the dystrophic phenotypes of *mdx* mice when introduced as a transgene[24] or by adeno-associated viral vectors.[25] We therefore inserted a 4.9 kb micro-dystrophin *CS1* into the lentiviral vector as a therapeutic gene. Freshly isolated *mdx*-SM/C-2.6$^+$ cells were transduced with lentiviral vectors expressing micro-dystrophin *CS1* under the control of a CMV promoter (**Figure 4a**) at a MOI of 200 for 16 hours. In this condition, 97% of the cells expressed micro-dystrophin *CS1* (data not shown). These transduced cells were injected into TA muscles of 5-week-old *mdx* mice at 2×10^4 cells per muscle. Four weeks after the injection, the muscle regeneration capacity of the cells was investigated by immunodetection of micro-dystrophin-positive fibers. Many myofibers expressed micro-dystrophin *CS1* on the sarcolemma at an average of 120 fibers per muscle (**Figure 4b**). Further, we examined the restoration of the dystrophin-associated protein complex in micro-dystrophin-positive fibers by immunodetection of α-SG, β-dystroglycan, and α1-syntrophin. As shown in **Figure 4c**, all these proteins were expressed at the sarcolemma of micro-dystrophin *CS1*-positive myofibers, thereby suggesting the recovery of dystrophin-associated protein complex by the introduction of micro-dystrophin. These results indicate that *mdx*-SM/C-2.6$^+$ cells transduced with lentiviral vectors expressing micro-dystrophin *CS1* efficiently contribute to regeneration of dystrophic muscles of *mdx* mice and restore the expression of the dystrophin/dystrophin-associated protein complex. It therefore follows that transplantation of autologous myogenic precursor cells prepared using the SM/C-2.6 antibody and genetically corrected by a lentiviral vector, is a possible approach for cell therapy in DMD or in localized forms of muscular dystrophy.

DISCUSSION

In vitro *passaging reduced the regenerative capacity of satellite cells*: In the present study, we directly isolated satellite cells from skeletal muscles of wild-type and *mdx* mice using SM/C-2.6, a novel monoclonal antibody,[35] and flow cytometry. Almost all satellite cells prepared from normal muscle are negative for MyoD, myogenin, and Ki67 immediately after isolation, thereby indicating that they are in a quiescent state. In contrast, approximately 20% of *mdx*-satellite cells are positive for MyoD and 35% are positive for Ki67 (**Table 1**). This result indicates that a fraction of *mdx*-satellite cells are already in an activated state.

Transplantation experiments showed that freshly isolated SM/C-2.6$^+$ satellite cells possess a higher capacity for muscle reconstitution when compared with SM/C-2.6$^+$ myoblasts passaged *in vitro* prior to transplantation. This result indicates that passaging and subsequent proliferation of satellite cells in culture reduce their intrinsic capacity for muscle reconstitution. In order to clarify the mechanisms of low myogenicity of passaged cells, we performed a colony-forming assay of freshly isolated satellite cells and passaged satellite cell-derived myoblasts (passage 1). When the cells were seeded at a density of 1 cell/well on 96-well plates, fresh satellite cells formed larger colonies than "passage 1" myoblasts (**Supplementary Figure S1**). In contrast, there was no difference in fusion index between these two cell populations (data not shown). Collectively, reduced efficiency of muscle fiber regeneration by passaged myoblasts can be partly explained by gradual loss of proliferative ability during passaging.

Importantly, we also found that satellite cells that were expanded *in vitro* without passaging showed regenerative capacity comparable to freshly isolated satellite cells. We therefore hypothesized that it might be possible to introduce therapeutic genes into satellite cells *in vitro* by a lentiviral vector before transplantation without causing any reduction in their regenerative capacity.

Comparison of regenerative capacity of SM/C-2.6$^+$ satellite cells with other reports: Previously, Montarras *et al.* directly isolated (Pax3) GFP-expressing satellite cells, which constitute a homogeneous population of small, non-granular, CD34$^+$ CD45$^-$ Sca-1$^-$ cells, from diaphragms of adult *Pax3*$^{GFP/+}$ mice by flow cytometry, and examined their regenerative capacity.[23] The researchers concluded that *in vitro* expansion of freshly isolated satellite cells for a few days prior to transplantation is a disadvantageous approach, because such satellite cell-derived myoblasts displayed considerably lower muscle regenerative efficiency than fresh satellite cells. In contrast, we observed no reduction in regenerative capacity as a result of *in vitro* expansion of freshly isolated satellite cells without passaging, although their capacity was remarkably reduced after passaging (**Figure 1d** and **e**). The discrepancy between the results of Montarras *et al.* and our results may be due to differences in the culture conditions employed. One possible explanation could be that our culture medium contained basic fibroblast growth factor. It has been reported that addition of basic fibroblast growth factor to culture medium improves transplantation efficiency.[42,43] The modification of culture conditions may enable maintenance of the intrinsic muscle regenerative capacity of satellite cells.

Previous muscle transplantation experiments utilized the progeny of satellite cells enzymatically dissociated from myofibers and extensively cultured to increase their numbers.[15,20–22] When 5×10^5 to 1×10^6 myoblasts taken from normal mice and prepared by the pre-plating method were transplanted into non-irradiated muscles of *mdx* mice, it resulted in fewer than 100 dystrophin-positive myofibers per muscle.[21] On the other hand, when 5×10^5 cells were injected into muscles of immunodeficient *mdx nu/nu* mice that had been pre-irradiated to ablate endogenous satellite cell function, they formed an average of 328 dystrophin-positive fibers.[18] Furthermore, grafting of 2×10^4 satellite cells freshly isolated from *Pax3*$^{GFP/+}$ mice into pre-irradiated TA muscles of *mdx nu/nu* mice led to dystrophin expression in an average of 587 fibers.[23] In our experiment, the same number (2×10^4) of satellite cells freshly isolated from adult normal mice gave rise to an average of only 277 myofibers in non-irradiated *mdx* muscles (**Figure 1d** and **e**). This shows that grafted muscle precursor cells form a far greater number of dystrophin-positive fibers in irradiated muscle than in non-irradiated muscle. The use of immunosuppressants such as FK506 also greatly improves the efficiency of transplantation.[7] In the present study, we injected myogenic cells into non-irradiated TA muscles of immunocompetent mice without any immunosuppressant. Therefore, in our experimental

conditions, the intrinsic function of SM/C-2.6$^+$ satellite cells may be underestimated.

