Published in final edited form as:

J Cell Physiol. 2010 March ; 222(3): 676–684. doi:10.1002/jcp.21989.

Defining the Heterogeneity of Skeletal Muscle-Derived Side and Main Population Cells Isolated Immediately Ex Vivo

KRISTEN M. KALLESTAD and LINDA K. MCLOON*

Department of Ophthalmology and Neuroscience, University of Minnesota, Minneapolis, Minnesota

Abstract

Myoblast transfer therapy for Duchenne muscular dystrophy (DMD) largely fails due to cell death and inability of transplanted cells to engraft in diseased muscles. One method attempting to enrich for cell subpopulations is the Hoechst 33342 dye exclusion assay, yielding a side population (SP) thought to be progenitor enriched and a main population (MP). However, in vitro and transplant studies yielded inconsistent results relative to downstream progeny. Cell surface markers expressed by skeletal muscle-derived MP and SP cells have not been fully characterized directly ex vivo. Using flow cytometry, MP and SP cells were characterized based on their expression of several well-accepted progenitor cell antigens. Both the MP and SP populations are heterogeneous and overlapping in the cells they contain. The percentages of cells in each population vary with species and specific muscle examined. MP and SP populations contain both satellite and multipotent progenitor cells, based on expression of CD34, Sca-1, Pax7, and M-cadherin. Thus, isolation using this procedure cannot be used to predict downstream differentiation outcomes, and explains the conflicting literature on these cells. Hoechst dye also results in significant mortality of sorted cells. As defined subpopulations are easily obtained using flow cytometry, sorting immediately ex vivo based on accepted myogenic precursor cell markers will yield superior results in terms of cell homogeneity for transplantation therapy.

Skeletal muscle possesses a remarkable ability to remodel and repair after injury and in disease. The repair process is mainly accomplished through activation of satellite cells along the myofibers, which differentiate and fuse into damaged areas. Although the satellite cell was described nearly 50 years ago (Mauro, 1961), the genotype and maintenance of these regenerative cells are not well understood. In dystrophic and sarcopenic muscles, the satellite cell pool becomes exhausted leading to severe and permanent muscle degeneration (Decary et al., 2000; Renault et al., 2000). Many investigations have sought a method of replacing the satellite cell population through transplantation. Adult-derived myoblasts have been investigated for their therapeutic potential in the treatment of dystrophies, but unfortunately, this approach has been largely unsuccessful due to relatively rapid death of the majority of the transplanted cells (Gussoni et al., 1997; Miller et al., 1997; Skuk et al., 2002). In part, this is due to immune rejection of the transplanted cells (Watt, 1990; Rando and Blau, 1994). However, immune system rejection only partly explains the failure of myoblast therapy, as even transplantation between monozygotic twin girl carriers of the Duchenne muscular dystrophy (DMD) gene does not result in long-term survival of the transplanted cells (Tremblay et al., 1993). In addition, transplanted myoblasts do not disperse very well within treated muscles (Lipton and Schultz, 1979; Morgan et al., 1987;

Huard et al., 1994), and their expansion in vitro is limited by replicative senescence (Karpati et al., 1993; Decary et al., 1997). Interestingly, a small percentage of the transplanted cells not only produce new myofibers but also enter the satellite cell niche within the transplanted muscles (Blaveri et al., 1999; Heslop et al., 2001). The cells that survive have been hypothesized to be multipotent precursors (Qu et al., 1998; Beauchamp et al., 2000; Collins et al., 2005). As a result, current research is focused on isolating subpopulations of mpcs for transplantation that may have greater proliferative and/or differentiation potential with concomitantly enhanced survival rates. Again, these studies have met with differential success (Qu et al., 1998; Gussoni et al., 1999; Torrente et al., 2001; Jiang et al., 2002; De Bari et al., 2003). Part of the difficulty rests in the inability to identify the most appropriate precursor cell within the extremely heterogeneous population of mononuclear cells within adult skeletal muscle.

Adult skeletal muscle is known to contain less committed progenitor cells that are not only capable of regenerating and repairing skeletal muscle, but also capable of osteogenic, adipogenic, hematopoietic, and possibly neurogenic differentiation (Goodell et al., 1996; Asakura et al., 2001; Kondo et al., 2006). While many studies have focused on defining specific myogenic progenitor populations, there is little consensus in the literature about their in vivo phenotypes, molecular markers, or activation requirements. One assay proposed to isolate distinct precursor cell populations from tissue is the Hoechst dye exclusion assay (Goodell et al., 1996), which divides cells into a main population (MP) of dye-retaining cells and a side population (SP) of dye-effluxing cells described as being multipotent (Asakura et al., 2002). However, if the literature on the fate of skeletal muscle-derived SP cells is examined, results are quite contradictory. In some studies, SP cells isolated from skeletal muscle display hematopoietic or endothelial potential in vitro (Asakura et al., 2002; Dell'Agnola et al., 2002; Howell et al., 2002), and myogenic fates when transplanted in vivo (Asakura et al., 2002; Dell'Agnola et al., 2002; Howell et al., 2002; Meeson et al., 2004; Muskiewicz et al., 2005). However, other work demonstrates that SP cells can form muscle in vitro (Gussoni et al., 1999; Tamaki et al., 2003; Tanaka et al., 2009). Depending on the study, SP cells analyzed by flow cytometry (FACS) were differentially positive and/or negative for the endothelial and hematopoietic lineage markers CD31 and CD45 (Montanaro et al., 2004; Uezumi et al., 2006; Motohashi et al., 2008). The same contradictory results are seen when SP cells are examined for markers considered indicative of myogenic lineage commitment (Fukada et al., 2004; Tanaka et al., 2009). Even the origin of SP cells within skeletal muscle is a source of controversy. Some studies suggest they are derived from hematopoietic stem cells (HSCs) that originate in bone marrow (Majka et al., 2003; McKinney-Freeman et al., 2003), while others show no evidence of bone marrow origin (Montanaro et al., 2004; Rivier et al., 2004). Depending on the method used to isolate cells from intact muscle, as well as in vitro conditions, different downstream populations can form. Studies thus far have not definitively shown differentiated descendants from a single defined SP cell. SP cells, therefore, represent a population that is not well understood. The MP population also has conflicting reports of its differentiation potential in the literature (Tamaki et al., 2003). Few studies have specifically addressed the co-localization characteristics of SP and MP cells derived from adult skeletal muscle relative to multiple satellite cell and precursor cell markers directly ex vivo (Fukada et al., 2004).

