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Materials and methods: We examined proliferation and subsequent differentiation of non-passaged MPCs isolated from skeletal muscles of 1-, 3- and 32-month old rats over a 72-h time course, using a serum cross-over design. Results and conclusions: We found no effect of serum age on MPC proliferation, but we did dis- cover that MPCs isolated from skeletal muscle of 32-month old rats had delayed onset of, and exit from proliferation, compared to MPCs isolated from skeletal muscle of 1-month old rats. Delayed proliferation of MPCs from 32-month old rats was associated with delayed p38 MAPK phosphorylation, and MyoD and p21Cip1 protein expression. We also demonstrate that MPCs from 32-month old rats exhibited lower levels of muscle creatine kinase mRNA compared to 1-month old rats, but elevated levels of myogenin mRNA, when stimulated to differentiate after 36 h proliferation. These findings suggest that delayed entry and exit of the cell cycle observed in MPCs from 32-month old rats may compromise their ability to respond to differentiation stimuli and subsequently impair myogenic potential of 32-month old skeletal muscle, in this model.

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Non-passaged muscle precursor cells from 32-month old rat skeletal muscle have delayed proliferation and differentiation

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Introduction

Ageing is associated with reduced skeletal muscle mass and strength, defined as sarcopenia. Sarcopenia can result in functional impairment (1,2), physical disability (1,3,4) and decreased quality of life (5,6). Decline in capacity to regenerate and repair skeletal muscle may contribute to sarcopenia (7,8). Multiple factors [for example, satellite cell dysfunction, muscle homeostatic niche (myofibres, basal lamina), extracellular matrix, microvasculature, neural, immune/inflammatory, fibro-adipogenic precursor cells, myokines, reactive oxygen species, energy metabolism, systemic factors and more] have been implicated in impaired capacity of aged skeletal muscle to regenerate and repair (8,9). The multiplicity of factors contributing to sarcopenia makes it unlikely that all factors can be considered in a single experiment.

Skeletal muscle cells (fibres) are multinucleate, but are terminally differentiated, hence their nuclei are unable to replicate (9–11). Satellite cells, a population of muscle progenitor cells located outside the fibres yet inside the basal lamina, function in repair and growth of skeletal muscle through proliferation, fusion into existing muscle fibres and differentiation. Some contend that dysfunctional satellite cells could negatively influence repair and growth, thus contributing to sarcopenia. Our current study described here, focuses on comparing proliferation and differentiation of satellite cells isolated from skeletal muscles of rapidly growing,

young (1- or 3-month old) and old, sarcopenic (32-month old) rats.

Sera from young and old animals have been shown to differentially alter proliferative phenotypes of their (young) and (old) satellite cells. Heterochronic, parabiotic pairings of 2- to 3-month old with 19- to 26-month old mice, rescued regeneration in the older individuals (12). These authors concluded that age-related decline of satellite cell activity could be modulated by systemic factors that change with age. Such findings contribute to the notion that the systemic milieu plays an important role by altering the stem cell niche or by acting directly on the stem cells to suppress regenerative potential, in aged skeletal muscle (8). Furthermore, Carosio *et al.* (7) summarized data suggesting that the internal systemic environment of a young animal is one that promotes successful regeneration, while that of old animals either fails to promote, or inhibits, muscle regeneration. However, other reports suggest that exceptions to the above conclusions may occur. Conboy *et al.* (12) reported that while serum from 2- to 3-month old mice enhanced proliferation of satellite cells from 19- to 26-month old mice, serum from 19- to 26-month old mice did not reduce proliferation of satellite cells from 2- to 3-month old mice. George *et al.* (13) reported no effect of age (23- to 36-year old versus 69- to 84-year old) of human serum on cultured human myoblasts 3, 5 or 7 days after plating, whereas Shavlakadze *et al.* (14) demonstrated that excellent myotube formation occurred on the 10th day after transplantation of muscle autografts from 27- to 29-month old mice, into old mice, which they interpreted as meaning that myogenic potential had been retained in this sarcopenic skeletal muscle.

One commonality among the above reports is assessment of either no, or one, time point within the first 48 h after plating satellite cells for culture. We wondered whether examining a broader time course within the first 48 h of plating satellite cells would reveal greater insights into satellite cell proliferation. We hypothesized that additional time points during the first 48 h might indicate differences in proliferation between satellite cells of young and old rats. Our approach was to limit known experimental variables by using a cross-over design with heterochronic pairings of young and old satellite cells with young and old sera.

Materials and methods

Animals

The Institutional Animal Care and Use Committee at the University of Missouri–Columbia, approved all procedures of this experiment. 1-, 3- and 32-month old Fischer 344 9 Brown Norway F1 hybrid male rats were obtained from the National Institute on Aging aged rodent colony. These ages were selected for their typical growth and skeletal muscle mass characteristics. For this study, the 3 animal age groups are defined as follows:

1-month old: rapidly growing
3-month old: slower growth
32-month old: growth impairment

according to growth curves (15) and muscle weights (16) previously reported for Fischer 344 9 Brown Norway F1 hybrid male rats. Animals were housed at 21 ° C, 12-h light:dark cycle, and allowed free access to food and water. At the time of sacrifice, animals were intraperitoneally injected with ketamine (80 mg/kg), xylazine (10 mg/kg) and acepromazine (4 mg/kg), and blood was collected by cardiac puncture; then muscles were excised.

Muscle precursor cell isolation procedure

Muscle precursor cells (MPCs) were isolated from the rat skeletal muscle using methods modified as described previously (17) at our laboratory (18,19). Briefly, cells were isolated from the gastrocnemius and plantaris muscles of both hind limbs by pronase digestion, and pre-plated in Dulbecco's Modified Eagle Medium (DMEM) (DMEM; containing 10% horse serum, 100 units/ml penicillin, 100 lg/ml streptomycin and 40 lg/ml gentamicin) for 2 h, on tissue culture-treated 150-mm plates. Due to the small size of 1-month old rats, muscles from three animals were combined to make one sample for 1-month old MPC isolations. We have previously published that our MPC isolation technique provides greater than 90% isolated cells express, thus stain positively, for both MyoD and desmin (18–22).

After 2-h pre-plating, cells were plated on Matrigel (BD Biosciences, San Jose, CA, USA)-coated plates (0.01 mg/ml Matrigel, 60 min at 37 °C) and cultured in growth media (GM; containing 10% rat serum, 100 units/ml penicillin, 100 lg/ml streptomycin, 40 lg/ml gentamicin in Ham's F-10) in a humidified incubator, 5% O_2 and 5% CO_2 at 37 °C (HERAcell, Thermo Scientific, Waltham, MA, USA). Greater than 90% of all cells contain desmin- and MyoD-positive immunoreactivity (markers for MPCs) using this protocol for MPC isolation (18–22). All experiments in the current study were performed on non-passaged (passage 0; P-0) MPCs and GM was refreshed every 24 h after plating.