The use of the Lentiviral vector is feasible for ex vivo *gene transfer*: In this study we showed that, at a MOI of 200, lentiviral vectors can introduce the rod-truncated micro-dystrophin gene *CS1* into more than 97% of *mdx*-satellite cells without detrimental effects on cell viability and regenerative capacity. But at a MOI of 300 we observed cell toxicity, whereas at a MOI of 100, the transduction efficiency was below 80% (data not shown). When we injected the transduced autologous myoblasts into *mdx* muscle, the cells contributed to regeneration of myofibers and expressed micro-dystrophin and dystrophin-associated proteins at the sarcolemma. Our results therefore suggest that *ex vivo* gene transfer into autologous myogenic cells by a lentiviral vector is feasible. On the other hand, direct intramuscular injection of vesicular stomatitis virus-G glycoprotein–pseudotyped lentiviral vectors led to relatively low expression of the transgene in mouse skeletal muscles.[30,44] Because the lentiviral vector genome is inserted into the host genome, the transduction of cells other than the target cell could introduce the risk of mutagenesis. Further, *in vivo* administration could induce undesirable immune responses to exogenous viral proteins. In effect, direct *in vivo* administration of lentiviral vectors poses a safety problem for clinical application. In contrast, *ex vivo* gene transfer has the merit of minimizing the risks of introducing free lentiviral vectors into the host. Transduced cells can efficiently proliferate and differentiate *invitro* (data not shown).

Limitations of ex vivo *gene therapy in DMD, using satellite cells*: One of the demerits of our procedure, as compared to *in vivo* gene transfer, is that only a part of the genetically modified myogenic precursor cells contributes to regeneration of the host muscle, given the poor survival rate of these cells. In fact, by using real-time polymerase chain reaction on transcripts from the transgenic enhanced GFP gene, we found that more than 90% of the injected cells were lost within the first 24 hours after injection (data not shown). In addition, migration of the surviving cells is limited in the host muscle after injection. Furthermore, because *in vitro* passaging greatly reduces their myogenicity, it is difficult to obtain a sufficient number of satellite cells or their progeny from a small muscle biopsy of a DMD patient. Therefore current myoblast transfer might be more realistic for localized forms of muscular dystrophy, such as oculo-pharyngeal muscular dystrophy or facio-scapulo-humeral muscular dystrophy.[45] Surprisingly, however, Collins *et al.* transplanted a single intact myofiber into irradiation-ablated muscles and demonstrated that as few as seven satellite cells associated with one transplanted myofiber can generate over 100 new myofibers containing thousands of myonuclei.[5] Their observations suggest that proper isolation and handling of satellite cells might greatly improve their myogenic potential.

In this study we have demonstrated transplantation of autologous satellite cells genetically corrected by a lentiviral vector *ex vivo* into *mdx* muscle. For treating DMD patients, however, it is necessary to find the optimum *in vitro* culture condition that will enable human muscle precursor cells to maintain their intrinsic myogenic potential. It would also be useful to identify the factors that support survival and/or proliferation of transplanted cells in the host muscle.

MATERIALS AND METHODS

Animals. All procedures used on the experimental animals were approved by the Experimental Animal Care and Use Committee at the National Institute of Neuroscience. Eight-to-twelve-week-old C57BL/6 mice were purchased from Nihon CLEA (Tokyo, Japan). C57BL/6-GFP Tg mice were kindly provided by Dr. Okabe (Osaka University, Japan). C57BL/10 mice and C57BL/10-*mdx* mice were maintained in our animal facility and propagated by allowing mating.

In order to induce muscle regeneration, 50 µl of CTX (10 µmol/l in saline; Wako Pure Chemical Industries, Tokyo, Japan) was injected into the TA muscle 24 hours before cell transplantation.

Cell preparation and FACS analysis. Freshly isolated muscle-derived cells were prepared from 8–12-week-old GFP-Tg mice, C57BL/6 mice, C57BL/10 mice, or 5-week-old *mdx* mice as previously described.[35] Hind-limb and fore-limb muscles were isolated and digested with 0.2% type II collagenase (Worthington Biochemical, Lakewood, NJ) for 90 minutes at 37 °C. The muscle slurries were filtered through 100 µm nitrex mesh (BD Biosciences, Franklin Lakes, NJ) and subsequently through 40 µm nitrex mesh (BD Biosciences, Franklin Lakes, NJ). Erythrocytes were eliminated by treatment with 0.8% NH_4Cl in Tris-buffer. Mononucleated cells were stained with biotinylated SM/C-2.6 monoclonal antibody,[35] and labeled by allophycocyanin-conjugated streptavidin (BD PharMingen, San Diego, CA). Mononucleated cells derived from *mdx* muscles were stained with antibodies to additional surface markers, phycoerythrin-conjugated anti-CD45 antibody (clone 30-F11; BD PharMingen, San Diego, CA), phycoerythrin-conjugated anti-CD31 antibody (clone 390; BD PharMingen, San Diego, CA), and phycoerythrin-conjugated anti-Sca-1 antibody (clone D7; BD PharMingen, San Diego, CA). After being washed, stained cells were re-suspended in phosphate-buffered saline (PBS) containing 2% fetal bovine serum (Trace Biosciences, New South Wales, Australia) and 2 µg/ml propidium iodide (BD PharMingen, San Diego, CA). Cell sorting was performed on a FACS VantageSE flow cytometer (BD Biosciences, Franklin Lakes, NJ). Debris and dead cells were excluded by forward scatter, side scatter, and propidium iodide gating. We used only propidium iodide-negative fractions for further experiments. We usually obtained approximately 1.5×10^5 sorted cells from 1 g of muscle of 8–12-week-old female C57BL/6 mice.

Cell culture and intramuscular transplantation. Freshly isolated SM/C-2.6$^+$ cells from GFP-Tg mice were seeded at a density of 1×10^5 cells per 35-mm dish coated with Matrigel (BD Biosciences, Franklin Lakes, NJ) in a growth medium, Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing 20% fetal bovine serum and 2.5 ng/ml basic fibroblast growth factor (Invitrogen, Carlsbad, CA), and expanded for 4 days. Further, these cells were passaged up to three times at 2-day intervals. Primary myoblasts isolated by the pre-plating method[39] from GFP-Tg mice were also cultured in growth medium. Freshly sorted cells, expanded and passaged cells, or cultured primary myoblasts were injected into TA muscles of 8–12-week-old CTX-treated C57BL/6 mice or 5-week-old *mdx* mice that show active cycles of the degeneration–regeneration process. The number of injected cells was 2×10^4 per TA muscle. Four weeks later, the injected muscles were isolated and fixed in 4% paraformaldehyde for 30 minutes, immersed sequentially in 10% sucrose/PBS and 20% sucrose/PBS, and frozen in isopentane cooled with liquid nitrogen.

Immunohistochemistry. Frozen muscle tissues were sectioned (6 µm) using a cryostat. The sections were blocked with 5% goat serum (Cedarlane, Hornby, Canada) in PBS and then reacted with anti-GFP antibody (1:100; Chemicon International, Temecula, CA) and/or anti-laminin α2 antibody (1:100; Alexis, San Diego, CA), or anti-α1-syntrophin antibody

(1:500)[46] at 4 °C overnight. Dystrophin (1:20; NCL-DYSB or DYS2; Novocastra, Newcastle, UK), α-SG (1:50; NCL-α-SARC; Novocastra, Newcastle, UK), and β-dystroglycan (1:50; NCL-β-DG; Novocastra, Newcastle, UK) were detected using monoclonal antibodies after blocking with a MOM kit (Vector Laboratories, Burlingame, CA). The sections were incubated with appropriate combinations of Alexa 488-, Alexa 568-, and Alexa 594-labeled secondary antibodies (Molecular Probes, Eugene, OR) for 30 minutes. The nuclei were counterstained with TOTO-3 (1:5,000; Molecular Probes, Eugene, OR). The stained sections were observed under the confocal laser scanning microscope system TCSSP (Leica, Heidelberg, Germany).

