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In vitro clonal analysis of murine pluripotent stem cells isolated from skeletal muscle and adipose stromal cells

Jamie Case^{a,b}, Tamara L. Horvath^a, Christopher B. Ballas^a, Keith L. March^{a,c}, and Edward F. Srouf^{a,b,d}

^aDepartment of Medicine, Indiana University School of Medicine, Indianapolis, Ind., USA

^bDepartment of Pediatrics, Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, Ind., USA

^cIndiana Center for Vascular Biology and Medicine Indiana University School of Medicine, Indianapolis, Ind., USA

^dDepartment of Microbiology/Immunology, Indiana University School of Medicine, Indianapolis, Ind., USA

Abstract

Objective—Possible clinical utility of pluripotent stem cells (PSCs) with multilineage differentiation capacity depends on their ability to adapt to tissue-specific differentiation conditions. Previous data from our laboratory suggest that putative PSCs exhibiting an immunophenotype of CD45[−]Sca-1⁺CD117[−]CD90⁺ can be isolated from multiple tissues. In the present study, the clonal in vitro differentiation potential of two isolates of PSCs was examined.

Materials and Methods—Clonal analysis of the differentiation potential of skeletal muscle-(SM) and adipose stromal cell (ASC)-derived PSCs into myogenic, adipogenic, and neurogenic cells was investigated by expanding single PSCs prior to specification under three separate differentiation conditions.

Results—Differentiation of SM- and ASC-derived PSCs into myotubes, adipocytes, and neuronal-like cells was evident in clonal cultures promoting differentiation along these lineages. A total of 2.0%, 1.0%, and 0.33% of SM-derived clones demonstrated unipotent, bipotent, and tripotent differentiation, respectively, into combinations of myocytes, adipocytes, and neuronal cells. As a percentage of SM-derived PSCs, tripotent clones comprised 0.016% of total muscle cells. Similar results were obtained with ASC-derived PSCs, suggesting phenotypic and functional similarities between PSCs from both tissues. Following differentiation of single PSCs into three lineages, a clear and complete commitment to tissue-specific gene expression accompanied by inactivation of lineage-unrelated genes could not be demonstrated in several SM- and ASC-derived clones.

Conclusions—These data demonstrate that phenotypically defined PSCs remain functionally heterogeneous at the single-cell level and illustrate that morphologic lineage commitment may be independent of exclusive expression and/or loss of associated lineage specific genes.

Until recently, it was believed that tissue-specific stem cells (SC) could only differentiate into cells of their tissue of origin. However, recent reports have demonstrated that SC can differentiate into a variety of cell types. Bone marrow-derived hematopoietic SC and mesenchymal SC have been shown, for example, to differentiate in response to diverse microenvironmental cues into many different lineages belonging to all three germinal layers

[1–4]. Furthermore, differentiation of putative adult SC from several tissues into different cell types has been also widely reported. Groups of skeletal muscle (SM)–derived cells differentiated into myogenic, adipogenic, hematopoietic, and osteogenic fates [5–9]. Similarly, SC from other tissues have also been proposed to differentiate outside their tissue of origin [10–13].

We previously demonstrated that adult murine SM contained a population of cells defined as CD45[−] Sca-1⁺CD117[−] CD90⁺ capable of pluripotent differentiation into at least three distinct cell types [14–16]. Furthermore, we also demonstrated that cells with an identical phenotypic makeup as those detected in SM were also present among adipose stromal cells (ASC) [17, 18]. These ASC-derived CD45[−] Sca-1⁺CD117[−] CD90⁺ cells displayed similar multi-lineage differentiation potentials under appropriate culture conditions [17]. Analysis of single-cell suspensions of multiple other murine tissues, including bone marrow [18], blood, intestinal epithelium, myocardium, and brain revealed that CD45[−] Sca-1⁺CD117[−] CD90⁺ cells could be detected in all these tissues, albeit at significantly different frequencies (E.F. Srour, unpublished observations). Detection of CD45[−] Sca-1⁺CD117[−] CD90⁺ cells in several tissues and the observed functional similarities between PSCs from SM and ASC prompted us to examine whether cells from these two tissues behaved similarly at the single cell level.

In order to characterize putative adult stem cells as bona fide SC, they must undergo clonal pluripotent differentiation [19]. While some studies carried out differentiation studies from a clonally derived group of cells [9], these studies could not quantitate the frequency of pluripotent stem cells within a tissue, nor did they demonstrate the clonality of the observed differentiation over a large number of clones. We previously demonstrated that single SM- or ASC-derived PSCs were capable of clonal pluripotent differentiation in vitro to produce morphologically distinct myogenic, adipogenic, and neuronal progeny cells [17]. However, these studies did not examine the genetic profile of differentiated cells and, therefore, failed to determine the level of genetic commitment of progeny cells along each of these three lineages. In the present studies, we analyzed the multilineage differentiation potential of single SM- and ASC-derived PSCs and assessed the expression of lineage-specific genes in each clone. Our results illustrate that morphologic differentiation of clonally derived cells may proceed independently from complete genetic lineage specification, including downregulation or deactivation of nonlineage-specific genes.