Muscle precursor cell culture experimental design

Originally, we hypothesized that (i) serum from 32-month old rats would attenuate proliferative potential of MPCs isolated from skeletal muscle of 3-month old rats and (ii) serum from 3-month old rats would enhance proliferative potential of MPCs isolated from skeletal muscle of 32-month old rats. Thus, serum cross-over experiments were performed using P-0 MPCs evenly plated across 8 Matrigel-coated 60-mm plates as follows: 4 plates 3-month old MPCs cultured in GM containing 10% rat serum from 3-month old rats (3-month old MPC/3-month old GM), and the other 4 plates of 3-month old MPCs cultured in GM containing 10% rat serum from 32-month old rats (3-month old MPC/32-month old GM) and *vice versa* (n = 4 rats/age group). A time-course experiment was performed and MPC proliferation was analysed at 18, 24, 36 and 48 h after plating. Due to non-significant differences in 3-month old and 32-month old serum cross-over time-course experiments, a second serum cross-over time-course experiment was performed in the same manner, but with a younger set of MPCs (1-month old) and a new set of 32-month old MPCs and serum (n = 4–5 rats/age group).

To analyse MPC proliferation 72 h after plating, the original time-course was extended. Thus, MPCs were isolated from additional skeletal muscles and cultured in GM containing 10% rat serum from the same aged rat (that is, 3-month old MPCs cultured in GM containing 3-month old serum) from 1-, 3- and 32-month old rats, to analyse MPC proliferation at 24, 48 and 72 h after plating. These data were combined with data for 1-, 3- and 32-month old MPCs cultured in GM containing 10% rat serum from the same aged rat in the serum cross-over experiment described above (n = 4–8 rats/age group across all time points; except 1-month-old at 72 h, n = 1).

To investigate potential mechanisms responsible for observed results in the MPC proliferation time-course experiment, MPCs were isolated from new groups of 1- and 32-month old animals' skeletal muscles only (n = 6 rats/age group), evenly plated on 3–100 mm Matrigel-coated plates, and cultured in GM containing 10% rat serum (same age as MPCs). MPCs (P-0) were harvested at 24, 36 and 48 h after plating and $p21^{Cip1}$, $p27^{Kip1}$, MyoD and p38 MAPK proteins, common indices for status of the cell cycle, were analysed using western blot techniques.

To determine whether differences in proliferation affect ability of 1- and 32-month old MPCs to differentiate, mRNA levels of muscle creatine kinase and myogenin, common markers of differentiation status, were measured during cell differentiation. MPCs were isolated from additional groups of 1- and 32-month old rats (n = 4 rats/age group) and plated on 60-mm Matrigel-coated plates. To control for potential seeding density and proliferation differences between 1- and 32-month old P-0 MPCs, cells were plated at fractions of 1/2 (high density), 1/3 (medium density) and 1/6 (low density) of total cells, with 2 plates per concentration. For each concentration, one plate was harvested and lysed 36 h after plating and served as proliferation control, while the other was induced to differentiate by replacing GM with differentiation medium (DM; 2% rat serum of same age as MPCs, in DMEM). Differentiating MPCs were harvested and lysed 24 h after switching to DM. The 36 h proliferation time point was chosen based on findings from the MPC proliferation time-course experiment. The 24 h differentiation time point was based on preliminary findings that had indicated that 24 h exposure to DM, after 36 h proliferation in GM, was sufficient to detect increases in muscle creatine kinase mRNA in MPCs, isolated from 6-month old rat skeletal muscle (data not shown). At the time of harvesting, MPCs were lysed in RLT buffer using QIAShredder columns (Qiagen, Valencia, CA) according to manufacturer's specifications, and cell lysates were stored at -80 °C until RNA isolation.

Additional 1- and 32-month old MPCs, plated at high, medium and low densities, were counted from images taken of five random fields of view using light microscopy at 49 objective lens magnification. Cell counts from the five images were averaged for each sample and four independent samples for each age were used for statistical analyses.

BrdUrd incorporation

DNA incorporation of 5-bromo-2′-deoxyuridine (BrdUrd) implies that a cell has entered S phase of the cell cycle and is actively replicating its DNA (23). Thus, incorporation of BrdUrd was measured to be an indication of state of MPC proliferation at the time of assessment (24). MPCs were incubated with 10 lM BrdUrd for 60 min before their harvest at designated time points after plating. Then they were washed twice in PBS, removed from the plates with TrypL Express (Invitrogen, Carlsbad, CA, USA) and centrifuged at 500 9 g for 5 min, followed by fixing in ice-cold 70% ethanol. As previously described (25,26), DNA was denatured using 2NHCl for 30 min, then BrdUrd incorporation was detected using FITC-conjugated monoclonal antibody raised against BrdUrd (5 lg/ml; Roche Applied Sciences, Indianapolis, IN, USA), in PBS with 0.1% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA). MPCs (20 000 cells) were analysed using a

FACScan flow cytometer and CellQuest Pro software, both from BD Biosciences (San Jose, CA, USA).

Protein sample preparation and western blot analysis

Total cell protein was isolated from MPCs at designated time points after plating, as described above. Cells were lysed using RIPA buffer with protease inhibitor (P8340; Sigma, St. Louis, MO, USA) and phosphatase inhibitor (P2850 and P5726; Sigma) cocktails. Cell lysates were incubated on ice for 30 min and then insoluble protein fraction was cleared by centrifugation at 12 000 9 g for 20 min at 4 °C. Cell lysates were stored at -80 °C until further processing. Due to large buffer volume required to harvest and lyse the MPCs, cell lysates were concentrated using Amicon Ultra-0.5 ml centrifugal filters (Millipore, Billerica, MA, USA) then protein content for each sample was determined using bicinchoninic acid (BCA Protein assay kit; Pierce, Rockford, IL, USA).

