In vitro induction of quiescence in isolated primary human myoblasts

Kirankumar B. Gudagudi [ID] · Niccolò Passerin d'Entrèves · Nicholas J. Woudberg [ID] · Paul J. Steyn · Kathryn H. Myburgh [ID]

Abstract Adult skeletal muscle stem cells, satellite cells, remain in an inactive or quiescent state in vivo under physiological conditions. Progression through the cell cycle, including activation of quiescent cells, is a tightly regulated process. Studies employing in vitro culture of satellite cells, primary human myoblasts (PHMs), necessitate isolation myoblasts from muscle biopsies. Further studies utilizing these cells should endeavour to represent their native in vivo characteristics as closely as possible, also considering variability between individual donors. This study demonstrates the approach of utilizing KnockOut[TM] Serum Replacement (KOSR)-supplemented culture media as a quiescence-induction media for 10 days in PHMs isolated and expanded from three different donors. Cell cycle analysis demonstrated that treatment resulted in an increase in G_1 phase and decreased S phase proportions in all donors ($p < 0.005$). The proportions of cells in G_1 and G_2 phases differed in proliferating myoblasts when comparing donors ($p < 0.05$ to $p < 0.005$), but following KOSR treatment, the proportion of cells in G_1 ($p = 0.558$), S ($p = 0.606$) and G_2 phases ($p = 0.884$) were equivalent between donors. When cultured in the quiescence-induction media, expression of CD34 and Myf5 remained constant above $> 98\%$ over time from day 0 to day 10. In contrast activation (CD56), proliferation (Ki67) and myogenic marker MyoD decreased, indicated de-differentiation. Induction of quiescence was accompanied in all three clones by fold change in p21 mRNA greater than 3.5 and up to tenfold. After induction of quiescence, differentiation into myotubes was not affected. In conclusion, we describe a method to induce quiescence in PHMs from different donors.

K. B. Gudagudi · N. P. d'Entrèves · N. J. Woudberg · P. J. Steyn · K. H. Myburgh (✉)
Department of Physiological Sciences, Stellenbosch University, Matieland,
Private Bag X1, Stellenbosch 7602, South Africa
e-mail: khm@sun.ac.za

K. B. Gudagudi
e-mail: gudagudik@gmail.com

N. P. d'Entrèves
e-mail: niccolo.pde@gmail.com

N. J. Woudberg
e-mail: njwoudberg@sun.ac.za

P. J. Steyn
e-mail: paul.steyn@uct.ac.za

P. J. Steyn
Department of Human Biology, University of Cape Town,
Anzio Road, Observatory, South Africa

Introduction

Postnatal adult muscle stem cells (satellite cells) reside in an inactive or quiescent state within a cellular niche between the sarcolemma and basal lamina of muscle fibers and are generally not actively proceeding through the cell cycle (Mauro 1961). Cell cycle inhibitors including cyclin-dependent kinase inhibitor p21 are highly expressed in satellite cells (Li et al. 2015). These negative regulators of the cell cycle, together with environmental cues including physical contact with the extracellular matrix (ECM), all function in concert to maintain satellite cells in a quiescent state (G_0) (Crist et al. 2012; Fuchs et al. 2004; Fukada 2011; Kitzmann and Fernandez 2001). Satellite cells are activated by mechanical stretch, the release of several cytokines, (Smith et al. 2008) and various growth factors including fibroblast growth factor (FGF) and hepatocyte growth factor (HGF) as reviewed by Kuang et al. (2008). These factors also promote entry into the cell cycle. The subsequent fate of satellite cells is largely controlled by muscle regulatory factors (MRFs) including MyoD and Myf5 (Asakura et al. 2007; Gayraud-Morel et al. 2007; Megeney et al. 1996; Olson 1990; Sabourin et al. 1999). These myogenic regulatory factors (MRFs) are differentially expressed by active satellite cells, and this determines their progress through different stages in the myogenic path (Boldrin et al. 2010). In order to preserve the population of satellite cells, despite rapid proliferation, some satellite cells can return to a state of quiescence in a tightly controlled process called self-renewal (Fukada 2011; Sacco et al. 2008) for which the MRF Myf5 plays an important role (Gayraud-Morel et al. 2007; Fukada 2011; Liu et al. 2013).

In a study where continuous Bromodeoxyuridine (BrdU) administration was used to label proliferating satellite cells, in growing rats soleus muscles, data revealed that 80% of the satellite cells readily entered the cell cycle while the other 20% of satellite cells entered the cell cycle at a much slower rate (Schultz 1996). In line with these findings, the long-term self-renewal abilities were retained in slow-dividing satellite cells, whilst fast dividing cells proceeded rapidly to differentiation (Ono et al. 2012). Further, donor to donor variation in terms of primary cell characteristics can be maintained after ex vivo isolation and expansion (Detela et al. 2018; Siddappa et al. 2007). Such variability suggests that studies aiming to investigate exogenous treatments should first synchronise the harvested cell population.

Isolation of primary human myoblasts (PHMs) from muscle biopsies is a well-established technique (Agley et al. 2015; Spinazzola and Gussoni 2017). However, prior to their use for experimental purposes, a crucial step is the expansion of these isolated cells, requiring extensive in vitro stimulation of proliferation. Therefore, following expansion, it may be necessary to manipulate myoblasts to re-enter a quiescent state. Some previous studies have considered the synchronisation of cell cycle of myoblasts prior to experimentation and current strategies employ serum deprivation to promote cell cycle synchronisation (Ashihara and Baserga 1979). Whilst this is an important consideration in all in vitro studies using mammalian cells, satellite cells reside in a specific niche where quiescence is tightly controlled in vivo, therefore in vitro experiments it would be more appropriate to induce synchronisation specifically in this basal condition.

Serum is an important part of in vitro culture. In myoblast culture, serum reduction tends to inhibit proliferation while promoting differentiation. Nonetheless, knockout serum has been utilized previously in in vitro culture to promote cell survival (Ishii et al. 2015) and in embryonic cultures (Xiang et al. 2019), for example Ishii et al. (2015) demonstrated the use of Knockout serum in a study devoted to cell survival and Xiang et al. (2019) employed Knockout serum in post-warming recovery of blastocyst. However, the concept of employing KOSR to induce quiescence in vitro in PHMs extracted and cloned from adult muscle is a novel approach. In the current study, KOSR was proposed as a supplement to negate influence towards differentiation despite serum deprivation. It was further proposed that over time, KOSR would induce quiescence.

The aims of the current study were to establish a model of induced quiescence in PHMs by assessing markers of stemness, myogenic commitment and proliferation and differentiation over a period of 10 days. Due to individual variability, we considered

cells isolated and expanded from three different volunteers, to determine if the technique not only induced quiescence but also synchronised myoblasts from different individuals to the extent that all clones entered a predominantly quiescent state characterized using cell cycle analysis. To test for subsequent myogenic capacity, cells were then induced to differentiate.

Materials and methods

Human myoblast isolation

The isolation and expansion of PHMs from young healthy male subjects, (KH3, KH1 and S6.3) aged between 22 and 27 years were performed using the micro-explant technique (Smith et al. 2008). Subjects gave written permission for the use of the biopsy samples and the study was approved by the human Ethics Committee of Stellenbosch University (HREC N12/08/051). Briefly, needle biopsy procedures (5 mm trephine biopsy needle with assisted suction) on the vastus lateralis of the subjects were carried out to harvest approximately 50–100 mg of skeletal muscle. The muscle sample was immediately transferred to a harvesting $1 \times$ phosphate buffered saline (PBS) solution containing 10% (v/v) penicillin/streptomycin (P/S) (Sigma-Aldrich, P43333) and 1% (v/v) gentamicin (GIBCO^{TM}, 15750-060). Muscle samples were then removed from the harvesting PBS and washed with 1x PBS before being transferred into entactin-collagen IV-laminin (ECL) (Merck, USA, 08-110), coated 96-well tissue culture plates. Individual muscle pieces were then transferred 96-well plates containing proliferation media [Ham's F-10 Nutrient Mixture Medium (Sigma-Aldrich, N6908), supplemented with 20% (v/v) foetal bovine serum (FBS) (Life Technologies, 10499-044), 1% (v/v) penicillin/streptomycin (Sigma-Aldrich, P43333) and 10 ng/mL recombinant fibroblast growth factor-2 (rh-FGF2) (Promega, G5071)]. The biopsy samples were removed from the culture dish 14 days post transfer and then transferred to a coated well for further expansion. Cells were then cryo-preserved in liquid nitrogen for long term storage at a p0 state/stage (Steyn et al. 2019). As previously published, in our hands this myoblast isolation method resulted in 93.4 ± 1.71% Pax7 cells. Upon defrost for the current

studies, cells were reestablished in culture and myogenic lineage was shown by Myf5 in over 99%.