Materials and methods

Animals

For these experiments, green fluorescent protein (GFP)⁺ C57BL/6 mice were utilized. SM was obtained from neonatal mice (7–14 days old), and adipose tissue was isolated from 6- to 9-month-old mice. GFP⁺ C57BL/6 mice were bred and maintained at Indiana University, according to protocols approved by the Laboratory Animal Research Facility of the Indiana University School of Medicine. All studies were approved by Laboratory Animal Research Facility and adhered strictly to National Institutes of Health guidelines for the use and care of experimental animals. Each experimental group of donor GFP⁺ C57BL/6 mice was obtained from the same litter.

Cell harvest

Skeletal muscle cells—For each experiment, five neonatal GFP⁺ C57BL/6 mice were euthanized by CO₂ inhalation and hind limbs were removed and placed in minimal essential medium plus Earle's salts (MEM; GIBCO, Grand Island, NY, USA). Adipose tissue and large vasculature (such as the femoral and popliteal arteries) were identified and removed carefully with fine forceps and discarded. SM (i.e., sartorius, quadriceps, thigh adductor, gastrocnemius,

and soleus muscles) was removed with fine forceps, excised from tendons and placed in 15 mL fresh medium. Using a sterile disposable scalpel, the muscle tissue was dissected and minced into a fine pulp and triturated by repeatedly pipetting the tissue with a 10-mL serologic pipette then centrifuged at 500 rpm for 10 minutes. The supernatant was discarded and the pelleted SM tissue was digested in 220 U/mL collagenase I (Worthington Biochemical Corporation, Lakewood, NJ, USA) and 33 U/mL dispase (BD Biosciences, Bedford, MA, USA) in 50 mL MEM for 45 minutes at 37°C with gentle agitation by a sterilized magnetic bar on a heated stir plate. After digestion, cells were filtered through a 40-µm cell strainer (BD Labware) into a sterile 50-mL conical tube then centrifuged at 500 rpm for 10 minutes. The supernatant was discarded, and viability was assessed by Trypan blue (Sigma, St. Louis, MO, USA) staining. Approximately 4 to 5×10^6 digested GFP⁺ SM cells were reproducibly obtained from each donor mouse.

Adipose stromal cells—For each experiment, between 5 and 10 sixto 9-month old GFP⁺ C57BL/6 mice were used. Adipose tissue was removed from the inguinal and lateral abdominal areas of the mice with fine forceps and placed in 15 mL MEM. Using a sterile disposable scalpel, the adipose tissue was dissected and minced into a fine pulp and triturated by repeatedly pipetting the tissue with a 10-mL serologic pipette. The adipose tissue was then digested and processed as described above. Viability was assessed by Trypan blue staining and approximately 3 to 4×10^6 digested GFP⁺ ASC were reproducibly obtained from each donor mouse.

Immunostaining of SM- and ASC-derived cells and flow-cytometric cell sorting

Freshly isolated GFP⁺ SM cells and ASCs were pelleted in phosphate-buffered saline (PBS) plus 1% fetal bovine serum (HyClone, Logan, UT, USA) and stained simultaneously with phycoerythrin–conjugated monoclonal anti-mouse Sca-1 (E13–161.7, BD Pharmingen, San Diego, CA, USA), allophycocyanin–conjugated monoclonal anti-mouse CD45 and CD117 (c-kit; 30-F11 and 2B8, respectively, BD Pharmingen) and biotin-conjugated anti-mouse CD90 (Thy-1; 53–2.1, BD Pharmingen). Secondary staining was performed using phycoerythrin–Cy7–conjugated streptavidin (Caltag Laboratories, Burlingame, CA, USA). All staining was performed at 4° C for 30 minutes and cells were washed with PBS plus 1% fetal bovine serum after staining.

Flow cytometry and cell sorting was performed on a FACS Aria (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA). Single GFP⁺ PSCs were sorted into individual wells of round-bottomed 96-well plates containing multipotent adult progenitor cell media [20] supplemented with 10 ng/mL platelet-derived growth factor (R&D Systems, Minneapolis, MN, USA), leukemia inhibitory factor (Chemicon International, Temecula, CA, USA) and epidermal growth factor (Sigma).

Cell-culture conditions

Tissue-specific cytokines and conditions were used to promote myogenic, adipogenic, and neurogenic differentiation. For clonality studies, single SM- or ASC-derived GFP⁺ PSCs were sorted into round-bottomed 96-well plates and expanded for 10 days in multipotent adult progenitor cell media as described previously. The 96-well plates were scored for expansion of cells, and on day 10, wells containing 30 or more cells were split into three equal parts and transferred to three individual wells of a flat-bottomed 96-well plate. Each set of three wells was conditioned with myogenic, adipogenic, and neurogenic conditioning media. For myogenic differentiation, cells were cultured in wells coated with 1% gelatin (Sigma) in MyeloCult M5300 medium (StemCell Technologies, Vancouver, BC, Canada) supplemented with 3 U/mL 5-azacytadine (Sigma). Cultures promoting adipogenesis were established in Dulbecco's modified Eagle medium (GIBCO) supplemented with 100 U penicillin/100 mg