Expressions of cell cycle inhibitors, $p21^{Cip1}$ and $p27^{Kip1}$, phosphorylated p38 MAPK and MyoD protein were analysed by standard Western blotting techniques. Protein cell lysates were diluted to 0.4 lg/lL in Laemmli buffer, boiled for 5 min and 30 ll (12 lg total) protein sample was separated by SDS-PAGE on 12% gels. Proteins were transferred to nitrocellulose membranes and stained with Ponceau S. To ensure equal protein loading across the lanes, images of Ponceau S-stained membranes were acquired using a flatbed scanner (Epson 3170 Photo Scanner; Epson America, Inc., Long Beach, CA, USA) and optical density of lane length was quantified using Kodak molecular imaging software (version 4.0; Eastman Kodak Company, Rochester, NY, USA); there were no significant differences in protein quantities across membranes (data not shown). Membranes were blocked in 5% non-fat dry milk (NFDM) for 1 h at room temperature and then incubated with primary antibody overnight at 4 °C. Primary antibodies were used in the following conditions: $p21^{Cip1}$ (Millipore, Billerica, MA, USA) 1:200 dilution in 5% NFDM; $p27^{Kip1}$ (Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1:200 in 5% NFDM; phospho-p38 MAPK (Cell Signaling, Danvers, MA, USA) 1:1000 dilution in 5% BSA and MyoD (Santa Cruz Biotechnology) 1:500 dilution in 5% NFDM. Immunoblots were incubated with ImmunoPure® peroxidase-conjugated secondary antibody (Pierce; 1:1000 dilution in 5% NFDM) for 1 h at room temperature. Antibody complexes were detected using SuperSignal® WestDura Western Blotting Detection reagent (Pierce) and a Kodak Image Station 4000R Digital Imaging System. Immunoblots were quantified using Kodak molecular imaging software.

RNA isolation and real-time PCR

Total cell RNA was isolated from MPCs using commercial RNA isolation columns (QIAShredder and RNeasy micro kit) according to manufacturer's specifications. RNA was quantified using a NanoDrop® 1000 spectrophotometer (Thermo Scientific) and stored at -80 °C until reverse transcription. RNA purity was verified by 260:280 ratios 2:1.8 (data not shown). Reverse transcription was performed on equal amounts of total RNA sample using High Capacity cDNA Reverse Transcription kit [Applied Biosystems (AB), Foster City, CA]. Muscle creatine kinase (MCK) and myogenin mRNA levels were determined using quantitative real-time PCR and reactions, performed in duplicate with SYBRGreen PCR master mix (AB), contained 25 ng cDNA and 900 nM each forward and reverse primers in 25 ll volume. An AB Prism 7000 sequence detection system was used for all real-time PCR reactions. Real-time PCR primers were designed with Primer Express 2.0 (AB) using specific BLAST sequences for rat genes. Information on primer sequences used for mRNA templates is provided in Table 1. All primers were validated and amplification efficiency was verified prior to experimentation (data not shown). Amplicon product size was confirmed by gel electrophoresis using 3.0% agarose gel stained by ethidium bromide and visualized using ultraviolet light (data not shown). Dissociation (melting) curves after SYBRGreen real-time PCR also served as validation for specific product amplification (data not shown). Relative changes in mRNA levels were determined using the DDCt method (AB User Bulletin no. 2) and normalized to 18SrRNA. Levels of 18S rRNA were unchanged over the various time points or between proliferating and differentiating MPCs (data not shown).

Statistical analyses

BrdUrd incorporation data from the two serum cross-over proliferation time-course experiments (3- versus 32-month old, Fig. 1 and 1- versus 32-month old, Fig. 2) were analysed using three-way ANOVA (serum 9 age 9 time). BrdUrd incorporation data from the proliferation time-course experiment comparing all three age groups in the same aged serum (1- versus 3- versus 32-month old, Fig. 3), as well as phosphorylated p38 MAPK, MyoD, p21 and p27 protein levels (Fig. 4), were analysed using two-way repeated measures ANOVA (age 9 time). A one-way ANOVA was performed on the area beneath the curve (AUC) data from the proliferation time-course experiment, comparing the three different ages. Following significant F-ratios with ANOVA analyses, Holm-Sidak or Fisher LSD post-hoc

Table 1. Primer sequences used for SYBR Green real-time PCR quantification of target RNA

Target	Forward primer	Reverse primer
Muscle creatine kinase	GACCACCTTCGCGTGATCTC	CGGCGGAAAACTTCCTTCA
Myogenin	GCCCCTGAGTTGGTGTCAGA	AGGACAGCCCCACTTAAAAGG
18S	GCCGCTAGAGGTGAAATTCTTG	CATTCTTGGCAAAGCTTTCG

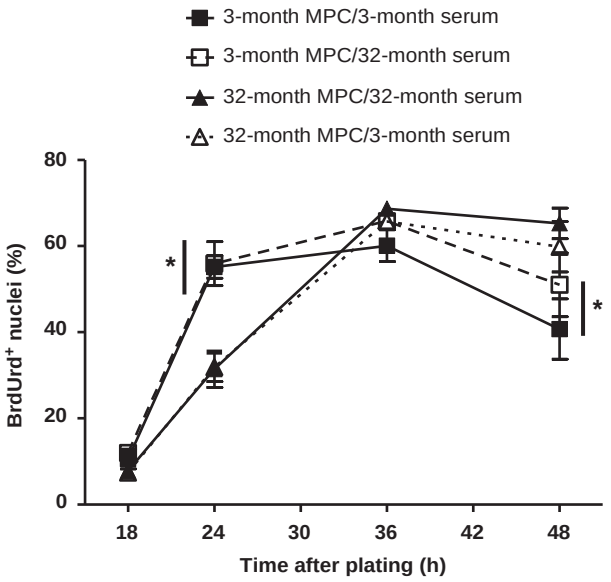

Figure 1. Three- and 32-month old P-0 MPCs in serum cross-over time-course experiment. P-0 MPCs isolated from 3- and 32-month old rat skeletal muscles were analysed for BrdUrd incorporation (as an index of proliferation) in a serum cross-over design. There was a significant main effect of age on BrdUrd incorporation. However, treatment of MPCs with different aged serum tended to be significant across the time course of BrdUrd incorporation ($P = 0.094$). *indicates significant difference from 32-month old MPCs at the same time point. Data presented as Mean ± SEM; $n = 4$ rats/each.

