Human fetal skeletal muscle contains a myogenic side population that expresses the melanoma cell-adhesion molecule

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Muscle side population (SP) cells are rare myogenic progenitors distinct from satellite cells, the known tissue-specific stem cells of skeletal muscle. Studies in mice demonstrated that muscle SP cells give rise to satellite cells in vivo. Given that muscle SP cells are heterogeneous, it has been difficult to prospectively enrich for myogenic progenitors within the SP fraction, particularly from human tissue. Further, conditions that favor the expansion of human muscle SP cells while retaining their myogenic potential have yet to be reported. In this study, human fetal muscle SP and main population (MP) cells were purified based on the expression of melanoma cell adhesion molecule (MCAM), a marker we previously reported to enrich for cells with myogenic potential. To define the relationship between MCAM expression and the degree of myogenic commitment, single cells were analyzed for the expression of myogenic-specific markers. Myogenic factors strongly associated with MCAM expression in single cells, particularly Myf5. Different MCAM+ populations, including SP cells, were expanded and assayed for fusion potential in vitro and engraftment potential in vivo. All MCAM1 subpopulations fused robustly into myotubes in vitro, whereas the MCAM− subpopulations did not. Further, MCAM+ SP cells exhibited the highest fusion potential in vitro and were the only fraction to engraft in vivo, although at low levels, following propagation. Thus, MCAM can be used to prospectively enrich for myogenic muscle SP cells in human fetal muscle. Moreover, we provide evidence that human MCAM+ SP cells have intrinsic myogenic activity that is retained after propagation.

INTRODUCTION

Skeletal muscle possesses an impressive ability to regenerate in response to injury or chronic disease. This remarkable regenerative capacity is attributed to its resident mononuclear myogenic progenitors, referred to as the satellite cells (1–3). Satellite cells are defined by their proximal position to mature myofibers underneath the basal lamina (4) and by expression of Pax7 (5–8). Upon stimulation following skeletal muscle damage, satellite cells undergo cell division to replenish the satellite cell compartment and generate myogenic precursor cells, which in turn undergo multiple rounds of cell division (9,10). These derivatives of satellite cells or myoblasts express Myf5 and/or MyoD and can be expanded in vitro, undergo further differentiation and eventually fuse to each other to form new myofibers or fuse to existing fibers to repair damage (11,12). Myoblasts have been tested for their muscle repair ability in vivo in both mice and humans (13–16), and although safe, direct myoblast injections have proved inefficient (17–22). This inefficiency might be partially due to the sub-optimal conditions used to expand satellite cell-derived myoblasts prior to their injection. Studies demonstrated that satellite cells injected without prior separation from their niche retain a greater reparative capacity, compared with cultured myoblasts (23–25). However, recent studies have optimized culture conditions that mimic more closely the environment of the satellite cell niche and demonstrated effective repair and self-renewal capacity in vivo following expansion under these conditions (26).

Since optimization of cell-based therapy for muscular dystrophy would preferably deliver donor cells through a vascular route, progenitors different from satellite cells have also been isolated and tested in animal models via systemic delivery. These include murine muscle-derived stem cells (27,28),

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human CD133+ progenitor cells (29), human myo-endothelial cells (30), human pericytes (31) and murine skeletal muscle side population (SKM SP) cells (32–35), which are isolated by FACs on the basis of Hoechst 33342 dye exclusion (36). Murine SkM SP cells demonstrated myogenic potential in vitro when co-cultured with primary myoblasts (33,37,38). Unfortunately, SkM SP cells are heterogeneous and require further fractionation to enrich for myogenic progenitors within the population (39–41). A strategy for the enrichment of myogenic SkM SP cells was developed for adult mouse cells by selecting for the expression of ABCG2, the transporter that marks all interstitial progenitors in murine muscle, including SP cells (42), in conjunction with the cell surface markers syndecan-4 and Sca-1 (43). These ABCG2+ Sca+ syndecan-4+ cells express Pax7 and exhibit myogenic potential in vitro and in vivo (43). Unfortunately, parallel studies for the characterization of muscle SP cells from human muscles have been lagging behind. Thus far, there has been little or no indication that SP cells in human skeletal muscle have intrinsic myogenic potential and can be expanded in vitro. One problem that hindered these studies is that ABCG2 does not identify human fetal SkM SP cells (44). Therefore, alternative strategies that aid in the selection of myogenic cells within the human muscle SP fraction are desired.