streptomycin (Bio-Whittaker, Walkersville, MD, USA), 10% fetal bovine serum, 2 mM L-glutamine (BioWhittaker) and 20 ng/mL murine insulin-like growth factor 1 (R&D Systems). For neural differentiation, cells were cultured in wells coated with 10 ng/mL fibronectin (Sigma) in serum-free conditions containing Dulbecco's modified Eagle medium supplemented with 100 U penicillin/100 mg streptomycin, 2 mM L-glutamine, 1 mM HEPES (BioWhittaker), 100 ng/mL basic bovine fibroblast growth factor (R&D Systems), and 10 ng/mL platelet-derived growth factor (R&D Systems). Plates were incubated after seeding at 37° C, 5% CO₂, and 5% O₂. Only sets in which GFP⁺ cells were detected in each of the three sister wells 4 days later were considered for further analysis. After 14 days (total of 24 days in culture), in vitro clonal analysis of the single sorted SM- or ASC-derived PSCs was assessed for unipotent, bipotent, and tripotent cell growth.

Photomicroscopy and fluorescence imaging

Cultured SM- and ASC-derived GFP⁺ PSCs were visualized using a Zeiss Axiovert 25 inverted microscope with a CP-ACHROMAT/0.12NA objective. Images were acquired using a SPOT RT color digital camera (Diagnostic Instruments, Sterling Heights, MI, USA) with the manufacturer's software.

Polymerase chain reaction and gel electrophoresis

Total cellular RNA was isolated from both freshly sorted day-0 SM- and ASC-derived PSCs, as well as the cultured clonal cells, using the RNeasy Micro kit (Qiagen, Valencia, CA, USA) as per manufacturer's instructions. First-strand complementary DNA (cDNA) synthesis was performed by reverse transcription according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Polymerase chain reaction (PCR) was performed using gene-specific primer pairs (Integrated DNA Technologies, Coralville, IA, USA) as shown in Table 1. All sequences were obtained from Genebank.

Samples were amplified and denaturation was performed at 95° C for 30 seconds, annealing temperature was either 60° C or 63.5° C for 30 seconds and extension was carried out at 72° C for 30 seconds for a total of 35 cycles. Nested PCR was then performed using the first-round PCR product and the subsequent gene-specific nested primer pairs (Integrated DNA Technologies) as shown in Table 2. Nested PCR samples were amplified and denaturation was performed at 95° C for 30 seconds, annealing temperature was 60° C for 30 seconds and extension was at 72° C for 30 seconds for a total of 35 cycles. Nested PCR products were analyzed by electrophoresis using 2% agarose gels and a 100-bp ladder (Promega, Madison, WI, USA), visualized with ethidium bromide (Sigma), photographed, and data acquired using the Kodak Electrophoresis Documentation and Analysis System 120 (Eastman Kodak, Rochester, NY, USA).

Immunohistochemistry

Cultured adipocytes were gently washed three times and fixed for 1 hour in 10% formalin (Sigma) at 37° C then washed three more times with water. Cells were then stained for 90 minutes with oil red O (Sigma), prepared at 0.21% w/v in a 3:2 v/v mixture of absolute isopropanol to distilled water and filtered twice. Excess dye was washed off and cells were maintained in PBS to be photographed. Neurogenic cells grown on chamber slides were fixed for 4 minutes in 4% paraformaldehyde at reverse transcription followed by 2 minutes with absolute methanol at 4° C then blocked with 3% (w/v) dry milk in PBS for 1 hour. Cells were incubated overnight with goat anti-mouse Tau antibody (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-pig 200 kDa neurofilament (NF-200) antibody (1:500) (InnoGennex, San Ramon, CA, USA) that were directly conjugated to Alexa Fluor 546 and 647, respectively (Molecular Probes, Eugene, OR, USA). Slides were rinsed thoroughly with

PBS, coverslipped, and examined for positive fluorescence under a Zeiss LSM-510 confocal microscope.

Results

PSCs can be isolated from SM and adipose tissue

As described in Materials and Methods, SM and adipose tissue were collected and purified in order to obtain a single cell suspension. Single cell suspensions were immuno-stained and analyzed. PSCs expressing GFP were detected in both SM (Fig. 1A) and among adipose stromal cells (Fig. 1B). However, the frequency of ASC-derived PSCs was almost half of that detected in SM-derived cells. Both types of PSCs displayed similar morphologic and growth characteristic properties (data not shown).

SM- and ASC-derived PSCs exhibit myogenic, adipogenic and neurogenic potential in vitro

We have previously shown [17] that SM- and ASC-derived cells expressing the phenotype $CD45^- Sca-1^+CD117^-$ can differentiate into adipogenic, myogenic, and neurogenic cells in response to specific culture conditions promoting differentiation along these three lineages. PCR analysis of lineage-specific gene expression by freshly isolated SM- and ASC-derived PSCs revealed that these cells did not express a large number of muscle-, adipose-, and neuronal-specific genes, nor did they express the embryonic markers Oct-4 and Rex-1 (Table 3). In addition, freshly isolated SM- and ASC-derived PSCs did not express the hematopoietic-specific genes CD45, stem cell leukemia (SCL), c-kit, GATA-1, and PU.1. However, as can be seen in Table 3, fresh isolates from both tissues were positive for desmin and adiponectin, while some isolates expressed β -tubulin.