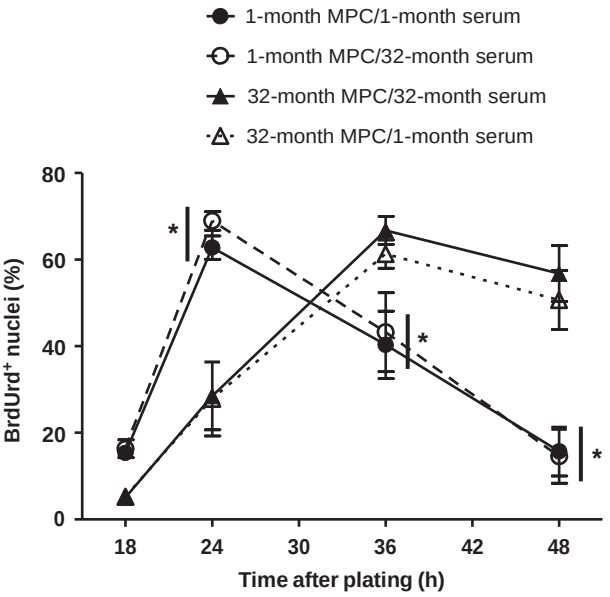

Figure 2. One- and 32-month old P-0 MPCs in serum cross-over time-course experiment. P-0 MPCs isolated from 1- and 32-month old rat skeletal muscles were analysed for BrdUrd incorporation (as an indicator of proliferation) in a serum cross-over design. There was a significant main effect of age on BrdUrd incorporation; however, there was no effect of treatment with different aged sera across the time course ($P = 0.344$).*indicates significantly different from 32-month old MPCs at the same time point. Data presented as Mean ± SEM; $n = 4$-5 rats/each.

analyses were performed to determine differences between groups. Student's unpaired *t*-tests were performed on cell density data (Table 2), as well as relative quantification data (using the DDC_T method) on MCK and myogenin mRNA levels from the differentiation experiments (Fig. 5). Statistical significance was set at P ::; 0.05; all data are presented as mean ± SEM.

Results

Serum cross-over experiments

Previous reports have obtained mixed results for responses in MPC proliferation in cross-over studies using sera from young and old animals with MPCs from young or old skeletal muscle (12–14,27). Common to these studies is the investigation of no early time point or only a single time point within the first 48 h of plating. We deduced that examining multiple time points within 48 h of plating P-0 MPCs might reveal resolution absent in previous reports. The initial objective of this study was to determine whether (i) serum from 32-month old rats would reduce proliferative potential of MPCs from 3-month old animals; and (ii) serum from 3-month old rats would increase proliferative potential of MPCs from 32-month old rats. Using BrdUrd incorporation as an index of the state of MPC proliferation, P-0 MPCs were isolated from 3-month old and 32- month old rat skeletal muscles and allowed to proliferate for up to 48 h in GM containing either serum from the

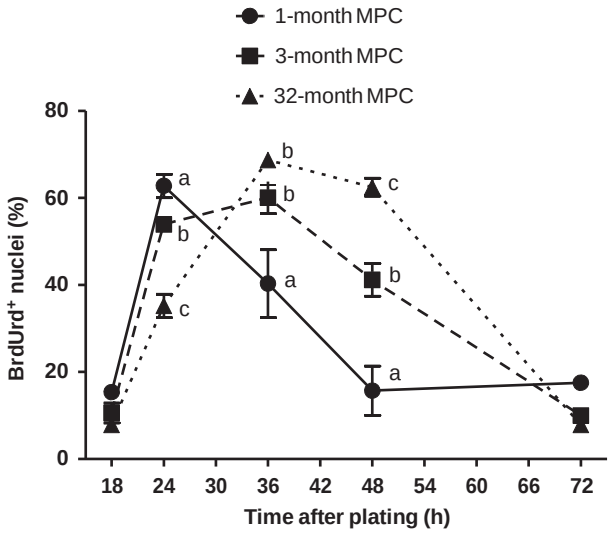

Figure 3. Expanded proliferation time course of 1-, 3- and 32-month old P-0 MPCs. The proliferation BrdUrd incorporation time-course data with 1-, 3- and 32-month old P-0 MPCs exposed only to media containing serum from same aged rats (1-mo MPCs with 1-mo serum, etc.) were combined and expanded to include more animals and a 72-h time point. BrdUrd incorporation of 1-month old MPCs peaked at the 24-h time point and was significantly higher than 3- and 32-month old MPCs. However, BrdUrd incorporation in 1-month old MPCs returned to baseline by 48 h. On the other hand, proliferation of MPCs from 3- and 32-month old skeletal muscles peaked at 36 h; and while 3-month old MPC proliferation started to decline at 48 h, BrdUrd incorporation in 32-month old MPC remained elevated at 48 h. BrdUrd incorporation in MPCs of all ages returned to baseline by 72h. Significant differences within the same time point are indicated by different letters. Data presented as Mean ± SEM; n = 4–8 rats/each, except for 1-month-old MPC at 72 h (n = 1 rat).

same aged rat or different aged rat serum (Fig. 1). There was a significant main effect of age on BrdUrd incorporation; however, treatment of MPCs with different aged serum only tended towards significance across the time course of BrdUrd incorporation (P = 0.094). The BrdUrd incorporation was significantly higher in 3-month old MPCs at 24 h, but lower at 48 h, compared with 32-month old MPCs at those same time points. There was no difference in BrdUrd incorporation between 3- and 32-month old MPCs at 18 and 36 h Importantly, our results revealed peak values for BrdUrd incorporation of ~60% of nuclei at 24 and 36 h for MPCs plated from 3-month old rats, but with peak BrdUrd incorporation delayed to 36 and 48 h for MPCs from 32-month old animals. This observation, along with the fact that 3-month old rats have lower percentage daily growth than 1-month old ones (28), led us to think that 1-month old MPCs may show even greater age differences in peak BrdUrd incorporation, compared to 32-month old MPCs.

Thus, the proliferation time-course serum cross-over experiment was repeated; however, this time 1- and 32-month old rats were used. As in Fig. 1, no treatment effect of serum age was observed across the time-course (P = 0.344). Importantly, however, times of peak BrdUrd incorporation into P-0 MPCs for 1-month old versus 32-month old serum cross-over experiment (Fig. 2) differed from peak times in our previous experiment (Fig. 1) using MPCs from 3- and 32-month old skeletal muscles. Again, as with the 3- and 32-month old comparisons, a significant main effect of age existed for BrdUrd incorporation between the 1- and 32-month old MPCs. BrdUrd incorporation was higher in 1-month old MPCs at 24 h, but lower at the 36- and 48-h time points, compared to 32-month old MPCs. There was no difference in BrdUrd incorporation between 1- and 32-month old MPCs at 18 h. Comparisons between Figs 1 and 2 led to two new observations: (i) at the time point of 36 h post-plating, no difference existed between 3- and 32-month old MPCs (Fig. 1), but significantly lower BrdUrd incorporation was present in 1-month old than in 32-month old
MPCs at this 36-h time point (Fig. 2); and (ii) at the time point of 48 h post-plating, ~60% of nuclei were BrdUrd-labelled in 32-month old cells, but only about ~15% of nuclei were labelled in 1-month old MPCs, which is essentially baseline labelling. These findings led to the question: Did 32-month old MPCs return to baseline at 72 h post-plating?