Studies in our laboratory have identified melanoma adhesion molecule (MCAM) as a cell surface marker expressed by myogenic progenitors in human fetal skeletal muscle (45). MCAM is robustly expressed in proliferating myogenic progenitors in vitro, and its expression is significantly downregulated during myogenic differentiation and fusion (45). During skeletal muscle development, MCAM is expressed in somitic cells that specify the myotome (46). In addition, the conversion of mesoderm-like 10T1/2 cells into committed muscle cells by 5-azacytidine resulted in concomitant expression of MCAM and the myogenic transcription factor, Myf5 (46). However, whether MCAM and Myf5 were co-expressed in the same cell was not determined. Our previous studies in human fetal skeletal muscle showed that MCAM partially co-localizes in cells expressing Pax7 or MyoD (45), but the relationship between MCAM expression and the degree of myogenic commitment of distinct MCAM+ subpopulations (such as SP and non-SP cells) was not addressed. Furthermore, the in vivo engrafment potential of specific MCAM+ subpopulations remains unknown.

Here, we utilize MCAM to fractionate the SP, main population (MP) and Total (SP + MP) populations within human fetal skeletal muscle and assess each fraction’s myogenic potential. We clearly demonstrate that MCAM-expressing cells are myogenic, whereas MCAM− cells are not. Specifically, MCAM+ SP and MP cells express myogenic markers, such as Myf5 and Pax7, at the single cell level, whereas MCAM− SP and MP cells do not. We also observe that expanded human MCAM+ SP, MCAM+ MP and Total MCAM+ cells form myotubes in vitro, whereas the respective MCAM− fractions do not. Assessment of the engrafment potential of expanded MCAM+ fractions by intramuscular injection shows that expanded MCAM+ SP cells engraf to a greater extent in vivo than Total MCAM+ or MCAM+ MP cells, although the overall level of engrafment only provides a proof-of-principle finding and is not therapeutically significant.

Taken together, these data indicate that MCAM specifies a potent myogenic progenitor population within human fetal skeletal muscle and that these myogenic progenitors, particularly enriched in the MCAM+ SP fraction, can be expanded in vitro and retain limited engrafment potential in vivo.

RESULTS

MCAM identifies myogenic cells in human fetal skeletal muscle

Previously, we have demonstrated that MCAM-expressing cells in human fetal skeletal can co-express myogenic markers such as Pax7 or MyoD (45). To investigate how robustly MCAM expression segregates with myogenicity and to determine whether early myogenic progenitors are contained within a specific subfraction of MCAM-expressing cells, such as muscle SP cells, we isolated four groups of cells on the basis of MCAM expression and Hoechst 33342 dye uptake (Fig. 1A). In 22 human fetal samples analyzed, MCAM+ cells constituted ~35–80% of the total mononuclear cells, 19–63% of the SP and 40–81% of the MP populations; the percentage of MCAM+ cells did not trend with gestational age (Supplementary Material, Table S1).

To determine whether the expression of myogenic factors correlates with MCAM expression and whether differences in the expression of myogenic markers exist between SP and MP cells positive for MCAM, RT-PCR analyses were performed at the single cell level. Single cells from four distinct populations (MCAM+ and MCAM− SP, MCAM+ and MCAM− MP) were freshly sorted from three distinct human samples and analyzed for the expression of several genes (Fig. 1B, Supplementary Material, Table S2). As a control for the presence of a cell and RNA integrity, beta 2 microglobulin (b2M) was amplified, whereas MCAM expression was amplified as a control for FACs sort purity. Only single cells that exhibited b2M and MCAM expression when appropriate were included in this analysis. In parallel, the expression of the myogenic markers Pax7, Myf5 and MyoD were also analyzed (Fig. 1B, Supplementary Material, Table S2). Control-positive and -negative reactions were also included in each set of amplifications to ensure that all primers were amplifying properly in the presence of the proper target template and that no reagent contamination had occurred. In the MP populations, more MCAM+ cells expressed Pax7 and Myf5. In single MCAM+ MP cells, 5 out of 77 were Pax7+ (6.5%), whereas no Pax7+ cells were found in the MCAM− MP population (n = 172 cells). Approximately 40% of MCAM+ MP cells were Myf5+ (31 out of 77), whereas only 1 Myf5+ out of 172 cells analyzed was found in the MCAM− MP cells (0.6%) (Fig. 1C). Of the SP populations, the MCAM+ SP fraction exhibited the highest percentage of Pax7+ single cells (8 out of 50 cells = 16%), whereas in MCAM− SP cells only 2 Pax7+ cells were detected out of 143 cells analyzed (2 out of 143 = 1.4%). Analysis of Myf5 expression revealed that 19 out of 50 cells in the MCAM+ SP fraction expressed Myf5 (38%), which was in significant contrast to the low percentage seen in the MCAM− SP population (n = 2 out of 143 = 1.4%). Furthermore, five out of the eight MCAM+ SP cells
expressing Pax7 were also co-expressing Myf5, whereas four out of five single cells in MCAM+ MP fraction expressing Pax7 also co-expressed Myf5. None of the four populations exhibited a high percentage of MyoD-expressing cells (0% for MCAM+ SP, 2.8% for MCAM− SP, 5.2% for MCAM+ MP and 1.7% for MCAM− MP; Fig. 1D). These results indicate that although the expression of myogenic markers in single cells obtained from the same cell fraction is heterogeneous, all MCAM+ fractions are significantly more enriched in cells expressing myogenic factors than the MCAM− fractions.