One complication in assessing the in vivo identity of mpcs, including SP and MP cells, is that cellular expression of various markers of cell identity change when the cells are placed in culture (Yoshida et al., 1998; Jankowski et al., 2002; Mitchell et al., 2005). Despite this, many of the hypotheses about SP and MP differentiation potential are based on in vitro examination rather than directly ex vivo. The main aim of this study was to more accurately determine the identity of cell subgroups within the SP and MP derived from skeletal muscle immediately after sorting with Hoechst dye. SP and MP cells from muscles of mice and

rabbits cells were fixed, stained for markers of mpcs and satellite cells, and analyzed using flow cytometry (FACS). CD34 and Sca-1 were used as general markers of progenitor cells (Jackson et al., 1999; Beauchamp et al., 2000), while Pax-7 or M-cadherin (Cornelison and Wold, 1997; Seale et al., 2000) was used in conjunction with CD34 to determine if cells were committed to the myogenic lineage. Cells were examined for co-expression of CD45 and Sca-1 to determine potential hematopoietic lineage fate. In addition, the effect of Hoechst 33342 dye on cell survival was assessed using 7-amino-actinomycin D (7AAD) staining. Thus, different muscles, species, and co-expression patterns were used to assess the composition of the SP and MP populations directly ex vivo and related to the usefulness of this tool to enrich for precursor cell subtypes from adult skeletal muscle.

Materials and Methods

New Zealand white rabbits were obtained from Bakkom Rabbitry (Viroqua, WI). BALB/c or C57/Bl-6 mice (National Cancer Institute) were used for all mouse experiments. All mice were fed ad libitum, and kept on a 12 h light/dark cycle. All animals were housed in the AALAC-approved animal facilities at the University of Minnesota. Experiments were approved by the Institutional Animal Care and Usage Committee and followed the National Institutes of Health and Association for Research in Vision and Ophthalmology guidelines for the use of animals in research.

Rabbits were anesthetized with a 1:1 ketamine/xylazine solution (10 and 2 mg/kg, respectively), then euthanized with a barbiturate overdose followed by a thoracotomy and exsanguination. BALB/c or C57/Bl-6 mice were sacrificed using CO₂ asphyxiation followed by a thoracotomy and exsanguination. Tibialis anterior (“limb”) and superior, inferior, lateral and medial recti extraocular muscles (“EOM”) were harvested immediately post-mortem and stored on ice in phosphate-buffered saline (PBS) prior to weighing and digestion. Samples were digested using a Collagenase I/Dispase (Roche Diagnostics, Indianapolis, IN) solution as previously described (Asakura et al., 2001). Briefly, tissue was minced into small pieces mechanically and collagenase/dispase mixture was added. Samples were triturated, incubated at 37°C for 15 min, triturated and incubated for another 15 min until no solid tissue remained. Samples were washed with cold Dulbecco's Minimal Essential Medium (DMEM) at pH 7.0–7.2 and supplemented with 10 mM HEPES buffer and 2% fetal calf serum (FCS) (Invitrogen, Carlsbad, CA) (DMEM+), centrifuged at 1,400 rpm for 5 min and maintained in cold DMEM+ until Hoechst or antibody labeling. Mononuclear cells were counted using trypan blue (Sigma–Aldrich, St. Louis, MO) and a hemacytometer (Sigma–Aldrich).

Cells were filtered over 70 µm nylon mesh filters to remove any larger cell masses. Labeling was previously described (Asakura et al., 2001). Briefly, cells were diluted to 1 million cells/ml in warm DMEM+ and incubated with 8 µg/ml Hoechst 33342 dye (Sigma–Aldrich) with or without 20 µM verapamil (Sigma–Aldrich) at 37°C for 30 or 60 min (Asakura et al., 2001). This is a higher concentration than used most frequently (5 µg/ml; Goodell et al., 1996; Asakura et al., 2002; Kondo et al., 2006), but lower than is now in use in some laboratories (12 µg/ml; Montanaro et al., 2004). In our hands, a dose of 12 µg/ml of Hoechst 33342 resulted in a significant reduction in SP cells as seen previously, but more importantly, these cells were not removed from the SP gate by verapamil (data not shown). Cells were washed with cold DMEM+, centrifuged at 1,400 rpm and resuspended in DMEM + to a final concentration of $1-2 \times 10^6$ cells/ml for flow cytometry (FACS).

Hoechst dye analysis and cell sorting were performed on a FACSDiVa or LSR flow cytometer (Becton Dickinson Biosciences, San Jose, CA) at the University of Minnesota Cancer Center Flow Core Facility. All Hoechst-sorted cells were immediately fixed in 2%

formaldehyde and subsequently stained with fluorescent antibodies. Analysis of fluorescent antibody staining was performed on a FACSCalibur cytometer. Freshly isolated or Hoechst-sorted cells were incubated with Fc Block (CD16/CD32, BD Pharmingen, San Diego, CA) and as necessary, avidin and biotin blocking agents (Vector Laboratories, Burlingame, CA). Cells were stained using antibodies against CD34 (Beauchamp et al., 2000), Sca-1 (Jackson et al., 1999), M-Cadherin (Cornelison and Wold, 1997), CD45, CD31 (all BD Pharmingen), and Pax-7 (Seale et al., 2000) (R&D Systems, Minneapolis, MN). CD34, Sca-1, CD31, and CD45 were directly conjugated to either phycoerythrin (PE), fluorescein isothiocyanate (FITC), phycoerythrin-Cy5 (PE-Cy5), or allophycocyanin (APC). Additionally a biotinylated Sca-1 was used and detected with streptavidin APC (BD Pharmingen). M-Cadherin and Pax-7 were detected with a biotinylated anti-mouse IgG (Vector Laboratories) followed by streptavidin APC. All antibody staining controls include appropriate isotype antibodies for primary and secondary reagents. Samples stained with secondary only or secondary and tertiary only reagents were also used as controls. Strict controls for every staining step were performed. Dead cells were always excluded using 7AAD (BD Pharmingen) both preand post-sorting, as dead and dying cells are known to bind antibodies non-specifically. Intracellular staining was performed as previously described (Khoruts et al., 1998). Briefly, after fixation in 2% formaldehyde for 20 min, samples were incubated with a saponin and FCS-supplemented PBS. Antibodies for the intracellular antigens were added at $3-9 \mu\text{g}/1 \times 10^6$ cells. Analysis of flow cytometric data was performed using CellQuest Pro Version 5.2 (BD Pharmingen). Gates were drawn based on fluorescence above isotype control staining.

Immunohistochemistry was performed on frozen, FACS-sorted SP cells cytospun onto slides. Samples were fixed in ice-cold acetone and air-dried. Avidin/biotin and antibody blocking steps were performed using manufacturer's protocols (Vector Laboratories; Molecular Probes, Eugene, OR). Pax-7 was visualized using a 1:500 dilution and fluorescent Mouse on Mouse FITC kit (Vector Laboratories). Sca-1 was visualized using a 1:500 dilution and the tyramide signal amplification-streptavidin kit (Molecular Probes) per manufacturer's protocol and as previously described (Wolnicka-Glubisz et al., 2005). Slides were mounted with Vectashield mounting media containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Appropriate isotype control and secondary antibody only controls were performed alongside test slides. Images were captured under a microscope (DMR; Leica Microsystems, Wetzlar, Germany) and analyzed using image analysis software (Bioquant Nova Prime, Nashville, TN).