Immunocytochemistry. Cells sorted using FACS were collected by Cytospin3 (Thermo Fisher Scientific, Waltham, MA). After being fixed with 4% paraformaldehyde for 10 minutes, the cells were blocked with 5% goat serum in PBS and then reacted with anti-Pax7 antibody (1:2; Developmental Studies Hybridoma Bank, Iowa, IA), anti-MyoD antibody (1:200; Dako, Glostrup, Denmark), anti-myogenin antibody (1:200; Developmental Studies Hybridoma Bank, Iowa, IA), and anti-Ki67 antibody (1:2, Ylem, Rome, Italy) at 4 °C overnight. Primary antibodies were detected by Alexa 488- or Alexa 568-labeled secondary antibodies (Molecular Probes, Eugene, OR) for 30 minutes. Stained cells were mounted in Vectashield with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) and observed with fluorescence microscopy IX70 (Olympus, Tokyo, Japan).

Generation of lentiviral vectors and* in vitro *transduction. The third-generation self-inactivated human immunodeficiency virus-1–based lentiviral vector, pCSII-CMV-IRES2-Venus, has been described previously.[47] The vector contains a CMV promoter; an internal ribosomal entry site (IRES) followed by *Venus*, which is a variant of yellow fluorescent protein[41]; and a woodchuck hepatitis virus post-transcriptional regulatory element. A rod-truncated micro-dystrophin *CS1* complementary DNA (four rod repeats, 4.9 kb) was excised from pCAG-*CS1*[24] and cloned into pCSII-CMV-IRES2-Venus, generating pCSII-CMV-*CS1*-IRES2-Venus. The lentiviral vectors expressing Venus only, or micro-dystrophin *CS1* followed by Venus, were generated by transient cotransfection of the pCSII-CMV-IRES2-Venus or pCSII-CMV-*CS1*-IRES2-Venus, respectively, with the packaging construct (pCAG-HIVgp), vesicular stomatitis virus-G protein, and Rev-expressing construct (pCMV-VSV-G-RSV-Rev) into 293T cells, using the calcium phosphate transfection method.[47–49] Two days after transfection, the vector-containing supernatant was collected, filtered through a 0.45-μm-pore-size filter (Thermo Fisher Scientific, Waltham, MA), and concentrated by centrifugation twice at 50,000*g* for 2 hours at 20 °C. The virus pellet was re-suspended in Hank's Balanced Salt Solution (Invitrogen, Carlsbad, CA) and stored at −80°C until use. The titer of the concentrated virus was 5×10^8 to 1×10^9 infectious units/ml when assayed on 293T cells, and infectivity was determined by Venus expression as analyzed on a FACS VantageSE (BD Biosciences, Franklin Lakes, NJ).

Sixty thousand freshly isolated SM/C-2.6$^+$ cells in 300 μl growth medium were seeded in each well of 24-well plates and cultured for 16 hours with viral vectors expressing Venus or micro-dystrophin *CS1* at MOI of 100 or 200, respectively. After removal of free viral vectors by changing the medium, the transduced cells were cultured for 2 or 3 days and trypsinized. A cell suspension containing 2×10^4 cells in 20 μl of PBS was injected into the TA muscles of *mdx* mice. The infection efficiency of the injected cells was evaluated using a FACS VantageSE (BD Biosciences, Franklin Lakes, NJ).

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SUPPLEMENTARY MATERIAL

Figure S1. Freshly isolated satellite cells give rise to larger colonies than passaged myoblasts *in vitro*.

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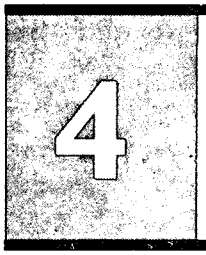

Side population (SP) cells and skeletal muscle differentiation

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Abstract

Side population (SP) cells are isolated from various tissues by their ability to efficiently exclude the vital DNA dye Hoechst 33342. The clearance of the dye from the cells is thought to be mediated by ABC transporters. Bone marrow SP cells are rich in hematopoietic stem cells and have been demonstrated to participate in muscle fiber repair. Similarly, SP cells from skeletal muscle were shown to reconstitute the bone marrow of lethally irradiated mice and, at the same time, participate in muscle fiber regeneration.

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Several reports, however, suggest that muscle-derived SP cells are heterogeneous in origin, gene expression, and function. To further elucidate their functions and relationships with the other myogenic cells identified to date and their potential as a tool for cell-based therapy of muscular dystrophies, it might be necessary to refine the protocol for SP cell preparation and combine Hoechst staining with identification of several molecular markers.

Introduction

Duchenne muscular dystrophy (DMD) is a progressive, ultimately lethal X-linked muscle disorder, caused by mutations of the DMD gene [1], which encodes a large cytoskeletal protein, named dystrophin. Dystrophin forms the large dystrophin/dystrophin-associated protein complex at the sarcolemma of myofibers, linking the basal lamina and cytoskeleton. Dystrophin deficiency causes structural weakness of the sarcolemma. The defective sarcolemma easily ruptures under mechanical stress, leading to muscle fiber necrosis, and finally results in loss of myofibers and reduced contractile power.

Skeletal muscle regenerates when injured. Muscle satellite cells, which are muscle progenitor cells located between the muscle basal lamina and myofibers, are largely responsible for this activity [2], and were expected to be a cell source for cell-based therapy of DMD. However, transplantation of satellite cells or their progeny (myoblasts) into skeletal muscle showed insufficient regenerative efficiency, and failed to ameliorate the dystrophic phenotypes of animal models and DMD patients (reviewed in [3, 4]).

On the other hand, several reports have suggested that stem cell-like activities are found in non-satellite cell fractions derived from adult skeletal muscle or in non-muscle tissues and participate in muscle fiber regeneration [5-8]. Therefore, stem cells other than satellite cells could be an alternative cell source for cell-based therapy of muscle diseases such as DMD.

Among the myogenic stem cells reported to date are side population (SP) cells. Originally, SP cells were isolated from bone marrow as highly purified hematopoietic stem cells on the basis of their ability to efflux Hoechst 33342 dye [9]. Since then, cells with the SP phenotype have been found in a wide variety of mammalian tissues, cell lines, and tumor cells, some of which have shown to possess stem cell-like properties (reviewed in [10, 11]).

In this chapter, we review papers characterizing the properties of bone marrow SP cells and muscle SP cells. Importantly, many reports show that SP cells are highly heterogeneous. To correctly understand the therapeutic potential of SP cells, it might be necessary to combine Hoechst staining with identification of several cell surface markers and perform functional analysis using a limited number of SP cells.