Muscle SP cells can be expanded *in vitro*

Robust *in vitro* expansion of human muscle SP cells has not yet been optimized, hindering the investigation of the potential of this cell fraction in cell-based therapy studies. Thus, in an effort to find culture conditions that would support human muscle SP cell expansion, we isolated muscle SP cells by FACS and seeded equal numbers of cells (75 cells/well) in 12 different media (Fig. 2). Examples of seeded wells on the day of sorting are shown in Figure 2A. Analysis of approximately 200 wells from two separate experiments indicated that an average of 59 ± 10.5 cells were seeded per well (Fig. 2B) in each experiment.

Muscle SP cells were cultured for 10 days *in vitro* and assessed for proliferation under each medium condition, accompanied by an assessment for minimal differentiation (i.e. low or no myotube formation) (Fig. 2C and D). Propagation of unfractionated human fetal SkM SP cells was significantly higher in several media relative to 20% FBS/DMEM, which was used as a standard (Fig. 2C), while three media did not
support the proliferation of SP cells to any significant extent in vitro (Fig. 2C and D). In several media conditions, human muscle SP cells had also differentiated significantly and formed fused myotubes (Fig. 2C). Thus, under the tested conditions, StemProMSC SFM was the medium that seemed to best support the propagation of human fetal SkM SP cells with minimal differentiation, as evidenced by the lack of fused myotubes (Fig. 2C, condition 2). StemPro MSC SFM is a serum-free medium and is thus completely defined, which should allow for better consistency from culture to culture, and removes the effect of different serum lot compositions on resulting cultures.

Expression of myogenic markers is retained in propagated MCAM+ populations

We next tested whether StemProMSC SFM could support the expansion of MCAM+ and MCAM− cell fractions in vitro and whether the myogenic potential of propagated cells might be retained and/or acquired. To do this, six cell fractions (MCAM+ SP, MCAM− SP, MCAM+ MP, MCAM− MP, Total MCAM+ and Total MCAM−) were purified by FACS, plated at equal number and propagated in parallel (Fig. 3A). After 10 days, all cell populations had propagated and adhered to the plate surface, displaying an elongated...
morphology (Fig. 3A). Sufficient cell numbers of each population, including the SP populations, could easily be obtained for subsequent RT-PCR and western blot analyses.

To determine whether these cell populations retained (in the case of the MCAM+ populations) or acquired (in the case of the MCAM- populations) myogenic potential after expansion in vitro, real-time quantitative RT–PCR analyses were performed to determine the expression of the myogenic factors Pax7, Myf5, MyoD, and myogenin, as well as β2M, which was used as an internal gene control. In agreement with the single-cell data collected from freshly isolated cells, all cultured MCAM+ populations expressed myogenic markers, whereas the expanded MCAM- populations did not (Fig. 3B). Notably, the cultured MCAM+ SP population expressed higher mRNA levels of Pax7, Myf5, and MyoD than the cultured MCAM+ MP population. These results were confirmed by western blot analysis on protein lysates obtained from all six cultured populations (Fig. 3C). Thus, cultured MCAM+ cells retained the expression of myogenic proteins, whereas the MCAM- populations did not gain the expression of myogenic markers upon expansion.