All data were analyzed for statistical significance using analysis of variance (ANOVA) and Dunn's multiple comparison tests using the Prism and Statmate software for Macintosh (Graphpad, San Diego, CA) or SigmaStat 2.03 (SPSS Science, Chicago, IL). For data collected as percentages, angular transformation of the data and ANOVA tests were performed using Microsoft Excel and GraphPad for Macintosh. Errors were calculated based on the propagation of errors associated with each measurement. An *F*-test was used to verify if the variances were significantly different. Data were considered significantly different if $P \leq 0.05$.

Results

Percentage of cells in the SP and MP cell populations depend on species and muscle type

Hindlimb and EOM from individual rabbits or pooled muscles from 5 to 10 mice were stained with Hoechst 33342 dye. EOM was used as a comparison muscle as it is considered to be a distinct allotype (Hoh and Hughes, 1988). The percentage of cells that fell within the MP or SP gate, as defined by verapamil inhibition of Hoechst 33342 dye efflux, varied depending on the species and the muscle type tested. SP gates were identical for each

experiment, but varied between experiments, based on normal staining variability (Fig. 1A). In rabbit experiments, $0.25 \pm 0.09\%$ of cells from EOM and $0.40 \pm 0.08\%$ of cells from hindlimb muscle were within the SP gate (Fig. 1B). When similar experiments were performed on mouse tissue, however, in EOM, $9.43 \pm 1.75\%$ fell within the SP gate and only $2.99 \pm 0.54\%$ of cells from the mouse hindlimb fell within the same gate (Fig. 1B). In EOM from human patients, only 0.25% of the cells fell within the SP gate (data not shown). Two-way ANOVA reveals a significant interaction of species and muscle type on the percentage of SP cells ($n = 11$ separate experiments). There were significant $\frac{1}{4}$ differences between the percentages of SP cells from different muscles obtained from the same animal(s), as well as significant differences in the percentages of SP cells obtained from the same muscle from the two different species (Fig. 1B).

Multipotent precursor and satellite cells are found in both the MP and SP fractions

Due to the striking differences in SP percentages from different muscles, we investigated the possibility that multipotent precursor cells may also be found in the main population (MP) of hindlimb muscles. Only mouse tissue was used for these experiments, as a greater number of species-specific antibodies targeting mouse precursor cell markers are commercially available. The cells were sorted and stained for several commonly used markers of multipotent myogenic precursor cells and satellite cells in order to better define the cell populations present. Live (7AAD-negative) MP and SP fractions of Hoechst labeled cell samples were separately fixed and labeled for the expression of the stem cell markers CD34 and Sca-1 as well as satellite cell markers Pax-7 and M-cadherin. Both the SP and MP fractions had subpopulations of cells positive for CD34 and/or Sca-1, indicating that they are present in each sorted group (Fig. 2A). Also, CD34+ cells and Sca-1+ cells that co-expressed either Pax7 or M-cadherin were present in both fractions (Fig. 2B). To confirm co-expression of Sca-1 with satellite cell markers, freshly isolated live mouse hindlimb and EOM SP cells were cytopun onto slides and stained for Sca-1 and Pax7. The immunohistochemistry clearly shows co-expression of the two molecules with DAPI-positive nuclei, indicating that many SP cells are both Sca-1⁺ and Pax7⁺ (Fig. 2C). There exists a subgroup of CD34+ cells that co-expresses Pax-7 or M-cadherin demonstrating that there is no enrichment of either population in the SP or the MP (Fig. 2D,E). There are also live mononuclear cells that do not take up the Hoechst dye at all, and thus are not MP or SP (Fig. 1A). This third population contains some Sca-1+ cells, but none of the other markers visualized in this study were observed in these cells (data not shown).

The MP and SP compartments lack Sca-1+/CD45+ cells

Controversy surrounds the origin of the SP cells; the possibility of bone marrow-derived multipotent precursor cells being present in the skeletal muscle SP was investigated by direct ex vivo analysis of potential co-expression on SP cells of Sca-1 and CD45, a marker of the hematopoietic lineage. There were very few CD45+ cells within either the SP or MP fractions of mouse hindlimb muscles. When cells were examined for co-expression of CD45 and Sca-1, <0.5% of the Sca-1+ SP cells co-expressed CD45 (Fig. 2F). Cells positive for CD45 were found in slightly greater numbers in the MP compared to SP, indicating that there was likely no enrichment of HSCs in the SP. Still, it remained possible that the CD45+ cells within the MP were actually contaminating blood cells, whereas the CD45+ cells from SP might reflect a small population of HSCs. In order to further identify the CD45+ cells, we co-stained them with lineage markers for myeloid, T cell, and B cell populations. More than 99.5% of CD45+ cells from sorted SP populations co-stained for at least one lineage marker (B220, CD11b, or CD3) confirming that they were not stem cells, but contaminating peripheral blood cells (data not shown). While it is possible that a very small fraction of Sca-1+ cells that co-express CD45 in the SP may represent HSCs within muscle, the percentage is so low that the physiological relevance may be questionable.

There are more muscle-derived precursor cells in the MP than the SP fractions

To determine if the density of mpcs was increased in the SP, the total numbers of cells staining for markers of each population were determined in both the MP and SP of mouse hindlimb muscle directly ex vivo. To calculate the number of cells per milligram of tissue, hindlimb muscles were weighed prior to digestion, and cells were counted before the Hoechst exclusion assay. The sorted cells were fixed and stained for the following markers: CD34, Sca-1, M-cadherin, and Pax-7 (Fig. 3). More cells in the MP stained for all of the analyzed markers compared with cells from the SP. Fold increases for each marker in MP compared with SP were as follows: CD34 = 3.45, Sca-1 = 5.46, CD45 = 2.78, pax7 = 2.72, and M-cadherin = 4.02 (Fig. 3). Therefore, not only does the SP fraction contain cells positive for satellite cell-markers, but there are more cells positive for progenitor cell markers within the MP than isolated in the SP via the Hoechst 33342 dye-exclusion assay.