I. Bone marrow side population cells

1. Discovery of SP cells as hematopoietic stem cells

Side population (SP) cells were discovered as highly purified hematopoietic stem cells [9]. While using Hoechst 33342 vital dye staining to study the cell cycle of bone marrow (BM) cells, Goodell et al. found that simultaneously displaying Hoechst fluorescence at two emission wavelengths (red 675 nm and blue 450 nm) localizes a distinct, small, non-stained cell population (0.1% of the total BM cells) that expresses markers of multipotent hematopoietic stem cells (HSC) (Sca1+lin$^{neg/low}$). *In vivo* competitive repopulation experiments revealed that HSC activities were enriched at least 1,000-fold in the SP fraction. The majority of BM-SP cells were not cycling: only 1-3 % of bone marrow SP cells were in S-G_2M stages of the cell cycle, whereas 20 % of main population (MP) cells were [9]. Because the SP fraction disappears when staining is performed in the presence of verapamil, Goodell et al. speculated that the exclusion of Hoechst 33342 by SP cells is an active process involving multidrug resistance protein (mdr) or mdr-like transporters [9]. Later, Zhou et al. demonstrated that breast cancer resistance protein (BCRP), also known ABCG2, is the molecular determinant of the SP phenotype [12, 13]. Interestingly, more detailed fractionation studies indicated that the SP tail can be further divided into subregions according to their dye efflux abilities, and that the tip of the SP cells (which have the highest Hoechst efflux activity) shows higher progenitor activity than the distal portion [14-16].

Although BM-SP cells are widely accepted as highly enriched hematopoietic stem cells, it seems that not all SP cells possess HSC activities [14]. Further, a recent study showed that hematopoietic stem cells are present in both SP and non-SP fractions [17]. Therefore, the properties of BM-SP and HSC cells are not completely identical.

2. Role of bone marrow SP cells in myogenesis

Ferrari et al. reported that BM-derived cells participated in repair of muscle fibers [8], suggesting that at least a fraction of myogenic precursor cells originate in the bone marrow, circulate throughout the body, and are mobilized to damaged muscle to regenerate muscle fibers. Later, Gussoni et al. injected BM-SP cells from wild-type male mice intravenously into lethally irradiated *mdx* female mice, and demonstrated that bone marrow SP cells contain both myogenic and hematopoietic precursors, i.e., they are multipotent stem cells with great plasticity [6]. These results gave us hope of recovering dystrophin expression in the whole musculature of patients with DMD by systemic delivery of BM-derived wild-type stem cells. Stimulated by these reports, researchers intensively investigated the properties of side population cells in bone marrow, especially the contribution of BM cells [18, 19] or BM-

SP cells [20] to muscle regeneration. BM cells and BM-SP cells prepared from GFP-transgenic or LacZ-expressing mice were indeed found to differentiate into muscle fibers *in vivo* after transplantation. Disappointingly, however, the percentage of myofibers formed by donor-derived cells delivered via the circulation was very low (1-2 %) and therapeutically not significant in most skeletal muscles.

Fusion or stepwise myogenic differentiation?

LaBarge and Blau reported that BM cells differentiate stepwise into myogenic precursor cells (e.g. satellite cells) and then, response to muscle injury, proliferate, fuse, and finally develop into mature myofibers [18]. Similarly, several reports suggested that BM-derived cells can differentiate into satellite cells [6, 19]. On the other hand, Sherwood et al. demonstrated that cells of bone marrow or hematopoietic origin did not give rise to functional adult myogenic progenitors [21]. Several reports provided evidence that the plasticity of hematopoietic stem cells shown in BM transplantation experiments can be explained simply as fusion events [22, 23]. Further, additional concerns have arisen from studies demonstrating that while BM cells or BM-SP cells are able to fuse with myofibers, a large proportion of incorporated cells do not actually enter the myogenic program [24, 25].

II. Muscle SP cells

1. Protocol for isolation of muscle SP cells

Although SP-like cells are found in mononuclear cells prepared from skeletal muscle (Figures 1 and 2), there are often discrepancies among reports in abundance, cell surface markers, and differential potentials of muscle SP cells (Table 1). This may be due to the many variables involved in the preparation and staining for isolation of SP cells by FACS. Montanaro et al. investigated the effects of isolation parameters on viability, yield, and phenotype of SP cells [26], and found that 1) the enzymatic dissociation procedure, 2) cell-counting method 3) Hoechst concentration, and 4) SP gating are important parameters to minimize the heterogeneity of SP cells prepared from bone marrow, skeletal muscle, or skin. They showed that when isolated using stringent criteria, muscle SP cells are CD45-negative and Sca1-positive, and show very low Hoechst uptake. The Hoechst concentration seems to be the most critical. For example, Hoechst 33342 staining at a concentration of 5 μg/ml allows contamination by CD45-positive and Sca-1-negative cells. In contrast, 12.5 μg/ml Hoechst reduces the yield of SP cells and increases the percentage of CD45-negative Sca-1-positive cells. Because the percentage of CD45-positive SP cells tends to decrease at higher concentrations of Hoechst 33342 in both BM and non-hematopoietic tissues, muscle- SP cells seem to

Preparation of SP cells from mouse skeletal muscle

Dissect muscles, remove non-muscle tissues and mince muscle tissues

↓

digest muscle tissues with enzymes (e.g. collagenases, trypsin, or dispase)

↓

Remove red blood cells by lysis buffer or by LympholyteR
(or Percoll gradient)

↓

Re-suspend mononuclear cells at 106 cells/ml and stain with Hoechst s solution
(5-12.5 mg/ml) at 37°C for 60-90 min with or without Verapamil

↓

Antibody staining & FACS analysis and sorting

Figure 1. Preparation of SP cells from mouse skeletal muscle.

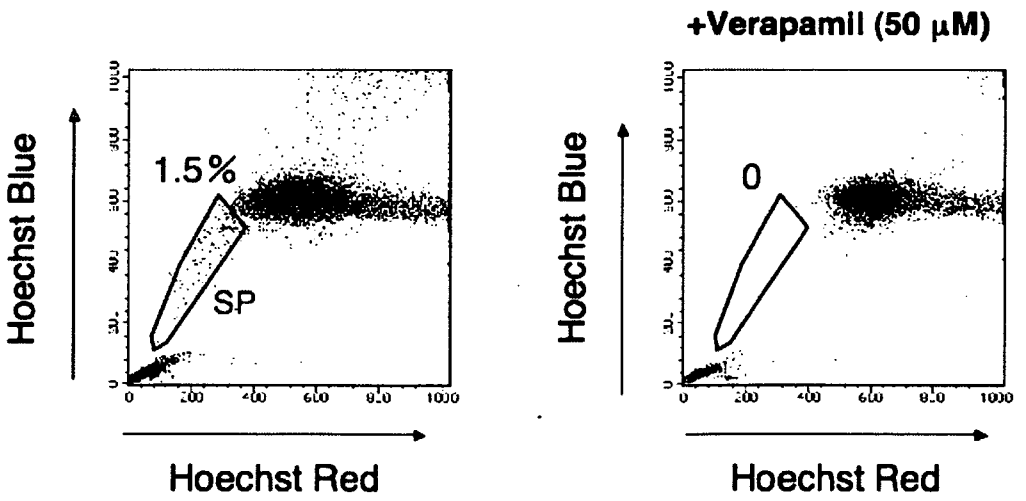

Figure 2. SP cells in adult skeletal muscle. Left panel: Muscle mononuclear cells were isolated from C57Bl/6 mice, stained with 5 mg/ml Hoechst 33342 dye, and analyzed on a dual laser FACS Vantage SE (Becton-Dickinson). Right panel: A calcium channel blocker verapamil was added to Hoechst solution to confirm the SP fraction.