Expanded MPC proliferation time-course

Proliferation time-course experiment with P-0 MPCs was expanded to include a 72-h time point and to include all three ages of MPCs from 1-month old, 3-month old and 32-month old skeletal muscles (plated in GM containing serum from the same aged rats, that is, 1-month old MPCs with 1-month old serum, and so on.). Combined data were analysed and plotted as seen in Fig. 3. Statistical analyses revealed significant overall interaction (P < 0.001) in proliferation among 1-, 3- and 32-month old MPCs. Interestingly, BrdUrd incorporation in 1-month old MPCs peaked at the 24-hr time point and was significantly higher than 3- and 32-month old MPCs; however, BrdUrd incorporation in 1-month old MPCs began to decline at 36 h and returned to baseline by 48 h. On the other hand, BrdUrd incorporation in 3- and 32-month old MPCs peaked at 36 h, while BrdUrd incorporation in 3-month old MPC started to decline at 48 h, 32-month old MPC BrdUrd incorporation remained elevated at 48 h. MPC BrdUrd incorporation at all ages returned to baseline by the 72-h time point. While it appears that 1-month old MPCs displayed

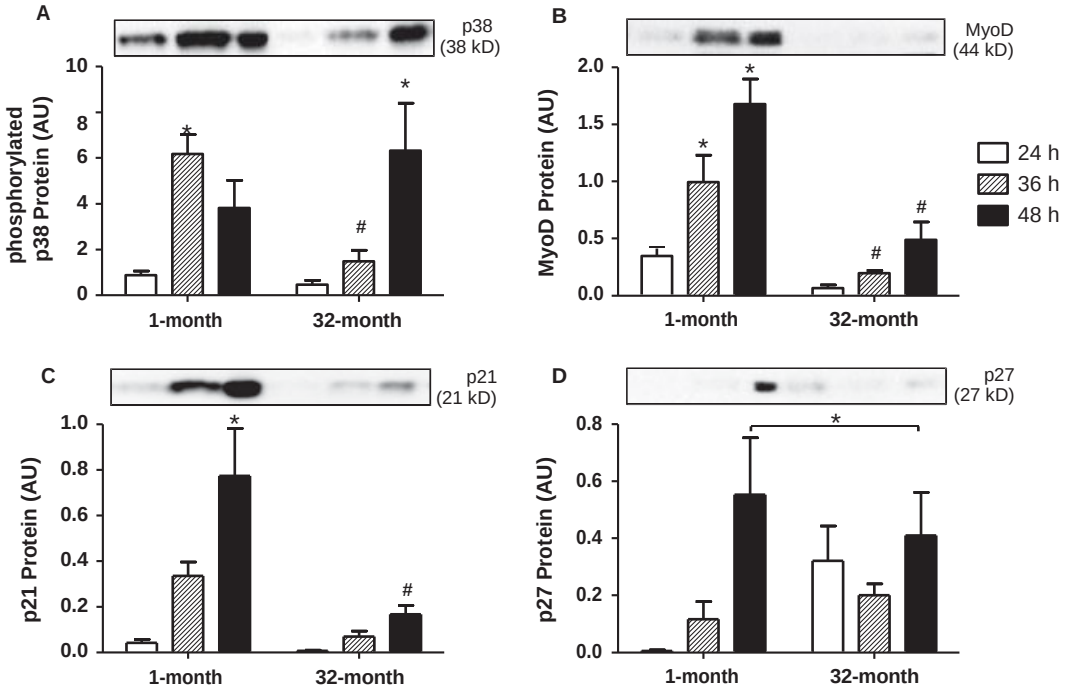

Figure 4. Time course of phosphorylated p38, MyoD, p21 and p27 protein expression. Phosphorylated p38 (A), MyoD (B) and cell cycle inhibitors, p21 (C) and p27 (D), were analysed at 24 (opens bars), 36 (hatched bars) and 48 (filled bars) hours in proliferating 1- and 32-month old P-0 MPCs exposed only to media containing serum from the same aged rats (1-month old MPCs with 1-month old serum, etc.). (a) Increased p38 phosphorylation was delayed in 32-month old MPCs, compared to 1-month old MPCs. (b) MyoD protein expression increased in 1-month old MPCs at 36 and 48 h, compared to 24 h, but not in 32-month old MPCs. (c) p21 protein expression significantly increased in 1-month old MPCs at 48 h, but not in 32-month old MPCs. D) p27 protein expression was significantly higher at 48 h (main effect of time). *indicating significant difference from 24 h within the same age group; #indicates significant difference from 1-month old MPCs within the same time point. Images of representative immunoblots are placed above each protein target. Densitometry data are presented as arbitrary units (AU) and displayed as Mean ± SEM. n = 6/each for p38; n = 5/each for MyoD and p27; n = 3/each for p21.

higher BrdUrd incorporation early in the time course and 32-month old MPCs displayed higher BrdUrd incorporation later in the time course, when areas under the curve (AUC) were analysed, 32-month old MPC BrdUrd incorporation AUC was significantly greater than that of 1-month old MPCs. Taken together, these data suggest that in this model of P-0 MPCs isolated from 1-, 3- and 32-month old skeletal muscles and plated in GM containing the same aged serum, younger MPCs began to increase BrdUrd incorporation earlier after plating than 32-month old MPCs, while 32-month old MPCs had a more prolonged BrdUrd incorporation period than young MPCs during the same time-course. Furthermore, 1-month old MPCs appeared to withdraw from the cell cycle earlier than 32-month old MPCs. As withdrawal from proliferation is required to begin differentiation and BrdUrd incorporation was 10–15% at 48 h post-plating in 1-month old MPCs, but nearly 60% at 48 h in 32-month old MPCs, we next examined markers for the cell cycle and for differentiation, with the generated hypothesis that these markers would associate with differences in BrdUrd incorporation between 1-month old and 32-month old MPCs.