Hoechst dye kills cells

Mononuclear cells exposed to Hoechst dye were more likely than unexposed cells to take up the cell death marker dye 7AAD (Fig. 4A). Samples of freshly isolated mononuclear cells from mouse hindlimbs stained with various antibodies and immediately analyzed by flow cytometry contained $11.1 \pm 1.20\%$ dead cells. Samples exposed to Hoechst dye, but otherwise prepared under similar conditions, contained $37.29 \pm 2.05\%$ dead cells (Fig. 4A). Using angular transformation with propagation of the measured errors, cell death due to Hoechst dye toxicity is calculated to be $26.19 \pm 3.24\%$ (Fig. 4A). While adding 7AAD prior to sorting cells with Hoechst dye excluded many of the dead cells, sorted cells continued to die between the sort time and time of fixation for immunostaining. This typically was 30–90 min, when the cells were maintained in DMEM+ on ice. In order to exclude dead cells from the final analysis of Hoechst sorted cells that were subsequently stained for cellular markers, 7AAD was added a second time after the sort, immediately before fixation. The percentage of cells that die between the sort and fixation is $14.11 \pm 0.89\%$ (Fig. 4B), a higher percentage even than cells that die from the isolation procedure. Liberation of mononuclear cells from muscle and sorting based on the Hoechst 33342 dye exclusion assay results in significant cell death, amounting to $37.29 \pm 1.96\%$ of the total starting population.

Discussion

The ability to separate defined populations of mpcs is critical to selection of the most appropriate cell for myoblast therapy. Hoechst 33342 dye showed great potential for sorting progenitor populations in a number of different tissues (Goodell et al., 1996; Jackson et al., 1999; Challen et al., 2006; Redvers et al., 2006). It is taken up quickly by live cells, can be visualized by flow cytometry, allows rapid sorting of stained cells, and was hypothesized to enrich for identified subgroups of precursor cells from heterogeneous cellular pools. Verapamil provided an easy negative control, allowing accurate identification of SP cells which efflux the dye (Goodell et al., 1996). The use of the Hoechst dye exclusion assay for the preparation of HSCs is well established, and this system appears to work well in separating populations of bone marrow-derived precursor cells (Goodell et al., 1996).

The quantity of cells in these two fractions varied considerably depending on the animal species as well as the particular muscles used for analysis. The presence of different muscle allotypes is well known (Hoh and Hughes, 1988), and muscle allotypes vary significantly in their protein and gene expression profiles (Porter et al., 2001; Caiozzo et al., 2003). They also vary considerably in their satellite cell composition, particularly total satellite cell number per myofiber, which can vary from 0 to 20 satellite cells per myofiber in limb skeletal muscle (Beauchamp et al., 2000; Zammit et al., 2002; Wozniak et al., 2003; Kadi et al., 2004; Collins et al., 2005) and 60–100 satellite cells per myofiber in EOM (McLoon et

al., 2007). This, in part, may explain why SP and MP cells derived from different skeletal muscles are more heterogeneous than cells derived from hematopoietic tissues in a similar manner. Despite significantly elevated numbers of mpcs in EOM compared to leg muscle, rabbit muscle analysis revealed a smaller percentage of cells within the SP gate from EOM compared with leg and the opposite was seen in the mouse muscles. Thus, one cannot predict the percentage and number of SP cells isolated even within a given muscle from two different species. Examination of satellite cells from adult human muscle supports the view that significant species differences exist between these populations in terms of percentages of distinct populations and the universality of assays (Reimann et al., 2004; Lindstrom and Thornell, 2009). The differences between these two species and between these two muscles prompted further investigation of the resident cells in each compartment.

The purity of both the MP and SP cell populations in skeletal muscle has not been well defined. In addition to the variable isolation based on species and muscle type, the present analyses of the SP and MP cells from skeletal muscle directly ex vivo demonstrate that identification and sorting of multipotent precursor cells from satellite cells in muscle does not result from the Hoechst 33342 dye efflux assay. We found no enrichment in either compartment of cells expressing common molecules that identify different populations of precursor cells, including CD34 (Beauchamp et al., 2000), Sca-1 (Epting et al., 2004; Mitchell et al., 2005), M-cadherin (Cornelison and Wold, 1997), or Pax 7 (Seale et al., 2000). Even the expression of the ATP-binding cassette superfamily of membrane transporters (ABCG-2), the multidrug-resistance transporter believed responsible for the Hoechst 33342 efflux, does not perfectly correlate with the SP phenotype in bone marrow-derived cells (Naylor et al., 2005). A recent paper found human pluripotent stem cells negative for ABCG-2 expression that actually retain Hoechst dye and end up in the MP gate (Zeng et al., 2009). In part, SP cells were considered to be enriched for more multipotent myogenic precursor cells because these cells were able to form hematopoietic, adipogenic, and osteogenic colonies in vitro (Asakura et al., 2002). This may reflect the differential survival of subsets of cell precursors within the heterogeneous population of SP cells based on the milieu in which they found themselves. This is supported by the demonstration that the SP cells did not form myocytes in vitro but were able to form both myocytes and satellite cells in vivo following transplantation (Asakura et al., 2002). However, it is well known that in vitro conditions alter cell-specific marker expression patterns over time, and various markers turn on and off (Goodell et al., 1997; Yoshida et al., 1998; Smythe and Grounds, 2000). Until the conditions for survival or differentiation of a specific cell type are absolutely defined, it is impossible to implicate a particular cell type from a heterogeneous pool as the progenitor forming a particular in vitro or in vivo population. In light of our current studies, the heterogeneity in the population of SP cells used in these experiments is a reasonable explanation for these apparently contradictory results.

It has been suggested that the variable results of cell fate in SP and MP cells in the literature using the Hoechst dye exclusion assay may simply be due to the significant differences in SP isolation protocols and the conditions used to assess cell fate after isolation (Gussoni et al., 1997; Montanaro et al., 2004). In the present study, 8 mg/ml was used. This is higher than the 5 μ g/ml used in the original studies in skeletal muscle SP and MP fractionation (Asakura et al., 2002), yet even at this higher concentration no population enrichment was obtained. Some groups have reported differences in SP populations based on the amount of effluxed dye (McKinney-Freeman et al., 2002; Camargo et al., 2006), but no clear strategy to define gating has been established. A previous study examined the conflicting results with this technique and hypothesized that Hoechst dye concentration was critically important (Montanaro et al., 2004). Using a very high concentration of Hoechst dye, 12 μ g/ml, resulted in significantly more cell death and a smaller SP fraction (Montanaro et al., 2004). The trade off for the higher doses was an apparent increase in SP cellular homogeneity; however, the

cells were still heterogeneous (Montanaro et al., 2004). In our hands, this Hoechst dye dosage resulted in SP cells that could not be removed from that gate by verapamil.

In addition to the lack of homogeneity in the SP and MP cells, what is most striking is that the MP gate actually contained more cells than the SP for all the markers that were analyzed, including Sca1 and CD34. Since a large percentage of progenitor cells are not found in the SP gate, the Hoechst 33342 dye efflux assay does not increase the percentage of purified mpcs isolated, nor does it even appear to capture a large percentage of these cells from adult skeletal muscle. Recent approaches using in vitro clonal analysis of single cells (Case et al., 2008) or single precursor cell transplants into muscle (Sacco et al., 2008) allow the clear determination of the lineage potential of defined cell populations as well as engraftment potential.