Myoblast cell culture

Myoblasts were cultured as described before (Steyn et al. 2019)The proliferation media consisted of Ham's F-10 Nutrient Mixture Medium (Sigma-Aldrich, N6908), supplemented with 20% (v/v) foetal bovine serum (FBS) (Life Technologies, 10499-044), 1 (v/v) penicillin/streptomycin (Sigma-Aldrich, P43333) and 10 ng/mL fibroblast growth factor-2 (Promega, G5071). CD34 was used to assess stemness on day 0 and proliferation assessment was carried out using Ki67. CD56 was used as an activation marker and Myf5, MyoD were used to assess myogenic regulation and at day 0.

Induction of cellular quiescence

To stimulate PHMs to return to a quiescent state, FBS was removed from the culture media and substituted with a synthetic, growth factor free serum replacement known as KnockOut^{TM} Serum Replacement (KOSR) (Life Technologies, 10828-010). The resulting media was termed quiescence-induction media and contained Ham's F-10 Nutrient Mixture Medium (Sigma-Aldrich, N6908), supplemented with 20% (v/v) KnockOut^{TM} Serum Replacement (KOSR) (Life Technologies, 10828-010) and 1% (v/v) penicillin/streptomycin (Life Technologies, 15140-122) Cells were cultured for 10 days in quiescence-induction media, with daily media changes, to clear pro-proliferative secretions, in order to induce cellular quiescence. The assessment of the same markers as day 0, was done on days 5 and 10 (see Table 1).

Cell cycle analysis

Cells in both proliferating and quiescence-induction media (at day 10) were trypsinised, pelleted and cell cycle dynamics were quantified using the BD Cycletest^{TM} Plus DNA Reagent Kit (BD Biosciences, 340242), which utilizes propidium iodide staining of DNA. Following staining according to kit specifications, cells were filtered through a 50 μm nylon mesh and immediately analysed using a flow cytometer (BD FACSAria^{TM} Cell Sorter, BD Biosciences, 95133) to determine the DNA ploidy. Proportions of cell cycle

Table 1 Summary of quiescence media-induced changes in expression of cell surface and nuclear markers from day 0 before KOSR treatment to day 10 as shown in Figs. 1, 2, 3

Phenotype markers	Days					
	0		5		10	
	Cells counted	% (+)	Cells counted	% (+)	Cells counted	% (+)
CD34	171,894	99.7	175,577	98.3	204,850	98.6
CD56	44,520	25.8	34,034	19.0	4456	2.1
Myf5	171,977	99.8	176,171	98.6	206,344	99.4
MyoD	34,642	20.1	25,252	14.1	19,418	9.3
Ki-67	145,691	84.5	97,847	54.8	95,643	46.1

fractions, G_1, S and G_2 were quantified with ModFitL 3.0 (Verity Software).

mRNA analysis

The StepOnePlus^TM Real-Time PCR system with SYBR Green dye was used to perform RT-qPCR. Triplicates (one set) per sample with one set of positive, negative and blank controls were loaded with every plate. Primers included: GAPDH (sense), 5′-TGGT GCTGAGTATGTCGTGGAGT-3′, and (antisense), 3′-AGTCTTCTGAGTGGCAGTGATGG-5′; Myf5 (sense), 5′-AATTTGGGGACGAGTTTGTG-3′, and (antisense), 3′-CATGGTGGTGGACTTCCTCT-5′; MyoD1 (sense), 5′-TGCACGTCGAGCAATCCAAA-3′ and (antisense), 3′-CCGCTGTAGTCCATCATGCC; MKI67 (sense), 5′-TGACCCTGATGAGAAAGCTCA A-3′ and (antisense), 3′-CCCTGAGCAACACTGTCTT TT-5′; and p21 (sense), 5′-TCTTGTACCCTTGTGCCT CG-3′ and (antisense), 3′-ATCTGTCATGCTGGTCT GCC-5′. Program temperature and time settings are described in Supplementary Table 1. RT-qPCR data was analyzed using delta delta Ct method ($\Delta\Delta$ct) to quantify fold change between control and test sample. GAPDH was used as a normalizer.

Imaging of differentiation

The myogenic capacity of PHMs after quiescence treatment was tested. PHMs were induced to differentiate after culture in quiescence-induction media to test myogenic differentiation capacity. To induce differentiation, cells were cultured in Dulbecco's modified Eagle's Medium (Sigma-Aldrich, D5671), supplemented with 2% horse serum (Life Technologies, 26-050-088) and 1% penicillin/streptomycin (Life Technologies, 15140-122). Staining of the differentiated PHMs was performed with Hoechst (ThermoFisher

62249) nuclei stain in blue and Cell tracker cytoplasmic (ThermoFisher, C2925) stain in green. Imaging was performed on Nikon (ECLIPSE E400). Cell tracker was used as a cytoplasmic stain to assess elongation of cells and together with Hoechst stain.

Antibody staining of PHMs and Flow cytometry

PHMs before KOSR and treated cells were fixed with 4% (v/v) for 15 min at room temperature and treated as described before (Steyn et al. 2019). The BD FACS Aria system was used. Triplicates (one set) per sample were analysed. The details of the antibodies used are provided in the Supplementary Table 2.

Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). All statistical analysis was performed using Statistica (Version 13.2, Dell Inc, 2016). Two-way repeated measures analysis of variance was used to compare the effects of myoblast origin (human donors) and the quiescent versus proliferative states. Values for quiescent cells were calculated as fold-change from proliferation condition and then comparisons of Ki67, Myf5 and p21 mRNA, one-way analysis of variance was used to compare myoblast origins. These tests were followed by Fischer post hoc testing. $p < 0.05$ was deemed statistically significant.

Results

Time dependent effect of quiescence-induction media on PHMs phenotypic markers and cell cycle