These data illustrate that phenotypically identical SM- and ASC-derived PSCs share the expression of a number of lineage-specific genetic markers and suggest that PSCs from at least these two tissues are similar. These results also suggest that PSCs isolates from both tissues may contain committed progenitor cells capable of lineage-specific differentiation in culture and that cells with clonal differentiation potential and other stem cell properties may not be exclusively contained within $CD45^- Sca-1^+CD117^- CD90^+$ cells.

Clonal analysis of PSCs differentiation

To investigate the clonal differentiation potential of SM- and ASC-derived PSCs, we initiated a series of single cell studies designed to examine the differentiation potential of clonal progeny of individual PSCs. Single SM cells or ASCs identified as $CD45^- Sca-1^+CD117^- CD90^+$ were deposited by cell sorting into individual wells of round-bottomed 96-well plates prepared with multipotent adult progenitor cell medium as described previously [20]. Cells that expanded within 10 days into clones of 30 cells or more, were separated into three equal aliquots and each was replated into a separate well conditioned as described in Materials and Methods to promote myogenic, adipogenic, or neurogenic differentiation. Cells from sister wells exhibiting distinct morphologies were harvested on day 24 and analyzed for expression of the same battery of mRNA transcripts used in the examination of freshly isolated PSCs.

Results of gene-expression profiling of three separate SM-derived PSCs clones, along with representative photomicrographs of the progeny of one of these clones under three different culture conditions can be seen in Table 4 and Figure 2A, respectively. As can be seen in Figure 2A, clonal progeny of SM-derived PSCs differentiated into morphologically discrete populations of cells when exposed to three different culture conditions. While some cells in each well failed to acquire all the morphological properties of the cell type contained in the well, the overwhelming majority of cells (visually approximated to be >70%) in each well displayed the unique physical attributes associated with differentiated cells within the particular

lineage. Cells that did not exhibit morphological properties of the expected cell lineage may have been progeny of cells that had lost their multilineage differentiation potential during the initial 10-day expansion period. These observations suggest that proliferation of single PSCs during the first 10 days of culture resulted in both self-renewal, as well as differentiation divisions, such that a substantial number of these cells retained their pluripotent differentiation potential.

In order to investigate whether differentiation of SM-derived PSCs into these three lineages was associated with lineage-specific gene expression, we examined the presence of mRNA of different tissue-specific genes by PCR. As outlined in Table 4, cells harvested from each of the three differentiation cultures initiated with cells expanded from three independent clones were analyzed for the expression of 12 different genes. Expression profiles of lineage-specific markers among these three clones demonstrated considerable heterogeneity both in the pattern of gene expression among cells within each clone and among cells generated in each of the three culture conditions (Table 4). While cells differentiated morphologically along one cell type in each defined condition, expression of tissue-specific genes was not restricted to the specific culture condition promoting this differentiation. Most noticeable was the expression of adipogenic gene markers in cells expanded in myogenic conditions, most likely due to the nonspecific impact of 5-azacytidine on gene activation. Interestingly, gene expression in the progeny of these clones was random, and no specific expression pattern could be detected, suggesting that, in addition to culture condition-induced differentiation, intrinsic stem cell differentiation outcomes may have influenced differentiation of these cells. Cells from the three clones shown in Table 4 were also examined for expression of CD45, SCL, c-kit, GATA-1, PU.1, Oct-4, and Rex-1, and none of these markers yielded positive results (data not shown). Similar results were obtained with ASC-derived clones, whereby three morphologically distinct cell types evolved from a single clone (Fig. 2B) and discordant genetic expression of lineage-specific genes was observed in clonal progeny of ASC-derived PSCs maintained under three different culture conditions (Table 5). Because it was logistically difficult to conduct both immunohistochemical and PCR analyses on the clonal progeny of SM- and ASC-derived PSCs, we maintained in parallel, bulk cultures of these cells and subjected these to immunohistochemical analysis 24 days later. Figure 3 depicts the oil red O staining of SM- and ASC-derived PSC maintained under adipogenic conditions as well as immunostaining of cells maintained under neurogenic differentiation conditions with Tau and NF-200. Data presented in Figure 3 confirm that the morphologic differentiation characteristics observed in clonal cultures (Fig. 2) were associated with immunohistochemical evidence in parallel bulk cultures of the same cells.

Overlapping coexpression of muscle, adipose, and neuronal markers was detected in fresh and cultured SM- and ASC-derived PSCs clones maintained under identical differentiation conditions. Therefore, a promiscuous expression of lineage-specific genes from multiple lineages was observed, suggesting that the accessibility of multiple lineage-specific differentiation programs may allow for greater flexibility in fate commitment and specification of these cells.

Frequency of unipotent and multipotent PSCs in SM and ASC

To determine the frequency of PSCs capable of multilineage differentiation, we visually assessed the proliferation potential of individual SM- and ASC-derived clones and determined morphologically their ability to differentiate into one, two, or three lineages in tissue-specific differentiation cultures. The same experimental design described above was used to initiate then separate progeny cells into three distinct cultures. Only clones demonstrating cell proliferation in all three sister wells on day 14 were considered for further evaluation.