Table 1. Variations in isolation protocols and properties of muscle SP cells.

Authors (year)	Preparation	Markers on SP cells	Properties	SP abundance
Gussoni et al., 1999	Hoechst 12.5 μg/ml	Sca-1+, lin-, c-kit-, CD45-, CD43-	hematopoietic stem cell-like activity produce myofibers after i.v. injection	1-1.5%
Jackson et al., 1999	Hoechst 5 μg/ml	Sca1+, c-kit+, CD45-	HSC activity (+)	1%
Asakura et al., 2002	Hoechst 5 μg/ml	Sca1+	CD45+ fraction: hematopoietic differentiate into muscle cells in co-culture differentiate into satellite cells and muscle fibers *in vivo*	CD45-SP: 0.3-0.5% CD45-SP: 2-3%
Majka et al., 2003	Percoll gradient Hoechst 5 μg/ml	c-met+, CD45-, Sca-1+, PE-CAM+, Tie-2+	bone marrow-derived vascular progenitor	0.2%
Meeson et al., 2004	Percoll gradient Hoechst 12.5 μg/ml	Sca-1+, c-kit+, CD31-, Abcg2+	increase during muscle regeneration	0.2%
Uezumi et al., 2006	Hoechst 5 μg/ml	heterogeneous	CD31+SP: Bcrp-1+, vessel-associated CD45+SP: bone marrow origin CD31-CD45-SP:differentiate into adipocyte, osteocyte, and myocyte	1-3% < 0.1% < 0.1%

have a much higher ability to exclude Hoechst 33342 than BM-SP cells. The study also points out that Hoechst 33342 is toxic to cells. This fact is important, because the cell toxicity of Hoechst dye makes it difficult to directly compare the biological properties of SP and non-SP cells.

In our opinion, one further parameter that has an effect on the heterogeneity of muscle SP cells is pre-fractionation by a Percoll gradient before Hoechst staining [27] (Table 1). In our experience, this procedure eliminates CD45-negative SP cells, resulting in enrichment of CD45-positive SP cells (our unpublished data).

The definition of SP cells for non-hematopoietic tissues is not clear. Therefore, it is difficult to determine which SP cells are the true muscle SP cells. We also think that Hoechst staining alone is insufficient to collect a homogeneous cell population. This approach should be combined with identification of other cell surface markers.

2. SP phenotype and ABC transporters

SP cells are generally defined as a cell population that actively and efficiently expels Hoechst 33342 dye. This property is thought to be mediated mainly by ATP-binding cassette (ABC) transporters. ABC transporters bind ATP as an energy source to transport endogenous or exogenous molecules, in most cases unidirectionally, across the cell membrane. Various tissues and cells express different combinations of ABC transporters, therefore, it is no wonder that SP cells from different tissues are heterogeneous in phenotypes and functions [10].

Muscle SP phenotype is not simply determined by Bcrp-1 expression

The ABCG2/Bcrp1 transporter is most often related to the SP phenotype. Bcrp-1-null mice show reduced numbers of SP cells in bone marrow and

skeletal muscle [13], but expression of this transporter is often found in non-SP cells [12]. Furthermore, isolation of human hematopoietic stem cells using an anti-ABCG2 antibody does not work [28]. These observations suggest that Bcrp-1 expression is not sufficient to endow the cell with the SP phenotype. In addition, it seems that a certain percentage of muscle SP cells are not Bcrp-1-dependent. When stained with an anti-Bcrp-1 antibody, the CD31-positive SP fraction is found to strongly express Bcrp-1, whereas two fractions, CD45-negative CD31-negative SP cells and CD45-positive SP cells reacted weakly with the anti-Bcrp-1 antibody [29]. Thus, transporters other than Bcrp-1 likely efflux Hoechst dye in a subset of muscle SP cells.

On the other hand, Meeson et al. examined the muscle SP fraction on a fluorescence-activated cell sorter (FACS) using both verapamil and fumitremorgin C (FTC), and confirmed that muscle-SP cells are highly sensitive to both of them [30]. Verapamil is a calcium channel blocker widely used to confirm the SP fraction on FACS profile, but shows no specificity toward a single transporter. FTC was shown to be a specific inhibitor of Abcd2, where it functions to inhibit Abcg2-associated ATPase activity [31]. Thus, the study showed that muscle SP cells are dependent on Abcg2/Bcrp-1 for the SP phenotype. SP preparation by Litman et al. employed a pre-fractionation of the muscle mononuclear cells using a Percoll gradient and a high concentration of Hoechst dye (12.5 μg/ml) for 90 min. The percentage of SP cells (0.19 %) was low, compared with other reports (see Table 1). Therefore, this result does not exclude the contribution of ABC transporters other than ABCG2/Bcrp-1 to the SP phenotype.

3. Other properties of muscle-derived SP cells

Most muscle SP cells are in quiescent stage

Freshly isolated SP cells from muscle are often reported to be non-adherent in culture, and are characterized by small cell size, stages G0/G1 of the cell cycle, and low metabolic activity, elements common to stem cells. Indeed, small size (6.6 ± 0.1 mm) and a high nucleus-to-cytoplasm ratio were reported for muscle-derived SP cells [30]. However, when we analyzed muscle SP cells during muscle regeneration, they discovered CD31-negative CD45-negative SP cells, which are large in size and Ki-67-positive. Thus, at least one subset of SP cells is not small and is cycling.

Cell surface markers on muscle SP cells

Reflecting the variety of experimental protocols for SP preparation, there are discrepancies in reported cell surface markers of SP cells (Table 1). In 1999, Gussoni et al. described the isolation of SP cells from skeletal muscle for the first time [6]. They used a higher concentration of Hoechst dye (12.5 μg/ml) than the original protocol (5 μg/ml) described by Goodell et al. [9], and

reported that SP cells in muscle are CD45-negative, c-kit-negative, and Sca-1-positive [6]. On the other hand, Jackson et al. reported that muscle-derived SP cells are Sca-1-positive (79 %), c-kit-positive (75 %), and CD45-negative [32]. Using a 5 μg/ml solution of Hoechst 33342, Asakura et al. reported that most muscle-derived SP cells are CD45-negative Sca-1-positive, but more than 10% of SP cells are CD45-positive [33]. Using an almost identical isolation procedure and Hoechst staining conditions, we reported that muscle-derived SP cells isolated from uninjured adult skeletal muscle have three phenotypically distinct SP subpopulations [29]. The report shows that approximately 90 % of muscle SP cells are CD31-positive CD45-negative. This fraction strongly expresses Bcrp-1 on the cell surface. About 5 % of muscle SP cells are CD45-negative CD31-negative, and approximately 3-5 % of muscle SP cells are CD45-positive.