It is possible that the Hoechst 33342 dye efflux assay may enrich for what is known to be a very small population of HSCs from muscle (Asakura et al., 2002). Other studies have conflicting data on whether cells from skeletal muscle with hematopoietic potential are derived from hematopoietic sources or are muscle-specific (McKinney-Freeman et al., 2002; Majka et al., 2003; Sherwood et al., 2004; Schienda et al., 2006). In our analysis, we were unable to resolve any increase in the percentage of CD45+/ Sca-1+ cells above isotype control staining in the SP or MP compartment. This contrasts with other studies of SP cells (Uezumi et al., 2006); however, CD45+ cells were previously shown to be a negligible cellular component when, as in the present study, higher doses of Hoechst dye are used (Montanaro et al., 2004). When the CD45+ cells were co-stained for other hematopoietic lineage markers such as CD11b, only a very small percentage (0.5%) were negative for all markers tested. Since CD45+ cells made up such a small percentage of the mononuclear cells extracted from skeletal muscle, and nearly all of those were identified by cell markers as contaminating peripheral blood cells, the muscle SP compartment does not appear to contain a physiologically relevant population of HSCs. If HSCs are present within mouse adult limb muscle, they are not adequately enriched by the Hoechst dye assay under the present conditions or are not identified by CD45 and Sca-1 (Spangrude et al., 1988; McKinney-Freeman et al., 2002). The observation that muscle-derived stem cells possess hematopoietic potential may be a phenomenon resulting from in vitro modifications, or although unlikely, a starting population that does not express the same markers as conventionally described HSCs.

An ideal method for sorting progenitor cell subpopulations from skeletal muscle would involve the ability to purify homogenous populations while maintaining the maximal viability of the cells. It has long been known that Hoechst dye is toxic (Durand and Olive, 1982; Zhang et al., 1999). Hoechst 33342 dye does not maintain the viability of all cells. This is not surprising, as the Hoechst assay was originally used as a method to measure cell death (Wallen et al., 1983; Ciancio et al., 1988). The procedure to release mononuclear cells from muscle tissue is disruptive and results in a baseline amount of cell death. The percentage of dead cells dramatically increases with incubation in media containing Hoechst 33342 dye. This dye has been used specifically to identify cells with fragmented DNA (Hardin et al., 1992). While some other groups used propidium iodide to exclude dead cells from their sorted populations (Goodell et al., 1996), our results show that 40% are killed during the assay, and cells that were alive at the time of cell sorting continue to die during the sort, with more than 10% incorporating 7AAD into their nuclei within the 30–90 min of sorting. Due to death caused by the process of mononuclear cell isolation, Hoechst 33342 incubation, and post-sorting, more than half of the starting population of mononuclear cells is dead after these procedures. This includes cells that express both CD34 and Sca-1. This is important to consider when differentiation potential is assessed in vitro.

Although the Hoechst 33342 dye efflux assay may be useful for some investigations of stem cells, particularly in the hematopoietic lineage, it does not appear to enrich for specific subpopulations in our directly ex vivo analysis of muscle-derived progenitor cells. Since this assay is increasingly being used in other tissue types, including tumors (Komuro et al., 2007), we suggest that careful analysis of the SP and MP compartments be performed to ensure that cells of interest are not being preferentially lost due to the sorting technique, and that other progenitor populations are not contaminating the sorted cells.

Current studies in our laboratory are directed at more carefully defining muscle-derived precursor cell populations by examination of co-expression patterns using a larger panel of markers directly ex vivo. It is hoped that this approach may be able to dissect out one or more specific subpopulations with greater ability to home, survive, and spread within injured, diseased or aging muscles after myoblast transplantation with improved restoration of muscle function.

Acknowledgments

The authors would like to thank Dr. Atsushi Asakura for his help in performing the Hoechst dye exclusion assay and the University of Minnesota Cancer Center Flow Core for sorting the SP and MP populations. Supported by EY055137 (L.K.M.) and EY11375 from the National Eye Institute, the Lew Wasserman Merit Award from Research to Prevent Blindness, Inc. (L.K.M.), Fight for Sight, (K.M.T.), the Howard Hughes Medical Institute (K.M.T.), the Nash Avery Search for Hope Research Fund (L.K.M.), and an unrestricted grant to the Department of Ophthalmology from Research to Prevent Blindness, Inc.

Contract grant sponsor: NIH;

Contract grant numbers: EY055137, EY11375.

Contract grant sponsor: Research to Prevent Blindness, Inc..

Contract grant sponsor: Howard Hughes Medical Institute.

Contract grant sponsor: Nash Avery Search for Hope Research Fund.

Contract grant sponsor: Fight for Sight.

Literature Cited

- Asakura A, Komaki M, Rudnicki MA. Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. *Differentiation* 2001;68:245–253. [PubMed: 11776477]
- Asakura A, Seale P, Girgis-Gabardo A, Rudnicki MA. Myogenic specification of side population cells in skeletal muscle. *J Cell Biol* 2002;159:123–134. [PubMed: 12379804]
- Beauchamp JR, Heslop L, Yu DS, Tajbakhsh S, Kelly RG, Wernig A, Buckingham ME, Partridge TA, Zammit PS. Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. *J Cell Biol* 2000;151:1221–1234. [PubMed: 11121437]
- Blaveri K, Heslop L, Yu DS, Rosenblatt JD, Gross JG, Partridge TA, Morgan JE. Patterns of repair of dystrophic mouse muscle: Studies on isolated fibers. *Dev Dyn* 1999;216:244–256. [PubMed: 10590476]
- Case J, Horvath TL, Ballas CB, March KL, Srour EF. In vitro clonal analysis of murine pluripotent stem cells isolated from skeletal muscle and adipose stromal cells. *Exp Hematol* 2008;36:224–234. [PubMed: 18023524]
- Caiozzo VJ, Baker MJ, Huang K, Chou H, Wu YZ, Baldwin KM. Single-fiber myosin heavy chain polymorphism: How many patterns and what proportions? *Am J Physiol Regul Integr Comp Physiol* 2003;285:R570–R580. [PubMed: 12738613]
- Camargo FD, Chambers SM, Drew E, McNagny KM, Goodell MA. Hematopoietic stem cells do not engraft with absolute efficiencies. *Blood* 2006;107:501–507. [PubMed: 16204316]