During quiescence induction the expression of markers of stemness in quiescent satellite cells (CD34),
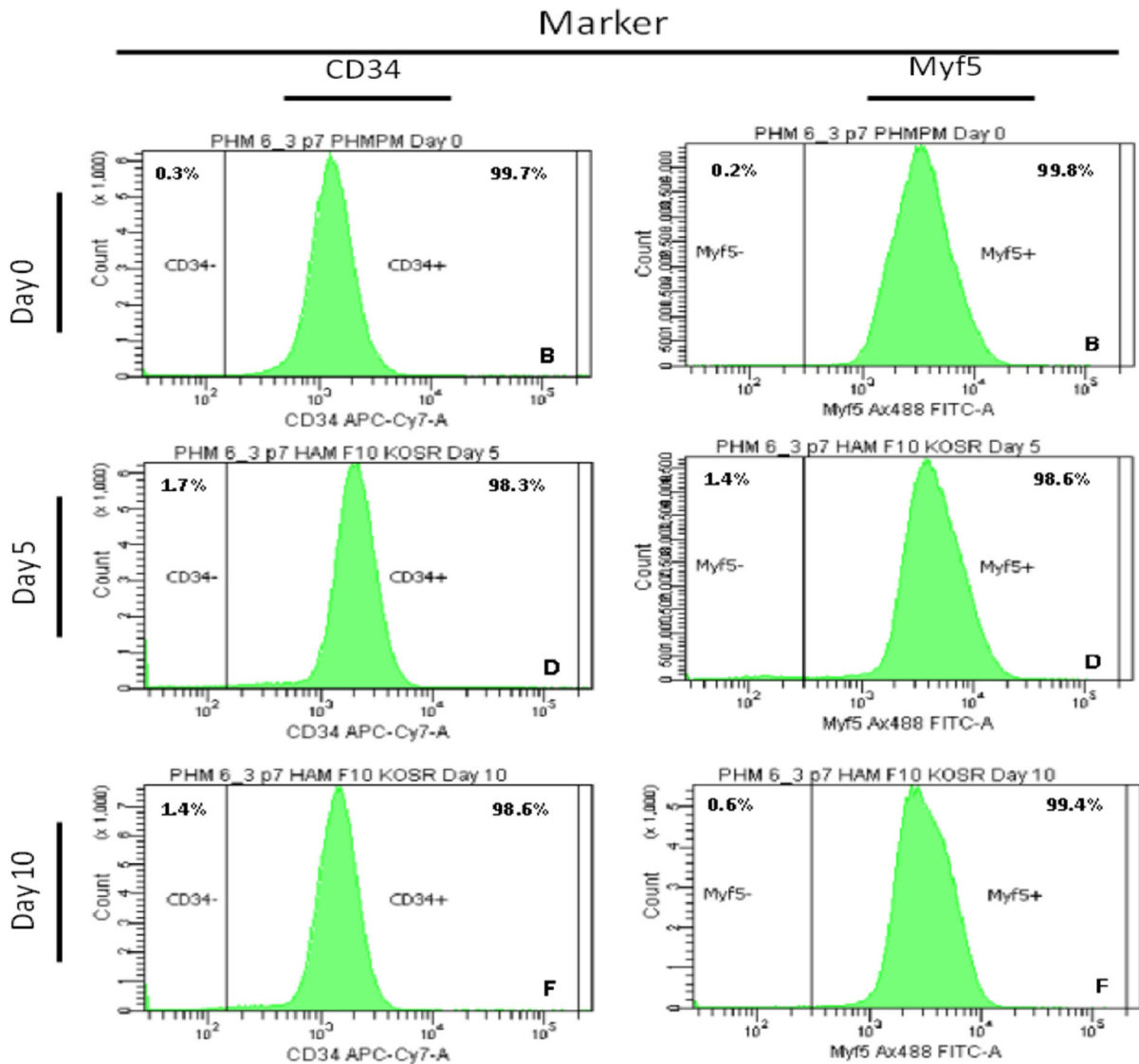
Fig. 1 Representative images of CD34 and Myf5 expression in PHMs over a 10 day culture period in quiescence-induction media. Cells were plated into a T75 tissue culture flask. **a** Day 0 expression of CD34 and Myf5 in PHMs cultured in quiescence-induction media. **b** Day 5 expression of CD34 and Myf5 in PHMs cultured in quiescence-induction media. **c** Day 10 expression of CD34 and Myf5 in PHMs cultured in quiescence-induction media. The culture media were changed daily, and cells were washed once with 1× PBS. A minimum of 1×10^5 satellite cells were analysed for each time point. Data analyses were performed on the FACSDiva™ software v6.1.3

myogenic commitment markers (Myf5 and MyoD), and proliferation marker (Ki67) in PHMs were evaluated over 10 days. On day 0, stemness marker CD34 and myogenic regulatory factor Myf5 were both > 98% in expanded PHMs (Fig. 1). Proliferation marker Ki67 was also high, > 80%, in expanded PHMs on day 0 (Fig. 2). In contrast, the cell surface activation marker CD56 and pro-differentiation marker MyoD were expressed in 26% and 20% of the cells analysed, respectively (Fig. 1). CD34 and Myf5 remained elevated from day 0 to day 10. These data show that PHMs that were induced to quiescence all maintained the expression of the marker of stemness (Fig. 1; Table 1). The proportion of cells expressing CD56, Ki67 and MyoD decreased from day 0 to day 5 and from day 5 to day 10 (Fig. 2, Table 1).
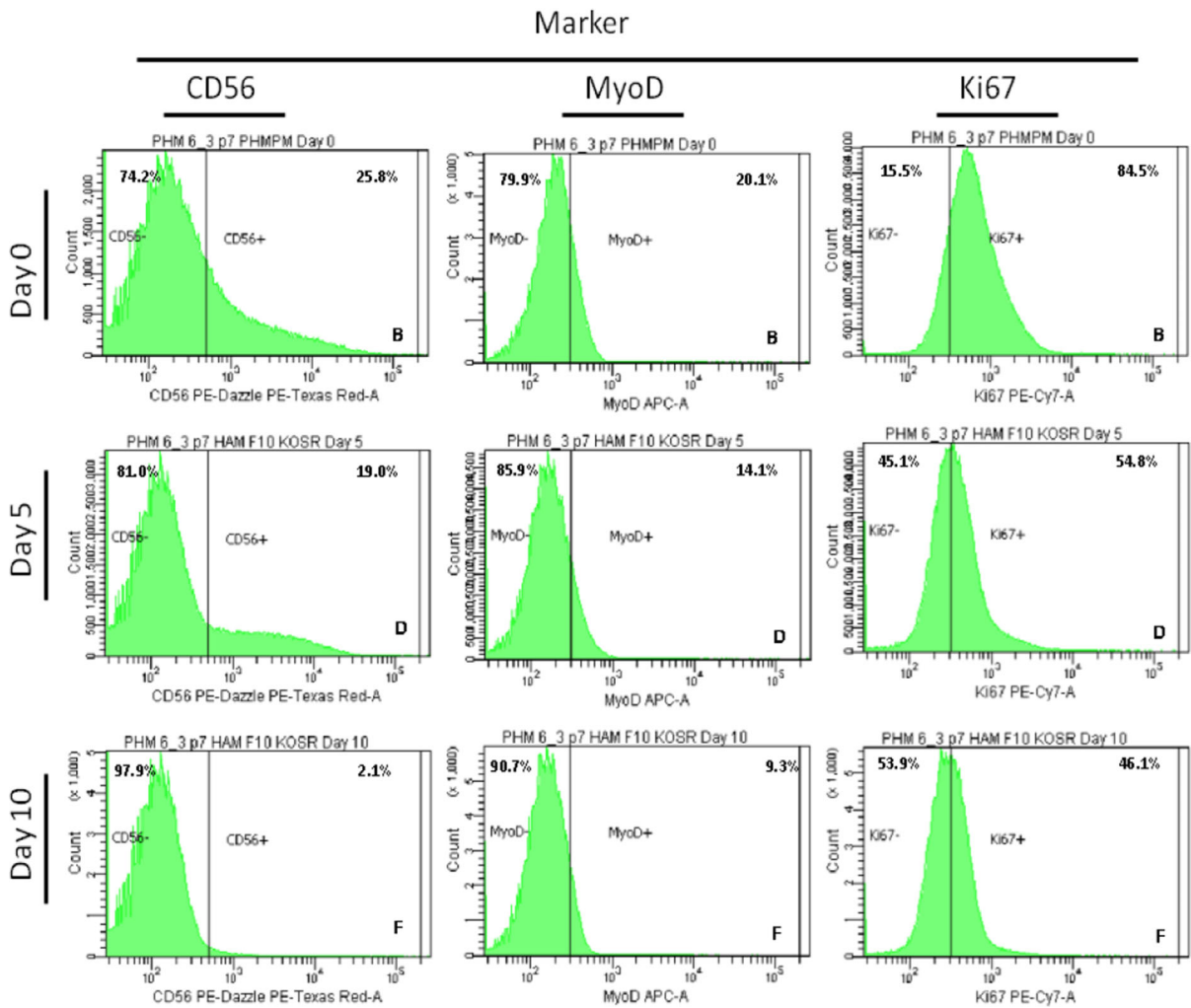
Fig. 2 Representative images of CD56, MyoD and Ki67 expression in PHMs over a 10 day culture period in quiescence-induction media. Cells were plated into a T75 tissue culture flask. **a** Day 0 expression of CD56, MyoD and Ki67 in PHMs cultured in quiescence-induction media. **b** Day 5 expression of CD56, MyoD and Ki67 in PHMs cultured in quiescence media. **c** Day 10 expression of CD56, MyoD and Ki67 in PHMs cultured in quiescence-induction media. The culture media were changed daily, and cells were washed once with 1x PBS. A minimum of 1×10^5 satellite cells were analysed for each time point. Data analyses were performed on the FACSDiva™ software v6.1.3