**MCAM+ cells undergo myogenic differentiation in vitro**

To evaluate the ability of expanded MCAM+ cell populations, including muscle SP cells, to fuse in vitro, equal numbers (7500 cells/well in 96-well plates) of expanded MCAM+ SP, MP, and Total (SP + MP) cells were placed in differentiation medium and assessed for their ability to form myotubes. A duplicate plate was fixed and stained on Day 0 to confirm equal plating density (Fig. 4A). After 3–4 days post-induction, all three MCAM+ populations were capable of myogenic differentiation in vitro, whereas the MCAM- populations were not (Fig. 4A, Day 3). Furthermore, cultured MCAM+ SP cells fused significantly better than the cultured MCAM+ MP or Total MCAM+ cell populations (76.2 ± 1.4, 41.4 ± 12.1, and 50.1 ± 1.2%, respectively; Fig. 4A and C). These results were further confirmed by assessment of myogenin expression, which is found in differentiating myocytes and myotubes (47) (Fig. 4B and D). Specifically, 53.2 ± 2.9% of differentiated MCAM+ SP nuclei were myogenin+, whereas 43.1 ± 14.8 and 46.4 ± 4.2% of MCAM+ MP and Total MCAM+ nuclei, respectively,
were myogenin+ (Fig. 4D). In contrast, all three MCAM− populations exhibited <7% myogenin+ nuclei (6.6 ± 4.9% for MCAM− SP, 0.2 ± 0.09% for MCAM− MP and 0.7 ± 0.7% for Total MCAM−). These results indicate that expanded MCAM+ cell fractions can undergo myogenic differentiation in vitro, whereas MCAM− cells cannot. Additionally, MCAM+ SP cells appear to possess a higher fusion potential than MCAM+ MP or Total MCAM+ cells following expansion.

Since these studies indicated that MCAM expression on freshly isolated cells is a strong predictor of myogenic activity, we sought to determine whether expanded cells would also maintain this positive correlation. To do this, we assayed all expanded cell fractions for the expression of MCAM by FACS and western blot analyses. Interestingly, these assessments indicated robust MCAM levels in all six cultured populations, regardless of whether the populations were originally sorted as MCAM+ or MCAM− (Supplementary Material, Fig S1). Given that the cultured MCAM− populations did not acquire myogenic potential, these results indicate that MCAM is highly specific for myogenic progenitor cells only in primary tissue and is not indicative of myogenicity upon culture in vitro.

Expanded MCAM+ SP cells are capable of engraftment in vivo

Given that expanded MCAM+ cell fractions exhibited myogenic differentiation potential in vitro, we next assessed whether they could also engraft in vivo. In total, 100 000 expanded MCAM+ SP, MCAM+ MP or Total MCAM+ cells were injected intramuscularly into the TA of NOD/Rag1null mdx5CV mice in a solution of BaCl2 in order to damage host muscle and induce myogenic regeneration. The NOD/Rag1null mdx5CV mouse is a model of Duchenne muscular dystrophy and does not express dystrophin; furthermore, the mouse is immunodeficient as it lacks T, B and NK cells and therefore it is a suitable host to receive human donor cells (48). Four to five mice were injected for each cell fraction (cultured MCAM+ SP, MCAM+ MP and Total MCAM+). Cell fractions from two unrelated individuals were tested in independent experiments.

One month after injection, muscles were harvested and assessed for engraftment by immunostaining tissue sections with antibodies specific to dystrophin and human spectrin (Fig. 5). Successful engraftment was defined as a myofiber that co-expressed dystrophin and human spectrin. Dystrophin
and human spectrin double-positive myofibers were detected in mice injected with cultured MCAM+ SP cells (28 ± 13 myofibers; n = 4 mice), whereas significantly fewer were detected in Total MCAM+ cell-injected mice (3 ± 5; n = 4 mice) and no double-positive myofibers were observed in MCAM+ MP-injected mice (0; n = 5 mice) (Table 1). Overall, despite the substantial in vitro myogenic activity exhibited by the expanded MCAM+ fractions, the engraftment yield in vivo was disappointing and too low to be considered effective. Nevertheless, these studies support the proof of principle that human MCAM+ muscle SP cells can be expanded in vitro and are capable of engraftment in vivo at yields higher than MCAM+ MP and Total MCAM+ cells tested in parallel.