Muscle-specific regulatory factors and cell cycle inhibitors in proliferating MPCs

To investigate potential mechanisms explaining differences in BrdUrd incorporation between 1- and 32-month old MPCs observed in the expanded MPC proliferation time-course experiment, MPCs were isolated from 1-month old and 32-month old rat skeletal muscles, plated in GM containing the same aged serum as MPCs and harvested at 24, 36 or 48 h, for determination of p38 MAPK phosphorylation and protein expression of MyoD and cell cycle inhibitors. These time points were chosen as peak BrdUrd incorporation values for both 1-month old and 32-month old MPCs were contained over this time frame. Phosphorylation of p38 MAPK was significantly elevated at 36 h and tended to remain high at 48 h (P = 0.099) in 1-month old MPCs. However, p38 MAPK phosphorylation was

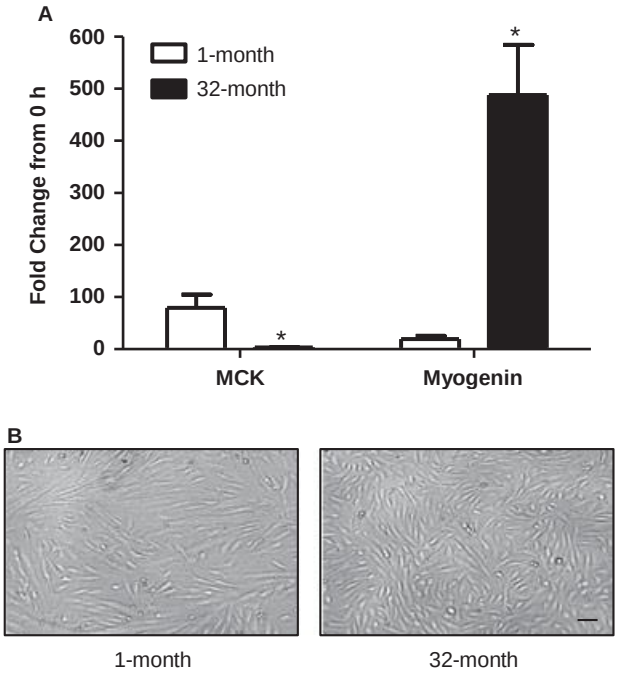

Figure 5. Muscle creatine kinase (MCK) and myogenin mRNA in differentiating MPCs. (a) MCK and Myogenin mRNA levels were measured as markers of differentiation in 1-month old (opens bars) and 32-month old (filled bars) P-0 MPCs after 36 h proliferation (control) and 24 h reduced serum conditions (differentiation). MCK mRNA was significantly higher in 1-month old MPCs after 24 h differentiation compared to 32-month old MPCs. However, myogenin mRNA was substantially higher in 32-month old MPCs compared to 1-month old MPCs. *indicates significant difference from 1-mo MPCs. Relative quantification data (DDC_T method) were normalized to 18S rRNA and are presented as fold change from control, and displayed as Mean ± SEM. *n* = 4 rats/each. (b) Representative light micrographs of 1- and 32-mo MPCs (seeded at medium density) after 24 h exposure to differentiation conditions. Cultures from 1-month old MPCs appear to have more fused cells after 24 h differentiation compared to 32-month old MPCs at the same time point. Scale bar = 100 ⌊m.

delayed and not significantly raised until the 48-h time point in 32-month old MPCs (Fig. 4a). In 1-month old MPCs, MyoD protein expression increased nearly 3- and 5-fold at 36 and 48 h, respectively, compared with 24 h. More importantly, MyoD was significantly higher in 1-month old MPCs at 36 and 48 h, compared to 32-month old MPCs, but not at 24 h (Fig. 4b).

Cell cycle inhibitors, $p21^{Cip1}$ and $p27^{Kip1}$, were also analyzed to determine whether their expression would be altered during the proliferation time-courses of 1-month old and 32-month old MPCs. In 1-month old MPCs, $p21^{Cip1}$ protein increased at 48 h compared to 24 and 36 h, while no increase in $p21^{Cip1}$ protein was observed in 32-month old MPCs (Fig. 4c). More importantly, $p21^{Cip1}$ protein was significantly higher in 1-month old MPCs at 48 h compared to 32-month old MPCs at the same time point. On the other hand, there was no effect of age on $p27^{Kip1}$ protein expression during the time course of proliferation, thus we did not make associations between p27 expression and proliferation indices over this time period. However, there was a significant main effect of time in expression of $p27^{Kip1}$ (Fig. 4d), where $p27^{Kip1}$ protein was higher at the 48-h time point, compared to 24 and 36 h. Taken together, these data suggest that increased p38 MAPK phosphorylation and MyoD and $p21^{Cip1}$ protein expression might play a role in early withdrawal from the cell cycle of 1- month old MPCs. Likewise, delayed phosphorylation of p38 MAPK and lower expression of MyoD and $p21^{Cip1}$ at the 48-h time point may explain why 32-month old MPCs displayed prolonged BrdUrd incorporation period and delayed exit from the cell cycle compared to 1- month old MPCs.

Markers of differentiation in MPCs from skeletal muscles of 1- and 32-month old rats

To determine whether differences in timing of cell cycle withdrawal from proliferation would alter induction of differentiation, levels of MCK and myogenin mRNA (markers of differentiation) were measured in 1- and 32-month old MPCs after 24 h exposure to differentiation media. To control for differences in proliferation and cell density, P-0 MPCs were plated at high, medium and low densities, allowed to proliferate for 36 h, then switched to differentiation media; cells were harvested after 24 h in differentiation conditions.

To test for which age group would progress most towards differentiation, prior to full differentiation and fusion into myotubes, MCK mRNA levels were found to be significantly higher in 1-month old MPCs after 24 h differentiation compared to 32-month old MPCs, regardless of the three cell plating densities. Data from medium density MPC cultures are displayed in Fig. 5a (data from high densities and low densities not shown, but results were similar to medium-density MPC cultures). On the contrary, myogenin mRNA levels were substantially higher in 32-month old MPCs at any den- sity compared to 1-month old MPCs. Figure 5b shows representative images from 1- and 32-month old MPCs seeded at medium density after 24 h in differentiation media. In addition, cells were counted from high-, medium- and low-density cultures after 36 h proliferation (immediately before switching to differentiation media). While not significant, cell counts from all 3 densities of 1-month old MPC cultures tended to be higher than those of 32-month old MPC cultures at that time point of proliferation (Table 2).