Cell surface markers and origin of muscle SP cells

Interestingly, the difference in cell surface markers on SP subfractions is clearly related to their developmental origins. CD45-positive SP cells are thought to originate from BM and home into skeletal muscle via the circulation [29, 34]. Further, the HSC activity found in skeletal muscle was proven to be almost completely limited to the CD45-positive SP fraction [34]. On the other hand, the majority, but not all, of the CD45-negative SP cells in limb muscles were shown to be derived from the hypaxial somite and to have higher myogenic potentials than CD45-positive SP cells [35]. Interestingly, the study also revealed that a certain percentage of CD45-negative SP cells are not likely derived from the somite and are less myogenic than SP cells of somitic origin.

4. Gene expression in skeletal muscle SP cells

No expression of myogenic regulators in SP cells

Asakura et al. showed that the muscle SP fraction expresses no desmin or Pax7. Furthermore, muscle SP cells prepared from Myf-5-nLacZ mice do not express β-galactosidase [33]. Using sensitive RT-PCR analysis, we also showed that muscle SP cells prepared from non-injured or regenerating muscles do not express Pax7, Pax3, Myf-5, or MyoD [29]. These observations suggest that muscle-SP cells are not committed to a myogenic lineage.

Molecular signatures of SP cells revealed by microarray analysis

Genome-wide gene expression analyses of SP fractions have been performed to elucidate the molecular regulation of SP cells. Meeson et al. examined the gene expression profiles of muscle SP cells isolated from uninjured and regenerating muscles, bone marrow-SP cells, and embryonic stem (ES) cells [30]. The results showed that skeletal muscle SP cells express Abcg2 (Bcrp-1) and endothelial and hematopoietic transcripts. They concluded

that muscle SP and BM-SP cells have distinct molecular programs. Liadaki et al. reported the gene expression of muscle SP and BM-SP cells. The analysis revealed that BM-SP and muscle SP cells share a transcriptome signature but at the same time express tissue-specific markers [36]. When compared with MP cells within the same tissues, SP cells were found to underexpress genes reflecting tissue-specific functions [36]. Rochon et al. reported gene expression analysis of SP cells isolated from adult mouse bone marrow, adult male germinal cells, muscle primary culture, and mesenchymal cells [37]. These four types of SP cells are proposed to be a "stem cell-like" population. Transcriptional profiles for SP and the more differentiated non-SP cells isolated from these four tissues were compared by microarray analysis. The authors reported that the genes commonly upregulated in SP cells are implicated in the quiescent status of cells, maintenance of their pluripotency, and capacity to undergo asymmetric division, and that the repression of lineage-affiliated genes in SP cells is responsible for their undifferentiated state.

5. Location of SP cells in skeletal muscle

SP cells are defined by FACS analysis, but their location in muscle is ill-defined. Because Bcrp-1 is largely responsible for the SP phenotype [12,13], it is informative to stain muscle tissues sections with an anti-Bcrp-1 antibody. Using a polyclonal antibody against Bcrp-1, we showed that CD31-positive SP cells on glass slides are strongly stained with the antibody after sorting. Interestingly, CD31-positive Bcrp-1-positive cells are found in capillaries and venous endothelium, suggesting that the majority of muscle SP cells are associated with blood vessels [29]. Meeson et al. also showed that Abcg2 (Bcrp-1)-expressing cells are closely associated with the vasculature [30]. However, Bcrp-1 positive cells are also found in the MP fraction [29]. Therefore, not all Bcrp-1-positive cells found on muscle cross sections have the SP phenotype [29]. On the other hand, CD45-positive SP cells express quite low levels of Bcrp-1 [29]. Currently CD45-positive SP cells lack a distinctive marker available for use on tissue sections. Therefore, it is difficult to see BM-derived CD45-posotive SP cells on muscle cross sections. Likewise, CD45-negative CD31-negative SP cells on glass slides are hardly stained with anti-Bcrp-1 antibody after cell sorting, so it is also difficult to find CD45-negative CD31-negative SP cells in muscle sections [29].

6. Origin of muscle SP cells

Satellite cells originate in somite

Repair of mature skeletal muscle is largely mediated by the muscle progenitor cells referred to as satellite cells [2]. Satellite cells reside beneath the basal lamina of adult skeletal muscle juxtaposed against skeletal muscle

fibers and account for 2-5 % of sublaminal nuclei in adult muscle. The developmental origin of satellite cells is reported to be the somite [35, 38, 39].

Satellite cells and muscle SP cells are distinct populations

In the beginning, the relationship between muscle SP cells and satellite cells was controversial. Seale et al. reported that Pax7-null mice have severely reduced numbers of satellite cells, but show normal levels of muscle SP cells [40]. Asakura et al. showed that satellite cells and muscle SP cells have distinct differentiation potentials both *in vitro* and *in vivo* [33]. Fukada et al. established an antibody, named SM/C-2.6, that can purify quiescent satellite cells from muscle efficiently [41], and directly showed that almost all satellite cells are found in the MP fraction [41]. Furthermore, it was shown that muscle SP cells are negative for Pax7 or Pax3 and hardly differentiate into myotubes *in vitro* without co-culturing with myoblasts [29, 33]. Taken together, satellite cells and muscle SP cells seem to be distinct populations. Some reports, however, suggest that when transplanted into skeletal muscle, a portion of muscle SP cells differentiate into Pax7-positive cells [6, 33] and express Pax7. Although a recent study demonstrated that satellite cells vigorously self-renew, denying the existence of muscle stem cells that replenish the satellite cell pool [42], muscle SP cells might slowly supply severely damaged muscle with myogenic precursor cells.

Majority of limb muscle SP cells are derived from hypaxial somite

The source of muscle SP cells has long been debated. Schienda et al. explicitly tested and quantified the contribution of embryonic somitic cells to side populations [35]. Chick somitic cells were labeled by using replication-defective retroviruses or quail/chick chimeras, and mouse cells were labeled by crossing somite-specific, Pax3-derived Cre driver lines with a Cre-dependent reporter line. The results showed that a significant number, but not all, of limb muscle SP cells are derived from the hypaxial somite. Notably, the developmental origin of SP cells is related to their potentials; somitically derived CD45-negative SP cells are intrinsically more myogenic than CD45-negative SP cells from other sources. As mentioned above, CD45-positive SP cells are thought to be derived from bone marrow [29, 34].

7. Differentiation potential of muscle SP cells

The differential potentials of muscle SP cells have long been controversial. However, their cell surface markers reveal their developmental origin and their differential potentials.