- Challen GA, Bertoncello I, Deane JA, Ricardo SD, Little MH. Kidney side population reveals multilineage potential and renal functional capacity but also cellular heterogeneity. *J Am Soc Nephrol* 2006;17:1896–1912. [PubMed: 16707564]
- Ciancio G, Pollack A, Taupier MA, Block NL, Irvin GL III. Measurement of cell-cycle phase-specific cell death using Hoechst 33342 and propidium iodide: Preservation by ethanol fixation. *J Histochem Cytochem* 1988;36:1147–1152. [PubMed: 2457047]
- Collins CA, Olsen I, Zammit PS, Heslop L, Petrie A, Partridge TA, Morgan JE. Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. *Cell* 2005;122:289–301. [PubMed: 16051152]
- Cornelison DD, Wold BJ. Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. *Dev Biol* 1997;191:270–283. [PubMed: 9398440]
- De Bari C, Dell'Accio F, Vandenabeele F, Vermeesch JR, Raymackers JM, Luyten FP. Skeletal muscle repair by adult human mesenchymal stem cells from synovial membrane. *J Cell Biol* 2003;160:909–918. [PubMed: 12629053]
- Decary S, Mouly V, Hamida CB, Sautet A, Barbet JP, Butler-Browne GS. Replicative potential and telomere length in human skeletal muscle: Implications for satellite cell-mediated gene therapy. *Hum Gene Ther* 1997;8:1429–1438. [PubMed: 9287143]
- Decary S, Ben Hamida C, Mouly V, Barbet JP, Hentati F, Butler-Browne GS. Shorter telomeres in dystrophic muscle consistent with extensive regeneration in young children. *Neuromuscul Disord* 2000;10:113–120. [PubMed: 10714586]
- Dell'Agnola C, Rabascio C, Mancuso P, Capillo M, Pruneri G, Gobbi A, Minucci S, Ronzoni S, Volorio S, Calabrese L, Tradati N, Martinelli G, Shultz L, Bertolini F. In vitro and in vivo hematopoietic potential of human stem cells residing in muscle tissue. *Exp Hematol* 2002;30:905–914. [PubMed: 12160842]
- Durand RE, Olive PL. Cytotoxicity, mutagenicity and DNA damage by Hoechst 33342. *J Histochem Cytochem* 1982;30:111–116. [PubMed: 7061816]
- Epting CL, Lopez JE, Shen X, Liu L, Bristow J, Bernstein HS. Stem cell antigen-1 is necessary for cell-cycle withdrawal and myoblast differentiation in C2C12 cells. *J Cell Sci* 2004;117:6185–6195. [PubMed: 15546912]
- Fukada S, Higuchi S, Segawa M, Koda K, Yamamoto Y, Tsujikawa K, Kohama Y, Uezumi A, Imamura M, Miyagoe-Suzuki Y, Takeda S, Yamamoto H. Purification and cell-surface marker characterization of quiescent satellite cells from murine skeletal muscle by a novel monoclonal antibody. *Exp Cell Res* 2004;296:245–255. [PubMed: 15149854]
- Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med* 1996;183:1797–1806. [PubMed: 8666936]
- Goodell MA, Rosenzweig M, Kim H, Marks DF, DeMaria M, Paradis G, Grupp SA, Sieff CA, Mulligan RC, Johnson RP. Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. *Nat Med* 1997;3:1337–1345. [PubMed: 9396603]
- Gussoni E, Blau HM, Kunkel LM. The fate of individual myoblasts after transplantation into muscles of DMD patients. *Nat Med* 1997;3:970–977. [PubMed: 9288722]
- Gussoni E, Soneoka Y, Strickland CD, Buzney EA, Khan MK, Flint AF, Kunkel LM, Mulligan RC. Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature* 1999;401:390–394. [PubMed: 10517639]
- Hardin JA, Sherr DH, DeMaria M, Lopez PA. A simple fluorescence method for surface antigen phenotyping of lymphocytes undergoing DNA fragmentation. *J Immunol Methods* 1992;154:99–107. [PubMed: 1401949]
- Heslop L, Beauchamp JR, Tajbakhsh S, Buckingham ME, Partridge TA, Zammit PS. Transplanted primary neonatal myoblasts can give rise to functional satellite cells as identified using the Myf5nlacZ⁺ mouse. *Gene Ther* 2001;8:778–783. [PubMed: 11420641]
- Hoh JF, Hughes S. Myogenic and neurogenic regulation of myosin gene expression in cat jaw-closing muscles regenerating in fast and slow limb muscle beds. *J Muscle Res Cell Motil* 1988;9:59–72. [PubMed: 2899091]