Table 2. Cell counts of 1- and 32-month-old P-0 MPCs that were plated at high (1/2 of the cells), medium (1/3) and low (1/6) densities and then allowed to proliferate for 36 h. Cell counts were performed from images taken using light microscopy (49 objective) on at least five random fields of view across the plate; fields of view must have contained at least 50 cells/cm^2 to be counted. n = 4 rats for each density/age group

Plating density	1-month-old MPC (cells/cm^2 ± SEM)	32-month-old MPC (cells/cm^2 ± SEM)	*P*-value
High	433 ± 91	227 ± 31	0.075
Medium	252 ± 52	135 ± 18	0.079
Low	121 ± 20	69 ± 12	0.065

Discussion

Several mechanisms, including satellite cell dysfunction, have been proposed to contribute to sarcopenia and impaired regrowth and regeneration observed in skeletal muscle with ageing (9–11). Satellite cells serve as a source of new myonuclei during muscle repair and growth because existing myonuclei are believed to have permanently withdrawn from the cell cycle. While discrepancies exist in the literature regarding whether systemic factors modulate satellite cell function with ageing, few studies have examined this with more than a single time point within 48 h of plating of non-passaged (P-0) MPCs, a time period when early phase events occur during *in vivo* muscle regeneration (29). In the present study, we have examined BrdUrd incorporation, and subsequent differentiation, of P-0 MPCs isolated from rapidly growing (1-month old) and growth-impaired, atrophying, sarcopenic, rat skeletal muscle (32-month old), over an acute time-course with multiple time points (18–72 h after plating), using a serum cross-over design cell culture model. The main findings of this study were: (i) No effect of serum age on MPC BrdUrd incorporation; (ii) Delayed onset of, and exit from, proliferation of MPCs isolated from 32-month old skeletal muscle compared to MPCs isolated from 1-month old skeletal muscle; (iii) Differences in BrdUrd incorporation among 1-month old, 3-month old and 32-month old individuals at given culture time points were associated with differences in p38 MAPK phosphorylation, as well as MyoD and $p21^{Cip1}$ protein expression; and (iv) Reduced levels of MCK mRNA (indication of reduced differentiation potential) in MPCs isolated from 32-month old skeletal muscle compared to MPCs isolated from 1-month old skeletal muscle, after 1-month old and 32-month old MPCs were subjected to differentiation conditions at the 36th hour post-plating. Taken together, we postulate that delayed entry into and exit from the cell cycle, observed in 32-month old MPCs may compromise their ability to respond within a given narrow time window to initiate differentiation, and thus subsequently impairing myogenic potential of old, sarcopenic skeletal muscle to support regeneration or muscle growth from atrophy.

Delayed entry into and exit from the cell cycle in MPCs from skeletal muscle of 32-month old rats

While the results from our serum cross-over experiments suggest that there is no extrinsic effect of serum age on BrdUrd incorporation in P-0 MPCs from skeletal muscles of 1-, 3- and 32-month old rats, distinct differences were evident in BrdUrd incorporation between these three ages. The results are unique due to our inclusion of 1-month old MPCs. We added the 1-month old category only after our initial investigation of groups at 3 or 32 month of age. The near similarity of 3- and 32 month old MPCs for peak BrdUrd incorporation after plating (Fig. 1) led us to question whether this similarity might be related to their both having relatively low percentages of daily changes in muscle mass. One-month old rats have higher percentage enlargement in skeletal muscle mass than 3-month old and 32-month old rats. Indeed, addition of the 1-month old rapid growth group revealed an earlier peak proliferation after plating MPCs than for growth-impaired, atrophying, sarcopenic 32-month old rats (Fig. 2), as described in more detail below.

Cells of all three age groups remained relatively inactive 18 h after plating in growth media (Fig. 3), as predicted from earlier reports (30). Assuming that the isolation process activates the MPCs to enter the cell cycle and begin proliferation (31), we observed a fairly synchronous and rapid entry into cell cycle in 1- and 3-month old MPCs 24 h after plating; however, entry into the cell cycle in 32-month old MPCs was started later. In addition, the time-course of BrdUrd incorporation in the current study from 1-month old MPCs was significantly shorter than that of 3- and 32-month old MPCs. After BrdUrd incorporation, 1-month old MPCs peaked at 24 h, and rapidly returned baseline levels by 48 h. However, BrdUrd incorporation in 3- and 32-month old MPCs remained high at 36 h and then gradually returned to baseline by 72 h. The shorter proliferative period we observed in 1-month old MPCs is similar to that reported in young animals *in vivo* by Schultz (23) and *in vitro* by Barani *et al.* (30), but our observations in 32-month old MPCs are opposite to those of Barani *et al.* (30), who reported a 28-hour proliferative period at the 7th day post-plating of muscle-derived cells from 24-month-old rats.

More recently, Lavasani *et al.* (32) demonstrated that muscle-derived stem cells isolated from young mice secrete factors that enhance proliferation and myogenic differentiation of muscle-derived stem cells from old skeletal muscle in a transwell insert co-culture system. However, these studies were executed over a much longer time frame than the current study, in cells that were passaged. Furthermore, Schultz and Lipton (33) examined numbers of progeny *in vitro* from satellite cells isolated from 6 days to 30 months of age and discovered that cells from 6- to 30-day old muscles replicated strikingly faster than cells from 3- to 30-month old muscles. These authors concluded that numbers of satellite cell progeny are inversely proportional to age, and suggested that there is clear delineation in satellite cell growth between rapidly growing and sarcopenic muscles. To the best of our knowledge, our study is the first to demonstrate differences in proliferation of non-passage MPCs isolated from very young, rapidly growing (1-month old) and growth-impaired, atrophying, sarcopenic (32-month old) skeletal muscles, of a time course within 72 h of plating.

Progressive delay in the time to peak BrdUrd incorporation after plating and its prolonged time to return to baseline for cultured non-passaged MPCs from 32-month old skeletal muscle, relative to 1-month old, demonstrates a direct relationship to age of animal, and inverse relationship to percentage increases in growth frequencies of muscle mass. For example, the longest time to peak BrdUrd incorporation and consequent return to relative baseline, was in MPCs from 32-month old skeletal muscle (Table 3). On the other hand, the shortest time to peak BrdUrd incorporation, followed by return to relative baseline was in MPCs from 1-month old skeletal muscle. Patterns for BrdUrd incorporation over the time-course were more comparable between 3-month old and 32-month old MPCs after plating than to 1-month old MPCs, suggesting importance of the environment of rapid growth status in skeletal muscle from which satellite cells were isolated. These associations led to two additional questions: (i) What changes in myogenic and cell cycle regulatory proteins might account for delays in proliferation?; and (ii) Was delayed proliferation associated with impaired differentiation of old satellite cells?