When 1500 single SM-derived PSCs were cultured individually, 50 clones demonstrated some degree of tissue-specific differentiation following the separation of all progeny cells into three distinct culture conditions (Table 6). From these 50 clones, 30 were unipotent, giving rise to mostly myogenic or adipogenic cells only, 15 were bipotent, and only 5 clones were tripotent, giving rise to morphologically distinct myogenic-, adipogenic-, and neuronal-like cells under each specific culture condition. Therefore, analysis of single sorted SM-derived GFP⁺ PSCs indicated that unipotent, bipotent, and tripotent clonal differentiation was observed in 2.0%, 1.0%, and 0.33% of the total number of plated single cells, respectively. Because SM-derived GFP⁺ PSCs represent 4.78% of total cells analyzed, tripotent PSCs therefore comprised 0.016% of total muscle cells (Table 6).

Similar results were also obtained in clonal analyses of ASC-derived PSCs, suggesting that both SM- and ASC-derived GFP⁺ PSCs may be phenotypically and functionally related (Table 6). Interestingly, very similar frequencies of unipotent and multipotent clones among ASC-derived PSCs compared to those isolated from SM were observed regardless of whether these calculations were based on the number of plated cells or the immunophenotypically defined CD45⁻ Sca-1⁺CD117⁻ CD90⁺ fraction of cells within each tissue. These results illustrate that single PSCs isolated from two distinct mesoderm tissues undergo morphologically evident pluripotent differentiation and suggest that CD45⁻ Sca-1⁺CD117⁻ CD90⁺ cells from both tissues are functionally similar and may be common PSCs in different tissues.

Discussion

Adult SC plasticity and their potential to differentiate (or transdifferentiate) into multiple cell types have been both controversial and divisive. While many investigations documented the ability of adult SC to differentiate across different tissues and cell lineages [5,21,22], the concept of adult SC plasticity has been challenged recently [23–25]. Central to the notion of SC plasticity is the ability of these cells to differentiate into functional cells within the target tissue and in large enough numbers to induce a measurable functional contribution. These two requirements have been difficult to ascertain in many models. Furthermore, cell fusion has been implicated as the mechanism responsible for detection and possibly attributed differentiation capacity of candidate adult SC in regenerating tissues [26–28]. Also missing in most of these reports are answers to two key questions. First, are adult SC from different tissues related, and second, is the pluripotential differentiation capacity of these cells clonal?

Here we demonstrate that cells with similar phenotypic characteristics possessing multipotential differentiation properties can be identified in and isolated from both skeletal muscle and adipose stromal cells. Although a rather limited number of surface markers were used to identify cells from both tissues, a striking phenotypic resemblance between the two cell types was noted. Furthermore, gene-expression profiles of freshly isolated PSCs from both sources were also very similar, suggesting a close genetic relatedness between the two cell isolates. Whether these cells constitute a common pluripotent SC present in multiple tissues requires further studies given that only two mesoderm-derived tissues were examined here. Our data are in agreement with previously published reports regarding the differentiation potential of adult SC isolated from these two tissues. Neural differentiation of adipose-derived SC was reported in both the murine [29] and non-human primate [30] models. Qu-Petersen et al. [8] isolated a muscle-derived group of cells with long-term proliferating potential characterized as Sca-1⁺, CD45⁻, and CD117⁻, which were capable of in vitro and in vivo differentiation into muscle, neural and endothelial lineages. Based on the expression pattern of these three markers and the origin of these cells, it is possible that cells described in our report are closely related to those described by Qu-Petersen et al. [8]

The concept of similar pluripotent stem cells present in multiple tissues is not without precedent. The possibility that adult bone marrow may be the common reservoir for SC from different tissues has been proposed previously [31]. Cells with similar in vitro hematopoietic potentials defined by their side population property following staining with Hoechst 33342 were detected in different adult tissues [32], suggesting shared functional properties. The nature, origin, and functional capacity of adult SM-derived SC have been hotly debated for years, with many studies suggesting a close relationship and/or trafficking of precursors from the bone marrow to muscle [33–37].

Surprisingly, clonal differentiation experiments revealed that morphological changes among progeny cells were not coupled to a complete adoption of molecular profiles associated with strict lineage differentiation and commitment. Such results may be explained by the presence of more than one sorted cell in wells displaying tripotent differentiation and subsequently analyzed for gene-expression profiles. However, the likelihood of this possibility is miniscule, for two reasons. First, wells were examined under a microscope for the presence of a single GFP⁺ cell immediately after sorting without identifying any well in any experiment with more than one cell. In fact, the only observed deviation from having a single cell deposited in each well was the detection of few empty wells, which normally results from an aborted sort of a selected cell due to the close proximity of an unselected cell in the flow stream. Second, using the doublet discriminator option during cell sorting ensures against the deposition of two attached cells into a single well.