- Howell JC, Yoder MC, Srour EF. Hematopoietic potential of murine skeletal muscle-derived CD45()Sca-1(b)c-kit() cells. *Exp Hematol* 2002;30:915–924. [PubMed: 12160843]
- Huard J, Acsadi G, Jani A, Massie B, Karpati G. Gene transfer into skeletal muscles by isogenic myoblasts. *Hum Gene Ther* 1994;5:949–958. [PubMed: 7948144]
- Jackson KA, Mi T, Goodell MA. Hematopoietic potential of stem cells isolated from murine skeletal muscle. *Proc Natl Acad Sci USA* 1999;96:14482–14486. [PubMed: 10588731]
- Jankowski RJ, Deasy BM, Cao B, Gates C, Huard J. The role of CD34 expression and cellular fusion in the regeneration capacity of myogenic progenitor cells. *J Cell Sci* 2002;115:4361–4374. [PubMed: 12376567]
- Jiang Y, Vaessen B, Lenvik T, Blackstad M, Reyes M, Verfaillie CM. Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. *Exp Hematol* 2002;30:896–904. [PubMed: 12160841]
- Kadi F, Charifi N, Denic C, Lexell J. Satellite cells and myonuclei in young and elderly women and men. *Muscle Nerve* 2004;29:120–127. [PubMed: 14694507]
- Karpati G, Ajdukovic D, Arnold D, Gledhill RB, Guttmann R, Holland P, Koch PA, Shoubridge E, Spence D, Vanasse M, Watters GV, Abrahamowicz M, Duff C, Worton RG. Myoblast transfer in Duchenne muscular dystrophy. *Ann Neurol* 1993;34:8–17. [PubMed: 8517684]
- Khoruts A, Mondino A, Pape KA, Reiner SL, Jenkins MK. A natural immunological adjuvant enhances T cell clonal expansion through a CD28-dependent, interleukin (IL)-2-independent mechanism. *J Exp Med* 1998;187:225–236. [PubMed: 9432980]
- Komuro H, Saihara R, Shinya M, Takita J, Kaneko S, Kaneko M, Hayashi Y. Identification of side population cells (stem-like cell population) in pediatric solid tumor cell lines. *J Pediatr Surg* 2007;42:2040–2045. [PubMed: 18082704]
- Kondo T, Case J, Srour EF, Hashino E. Skeletal muscle-derived progenitor cells exhibit neural competence. *Neuroreport* 2006;17:1–4. [PubMed: 16361939]
- Lindstrom M, Thornell LE. New multiple labelling method for improved satellite cell identification in human muscle: Application to a cohort of power-lifters and sedentary men. *Histochem Cell Biol* 2009;132:141–157. [PubMed: 19484472]
- Lipton BH, Schultz E. Developmental fate of skeletal muscle satellite cells. *Science* 1979;205:1292–1294. [PubMed: 472747]
- Majka SM, Jackson KA, Kienstra KA, Majesky MW, Goodell MA, Hirschi KK. Distinct progenitor populations in skeletal muscle are bone marrow derived and exhibit different cell fates during vascular regeneration. *J Clin Invest* 2003;111:71–79. [PubMed: 12511590]
- Mauro A. Satellite cell of skeletal muscle fibers. *J Biophys Biochem Cytol* 1961;9:493–495. [PubMed: 13768451]
- McKinney-Freeman SL, Jackson KA, Camargo FD, Ferrari G, Mavilio F, Goodell MA. Muscle-derived hematopoietic stem cells are hematopoietic in origin. *Proc Natl Acad Sci USA* 2002;99:1341–1346. [PubMed: 11830662]
- McKinney-Freeman SL, Majka SM, Jackson KA, Norwood K, Hirschi KK, Goodell MA. Altered phenotype and reduced function of muscle-derived hematopoietic stem cells. *Exp Hematol* 2003;31:806–814. [PubMed: 12962727]
- McLoon LK, Thorstenson KM, Solomon A, Lewis MP. Myogenic precursor cells in craniofacial muscles. *Oral Sci* 2007;13:134–140.
- Meeson AP, Hawke TJ, Graham S, Jiang N, Elterman J, Hutcheson K, Dimaio JM, Gallardo TD, Garry DJ. Cellular and molecular regulation of skeletal muscle side population cells. *Stem Cells* 2004;22:1305–1320. [PubMed: 15579648]
- Miller RG, Sharma KR, Pavlath GK, Gussoni E, Mynhier M, Lanctot AM, Greco CM, Steinman L, Blau HM. Myoblast implantation in Duchenne muscular dystrophy: The San Francisco study. *Muscle Nerve* 1997;20:469–478. [PubMed: 9121505]
- Mitchell PO, Mills T, O'Connor RS, Kline ER, Graubert T, Dzierzak E, Pavlath GK. Sca-1 negatively regulates proliferation and differentiation of muscle cells. *Dev Biol* 2005;283:240–252. [PubMed: 15901485]

- Montanaro F, Liadaki K, Schiend J, Flint A, Gussoni E, Kunkel LM. Demystifying SP cell purification: Viability, yield, and phenotype are defined by isolation parameters. *Exp Cell Res* 2004;298:144–154. [PubMed: 15242769]
- Morgan JE, Coulton GR, Partridge TA. Muscle precursor cells invade and repopulate freeze-killed muscles. *J Muscle Res Cell Motil* 1987;8:386–396. [PubMed: 3480896]
- Motohashi N, Uezumi A, Yada E, Fukada S, Fukushima K, Imaizumi K, Miyagoe-Suzuki Y, Takeda S. Muscle CD31(–) CD45(–) side population cells promote muscle regeneration by stimulating proliferation and migration of myoblasts. *Am J Pathol* 2008;173:781–791. [PubMed: 18669618]
- Muskiewicz KR, Frank NY, Flint AF, Gussoni E. Myogenic potential of muscle side and main population cells after intravenous injection into sub-lethally irradiated mdx mice. *J Histochem Cytochem* 2005;53:861–873. [PubMed: 15995145]
- Naylor CS, Jaworska E, Branson K, Embleton MJ, Chopra R. Side population/ABCG2-positive cells represent a heterogeneous group of haemopoietic cells: Implications for the use of adult stem cells in transplantation and plasticity protocols. *Bone Marrow Transplant* 2005;35:353–360. [PubMed: 15608658]
- Porter JD, Khanna S, Kaminski HJ, Rao JS, Merriam AP, Richmonds CR, Leahy P, Li J, Andrade FH. Extraocular muscle is defined by a fundamentally distinct gene expression profile. *Proc Natl Acad Sci USA* 2001;98:12062–12067. [PubMed: 11572940]
- Qu Z, Balkir L, van Deutekom JC, Robbins PD, Pruchnic R, Huard J. Development of approaches to improve cell survival in myoblast transfer therapy. *J Cell Biol* 1998;142:1257–1267. [PubMed: 9732286]
- Rando TA, Blau HM. Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. *J Cell Biol* 1994;125:1275–1287. [PubMed: 8207057]
- Redvers RP, Li A, Kaur P. Side population in adult murine epidermis exhibits phenotypic and functional characteristics of keratinocyte stem cells. *Proc Natl Acad Sci USA* 2006;103:13168–13173. [PubMed: 16920793]
- Reimann J, Brimah K, Schroder R, Wernig A, Beauchamp JR, Partridge TA. Pax7 distribution in human skeletal muscle biopsies and myogenic tissue cultures. *Cell Tissue Res* 2004;315:233–242. [PubMed: 14648195]
- Renault V, Piron-Hamelin G, Forestier C, DiDonna S, Decary S, Hentati F, Saillant G, Butler-Browne GS, Mouly V. Skeletal muscle regeneration and the mitotic clock. *Exp Gerontol* 2000;35:711–719. [PubMed: 11053661]
- Rivier F, Alkan O, Flint AF, Muskiewicz K, Allen PD, Leboulch P, Gussoni E. Role of bone marrow cell trafficking in replenishing skeletal muscle SP and MP cell populations. *J Cell Sci* 2004;117:1979–1988. [PubMed: 15054107]
- Sacco A, Doyonnas R, Kraft P, Vitorovic S, Blau HM. Self-renewal and expansion of single transplanted muscle stem cells. *Nature* 2008;456:502–506. [PubMed: 18806774]
- Schiend J, Engleka KA, Jun S, Hansen MS, Epstein JA, Tabin CJ, Kunkel LM, Kardon G. Somitic origin of limb muscle satellite and side population cells. *Proc Natl Acad Sci USA* 2006;103:945–950. [PubMed: 16418263]
- Seale P, Sabourin LA, Girgis-Gabardo A, Mansouri A, Gruss P, Rudnicki MA. Pax7 is required for the specification of myogenic satellite cells. *Cell* 2000;102:777–786. [PubMed: 11030621]
- Sherwood RI, Christensen JL, Conboy IM, Conboy MJ, Rando RA, Weissman IL, Wagers AJ. Isolation of adult mouse myogenic progenitors: Functional heterogeneity of cells within and engrafting skeletal muscle. *Cell* 2004;119:543–554. [PubMed: 15537543]
- Skuk D, Caron N, Goulet M, Roy B, Espinosa F, Tremblay JP. Dynamics of the early immune cellular reactions after myogenic cell transplantation. *Cell Transplant* 2002;11:671–681. [PubMed: 12518894]
- Smythe GM, Grounds MD. Exposure to tissue culture conditions can adversely affect myoblast behavior in vivo in whole muscle grafts: Implications for myoblast transfer therapy. *Cell Transplant* 2000;9:379–393. [PubMed: 10972337]
- Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. *Science* 1988;241:58–62. [PubMed: 2898810]