To address the first question, we examined different myogenic regulatory factors (MRFs) related to cell cycle progression and withdrawal. As myoblasts withdraw from the cell cycle and commit to differentiation, there is timely coordination of intracellular signalling with a number of MRFs (Myf5, MyoD, myogenin and MRF4) that regulate different myogenic events during differentiation. p38 MAPK plays a critical role in the ability of myoblasts to exit the cell cycle and differentiate (31). Activation of p38 MAPK is critical for muscle cells to express MyoD (34); further p38 MAPK activation also serves to guarantee complex and timely activation of muscle differentiation (34). Here, for the first time, under our experimental conditions, we report the novel finding that delayed p38 MAPK activation is associated with delayed expression of MyoD protein in 32-month old MPCs, compared to 1-month old MPCs.

Cyclin-dependent kinase inhibitors, $p21^{Cip1}$ and $p27^{Kip1}$, play a critical role in regulation of cell cycle progression through the G_1-S checkpoint. It has been suggested that $p27^{Kip1}$ acts as the trigger to initiate myoblast exit from the cell cycle, while $p21^{Cip1}$ maintains cell cycle arrest and progression to a post-mitotic state, and subsequent differentiation (35). Inhibiting $p27^{Kip1}$ and $p21^{Cip1}$ expression prevents the ability of myoblasts to withdraw from the cell cycle (36) and overexpression
of them induces G1 cell cycle arrest (37). Furthermore, upregulation of MyoD induces blockade of cell cycle progression, as shown by the ability of MyoD to induce $p21^{Cip1}$ expression (38). It has been reported that MyoD protein in satellite cells from old skeletal muscle is higher (39), lower (40) or is the same (10) compared to young. Despite discrepancies between relative expression of MyoD of young and old satellite cells, MyoD is

Table 3. Associations of age, changes in skeletal muscle mass, characteristics of satellite cell proliferation, peak BrdUrd incorporation and return of BrdUrd incorporation to baseline (taken from Fig. 3)

			Plating time for	
Age	Changes in growth and skeletal muscle mass	Characteristics of satellite cell proliferation	Peak BrdUrd incorporation	Return to baseline BrdUrd incorporation
1-month	Rapid growth	Active	24 h	48 h
3-month	Slower growth	Relatively quiescent	24–36 h	72 h
32-month	Sarcopenic atrophy	Quiescent	36–48 h	72 h

required for differentiation of myogenic cells (41,42). Our results show that $p21^{Cip1}$ protein was lower in the 32-month old MPCs compared to 1-month olds at the 48-h time point. Interestingly, $p21^{Cip1}$ protein levels appear to be inversely proportional to proliferation at the same time point during the time-course, suggesting that lower $p21^{Cip1}$ expression may play a role in delayed exit from the cell cycle observed in 32-month old MPCs.

Taken together, these observations suggest that delayed p38 MAPK activation in MPCs from non-treated muscles leads to delayed MyoD and $p21^{Cip1}$ protein expression, thus impairing the ability of MPCs from 32-month old skeletal muscle to withdraw from the cell cycle. Based on studies in skeletal muscle regeneration, satellite cells are first activated to proliferate, then are induced to differentiate and fuse to regenerate myofibres. Signals that stimulate proliferation and induce differentiation of satellite cells, while not yet fully identified, are thought to be mutually exclusive (43). It is well known, however, that exit from the cell cycle is prerequisite to terminal differentiation and fusion of satellite cells (44). The time-course utilized in the current study and progression of signalling events observed during proliferation and exit from the cell cycle, are consistent with timing of early phase events that occur during muscle regeneration *in vivo* (29). Thus, we hypothesize that if the phenomenon of delayed MPC exit from the cell cycle pre-existed in old skeletal muscle in response to a myogenic stimulus (regeneration, impaired regrowth from atrophy and so on), responsiveness of old MPCs might be too slow to activate and thus miss that window for differentiation, resulting in impaired/incomplete muscle regeneration. While limited data support such a concept, Schuierer *et al.* (45) speculate that if reduced rates of differentiation were to occur *in vivo*, this could have serious consequences for muscle repair.

Reduced markers of differentiation in MPCs from old skeletal muscle

Given that exit from the cell cycle is required for myoblasts to differentiate and fuse into myofibres (44), we questioned whether delay in cell cycle exit observed in 32-month old MPCs was associated with less potential for differentiation. Thus, we employed a unique experimental design where P-0 MPCs were plated after isolation (as with the proliferation time-course experiments), allowed to proliferate for 36 h, and then were experimentally induced to differentiate. MPCs were allowed to differentiate for only 24 h from the 36th hour, and then cells were harvested and myogenic factors involved in differentiation were analysed. The 36-h proliferation time point was chosen as it represents a time, in our model, where 1-month old MPCs have lower proliferation by approximately one-half and 32-month old MPCs have just peaked proliferating. Furthermore, the time of 24 h differentiation was selected as it was sufficient time to detect early events of differentiation, but cells had not yet fully fused into multinucleate myotubes. As expected, 32-month old MPCs displayed lower MCK mRNA after 24 h differentiation compared to 1-month old MPCs. Myogenin is an important MRF that can bind to E-box sequences to promote expression of several muscle-specific genes, such as *MCK* (46); however, increased myogenin expression and promotion of MCK expression, based on our observation of substantially higher levels of myogenin mRNA in differentiating MPCs from old skeletal muscle, appear to be disconnected. These findings are consistent with previous reports concerning old skeletal muscle with impaired regrowth/repair (18,47,48) in which increased myogenin expression has been suggested to be a compensatory mechanism to maintain differentiation (48). Complete regeneration of muscle fibres is dependent upon differentiation and fusion of myogenic cells into functional myofibres. Previous studies have reported similar findings of reduced differentiation and myotube formation in MPCs from old skeletal muscle (18,49–51), but the study described here is the first to associate attenuated differentiation with delayed exit from the cell cycle, in P-0 MPCs from skeletal muscle of old subjects.

In conclusion, our findings of delayed p38 MAPK phosphorylation with lower MyoD and $p21^{Cip1}$ protein expressions in 32-month old P-0 MPCs may posit a causal explanation to why MPCs from old skeletal muscle display prolonged proliferation/delayed exit from the cell cycle with consequential reduced differentiation potential. Based on our observations, a possible interpretation may be that at a time during muscle regeneration, when differentiation stimuli are abundant in the muscle, old MPCs (which are still in a highly proliferative state, compared to young), are likely to be unable to respond to cues that induce differentiation before these cues subside, thus, potentially impairing muscle regeneration. While these findings enhance our understanding of the intrinsic machinery within old MPCs, future research will be necessary to determine whether mechanisms suggested in this report play a role in *in vivo* models of muscle regeneration with ageing.

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