DISCUSSION

The presence of progenitor cells within adult tissues is crucial for postnatal growth and regeneration, particularly in a highly dynamic tissue such as skeletal muscle. Satellite cells are the cell type responsible for efficient repair of damaged muscle throughout postnatal life (1–3). Other progenitors with myogenic potential have been identified (49), but their role in muscle maintenance and their potential for cell therapy studies have been debated (50–52). In particular, mouse muscle SP cells first gained interest for their ability to fuse and express dystrophin, following intravenous injection in mdx mice (32,33). Studies demonstrated that muscle SP cells are present in normal proportion in Pax7 knockout mice, which exhibit severe postnatal muscle growth and lack satellite cells (5). In addition, murine muscle SP cells have been shown to generate satellite cells in vivo and express myogenic-specific factors (37,43). Altogether, these studies supported the idea that muscle SP cells could be progenitors that contribute to muscle during development, although they are inactive or quiescent in postnatal muscle. Studies on muscle SP cells have been hindered by their low frequency, heterogeneity and the inability to efficiently expand these cells in vitro, particularly from human skeletal muscle. Based on our previous observations that MCAM is expressed in myogenic progenitors within human fetal muscle (45), we hypothesized that muscle SP cells with myogenic potential might be enriched via positive selection for MCAM and Hoechst dye exclusion stain. Our analysis of single cells freshly isolated from MCAM+ SP and MP fractions demonstrates that MCAM+ SP and MCAM+ MP cells are more likely to express myogenic-specific markers than the respective MCAM− populations.

The myogenic potential of MCAM+ cells from SP and MP fractions was confirmed following cell propagation in vitro. Our culture conditions utilized a serum-free medium and did not necessitate the use of feeder cells, both of which are desirable features for large-scale expansion of cells to be used for cell therapy applications. Despite the enrichment for myogenic cells achieved via MCAM selection in both SP and MP populations, which was confirmed by the in vitro fusion studies, the purified cell fraction is still heterogeneous. For example, the expression of Pax7, Myf5 or MyoD was detected only in ~50% of single freshly isolated cells, although the remaining were indeed positive for MCAM, but negative for the expression of myogenic markers. Thus, identification of additional markers is necessary to further enrich prospectively for myogenic human muscle SP cells. Previous studies in adult murine SkM SP cells did not detect Pax7+ cells (37,38) and only a more recent enrichment based on co-expression of ABCG2, syndecan-4 and Sca-1 demonstrated strong myogenic potential of this cell fraction in vitro and in vivo (43). Unfortunately, this isolation strategy might not be adapted to isolate human fetal SkM SP cells, given that ABCG2 was not detected (44).
In addition to myogenic cells, transient expression is upregulated when quiescent satellite cells become activated (10). In developing myogenic progenitors (53–55) and its expression has also been reported in non-muscle mesoderm (56). Differences in marker consistency between species are not uncommon and it has been shown for several murine satellite cell markers, including CD34 and M-cadherin (49).

Among the myogenic markers analyzed, Myf5 expression has the highest association with MCAM expression in both SP and MP single cells. Myf5 clearly plays important roles in developing myogenic progenitors (53–55) and its expression is upregulated when quiescent satellite cells become activated (10). In addition to myogenic cells, transient expression of Myf5 has also been reported in non-muscle mesoderm (56). Thus, Myf5 expression in human fetal muscle SP and MP cells could result from a myogenic or non-myogenic developmental origin of these cells. Studies in avian somites have reported the expression of MCAM in myogenic progenitors (46). In addition, specification of the mesenchymal cell line 10T1/2 cells toward the myogenic lineage is characterized by upregulation of MCAM expression, which is also accompanied by an increase in Myf5 expression (46). Collectively, these previous studies point to the possibility that the expression of MCAM and Myf5 occur at similar times, but whether they are co-expressed in the same cells was not addressed. Our single-cell analysis demonstrates that MCAM and Myf5 are indeed co-expressed in human fetal SkM SP and MP cells, and that these cells exhibit strong myogenic potential in vitro compared with cells that do not express MCAM. Whether MCAM expression is directly regulated by Myf5 or, conversely, whether MCAM triggers a signaling cascade that induces downstream expression of Myf5 is not clear from the current data. In metastatic melanoma, MCAM has been shown to positively regulate the expression of Id1 via binding of ATF-3 to the Id-1 promoter (57).