These results mean that PHMs were being deactivated as they moved towards quiescence (Table 1). PHMs cultured in quiescence-induction media highly expressed Myf5 but expressed low levels of MyoD (Fig. 2; Table 1). High expression of Myf5 suggested that quiescent-induced PHMs maintained the ability to differentiate into myogenic cells should then subsequently be activated. Decrease in MyoD expression over the 10 days confirms a reversal of commitment to myogenic differentiation, meaning cells were being inducted into quiescence. PHMs cultured in quiescence-inducing media also decreased the initial high levels of Ki67 over time (Fig. 2; Table 1), confirming loss of proportion of proliferative cells over time, suggesting induced quiescence. To substantiate loss of proliferation, cell cycle analysis of PHMs incubated in quiescence-induction media showed decreased cells in S phase over the 10 days of incubation (Fig. 3). Between day 0 and day 3, proportion of cells in G_1 phase did not change. However, proportion of cells in G_2 phase declined, resulting in more cells in S phase (Fig. 3). Decrease in
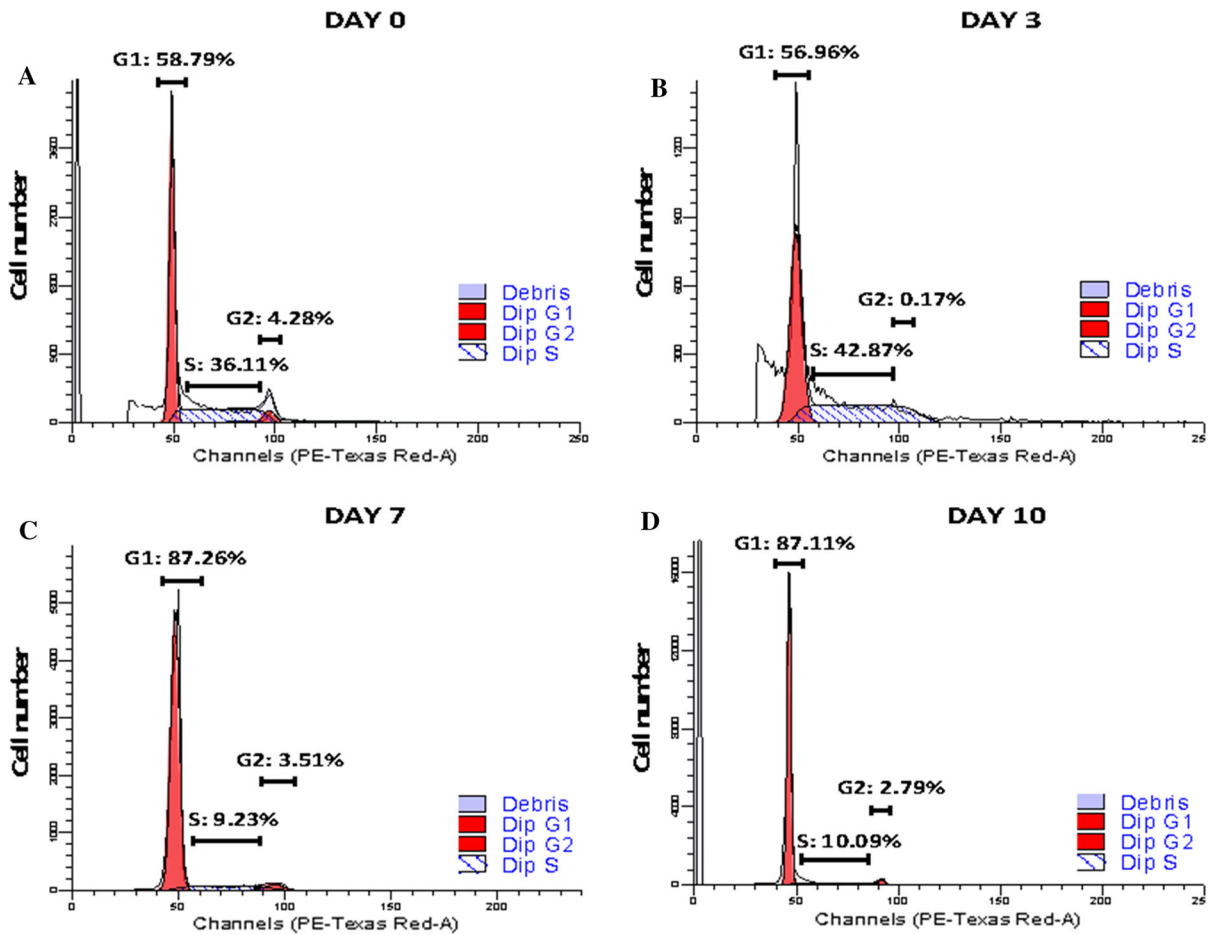
Fig. 3 Representative images of cell cycle analysis of PHMs cultured in quiescence-induction media for 10 days. Single cells were selected based on the size (FSC-A) and granularity (SSC-A). The propidium Iodide (PI) stained (PE-Texas Red-A) nuclei of the PHMs were analysed for cell cycle proportions on day 0, 3, 7, and 10 by the ModfitLT 3.0 software. The software assumes a Gaussian distribution (bell-shaped curve). The G_0/G_1 Proportion is first identified by the peak with single copies of DNA (*n*) and shows lower PI staining (around 45). The S phase ranges from 45 to 90, as cells will contain a range of DNA from *n* to *2n*. Cells in G_2 will have a peak double to that of G_1 as they have diploid nuclei (*2n*)

cells in S phase was coupled to increase in cells in G_0/G_1 phase, clearly demonstrating that there was G_0/G_1 phase cell cycle arrest (Fig. 3). From day 3 to day 7 there was a large increase in cells in G_1 phase, which did not increase further from day 7 to day 10. Overall, the expression of quiescent cell surface markers and nuclear factors point to PHMs induced into quiescence over time.