Hematopoietic activities of muscle SP cells

Skeletal muscle-derived cells have the potential to repopulate the major peripheral blood lineages of lethally irradiated mice and thus behave like

HSC [6, 32]. To further clarify the properties of muscle-derived HSC, skeletal muscle-derived cells were fractionated based on the expression of CD45 and c-kit and Hoechst 33342 efflux, and examined for HSC activity in vivo [34]. The results revealed that muscle-derived HSC activities fall exclusively in the c-kit (dim) CD45 (pos) compartment of the muscle side population (msSP). Furthermore, it was shown that the CD45-positive msSP compartment of skeletal muscle is derived from whole bone marrow HSC. CD45-positive muscle SP cells are, however, shown to be much less potent in HSC activity than bone marrow HSC cells in competitive repopulation assays [34].

Muscle SP cells contain vascular progenitors

Muscle SP cells have also been shown to contribute to vascular regeneration after local injection into chemically damaged regenerating muscle [27]. Majka et al. showed that more than 70 % of muscle SP cells are CD45-positive and derived from bone marrow. The higher percentage of CD45-positive cells in the muscle-derived SP fraction, compared with those reported by other laboratories, may be due to pre-fractionation by a Percoll gradient of crude muscle-derived mononuclear cells prior to Hoechst staining (Table 1).

Myogenic potential of muscle SP cells

Gussoni et al. reported that muscle SP cells fail to settle on the plate during the first two weeks after cell sorting and that they then differentiate as a mixture of myoblasts and fibroblasts [6]. In contrast, Asakura et al. showed that skeletal muscle SP cells cultured in myoblast growth medium do not give rise to myogenic progenitors unless cultured with primary myoblasts [33]. We also observed that muscle SP cells alone hardly form myotubes *in vitro* [29]. After transplantation into muscle, however, muscle-SP cells generate both satellite cells [6, 33] and mature myofibers [6, 29, 33].

Mesenchymal potential of muscle SP cells

We showed that a minor subset of muscle SP cells (the CD31-negative CD45-negative fraction) differentiate into adipocytes or osteocytes *in vitro* upon induction. Furthermore, when these cells are transplanted into irradiated muscle, muscle fiber regeneration is severely impaired, and transplanted CD31-negative CD45-negative SP cells differentiate into many adipocytes and fibrotic cells. This observation suggests that they might be a source of the adipogenesis seen in advanced muscular dystrophy. CD31-positive CD45-negative SP cells uptake 1,1'-dioctadecyl- 3,3,3',3'- tetramethylindo-carbocyanine perchlorate-acetylated-low density lipoprotein (Dil Ac-LDL), suggesting that they possesses endothelial cell-like properties [29], but their functions *in vivo* remain to be determined.

8. Relationship to other muscle stem cells

Several types of stem cells have been isolated from skeletal muscle, including SP cells, muscle-derived stem cells (MDSC) [43], multipotent adult precursor cells (MAPC) [44], myogenic-endothelial progenitors [45], CD45-positive Sca-1-positive cells [46], mesoangioblasts [7], and pericytes [47]. Although all these stem cells were isolated from skeletal muscle and showed multi-lineage differentiation potential, use of different cell isolation techniques, different culture conditions, and partial characterization make it difficult to clarify the interrelationships among them. For establishment of effective and safe cell therapy for muscular dystrophies, we must establish a standard protocol for isolation of muscle stem cells.

9. Physiological role of SP cells in muscle regeneration

Interestingly, the number of muscle SP cells increases during muscle regeneration [29,30] (Figure 3). In particular, CD45-negative, CD31-negative SP cells actively proliferate during muscle regeneration. In the later stage of muscle regeneration, however, the double-negative SP cells decrease in number and return to the normal level [29]. Although double-negative SP cells barely differentiate into muscle cells when cultured alone [29, 33], they form myofibers *in vivo* after injection into regenerating muscle of immunocompetent mice, suggesting that endogenenous muscle SP cells participate in muscle fiber repair. Intriguingly, CD31-negative CD45-negative SP cells express the genes that are expected to regulate muscle regeneration [29]. These results suggest that CD31-negative CD45-negative SP cells not only act as myogenic progenitors but also regulate muscle regeneration by acting on satellite cells or inflammatory cells. Contrary to expectation, CD31-positive SP cells (the major SP subset in muscle) do not expand in response to muscle injury. Their roles in muscle regeneration remain to be determined. Figure 4 shows our model of the roles for muscle SP cells in muscle regeneration.

10. Muscle SP cells as a tool for cell therapy of muscular dystrophy

Cell-based therapy continues to be a promising avenue for the treatment of DMD, an X-linked skeletal muscle-wasting disease. However, myoblast transplantation into animal models results in local restoration of dystrophin expression [48], and human clinical studies using myoblast transfer performed on DMD patients failed to improve the muscle strength of the treated group [49-51]. An alternative to myoblast transfer is a systemic delivery of precursor cells with myogenic potential. Recently, it was demonstrated that skeletal

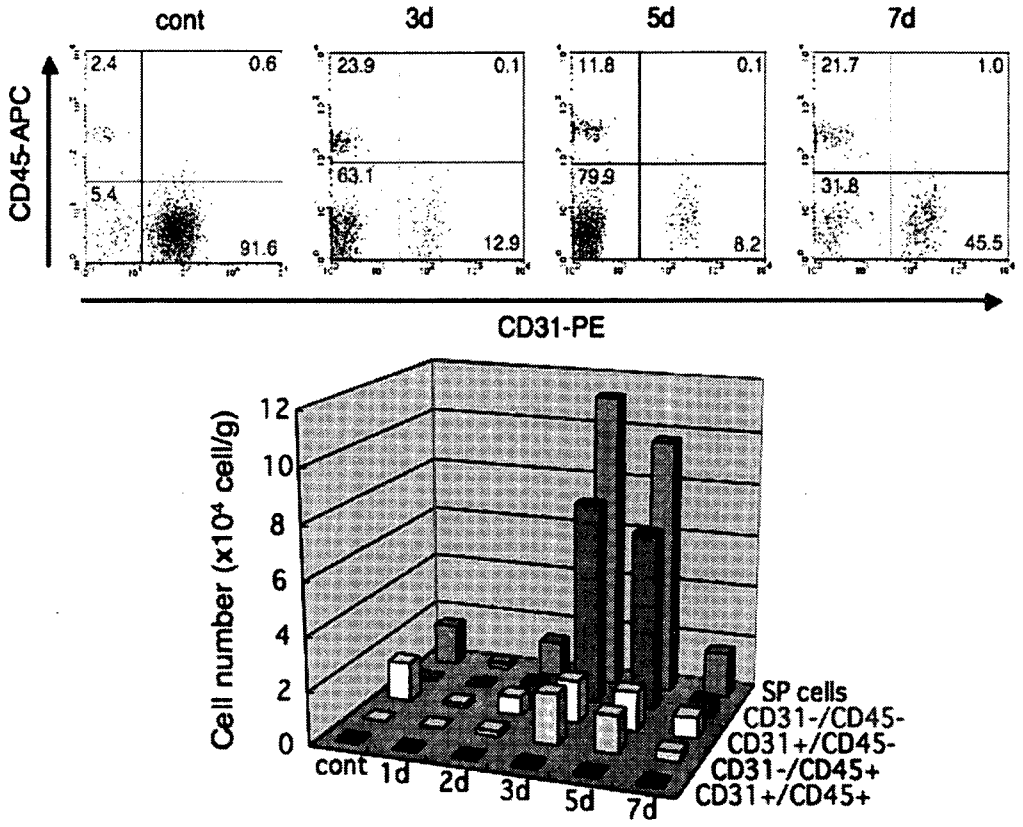

Figure 3. Number of CD31-negative CD45-negative SP subpopulation cells increases during muscle regeneration. Cardiotoxin was injected into hind limb muscles of 8-week-old C57BL/6 mice, muscles were dissected, and SP fractions were analyzed by FACS1, 2, 3, 5, and 7 days after injection. Upper panel: Muscle SP fraction was further analyzed by CD31 and CD45 expression. Lower panel: The cell numbers of each SP fraction were counted and plotted. Note that CD45-negative CD31-negative SP cells are a minor SP subset, but are significantly increased in response to muscle injury. Reproduced from [29] by permission.