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These samples were collected under a protocol approved by the Committee of Clinical Investigation at Brigham and Women’s Hospital and Boston Children’s Hospital. Limb skeletal muscle tissue was removed of skin and bone prior to being minced into fine pieces and enzymatically dissociated with 0.5 mg/ml of collagenase (Worthington Biochemicals) and 0.6 U/ml dispase II (Roche Applied Science) for 45–60 min at 37°C as previously described (61, 62). Cell suspensions were frozen and stored at −150°C for later use.

Flow cytometry

Human fetal muscle cells were thawed 8–24 h prior to analysis/cell sorting. Both floating and adherent cells were collected for FACS analysis and sorting. Cells were washed once with 1× Hank’s balanced salt solution (HBSS) and adherent cells were lifted using cell dissociation buffer (Intravenous Gibco). Samples were filtered and resuspended at 10⁶ cells/ml in warm 0.5% bovine serum albumin (BSA)/HBSS and then incubated for 1 h and 15 min at 37°C with 3.5 μg/ml Hoechst 33342 (Sigma) in the presence or absence of 5 μM reserpine (Sigma). For MCAM staining, samples were then washed and resuspended in ice-cold 0.5% BSA/HBSS at a concentration of 10⁶ cells/ml and then incubated on ice for 30 min with unconjugated MCAM antibody (1:100; Millipore). After washing with 0.5% BSA/HBSS, samples were incubated with AF488-conjugated goat anti-mouse antibody (1:100; Invitrogen) for 30 min on ice. Finally, samples were washed, resuspended in 2 μg/ml propidium iodide (PI)/0.5% BSA/HBSS and filtered through a 40 μm filter prior to FACS analysis/sorting.

Flow cytometric analysis and cell sorting were performed at the Dana Farber Cancer Institute/Hematologic Neoplasia Flow Cytometry Core Facility, using a BD FACS Aria II SORP flow cytometer equipped with 355 nm (20 mW UV) and 488 nm (100 mW) lasers, a temperature-controlled collection chamber and a plate collection attachment option. The following filters were utilized for Hoechst visualization: 450 ± 25 nm band pass (Hoechst Blue) and 660 ± 20 nm band pass with 635 nm long-pass dichroic mirror (Hoechst Red). Live cells were first selected using side scatter (SSC) versus forward scatter gating, followed by gating on the PI− population. Live cells were then viewed on a Hoechst Blue versus Hoechst Red plot for SP gating. The reserpine sample, in which the SP profile is absent, was used to accurately set the SP gate. For MCAM selection, gating of live cells was performed on an AF488 versus SSC plot.

Single-cell RT–PCR analysis on freshly isolated cells

Single cells were directly sorted by FACS into wells of a 384-well small-volume plate (Greiner) containing 5 μl of lysis buffer solution (0.15% IGEPAL (Sigma), Protector RNase Inhibitor (Roche), 2× RT-PCR Buffer (Superscript III Platinum One-Step RT-PCR System w/Platinum Taq; Invitrogen)); the plate was kept at 4°C at all times. After sorting, an additional 4 μl of lysis buffer solution was added to each well containing a single cell. The plate was then sealed with sealing film (Applied Biosystems) and shock-frozen on dry ice. Samples were stored at −80°C for subsequent analysis.

For nested, multiplexed RT–PCR, cell lysates were thawed on ice and transferred to 96-well PCR plates (VWR) with the addition of gene-specific external primer pairs (Supplementary Material, Table S2), RNase inhibitor and RT–PCR reaction mix as specified by the manufacturer. Samples were reverse-transcribed and subsequently amplified in an initial round of PCR amplification. Expected PCR product sizes ranged from 250 to 300 bp. PCR product from this reaction was then diluted 1:2 with water, and 1 μl of diluted product was subjected to a second round of PCR amplification using nested, gene-specific internal primer pairs (Supplementary Material, Table S1) and Taq-PRO Red Complete reaction mix (Denville) according to the manufacturer’s instructions. Expected second round PCR product sizes ranged from 120 to 170 bp. PCR products were visualized using gel electrophoresis on a 2% agarose gel.