Induction of quiescence synchronises cell cycle phase in isolated myoblasts

Myoblasts isolated from three separate donors were first cultured in standard proliferation media in order to expand the line. Thereafter, cells were induced to enter quiescence for 10 days as above. Cells from both proliferation and quiescence-induction media were then extracted and analysed using flow cytometry to quantify the cell cycle dynamics. The proportions of each of the donor myoblasts lines in G_0/G_1, S and G_2 phases in both proliferation and quiescence-induction media is presented (Fig. 4). Representative spectrograms of cell cycle analysis are shown in Supplementary Figure 1. During proliferation, the three donor cell lines differed in proportions in both G_0/G_1 and G_2 ($78.6 \pm 2.8\%$ vs $84.9 \pm 1.3\%$ vs $75.1 \pm 1.4\%$, $p = 0.017$ and $8.0 \pm 1.6\%$ vs $4.2 \pm 1.0\%$ vs $12.1 \pm 0.7\%$, $p = 0.003$ for KH3, KH1 and S6.3
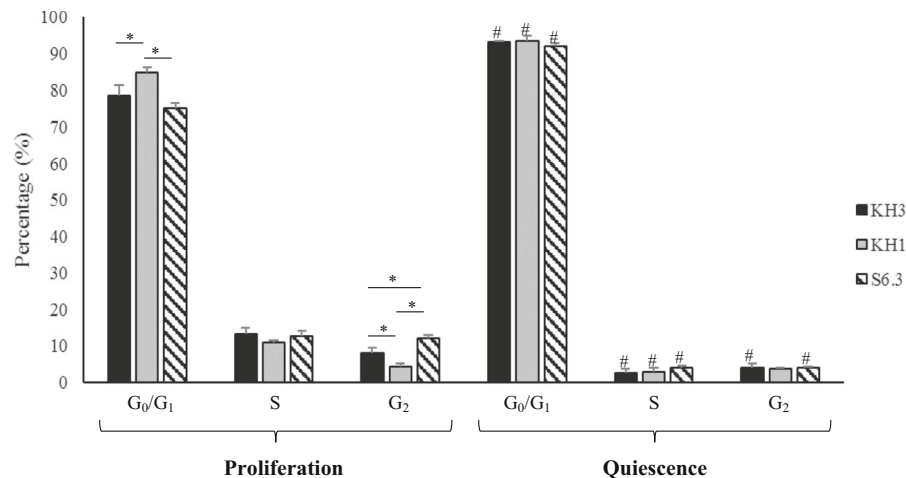
Fig. 4 Effect of proliferation media and quiescence-inducing media on cell cycle dynamics of PHMs. Myoblasts isolated from muscle biopsies (KH3, KH1 and S6.3) were cultured in proliferation media prior to induction of quiescence in Ham's F-10 Nutrient Mixture Medium with KnockOut[TM] Serum Replacement (KOSR). Propidium iodide staining of myoblast nuclei was analysed for cell cycle proportions using ModfitLT

3.0 software. The G_0/G_1 proportion corresponds to single copies of DNA (n); S corresponds to a range of n–2n and G_2 corresponding to diploid nuclei (2n). Results represent mean $\pm$ SEM (n = 4 replicates for each). *$p < 0.05$ comparing differences between donors while #$p < 0.05$ comparing quiescent versus corresponding cell cycle proportion in proliferating cells

respectively, Fig. 4). There was no difference in the proportion of myoblasts in the S phase between donors (13.3 $\pm$ 1.6% vs 10.8 $\pm$ 0.8% vs 12.8 $\pm$ 1.3%, p =0.371 for KH3, KH1 and S6.3 respectively, Fig. 4).

Importantly, in myoblasts isolated from each donor, quiescence-induction media resulted in a significant shift in cell cycle dynamics compared to the same donor cells in proliferation media. Quiescence-induction media treatment resulted in an increase in G_1 phase proportions (78.6 $\pm$ 2.8% vs 93.1 $\pm$ 0.5% for KH3; 84.9 $\pm$ 1.3% vs 93.5 $\pm$ 1.4% for KH1 and 75.1 $\pm$ 1.4% vs 92.1 $\pm$ 0.7% for S6.3, $p < 0.001$ for all donors, Fig. 4). Quiescence-induction media treatment resulted in a decrease in S phase proportions (13.3 $\pm$ 1.65 vs 2.7 $\pm$ 0.9% for KH3; 10.8 $\pm$ 0.8% vs 2.9 $\pm$ 1.2% for KH1 and 12.8 $\pm$ 1.3% vs 4.0 $\pm$ 0.7%, for S6.3, $p < 0.005$ for all donors, Fig. 1). Finally, quiescence-induction media treatment resulted in a decrease in G_2 phase proportions for KH3 and S6.3 donors, but no difference for KH1 (8.0 $\pm$ 1.6% vs 4.2 $\pm$ 1.1% for KH3; 4.2 $\pm$ 1.0% vs 3.7 $\pm$ 0.4% for KH1 and 12.1 $\pm$ 0.7% vs 3.9 $\pm$ 0.2%, for S6.3, $p < 0.001$, $p = 0.678$ and $p < 0.001$, respectively, Fig. 4). KH1 was already quite low in proportion of cells in G_2 in proliferation media.

Whilst there were differences in cell cycle dynamics with differing proportions of G_1 and G_2 phase in proliferating myoblasts, induction of quiescence resulted in synchronisation of cell cycle phases when comparing the three myoblast lines. The proportion of cells in G_1 phase ($p = 0.558$), S phase ($p = 0.606$) and G_2 phase ($p = 0.884$) were equivalent between donors.

mRNA levels of myogenic and cell cycle regulatory factors in quiescent myoblasts

Following the induction of quiescence over 10 days, isolated myoblasts were harvested and the expression levels of cell cycle and MRFs were quantified and expressed relative to proliferation (see Fig. 5). Inspection of fold changes comparing the different myoblast lines indicated that positive and negative fold changes occurred. Fold change in Ki67 mRNA indicated similar reduction in two myoblasts lines (KH1 and S6.3) with quiescence-induction media reducing Ki67 fold change to below 1. For one myoblast line (donor KH3) there was a twofold increase. p21 mRNA levels were elevated in KH1 and S6.3 myoblast lines, about fourfold. Despite this increase in mRNA of proliferation marker Ki67 (donor KH3) which could suggest quiescence was not induced, the mRNA of cell cycle inhibitor p21 were greatly elevated by tenfold. Which
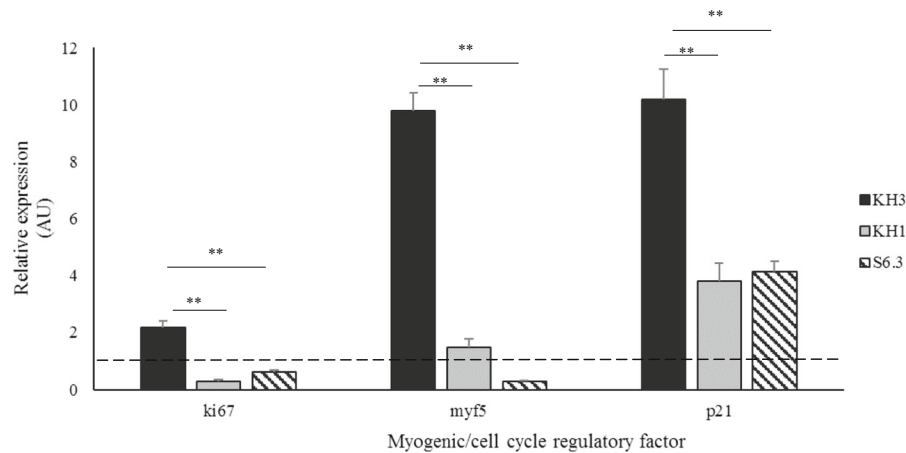
Fig. 5 Expression of myogenic and cell cycle regulatory factors PHMs. Myoblasts isolated from muscle biopsies (KH3, KH1 and S6.3) were cultured in proliferating media prior to induction of quiescence in Ham's F-10 Nutrient Mixture Medium with KnockOut™ Serum Replacement (KOSR). Cells in induced quiescence were harvested and the expression of proliferating marker (Ki67), myogenic marker (Myf5) and cell cycle inhibitor (p21) was quantified using quantitative polymerase chain reaction (PCR). Results represent mean ± SEM (n = 4). **$p < 0.005$ comparing differences between donors. Results indicate fold change—quiescence/proliferation. Dash line indicate no fold change = 1

was a significantly higher elevation compared to KH1 and S6.3. Myf5 was reduced in S6.3 and remained unchanged in KH1. In KH3 myoblasts Myf5 mRNA was significantly increased compared to the other two lines.

Induction of quiescence does not alter subsequent myogenic differentiation

It was important to ascertain that 10 days of culturing myoblasts in quiescent media didn't affect their inherent capabilities to become activated, proliferate and differentiate. Since differentiation is the final step in the myogenic path, it was elected to test the effect of quiescence-induction media on subsequent differentiation. Following induction of quiescence, myoblasts were cultured for 2 days in proliferation media, before to 5 days in differentiation media. Representative images are shown in Fig. 6a, b. Qualitative observations indicated that myoblast fusion and myotube formation occurred after 5 days (Fig. 6c). These data suggested that prolonged culture of PHMs in quiescence-induction media did not affect their subsequent ability to differentiate into myotubes, when exposed to pro-differentiation conditions.