The observed genetic promiscuity among progeny of these cells poses two interesting possibilities. First, differentiating stem cells may partially retain their ancestral genetic profile until a complete and final lineage commitment has been concluded. Second, simultaneous activation of transcriptional pathways and their related genes across different lineages allows progeny cells to adopt any of several commitment pathways as the need arises. While use of quantitative PCR would have been more sensitive to small changes in gene expression and thus more informative as far as the genetic makeup of progeny cells, conversion from negative gene expression among freshly isolated cells to positive readouts among expanded cells was an adequate indicator of specific gene activation in the context of the described morphological differentiation. Unfortunately, the limited numbers of cells in the clonal progeny of SM- and ASC-derived PSCs precluded us from obtaining concurrent immunohistochemical evidence for the clonal differentiation of these cells. However, such evidence was obtained from bulk cultures maintained in parallel under the same differentiation culture conditions.

That cells can differentiate into unexpected lineages while retaining their original differentiation potential has been recognized previously [38]. Cells defined by Cao et al. [38] are phenotypically very similar to PSCs described here. Both cell types are Sca-1⁺ and do not express CD117 and CD45. Whereas muscle-derived cells described by Cao et al. were capable of in vivo differentiation into hematopoietic lineages as we described previously [14], they also retained their potential to differentiate into myotubes after acquiring a hematopoietic fate [38]. Although not formally demonstrated by Cao et al. [38], it is probable that these muscle-derived stem cells retained their expression of myogenic-specific genes during their hematopoietic differentiation, reminiscent of the gene-expression profile of clones described here. This pattern of gene-expression infidelity has been described by many investigators. Goolsby et al. [39] demonstrated that hematopoietic progenitors express neural genes. Human bone marrow stem cells were determined to express neuroglial transcripts prior to any in vitro treatment or induction [40] including, as shown for our PSCs, β -tubulin. Of interest, is that human-derived stem cells described in these studies [40] expressed CD90, a marker also expressed by SM- and ASC-derived PSCs [17] (Fig. 1). Under different conditions, it has been also shown that fetal neural SC express nonneural markers when cocultured with embryoid bodies [41]. Promiscuous gene expression during human embryonic SC differentiation was

also noted in other systems [42]. More recently, Shiota et al. [43] described bone marrow–derived spheres, which constitutively expressed adipocytic, osteoblastic, and skeletal myeloblastic genes prior to in vitro specification and which clonally expanded into functional clones with varied gene-expression patterns as described here for SM- and ASC-derived clones.

Zipori introduced in 2004, the concept of open expression of genes from different lineages within a single stem cell population [44]. Under this scenario, a stem cell has all options open and stemness becomes a state, not an entity. In this model, stem cells coexpress multiple genes from different lineages and downregulate irrelevant genes as they commit to a particular differentiation pathway. Egusa et al. [45] documented gene silencing among discordant phenotypes of bone marrow–derived stromal cells as those differentiated along a neuronal pathway. Expression of neural genes among human mesenchymal stem cells, albeit at low levels in many cases, was considered by Blondheim et al. [46] as a “predisposition” of these cells for neuronal differentiation. Whether expression of some genes by freshly isolated PSCs described here or the unexpected expression of lineage-specific genes among their differentiated progeny is a manifestation of the stem cell state proposed by Zipori [44] remains to be investigated. It is important to stress that the observed promiscuous expression of lineage-specific genes among differentiated cells may have resulted from early analysis of these cells before differentiation was completed or because differentiation was arrested due to the absence of critical differentiation signals. Clearly, more detailed analyses of these clones are required for a better understanding of the differentiation potential of the cells and a more comprehensive assessment of the functional properties of progeny cells.

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Figure 1.

Flow cytometric isolation of pluripotent stem cells (PSCs) from skeletal muscle (**A**) and adipose (**B**) tissues. Single cell suspensions from both tissues were stained for CD45, CD117, Sca-1, and CD90. Green fluorescent protein (GFP)⁺ cells not expressing CD45 and CD117 were first identified (gate R2 in the top and bottom left dot plots) and these cells were then analyzed for expression of CD90 (X axis) and Sca-1 (Y axis) simultaneously. CD45⁻ Sca-1⁺CD117⁻ CD90⁺ cells (gate R3 in the top and bottom right dot plots) were identified and selected as PSCs. The purity of sorted cells always exceeded 96% and viability was >95%.

Figure 2.

Photomicrographs of clonally derived progeny of skeletal muscle (**A**) and adipose stromal cells (**B**) green fluorescent protein positive pluripotent stem cells differentiated in three different tissue differentiation media after 10 days of initial expansion in multipotent adult progenitor cell medium. Each set of three micrographs showing myogenic (top), adipogenic (middle), and neuronal (bottom) differentiation was obtained from the same clone.

Figure 3.

Immunohistochemical analysis of adipogenic and neurogenic differentiation of skeletal muscle (SM)- and adipose stromal cells (ASC)-derived pluripotent stem cells (PSCs). (A, B) Oil red O staining of SM- and ASC-derived adipocytes, respectively, following differentiation in adipogenic differentiation media for 24 days. (C) Immunofluorescent staining of SM-derived neuronal cells with Tau. (D) Immunofluorescent staining of ASC-derived neuronal cells with neurofilament; (NF-200). Cells in both (C) and (D) were also stained with DAPI. (E-G); ASC-derived neuronal cells stained with DAPI (E), with NF-200 (F) and the merged image (G) of both. Cells shown in (C) through (G) were maintained in neuronal differentiation cultures for 24 days.