Media testing assay for expansion of human muscle SP cells

Human fetal skeletal muscle SP cells were directly sorted into 384-well Small Volume™ μclear™ plates (Greiner BioOne); triplicate plates were seeded with 75 cells/well. Plates were pre-coated with 0.15% gelatin for 1 h at 37°C, washed once with 1× HBSS and loaded with 5 μl/well medium. Sixteen wells on each plate were seeded per medium condition and cells were allowed to settle at 37°C for 1–3 h before an additional 15 μl of medium was gently added to each well. One plate was immediately fixed as described below and stained with 2 μg/ml PI for analysis of seeding variability between wells. For the two remaining plates, cells were propagated in vitro for 10 days, with media replenished every other day as follows: 10 μl of medium was gently removed and replaced with 15 μl of fresh medium. Cells were maintained in a humidified 5% CO² incubator at 37°C.

After 10 days of culture in vitro, wells were fixed as follows: (i) medium from each well was removed such that ~5 μl of medium remained per well; (ii) 20 μl of 5% paraformaldehyde/1× phosphate buffered saline (PBS) was gently added to each well; (iii) cells were fixed for 20 min at room temperature; and (iv) cells were washed three times gently with 1× PBS. Cells were then permeabilized with 0.5% Triton X-100/PBS for 3 min, and subsequently blocked with 10% FBS/0.1% Triton X-100/PBS (blocking solution) for 30 min at room temperature. For primary antibody staining, cells were incubated overnight at 4°C with a mouse anti-desmin (1:100; DAKO D33) antibody diluted in blocking solution. Cells were then washed three times with PBS and incubated for 1 h at room temperature with DyLight488-conjugated donkey anti-mouse antibody (1:1000; Jackson ImmunoResearch) diluted in blocking solution. Cells were washed three times again in PBS before storage at 4°C in DAPI/PBS.

Images of each plate (before and after in vitro culture) were obtained at 4× magnification using a Nikon Eclipse TS100 microscope fitted with a Spot RT3 camera and appropriate filter sets (Day 0 plate: Texas Red/Pi; Day 10 plates: ultraviolet/DAPI, and FITC/GFP). Images were analyzed using ImageJ (NIH free software) with the Cell Counter plugin. Eight of the 16 initially seeded wells were assessed for each
medium condition, and three experiments were performed on different human fetal skeletal muscle cell isolations.

**Data analysis**

Overall growth efficiency was corrected for by summing the number of cells in the eight wells for each medium condition in a given experiment and then calculating a global rescaling factor for each experiment by fitting the best α to the following: \( \log(N_j) = \log(N_i) + \log(\alpha_i) \), where \( N \) is the total cell count for a given medium condition in a given experiment, \( \alpha \) is the global rescaling factor, \( j \) is the experiment being normalized and \( i \) is the ‘standard’ experiment to which the other two experiments are being normalized. The global rescaling factor was then utilized to normalize the total cell count for each medium condition for the given experiment. Second, the average fold change in growth compared with the 20% FBS/DMEM condition was calculated by taking the logarithm of the total normalized cell count for each medium condition (\( \log_2 N_{\text{normalized}} \)) in a given experiment and then averaging the \( \log_2 N_{\text{normalized}} \) values for each medium condition from all experiments. The standard deviation of the fold change in growth was also calculated from the \( \log_2 N_{\text{normalized}} \) values. Finally, statistical significance in growth rate in a given medium condition when compared with the 20% FBS/DMEM condition was calculated using Welch’s \( t \)-test on the \( \log_2 N_{\text{normalized}} \) values.

**Cell expansion**

Sorted MCAM ± SP, MP and Total cells were plated at 7500–20 000 cells per well in 48-well culture plates coated with 0.15% gelatin. Cells were grown in 1% penicillin–streptomycin–glutamine (PSG; Invitrogen Gibco)/StemPro® MSC SFM (Invitrogen). Cells were passaged at 60–70% confluency onto successively larger plates and maintained *in vitro* for ~2 weeks. After propagation, samples were harvested for RNA and protein analyses (*real-time* quantitative RT–PCR and western blot, respectively), used for *in vitro* fusion assays or intramuscular injections or frozen at -150°C for future use.