Discussion

Isolated cells such as primary human myoblasts have been used in in vitro studies to investigate quiescence, myogenesis and other muscle related processes over time and in response to a variety of factors added to culture media. These studies have provided significant insights into muscle physiology and associated pathological conditions. However, conflicting data exist and this has partly been attributed to cellular heterogeneity and partly to duration of culture in the pre-experiment conditions. In the present study, we aimed to establish a model of cellular quiescence in PHMs isolated from biopsies taken from different individuals. Successful induction of quiescence in isolated PHMs would allow scientist to use a pool of cells homogenous for cell cycle phases in in vitro experiments. A successful model would need to satisfy two primary requirements: (1) Induction of quiescence in a reproducible manner which did not compromise the myogenic phenotype of isolated cells and (2) In vitro synchronisation of cells isolated from different individuals. The method/protocol we describe in this manuscript fulfils both criteria (see Table 2).
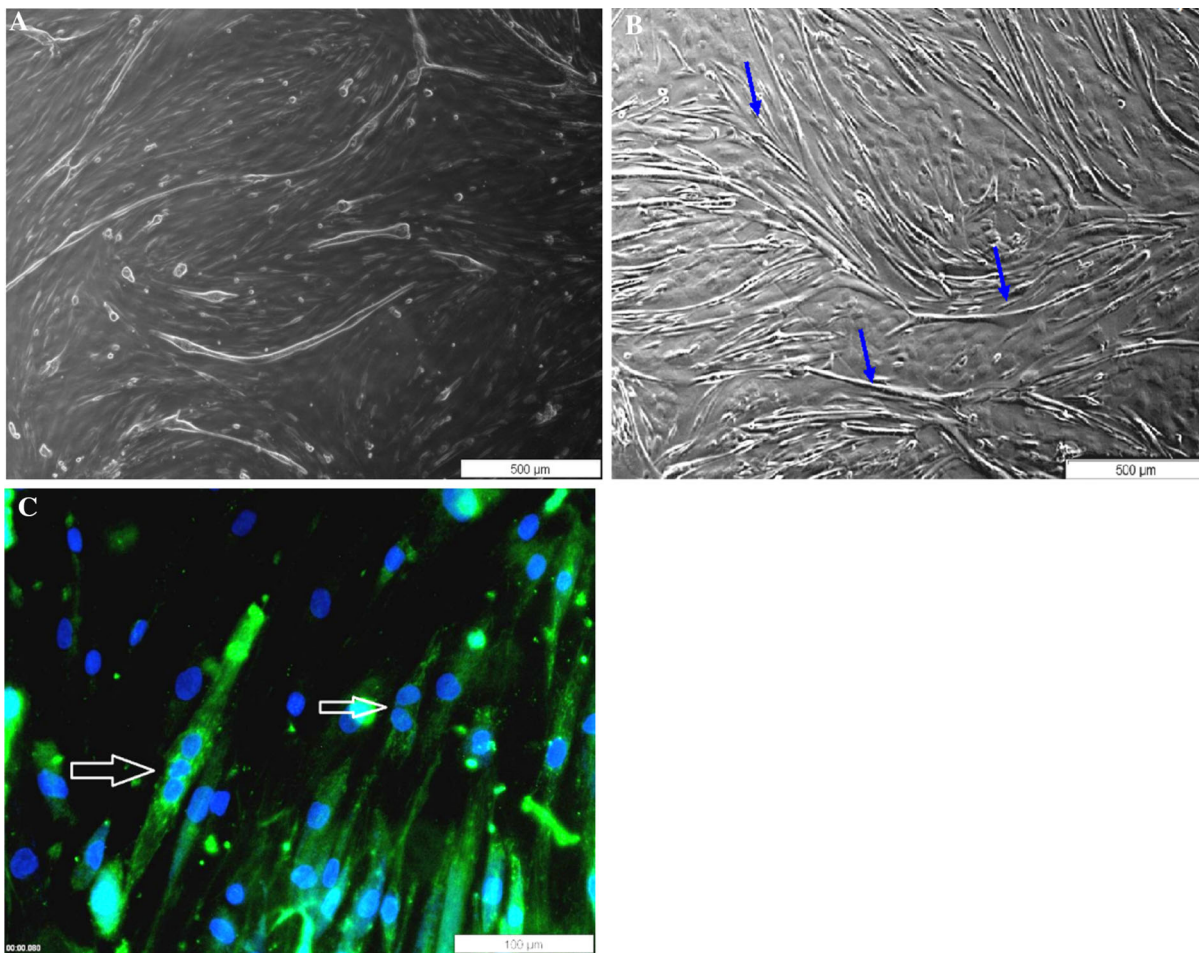
Fig. 6 Representative images of myoblasts cultured in differentiation media after 10 days in quiescence-induction media. Cells were plated into 6-well tissue culture plates (35 mm) and cultured for 10 days quiescence-induction media, followed by a 5 day culture in differentiation media. For fluorescence microcopy, cells were stained with Hoechst nuclear stain (blue) and cell tracker cytoplasmic stain (green). Representative image of myoblast morphology using Brightfield day 2 (**a**) and day 5 (**b**) fluorescence microscopy and image (**c**) white arrows representing multinucleated myotubes with Blue (nucleus) and green (cytoplasm)

Table 2 Trend of quiescence media-induced changes in expression of cell surface and nuclear markers over 10 days as shown in Figs. 1, 2, 3

Marker	Trend of gene expression over 10 days	Effect
CD34	Remained high	Induction into satellite cells
Myf5	Remained high	Induced quiescent cells are committed to myogenic differentiation-maintain ability to differentiate into myogenic cells
MyoD	Low and decreased over time	Loss of commitment to myogenic differentiation
Ki67	Initially high but decreased over time	Induced quiescent cells losing proliferative abilities—cells moving towards quiescence
CD56	Low and decreased over time	Deactivation of PHMs due to induction of quiescence

It is also vital to consider that while myoblasts display equivocal cell cycle proportions, expression of myogenic markers may still differ. Our method/protocol shows that induction of quiescence synchronised cell cycle phase in the cultured cells. Whilst there are some differences in the expression of some markers, overall our quiescence induction protocol was able to direct isolated PHMs towards quiescence as shown by the decrease in myogenic and proliferation markers and increase in cell cycle inhibitor p21. Differences in expression of some markers could possibly result from differences between donors.

In vivo, cells exist in specific niches that regulate their functions via interactions with other cells, biomolecules and the extracellular matrix. Once removed from this niche, cells can be activated and proliferate differently than would be expected for the influence present in the niche. When grown in vitro, cells in culture require the use of FBS to ensure cell survival, as it contains a vast array of growth factors essential for supporting cellular processes (Brunner et al. 2010). Whilst high percentage of serum is needed to allow cells, especially primary cells, to proliferate and grow in vitro, serum can also influence specific cellular processes, skewing results of further experimental interventions in the process. Serum deprivation is a common technique in mammalian cell culture to arrest cells in specific cell cycle phases in a process termed in vitro synchronisation (Ashihara and Baserga 1979; Langan and Chou 2011). Thus, serum deprivation is commonly used in an attempt to normalize cells before doing in vitro experimentation. In the present study, serum deprivation, by substitution of foetal bovine serum with KOSR, was used not only to synchronise cells but also to induce a state of quiescence. Therefore, the aim was to establish a reproducible model which could achieve in vitro synchronisation in the G_0/G_1 state.