Table 1

Gene-specific primers

Tissue	Gene	5' Sequence	3' Sequence	Size (bp)
Muscle	Desmin	CAG CCA CTC TAG CTC GTA TT	CTG GAT CTG GTG TCG GTA TT	338
	Myogenin	GAG AAG CGC AGG CTC AAG AA	GCT GTC CAC GAT GGA CGT AA	354
	MyoD	GCT CGT GAG GAT GAG CAT GT	ACT GTA GTA GGC GGT GTC GT	471
Adipose	Ap-2	TTG TCT CCA GTG AAA ACT TCG	ATG CAA ATT TCC ATC CAG GC	401
	Adipsin	ATG GAT GGA GTG ACG GAT GA	ATT GCC ACA GAC GCG AGA GA	495
	Lipoprotein lipase	CAA GTT TTA GAG CAG GAC CAT	TGA TGA AAT CGG TCA CCT TTG	504
	Adiponectin	AGG ACA TCC TGG CCA CAA T	TAG AGT CCC GGA ATG TTG CA	342
Neural	β -Tubulin	ATG GGC ACA CTG CTC ATC A	TGG GCA TTG AGC TGA CCA A	308
	Tau	AAG AAG CAG GCA TCG GAG AC	AGG CGG CTC TTA CTA GCT GA	418
	GFAP	AAT GCT GGC TTC AAG GAG ACA	AGA ACT GGA TCT CCT CCT CCA	424
	Synaptophysin	GTT GGA TCC AGC ATC TCT AGA A	AGC CCC ACA GAC CTG TAG A	457
	NF-200	TCC CAA CTA GGC TCT TGT TGA	ACT GCC TAC ACT GAA CGT CCC	503
	CD45	CCT GAG TCT GCA TCT AAA CCC C	TGC TTG GCC AGT ATT CTG GCA	294
Hematopoietic	SCL	CAG CCG CTC GCC TCA CTA GG	CTT CAT GGC AAG GCG GAG GA	279
	c-kit	GTG CAT TGA TCC CGA CTT TG	ATT GGA CAC CTG TGG GTC TG	268
	GATA 1	ACT CGT CAT ACC ACT AAG GT	AGT GTC TGT AGG CCT CAG CT	260
	PU.1	ACA GAT GCA CGT CCT CGA TAC T	TCC TTG TGC TTC GAC CAG AAC T	330
	Oct-4	CAA CAA TGA GAA CCT TCA GGA	TTG CCT TGG CTC ACA GCA T	484
Embryonic	Rex-1	ATG AAA GTG AGA TTA GCC CCG	TGC ACC AGA AAA TGT CGC TT	496

GFAP = glial fibrillary acidic protein; NF-200 = neurofilament; SCL = stem cell leukemia.

Table 2

Nested gene-specific primers

Tissue	Gene	Nested 5' sequence	Nested 3' sequence	Size (bp)
Muscle	Desmin	GGA GAT CGC GTT CCT TAA GA	TCT TAT TGG CTG CCT GAG TC	227
	Myogenin	CAG CGC CAT CCA GTA CAT TG	GAC CGA ACT CCA GTG CAT TG	173
Adipose	MyoD	CCA TCC GCT ACA TCG AAG GT	GCC GCT GTA ATC CAT CAT GC	185
	Ap-2	TTG GTC ACC ATC CGG TCA GA	CAT TCC ACC ACC AGC TTG TC	209
	Adipsin	GAG TGA CGG ATG ACG ACT CT	GAT TGC AGG TTG TCC GGT TC	336
	Lipoprotein Lipase	GAT TGT TGC CGC TGT T	TGT GGG TTG GTG TTC A	200
	Adiponectin	ATG GCA CAC CAG GCC GTG AT	AAG CGG CTT CTC CAG GCT CT	177
Neural	β -Tubulin	AGT GTC AGA CAC CGT GGT AG	GCA GAC ACA AGG TGG TTG AG	171
	Tau	CAA GCT GCT GTG GCC AGC AA	AGC CGC TTC GTT CTC CGG AT	220
	GFAP	AAC CAG CTT CGA GCC AAG GA	ACG CAG CCA GGT TGT TCT CT	211
	Synaptophysin	GAT CCA GCA TCT CTA GAA GG	CCA CAA TAC TTG GCT CTT CC	366
	NF-200	CCG TTC TGC TTG CTC CAG AT	CAG GCA CAT GGT GGC ACC TT	230
Embryonic	Oct-4	GAT CAC TCA CAT CGC CAA TC	CCT GTA GCC TCA TAC TCT TC	129
	Rex-1	CCT GAC GGA TAC CTA GAG TG	CTT GCG TGA CCT CTG TCT TC	205

GFAP = glial fibrillary acidic protein; NF-200 = neurofilament.