**Real-time quantitative RT–PCR analysis on expanded cells**

Total RNA from cultures of sorted human fetal skeletal muscle cells was obtained using the RNaseasy Mini Kit (Qiagen) as described by the manufacturer. After RNA quantification, 5 μg of total RNA was reverse-transcribed using oligo(dT) primers and the Superscript III First-Strand Synthesis System (Invitrogen), according to the manufacturer’s instructions. Resulting cDNA product was diluted 1:4 with water and then subjected to real-time qPCR analysis using gene-specific primers (Supplemental Material, Table S1; internal primer pairs only), SYBR green PCR master mix (Applied Biosystems) as described by the manufacturer and a 7900HT fast real-time PCR system (Applied Biosystems). Expected product sizes ranged between 120 and 170 bp. β2M was utilized as an internal control for each sample. Data were analyzed using the 2^−ΔΔC_T method as previously described (63). ΔC_T values for three independent experiments were calculated for each myogenic gene, using β2M as an internal control, and then averaged prior to calculation of average 2^−ΔΔC_T values. All 2^−ΔΔC_T values were calculated using the MCAM−MP population as the normalization control. Statistical significance of average gene expression was calculated using Welch’s \( t \)-test.

**In vitro fusion assays**

Samples were plated at 7500 cells per well in 96-well culture dishes as described above and maintained in StemPro® medium overnight. Fusion was induced using differentiation medium (2% horse serum/1% PSG/low-glucose DMEM), which was subsequently changed every 24 h. Time points were taken at 0, 1, 2, 3 and 4 days post-induction and processed for immunocytochemistry as below.

Images were taken using a 20× objective on a Nikon Eclipse TS100 microscope fitted with a Spot RT3 camera. Fusion indices were calculated as the number of nuclei in fused myotubes over the total number of nuclei in a given field. The percentage of myogenin+ nuclei was calculated as the number of myogenin+ nuclei over the total number of nuclei in a given field. For each experiment, four fields from two wells for each sample were assessed, resulting in 1500–2000 total nuclei counted per sample. Standard deviation was calculated for the two wells for each sample in a given experiment. The experiment was independently performed four times, using different human fetal samples.

To combine and compare the fusion index results of the individual experiments, the fusion indices across the different experiments were first determined to be proportional to each other, using the Pearson correlation method (\( r = 0.96, P \leq 0.002 \)). Since they were found to be proportional, fusion indices from one experiment can be rescaled to the levels of another experiment (called ‘standard experiment’), using a proportionality constant \( \alpha \). After rescaling, average fusion index results for each cell population as well as the average standard deviation were calculated using standard propagation of error calculations.

To combine and compare the percentage of myogenin+ nuclei of the individual experiments, myogenin expression was determined to depend on the fusion index in a Michaelis–Menten way, with the dissociation constant \( K_0 \) being experiment-independent. Thus, myogenin expression results, \( M \), were rescaled using the Michaelis–Menten relation between the myogenin expression and the fusion index: \( M_{\text{scaled}} = M \times (\alpha F / (\alpha F + K_0)) \), where \( F \) is the fusion index of a given experiment and \( \alpha \) is the proportionality constant between the fusion indices of the given experiment and the ‘standard experiment’.

\( P \)-values for pairwise comparisons between each of the cell populations were calculated using Welch’s \( t \)-test method and tested for multiple hypothesis false discovery, using the Benjamin–Hochberg method (B-H). For the B-H method: (i) uncorrected \( P \)-values were rank-ordered from smallest to largest values, (ii) \( P \times (i/N) \) was calculated for each comparison, where \( N \) is the number of comparisons (15 in this case), \( i \) is the rank order and \( P \) is the significance level (0.05 in this case), and (iii) \( P \times (i/N) < 0.05 \) was tested for each comparison. Eleven of 15 comparisons for the fusion indices and 9 of
the 15 comparisons for the percentage of myogenin+ nuclei passed the B-H method test. All P-values were thus corrected by multiplying N/i for each comparison.

Immunocytochemistry of cultured cell populations

Slides or plates were washed once in PBS and fixed for 20 min in 4% paraformaldehyde in PBS at room temperature. Cells were then permeabilized with 0.5% Triton X-100/PBS for 3 min and subsequently blocked with 10% FBS/0.1% Triton X-100/PBS for 30 min at room temperature. For primary antibody staining, cells were incubated overnight at 4°C with anti-desmin (1:100; Dako) or anti-myogenin (1:50; Dako) antibodies diluted in blocking solution. Cells were then washed three times with PBS and incubated for 1 h at room temperature with DyLight488-conjugated donkey anti-mouse antibody (1:1000; Jackson ImmunoResearch) diluted in blocking solution. Cells were washed again in PBS before storing samples at 4°C in DAPI/PBS.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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