Different approaches have been used to induce quiescence in PHMs (Sellathurai et al. 2013; Dhawan and Farmer 1990; Dike and Farmer 1988). One such approach utilizing suspension culture techniques has been described and successfully induced isolated PHMs into a quiescent state (Dhawan and Farmer 1990; Dike and Farmer 1988; Milasincic et al. 1996; Sellathurai et al. 2013). Instead of removing mitogens and growth factors from the culture media of adherent cultures (as described above for synchronisation), Sellathurai et al. (2013) utilized a suspension culture

technique to induce cellular quiescence. This had previously been reported to efficiently arrest C2C12 myoblasts proliferation and maintain cells in a quiescent (G_0) phase (Milasincic et al. 1996). These studies employed a semi-solid suspension medium (DMEM supplemented with 10% FBS, 1% P/S and 2% methylcellulose) to maintain myoblasts in suspension. Substantial loss of cell–cell interaction triggers the 'suspended' myoblasts to become quiescent (G_0) (Sellathurai et al. 2013). However, lack of attachment is not representative of the in vivo state of satellite cells as they are always in contact with the basal lamina, the sarcolemma of muscle fibres and other ECM components. In addition, for the cells to be used in experiments there is need to first re-plate on a surface, meaning that quiescent satellite cells generated via suspension culture will lose their quiescence upon attachment. In a suspension culture model, treating of cells and visualizing the phenotypic effect could be challenging. Additionally, live cell flow cytometry is not feasible with this model. Despite these limitations, the suspension protocol allows (Sellathurai et al. 2013) rapid induction into G_0. We also observed significantly higher proportion of cells in G_0/G_1 phase. The KOSR condition allowed for absence of an amorphous FBS environment, cells could be harvested easily and quickly for flow cytometry. It was clear that the protocol successfully induced quiescence in all isolated cells and similarly for 3 donors. Indeed, the adherent myoblasts in quiescence-induction media had proportion of cells in G_0/G_1 phase to above 90% and decreases in the S and G_2 phases. In addition, the proportion of cells in G_0/G_1 phase, which was different between donors in proliferation media, was synchronised in quiescence-induction media. This shift would be indicative of cell cycle arrest and entry into quiescence (Kitzmann and Fernandez 2001).

Specifically in the present study the proportion of PHMs cells in G_0/G_1 state increased 30% with 32% decrease in the proportion of S phase cells, compared to PHMs cultured in PHM-PM. Cell cycle analysis that assessed the time course supported by cell cycle flow cytometer data showing that both Ki67 and CD56 expression steadily decreased over the 10 day culture period in quiescence-induction media. This reduction in proliferation marker was not due to PHM's differentiation, as the expression of the stem cell marker, CD34, was maintained (stem cells that

differentiate loose expression of this marker). Sellathurai et al. (2013) showed that when PHMs were placed in suspension medium, levels of Ki-67 and BrdU (pulse labelling) expression steadily decreased over a 48 h period (12 h, 24 h, 48 h) and even disappeared after 96 h in suspension media (Sellathurai et al. 2013). Thus, both methodologies induce PHM quiescence effectively. Although the suspension media reduced Ki67 more than KOSR. Therefore, current study examined potential differences in further variables related to cell cycle in cells isolated from different donors. To this end, quiescent cells from the three donors were assessed for the mRNA expression of Myf5 and p21. Our data indicated, expression of p21, a cell cycle inhibitor, (Vousden and Prives 2009) was significantly increased. These data complement the cell cycle data, with both indicating that the level of actively proliferating myoblasts was reduced. With these observations, it was confirmed that using KOSR with Ham's F-10 nutrient media mixture inhibited cellular proliferation to a large extent without inducing differentiation. Specific to myoblasts, we also considered Myf5, a MRF, whose downregulation would be indicative of an undifferentiated, quiescent state, reviewed by (Olson 1990). Indeed the expression of Myf5 confirmed that PHMs cultured in quiescence induction media were induced towards/backwards to undifferentiated muscle precursor cells. Coupled to decrease in MyoD+ cells in the time-course experiment, this means that cells are withdrawing from their commitment to myogenic differentiation. We also measured selected markers to compare the state of activation and quiescence in the cells isolated from three different donors. Despite not differing in cell cycle proportions following KOSR treatment, KH1 and S6.3 had similar expression levels of Ki67, Myf5 and p21 while KH3 had higher levels for all three markers. This is in contrast to (Sellathurai et al. 2013) who demonstrated similar expression levels of Ki67, Myf5, p21 and other markers in three isolated human myoblast cultures. This may demonstrate the heterogeneity of the gene expression levels in different donor cell populations. Similarly, (De La Garza-Rodea et al. 2012) demonstrated how mesenchymal stem cells from different donors greatly differed in their differentiative capacity. Whilst a different primary cell was used, study alludes to donor-specific differences in stem cell characteristics.

For this model to be applied to studies examining the effects of exogenous factors on primary human myoblast myogenesis, it would be necessary to know that there were no adverse consequences for the myogenic phenotype. Cellular differentiation was qualitatively confirmed in cells exposed to different conditions after 10 days in quiescence-induction media. These findings indicated that treatment did not compromise the myogenic potential of isolated cells.

Limitations of study

Different methods have been used in the literature to obtain pure primary myoblasts from muscle samples. These include (Hindi et al. 2017; Shahini et al. 2018; Rando and Blau 1994). Here we used the adhesion to ECL. This is valid but does not rely on an actual myoblast marker for selection from the cell type. None of the subsequent analysis indicated > 98% Myf5+ cells. For identification of myotubes after 5 days in differentiation media (following the 10 days in KOSR) we identified morphologically elongated multinucleated cells. A myotube specific marker of early differentiation, such as desmin, would have strengthened this.

Given that we observed differences between the three donors for mRNA of key markers, it suggests that it would be interesting in future to determine variability between a larger number of donors. How in vitro primary myoblast behaviour is influenced by the in vivo satellite cell niche would require an additional aspect of comprehensive immunocytometric analysis of the same muscle biopsy from which the PHMs are isolated.

Conclusion

Using substitution of foetal bovine serum with KOSR, we have demonstrated a 10 days culture treatment regime to induce quiescence in isolated PHMs. Importantly, this protocol allows for expansion of myoblasts following isolation and induction of quiescence in this population prior to any subsequent studies. This provides a representative model in vitro of the in vivo environment. Our protocol successfully synchronised the cell cycle phases of cells isolated

from three different donors although there was individual variability in the expression of myogenic and cell cycle regulatory factors. Thus, we managed to describe a novel method/protocol which can be used by scientists to induce quiescence and generate a homogenous pool of cells in isolated PHMs before any experiments can be performed. This would be particularly useful to investigate factors influencing activation. We believe this method/protocol, as opposed to those requiring cells to be in suspension as removal from suspension will result in activation, and will be useful in experiments requiring quiescent satellite cells.

Compliance with ethical standards

Conflict of interests The authors declare no conflicts of interests.

References

Agley CC, Rowlerson AM, Velloso CP, Lazarus NL, Harridge SDR (2015) Isolation and quantitative immunocytochemical characterization of primary myogenic cells and fibroblasts from human skeletal muscle. JoVE J Vis Exp. https://doi.org/10.3791/52049

Asakura A, Hirai H, Kablar B, Morita S, Ishibashi J, Piras BA, Christ AJ, Verma M, Vineretsky KA, Rudnicki MA (2007) Increased survival of muscle stem cells lacking the MyoD gene after transplantation into regenerating skeletal muscle. Proc Natl Acad Sci 104:16552–16557. https://doi.org/10.1073/pnas.0708145104

Ashihara T, Baserga R (1979) Cell synchronization. Methods in enzymology, cell culture. Academic Press, Cambridge, pp 248–262. https://doi.org/10.1016/S0076-6879(79)58141-5

Boldrin L, Muntoni F, Morgan JE (2010) Are human and mouse satellite cells really the same? J Histochem Cytochem 58:941–955. https://doi.org/10.1369/jhc.2010.956201

Brunner D, Frank J, Appl H, Schöffl H, Pfaller W, Gstraunthaler G (2010) The serum-free media interactive online database. ALTEX Altern Anim Exp 27:53–62. https://doi.org/10.14573/altex.2010.1.53