Table 3

Expression of tissue-specific markers in freshly isolated skeletal muscle— and adipose stromal cell—derived pluripotent stem cells

Tissue	Genes	SM-derived PSCs (day 0) ^a	ASC-derived PSCs (day 0) ^a
Muscle	Desmin	+	+
	Myogenin	—	—
	MyoD	—	—
Adipose	Ap-2	—	—
	Adipsin	—	—
	Lipoprotein lipase	—	—
Neuronal	Adiponectin	+	+
	β-Tubulin	±	±
	Tau	—	—
	GFAP	—	—
	Synaptophysin	—	—
Hematopoietic	NF-200	—	—
	CD45	—	—
	SCL	—	—
	c-kit	—	—
	GATA 1	—	—
Embryonic	PU.1	—	—
	Oct-4	—	—
	Rex-1	—	—

GFAP = glial fibrillary acidic protein; NF-200 = neurofilament.

^a Analysis of the day-0 samples was performed using nested polymerase chain reaction. The ± designation indicates that β-tubulin was detected as positive in some, but not all isolates. The + designation indicates that all isolates were positive. These data were derived from the analysis of three independent isolates of skeletal muscle (SM)— and adipose stromal cell (ASC)—derived pluripotent stem cells (PSCs).

Table 4
Expression of tissue specific markers in lineage specific expansion cultures of skeletal muscle—derived pluripotent stem cell clones

Gene	SM-derived PSCs (day 0) ^a	Differentiated SM-derived PSCs clones								
		Clone 1			Clone 2			Clone 3		
		Myo	Adip	Neu	Myo	Adip	Neu	Myo	Adip	Neu
Desmin	+	+	+	+	+	-	-	+	+	-
Myogenin	-	+	-	-	-	-	-	+	-	-
MyoD	-	-	-	-	-	-	-	-	-	-
Ap-2	-	+	+	-	+	+	-	+	+	-
Adipsin	-	+	+	-	+	+	-	-	+	-
Lipoprotein lipase	-	+	+	-	-	+	-	-	+	-
Adiponectin	+	+	+	-	+	+	-	+	+	-
β-tubulin	±	+	-	+	+	-	+	+	+	+
Tau	-	-	-	+	-	-	-	-	-	-
GFAP	-	-	-	+	-	-	-	-	-	-
Synaptophysin	-	-	-	-	-	-	-	-	-	-
NF-200	-	+	+	-	-	-	-	+	+	-

Adip = adipogenic; GFAP = glial fibrillary acidic protein; Myo = myogenic; Neu = neurogenic; NF-200 = neurofilament; PSC = pluripotent stem cells; SM = skeletal muscle.

^a Analysis of the day-0 samples was performed using nested polymerase chain reaction. The ± designation indicates that β-tubulin was detected as positive in some, but not all isolates. The + designation indicates that all isolates were positive.

Table 5
Expression of tissue specific markers in lineage specific expansion cultures of adipose stromal cell—derived pluripotent stem cell clones

Gene	ASC-derived PSCs (day 0) <i>a</i>	Differentiated ASC-derived GFP ⁺ PSCs clones								
		Clone 1			Clone 2			Clone 3		
		Myo	Adip	Neu	Myo	Adip	Neu	Myo	Adip	Neu
Desmin	+	+	—	+	—	—	—	—	+	—
Myogenin	—	+	—	—	+	—	—	—	—	—
MyoD	—	—	—	—	—	—	—	+	—	—
Ap-2	—	—	+	—	+	+	—	—	+	—
Adipsin	—	+	—	—	—	—	—	—	—	—
Lipoprotein lipase	—	—	—	—	+	+	—	—	—	—
Adiponectin	+	+	—	—	—	+	—	—	+	—
β-tubulin	±	—	+	+	—	+	+	—	+	+
Tau	—	—	—	—	—	+	—	—	—	—
GFAP	—	—	—	—	—	—	—	—	—	—
Synaptophysin	—	—	—	—	—	—	—	—	—	—
NF-200	—	—	+	—	—	—	—	+	—	—

Adip = adipogenic; ASC = adipose stromal cells; GFAP = glial fibrillary acidic protein; GFP = green fluorescent protein; Myo = myogenic; Neu = neurogenic; NF-200 = neurofilament; PSC = pluripotent stem cells.

a Analysis of the day-0 samples was performed using nested polymerase chain reaction. The ± designation indicates that β-tubulin was detected as positive in some, but not all isolates. The + designation indicates that all isolates were positive.

Table 6
Frequency of unipotent and multipotent clones among skeletal muscle— and adipose stromal cell—derived pluripotent stem cells

Method ^a	Frequency of (%)					
	Plated wells			CD45 ⁺ Sca-1 ⁺ CD117 ⁺ CD90 ⁺ ^b		
	Unipotent	Bipotent	Tripotent	Unipotent	Bipotent	Tripotent
SM (1500 wells)	2.0	1.0	0.33	0.096	0.048	0.016
ASC (1500 wells)	2.08	0.85	0.45	0.047	0.019	0.010

ASC = adipose stromal cells; SM = skeletal muscle.

^aData were collected from three separate experiments per tissue (1 GFP⁺ cell per well).

^bCalculations based on a frequency of 4.78% and 2.27%, respectively, for SM and ASC-derived CD45⁺Sca-1⁺CD117⁺CD90⁺ cells.