- Tamaki T, Akatsuka A, Okada Y, Matsuzaki Y, Okano H, Kimura M. Growth and differentiation potential of main- and side-population cells derived from murine skeletal muscle. *Exp Cell Res* 2003;291:83–90. [PubMed: 14597410]
- Tanaka KK, Hall JK, Troy AA, Cornelison DD, Majka SM, Olwin BB. Syndecan-4-expressing muscle progenitor cells in the SP engraft as satellite cells during muscle regeneration. *Cell Stem Cell* 2009;4:217–225. [PubMed: 19265661]
- Torrente Y, Tremblay JP, Pisati F, Belicchi M, Rossi B, Sironi M, Fortunato F, El Fahime M, D'Angelo MG, Caron NJ, Constantin G, Paulin D, Scarlato G, Bresolin N. Intraarterial injection of muscle-derived CD34(+)Sca-1(+) stem cells restores dystrophin in mdx mice. *J Cell Biol* 2001;152:335–348. [PubMed: 11266450]
- Tremblay JP, Bouchard JP, Malouin F, Theau D, Cottrell F, Collin H, Rouche A, Gilgenkrantz S, Abbadi N, Tremblay M, Tomé FMS, Fardeau M. Myoblast transplantation between monozygotic twin girl carriers of Duchenne muscular dystrophy. *Neuromuscul Disord* 1993;3:583–592. [PubMed: 8186717]
- Uezumi A, Ojima K, Fukada S, Ikemoto M, Masuda S, Miyagoe-Suzuki Y, Takeda S. Functional heterogeneity of side population cells in skeletal muscle. *Biochem Biophys Res Commun* 2006;341:864–873. [PubMed: 16455057]
- Wallen CA, Higashikubo R, Roti Roti JL. Comparison of the cell kill measured by the Hoechst-propidium iodide flow cytometric assay and the colony formation assay. *Cell Tissue Kinet* 1983;16:357–365. [PubMed: 6190563]
- Watt DJ. A comparison of long-term survival of muscle precursor cell suspensions and minced muscle allografts in the non-tolerant mouse. *Adv Exp Med Biol* 1990;280:35–38. [PubMed: 2248153]
- Wolnicka-Glubisz A, King W, Noonan FP. SCA-1+ cells with an adipocyte phenotype in neonatal mouse skin. *J Invest Dermatol* 2005;125:383–385. [PubMed: 16098051]
- Wozniak AC, Pilipowicz O, Yablonka-Reuveni Z, Greenway S, Craven S, Scott E, Anderson JE. C-met expression and mechanical activation of satellite cells on cultured muscle fibers. *J Histochem Cytochem* 2003;51:1437–1445. [PubMed: 14566016]
- Yoshida N, Yoshida S, Koishi K, Masuda K, Nabeshima Y. Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates “reserve cells”. *J Cell Sci* 1998;111:769–779. [PubMed: 9472005]
- Zammit PS, Heslop L, Hudon Rosenblatt JD, Tajbakhsh S, Buckingham ME, Beauchamp JR, Partridge TA. Kinetics of myoblast proliferation show that resident satellite cells are competent to fully regenerate skeletal muscle fibers. *Exp Cell Res* 2002;281:39–49. [PubMed: 12441128]
- Zeng H, Park JW, Guo M, Lin G, Li XJ, Chen FP, Xu RH. Lack of ABCG2 expression and side population properties in human pluripotent stem cells. *Stem Cells* 2009;27:2435–2445. [PubMed: 19670287]
- Zhang X, Chen J, Davis B, Kiechle F. Hoechst 33342 induces apoptosis in HL-60 cells and inhibits topoisomerase I in vivo. *Arch Pathol Lab Med* 1999;123:921–927. [PubMed: 10506445]

Fig. 1.

Comparison of SP and MP cells isolated from EOM and TA of mice and rabbits. Whole cell suspensions from EOM and TA muscles from individual rabbits or 5–10 mice were labeled with Hoechst 33342 dye with or without verapamil (A). Analyses were performed using flow cytometry. Plots shown represent all live mononuclear cells. Percentage of cells falling within the SP gate in each species and muscle type were determined by flow cytometry (B). SP gate was determined as in A, using verapamil-treated cells as a negative control. n U 11 for each species.

Fig. 2.

Comparison of specific subpopulations of myogenic precursor cells found in the SP and MP gates. Mouse hindlimb muscle MP and SP cells were sorted, re-stained with 7AAD and immediately fixed. A: Cells were stained with antibodies against CD34 and Sca-1 and analyzed using flow cytometry. Plots represent cells that were alive just prior to fixation. Gray lines show isotype control staining. B: Cells from MP and SP stained for CD34 and Pax-7 or CD34 and M-cadherin. Gray lines represent isotype control staining. C: FACS plot showing CD34 cells examined for Sca-1 expression (X-axis) and M-cadherin expression (Y-axis). D: Immunohistochemistry confirming co-expression of Sca-1 and Pax7 with DAPI counterstain. E: The percentage of CD34+ cells co-expressing M-cadherin was analyzed by flow cytometry. F: The percentage of CD34+ cells co-expressing Pax-7 was analyzed using flow cytometry. $n = 3$ for each antigen examined. G: MP and SP cells examined for co-expression of Sca-1 and CD45. Gray lines show isotype control staining.

Fig. 3.

Determination of cell density of specific subpopulations of cells isolated by Hoechst. Cells isolated from mouse hindlimb muscle by the Hoechst dye exclusion assay. MP and SP cells were stained for expression of Sca-1, CD34, M-cadherin, Pax7, or CD45. Number of positive cells per mg tissue weight of the original muscle sample were determined. Sca-1, M-cadherin, CD45: n = 3, CD34: n = 6, Pax-7: n = 4.

Fig. 4.

Determination of amount of cell death due to the Hoechst assay. Cell death was analyzed using 7AAD-staining of whole cell suspensions from digested mouse hindlimb muscle. A: Cell death was significantly elevated in cells that were dissociated cells and exposed to Hoechst 33342 compared to cells not exposed to Hoechst. B: The percentage of cells that died with the addition of Hoechst dye at the time of the sort (“Pre-Sort”) were added to the percentage of cells that died within 30–90 min after the sort (“Post-Sort”) to calculate the total percentage of non-viable cells after both dissociated and sorting.