Crist CG, Montarras D, Buckingham M (2012) Muscle satellite cells are primed for myogenesis but maintain quiescence with sequestration of myf5 mRNA targeted by microrna-31 in mrnp granules. Cell Stem Cell 11:118–126. https://doi.org/10.1016/j.stem.2012.03.011

De La Garza-Rodea AS, Van Der Velde-Van Dijke I, Boersma H, Gonçalves MAFV, Van Bekkum DW, De Vries AAF, Knaän-Shanzer S (2012) Myogenic properties of human mesenchymal stem cells derived from three different sources. Cell Transplant 21:153–173. https://doi.org/10.3727/096368911X580554

Detela G, Bain OW, Kim H-W, Williams DJ, Mason C, Mathur A, Wall IB (2018) Donor variability in growth kinetics of healthy hmscs using manual processing: considerations for manufacture of cell therapies. Biotechnol J. https://doi.org/10.1002/biot.201700085

Dhawan J, Farmer SR (1990) Regulation of alpha 1 (I)-collagen gene expression in response to cell adhesion in Swiss 3T3 fibroblasts. J Biol Chem 265:9015–9021

Dike LE, Farmer SR (1988) Cell adhesion induces expression of growth-associated genes in suspension-arrested fibroblasts. Proc Natl Acad Sci 85:6792–6796. https://doi.org/10.1073/pnas.85.18.6792

Fuchs E, Tumbar T, Guasch G (2004) Socializing with the neighbors: stem cells and their niche. Cell 116:769–778. https://doi.org/10.1016/S0092-8674(04)00255-7

Fukada S (2011) Molecular regulation of muscle stem cells by "quiescence genes". Yakugaku Zasshi 131:1329–1332. https://doi.org/10.1248/yakushi.131.1329

Gayraud-Morel B, Chrétien F, Flamant P, Gomès D, Zammit PS, Tajbakhsh S (2007) A role for the myogenic determination gene myf5 in adult regenerative myogenesis. Dev Biol 312:13–28. https://doi.org/10.1016/j.ydbio.2007.08.059

Hindi L, McMillan JD, Afroze D, Hindi SM, Kumar A (2017) Isolation, culturing, and differentiation of primary myoblasts from skeletal muscle of adult mice. Bio Protoc. 7(9):e2248. https://doi.org/10.21769/BioProtoc.2248

Ishii Y, Nhiayi MK, Tse E, Cheng J, Massimino M, Durden DL, Vigneri P, Wang JY (2015) Knockout serum replacement promotes cell survival by preventing BIM from inducing mitochondrial cytochrome C release. PLoS ONE. 10(10):e0140585. https://doi.org/10.1371/journal.pone.0140585

Kitzmann M, Fernandez A (2001) Crosstalk between cell cycle regulators and the myogenic factor MyoD in skeletal myoblasts. Cell Mol Life Sci CMLS 58:571–579. https://doi.org/10.1007/PL00000882

Kuang S, Gillespie MA, Rudnicki MA (2008) Niche regulation of muscle satellite cell self-renewal and differentiation. Cell Stem Cell 2:22–31. https://doi.org/10.1016/j.stem.2007.12.012

Langan TJ, Chou RC (2011) Synchronization of mammalian cell cultures by serum deprivation. In: Banfalvi G (ed) cell cycle synchronization: methods and protocols, methods in molecular biology. Humana Press, Totowa, pp 75–83. https://doi.org/10.1007/978-1-61779-182-6_5

Li J, Han S, Cousin W, Conboy IM (2015) Age-specific functional epigenetic changes in p21 and p16 in injury-activated satellite cells. Stem cells (Dayton, Ohio) 33(3):951–961. https://doi.org/10.1002/stem.1908

Liu L, Cheung TH, Charville GW, Hurgo BMC, Leavitt T, Shih J, Brunet A, Rando TA (2013) Chromatin modifications as

determinants of muscle stem cell quiescence and chronological aging. Cell Rep 4(1):189–204. https://doi.org/10.1016/j.celrep.2013.05.043

Mauro A (1961) Satellite cell of skeletal muscle fibers. J Biophys Biochem Cytol 9:493–495

Megeney LA, Kablar B, Garrett K, Anderson JE, Rudnicki MA (1996) MyoD is required for myogenic stem cell function in adult skeletal muscle. Genes Dev 10:1173–1183. https://doi.org/10.1101/gad.10.10.1173

Milasincic DJ, Dhawan J, Farmer SR (1996) Anchorage-dependent control of muscle-specific gene expression in C2C12 mouse myoblasts. Vitro Cell Dev Biol Anim 32:90–99. https://doi.org/10.1007/BF02723040

Olson EN (1990) MyoD family: a paradigm for development? Genes Dev 4:1454–1461. https://doi.org/10.1101/gad.4.9.1454

Ono Y, Masuda S, Nam H, Benezra R, Miyagoe-Suzuki Y, Takeda S (2012) Slow-dividing satellite cells retain long-term self-renewal ability in adult muscle. J Cell Sci 125:1309–1317. https://doi.org/10.1242/jcs.096198

Rando TA, Blau HM (1994) Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. J Cell Biol 125(6):1275–1287. https://doi.org/10.1083/jcb.125.6.1275

Sabourin LA, Girgis-Gabardo A, Seale P, Asakura A, Rudnicki MA (1999) Reduced differentiation potential of primary MyoD−/− myogenic cells derived from adult skeletal muscle. J Cell Biol 144:631–643. https://doi.org/10.1083/jcb.144.4.631

Sacco A, Doyonnas R, Kraft P, Vitorovic S, Blau HM (2008) Self-renewal and expansion of single transplanted muscle stem cells. Nature 456:502–506. https://doi.org/10.1038/nature07384

Schultz E (1996) Satellite cell proliferative compartments in growing skeletal muscles. Dev Biol 175:84–94. https://doi.org/10.1006/dbio.1996.0097

Sellathurai J, Cheedipudi S, Dhawan J, Schrøder HD (2013) A novel in vitro model for studying quiescence and activation of primary isolated human myoblasts. PLoS ONE. https://doi.org/10.1371/journal.pone.0064067

Shahini A, Vydiam K, Choudhury D, Rajabian N, Nguyen T, Lei P, Andreadis ST (2018) Efficient and high yield isolation of myoblasts from skeletal muscle. Stem Cell Res. 30:122–129. https://doi.org/10.1016/j.scr.2018.05.017

Siddappa R, Licht R, van Blitterswijk C, de Boer J (2007) Donor variation and loss of multipotency during in vitro expansion of human mesenchymal stem cells for bone tissue engineering. J Orthop Res Off Publ Orthop Res Soc 25:1029–1041. https://doi.org/10.1002/jor.20402

Smith C, Kruger MJ, Smith RM, Myburgh KH (2008) The inflammatory response to skeletal muscle injury. Sports Med 38:947–969. https://doi.org/10.2165/00007256-200838110-00005

Spinazzola J, Gussoni E (2017) Isolation of primary human skeletal muscle cells. Bio-Protoc 7:1–19. https://doi.org/10.21769/BioProtoc.2591

Steyn PJ, Dzobo K, Smith RI, Myburgh KH (2019) Interleukin-6 induces myogenic differentiation via JAK2-STAT3 signaling in mouse C2C12 myoblast cell line and primary human myoblasts. Int J Mol Sci. https://doi.org/10.3390/ijms20215273

Vousden KH, Prives C (2009) Blinded by the light: the growing complexity of p53. Cell 3:413–431. https://doi.org/10.1016/j.cell.2009.04.037

Xiang C, Jia BY, Quan GB, Zhang B, Shao QY, Zhao ZY, Hong QH (2019) Wu GQ (2019) Effect of Knockout serum replacement during postwarming recovery culture on the development and quality of vitrified parthenogenetic porcine blastocysts. Biopreserv Biobank. 17(4):342–351. https://doi.org/10.1089/bio.2018.0132