically, cyclin B1 protein is 27% higher in muscle biopsies from older (65–75years) versus young adults (20–35years), and cyclin D1 is higher in older versus younger females [Kim et al., 2005]. p57 a cyclin dependent kinase inhibitor was reportedly decreased in senescent human myoblasts and associated with reduced expression of both myoD and myogenin (both of which were also reduced in the present MPD model) suggesting a dis-regulation in the ability for cells to exit the cell cycle with age [Bigot et al., 2008]. Overall the reported evidence suggests a sensitive mechanism, whereby, cells from aged individuals that have potentially undergone multiple divisions and self-renewal in-vivo reduce their ability to exit the cell cycle and differentiate; a phenotype observed after multiple divisions in-vitro in the present model. Indeed, as shown by Bigot et al. (2008) and in the present study the reduction of myoD and myogenin may regulate this process via a feedforward loop, as these muscle specific

transcription factors reportedly are involved in up-regulating the cyclin dependent kinase inhibitor, $p21^{cip}$ [Guo et al., 1995; Halevy et al., 1995].

Activation of ERK1/2 was investigated in the current model as these proteins are known to be important in myoblast proliferation [Coolican et al., 1997]. There was no change in the signaling activity of ERK1/2, yet previous research suggests that biopsies from old men had higher levels of MAPK proteins, including ERK1/2 phosphorylation, compared with young men but that cultured cells had lower levels in older versus younger samples [Williamson et al., 2003; Carlson et al., 2009; Beccafico et al., 2010] raising an area of controversy.

Further, it is widely accepted that the stem cell niche/environment is a key regulator of ageing muscle and that satellite cells of old mice can be rejuvenated following the parabiotic sharing of their circulatory system with young mice [Conboy and Rando, 2002; Conboy et al., 2005; Gopinath and Rando, 2008], suggesting that the ability for the cells to respond to environmental cues is not lost and that their ability to be activated and proliferate is not impaired. Our studies illustrate that the cells themselves alter their behavior following extensive doublings and that cellular adaptation as well as cell–environment interactions may both be critical for stem cell behavior with age.

Focusing on cellular adaptations, our studies revealed that altered IGFBP5 may contribute to the dampening of already reduced IGF-I function in MPD cells. IGFBP5 has been previously linked to the survival of C_2 myoblasts in the presence of TNF-α [Meadows et al., 2000] but also in dampening the progress of their differentiation, when constitutively over-expressed [James et al., 1996; Mukherjee et al., 2008] via inhibition of IGF actions [Mukherjee et al., 2008] or independent of IGF effects (James et al., 1996). In the present study we showed a significant increase in IGFBP5 at 48 h in MPD versus CON, which would hamper differentiation either directly or via inhibition of IGF-I action. As a consequence, reduced IGF action may result in the reduced Akt phosphorylation observed as previousy discussed. Furthermore, because of previous findings in parental C_2 and daughter C_2C_{12} cells [Sharples et al., 2010], with inverse expression patterns of IGFBP2 in the two cell lines leading to reductions in differentiation in parental C_2 cells, we could hypothesize that because of the reductions in differentiation observed in MPD cells versus controls, that IGFBP2 would have had a similar role here. There was, indeed, a steeper decline in IGFBP2 expression in MPD versus CON cells (graph not depicted) suggesting that altered temporal expression of the IGFBPs may enable differentiation in CON versus MPD cells, which undergo proliferation and survival as an alternative outcome, however, this was not statistically significant.

JNK1/2 activation was increased at 24 h in MPD cells versus controls. Activation of this protein has been linked to stress induced pathways, for example, downstream of TNF-α in skeletal muscle cells [Stewart et al., 2004]. TGF-β, which is involved in blocking differentiation, has also been shown to signal via JNK pathways [Rousse et al., 2001]. Furthermore, an inhibitor for JNK1/2 (SP600125) was able to attenuate reductions in IGF-I mRNA following TNF-α administration in C_2C_{12} cells, where MEK and p38 MAPK inhibitors were unable to rescue IGF-I levels [Frost et al., 2003]. As we showed large reductions in IGF-I expression at 48 and 72 h in MPD cells versus controls, these data suggest that the reduction may be due to increased JNK1/2 signaling at 24 h. Interestingly, JNK1/2 protein is reportedly increased in aged muscle biopsies from 36-month-old Fischer rats versus 3-month-old Fischer rats, underpinning a potential role for this protein in models of muscle ageing [Gupte et al., 2008]. Subsequent studies confirmed the presence of increased JNK protein in old sedentary rat muscle versus young controls, and extended these findings by demonstrating that levels were attenuated in old animals following aerobic exercise [Pauli et al., 2010]. Despite these compelling data further recent studies in cells isolated form old and young humans, suggests that JNK expression is reduced with age [Beccafico et al., 2010], the reasons for this controversy requires further investigation. Therefore, inhibition of JNK using SP600125 in this model was performed, however, was unable to significantly increase IGF-I mRNA and differentiation. Suggesting that the increase in JNK is not the mechanism for the reduction of differentiation seen between the MPD and control cells, and that the IGF-I/Akt pathway maybe more prominent with respect to the reduction of differentiation.

Finally, p38 MAPK was increased at 0 and 15 min in aged versus un-aged cells, however, a transient decrease was also observed at 5 min (although not significant). These adaptations may be indicative of p38 MAPK's dual roles in both stress related mechanisms induced by oxidative stress (H_2O_2) and cytokines [Li et al., 2005], and a positive regulator of differentiation [Li et al., 2000; Al-Shanti and Stewart, 2008; Lassar, 2009]. Therefore, although, there are changes in p38 MAPK, due to their alternate nature with respect to the changes in both cell types, it is unclear as to whether this is directly involved in the reduced differentiation observed using the present model and would make inhibition of this pathway difficult in the present model.

Overall, reduced IGF-I expression and myogenic regulatory factors and elevated IGFBP5 underpin the reduction in differentiation observed in aged myoblasts. Signaling studies suggest that decreased phosphorylation of Akt and early in the progression of differentiation may hamper the ability of aged cells to exit the cell cycle forcing their continued progression through S and G2. Later increases in the phosphorylation of JNK1/2 in aged cells may signal a stress event to the aged cells, however, does not contribute to the reduced differentiation observed.

CONCLUSION

This model potentially provides an interesting model to investigate whether multiple divisions by myoblasts in-vitro could recapitulate the rounds of population expansion and self-renewal that occur in-vivo (albeit potentially at an accelerated rate in the former). The model presents similarities with in-vivo human muscle and isolated muscle cells from elderly individuals, where reduced differentiation/regeneration are associated with reduced IGF/Akt, myoD, and myogenin expression and inappropriate cell cycling. Importantly, IGFBP5 and JNK activity seem to play important roles in this and other ageing models. However, JNK inhibition ruled out the protein as the major contributor to the reduced differentiation observed.

Overall, this study provides strong evidence for deterioration in differentiation capacity as a consequence of multiple population doublings in skeletal muscle cells that would potentially contribute to the ageing process and presents an interesting model for future investigations of the impact and need for prolonged survival and growth at the expense of efficient myoblast fusion.

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