muscle SP cells engraft into dystrophic fibers of nonirradiated mdx(5cv) mice after intravenous or intraarterial delivery [52, 53]. SP cells prepared from *mdx*(5cv) muscle were transduced with a recombinant lentivirus encoding microdystrophin or GFP, and transplanted into the femoral artery of non-injured *mdx*(5cv) mice. Sections of the recipient muscles demonstrated that 5-8 % of skeletal muscle fibers expressed donor-derived transgenes. Further, donor muscle SP cells, which did not express any myogenic markers prior to transplant, expressed a satellite cell transcription factor, Pax7, and a muscle-specific intermediate filament, desmin, after extravasation into host muscle. These results indicate that systemic autologous transplantation of SP cells genetically corrected *ex vivo* could be an option for cell therapy of DMD.

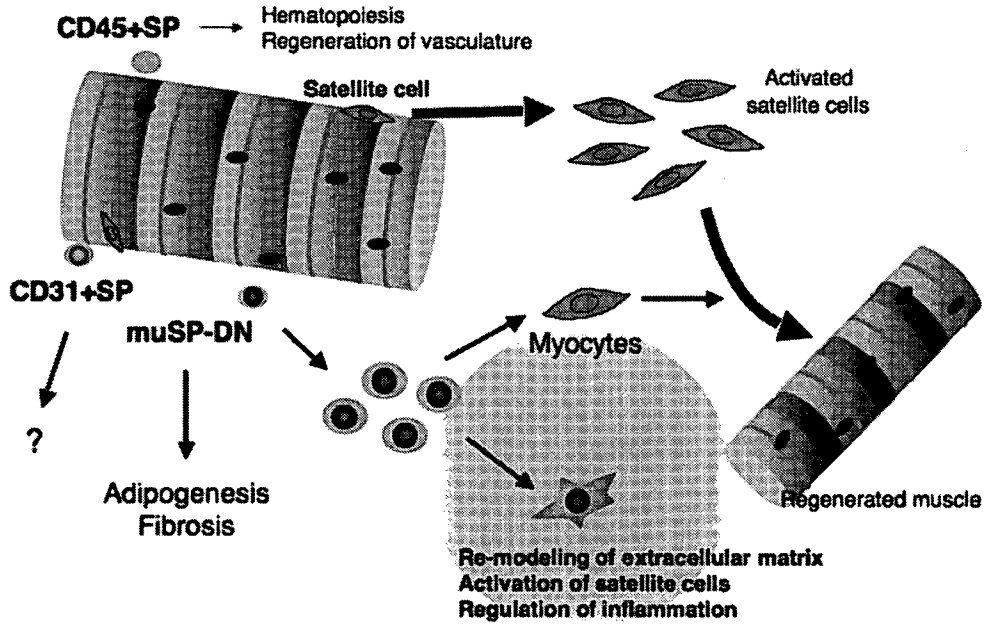

Figure 4. Roles of SP cells in muscle regeneration. Muscle-derived SP cells stained with Hoechst (5 μg/ml) are divided into three distinct subpopulations based on the expression of CD31 and CD45. The main population is CD31-positive. Their roles in muscle remain unclear. CD45-positive SP cells (less than 10% of total muscle SP cells) are shown to be bone marrow-derived and account for hematopoietic activity found in muscle. They are also reported to re-constitute the vasculature after muscle injury. The CD31-negative CD45-negative SP subset actively proliferates during muscle regeneration. These cells differentiate into adipocytes, osteocytes, and myocytes *in vitro*. Their roles in muscle regeneration are just beginning to be elucidated.

III. SP fraction in cultures of primary myoblasts and myogenic cell line C2C12 cells

One can identify a subset of cells with the SP phenotype in both primary myobasts and cells of a myogenic cell line, C2C12. Benchaouir et al. stained myoblasts and C2C12 cells with a variety of concentrations of Hoechst (5-30 μg/ml) and analyzed them on a dual-laser MoFlo flow cytometer; they found that 2-3 % of the cells show SP-like phenotypes [54]. Interestingly, gene expression analysis of C2C12-SP cells suggested that mdr1a, an ABC transporter is likely responsible for the SP phenotype of C2C12-derived SP cells. Cell cycle analysis by propidium iodide staining revealed that more than 90 % of SP cells in C2C12 cells are in the G0/G1 phase. Electron microscopy showed that they have a high nucleus-to-cytoplasm ratio. C2C12 SP cells express reduced levels of MyoD and exhibit delayed differentiation compared to main population cells in differentiation-promoting conditions. Because

forced expression of mdr1a increases the number of SP cells and blocks the differentiation of C2C12 cells, mdr1a may regulate the differentiation of C2C12 cells. One possible mechanism for this regulation is that mdr1a excludes certain differentiation-promoting substances from the cells. Interestingly, the study also demonstrated that SP and MP cells in C2C12 cells are in a dynamic equilibrium and largely reversible. Moreover, addition of FGF6 to the culture medium increased the ratio of SP cells [55]. C2C12 SP cells might be a useful *in vitro* model to study a reserve pool of muscle progenitor cells in skeletal muscle.

IV. Others

Roles for ABC transporters in SP cells

SP cells in several tissues represent immature, undifferentiated cell fractions [10]. SP-like cells are also found in cancer cells, and are considered to be cancer stem cells [11]. Although not all SP cells are stem cells, the Hoechst efflux phenomenon correlates well with stem cell activities in several tissues or organs. ABC transporters would protect stem/progenitor cells from toxic substances, hypoxia, or other stresses, ensuring long-term survival of the cells, and keeping stem/progenitor cells in an undifferentiated state.

Conclusions

Muscle SP cells are thought to be enriched in stem cells, but they are highly heterogeneous. In addition, variations in techniques for isolation of the muscle SP fraction make the properties of muscle SP cells elusive. To further characterize the origins, phenotypes, the functions of muscle SP cells in physiological and pathological conditions and their therapeutic potential, it might be necessary to find more reliable SP-specific markers and use limited numbers of SP cells in a functional assay to equate muscle SP cells with stem cells.

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