Systemic delivery of human microdystrophin to regenerating mouse dystrophic muscle by muscle progenitor cells

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Cell-based therapy for Duchenne muscular dystrophy patients and *mdx* mice has proven to be a safe but ineffective form of treatment. Recently, a group of cells called muscle side population (SP) cells have been isolated based on their ability to efflux the DNA-binding dye Hoechst. To understand the potential of skeletal muscle SP cells to serve as precursors for muscle, SP cells from the two mice strains *mdx*^{5cv} and C57BL/6N were isolated, transduced, and transplanted. Under coculture conditions with myogenic cells, some cells within the SP cell population can give rise to early Pax7-positive satellite cells and other later stage myogenic cells. Transduced SP cells were transplanted via the tail vein and were shown to successfully deliver enhanced GFP and human microdystrophin to the skeletal muscle of nonirradiated *mdx*^{5cv} mice, thus demonstrating their ability to travel through the capillaries and enter into damaged muscle. These results demonstrate that i.v. delivery of genes via SP cells is possible and that these SP cells are capable of recapitulating the myogenic lineage. Because this approach shows definitive engraftment by using autologous transplantation of noninjured recipients, our data may have substantial implications for therapy of muscular dystrophy.

Duchenne muscular dystrophy (DMD), a recessive X-linked disorder and the most common of a class of progressive muscle-wasting diseases, is characterized by the lack of dystrophin at the muscle cell membrane (1). Replacement of absent dystrophin in *mdx* mice by transgenic expression leads to complete restoration of normal muscle cell membrane function (2). Muscle is known to be a regenerative tissue, and this regeneration is accomplished via a heterogeneous population of cells called satellite cells or myoblasts. These cells divide upon damage to muscle, fuse to one another and with existing myofibers, and create new muscle fibers. One approach to therapy for DMD was the intramuscular injection of normal myoblasts into the skeletal muscle of DMD patients or *mdx* mice, which lack full-length functional dystrophin. Although early results in *mdx* mice were promising, the human clinical trials proved safe but ineffective, with little or no new expression of dystrophin documented (3, 4). Originally, it was thought that the cells were cleared by the immune system; however, follow-up studies indicated that the developmental status of the cells was also important (5–7). This finding led to efforts to improve methods of cell isolation for transplantation.

The recent discoveries of adult stem cells in most tissues has led to the anticipation of extraordinary advances in cell-based therapy approaches for genetic diseases (for review, see ref. 8). However, our modest understanding of adult stem cells and their plasticity leaves many questions unanswered. Muscle has been the target of a number of studies using various techniques for cell isolation from skeletal muscle. These approaches have identified a population of muscle cells that seem to function as stem cells due to their capacity to regenerate muscle and in some cases other tissues, *in vitro* and/or *in vivo* (6, 9–11). The hematopoietic system has served as a model for stem cell lineages and stem cell purification. One method developed to purify hematopoietic stem cells takes advantage of their ability to efficiently exclude the dye Hoechst 33342 by using an ATP-binding cassette (ABC) transporter (12). The so-called side population (SP) cell (13) has been purified from a number of adult tissues including muscle (9, 14–19). Cells within the SP gate seem to be a heterogeneous population that expresses different cell surface markers (20, 21). This heterogeneity may be due to different methods of isolation, but it may also reflect tissue origin, which may contribute to the observed differences in cell surface characteristics. We have already demonstrated the existence of SP cells, in murine skeletal muscle, that have the capacity to regenerate not only muscle but also hematopoietic cells *in vivo* (9). These cells are different from satellite cells, a subpopulation of muscle-derived cells that may have stem cell-like characteristics (22, 23). Moreover, based on the location of donor cells after i.v. injection into irradiated mice, we proposed that muscle SP cells could differentiate into satellite cells (9). After transplantation, these cells were found juxtaposed to the cell membrane, but outside the fibers. Studies by others have shown that SP cells are independent of Pax7 expression because Pax7 null mice have normal levels of SP cells in skeletal muscle (22). Similar to other studies of adult muscle stem cells, the SP cell population from muscle has been shown to become muscle under myogenic culture conditions (21). However, it was never clear that the cells that formed muscle were not a group of satellite cells within the SP population. These same studies were also done *in vivo* by using intramuscular injection of SP cells where Pax7-negative cells were shown to now express it within the muscle (21).

In this study, we compared the muscle SP population isolated from *mdx*^{5cv} mice and wild-type C57BL/6N mice. The *5cv* allele of *mdx* has a much lower spontaneous revertant phenotype and exhibits a slightly more severe disease than the *mdx* allele (24, 25). Comparing the SP cells isolated from wild-type mice to those prepared from *mdx*^{5cv} mice revealed differences in the potential to differentiate into skeletal muscle *in vitro*. The *mdx*^{5cv} SP cells seemed primed for myogenesis just due to residence within dystrophic tissues. The dystrophic muscle-derived SP cells contribute to muscle formation by differentiating into myogenic cells. The skeletal muscle SP cells from *mdx*^{5cv} mice strain were transduced *in vitro* by self-inactivating lentiviral vectors as a better model through which to study the effectiveness of dystrophin replacement in cell-based therapy and to better follow the donor cells. Some of these genetically modified-muscle SP cells were found to reside in host muscle tissue 4 to 10 weeks after i.v. delivery. They fused into host myofibers and expressed the transgene, indicating that these cells can also contribute to muscle formation *in vivo*. Cells capable of expressing human microdystrophin from a lentiviral vector were detected in few scattered fibers in all muscle studied when human microdystrophin-expressing-SP cells were systemically delivered

Abbreviations: SP, side population; MP, main population; eGFP, enhanced GFP; FACS, fluorescence-activated cell sorter; μdys, microdystrophin.

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into mdx^{5cv} mice. These studies represent direct systemic delivery of dystrophin expressed from a viral vector to muscle and suggest that the transplantation of skeletal muscle SP cells may be a successful form of cell-based dystrophin replacement therapy.

Methods

Mouse Strains. C57BL/6N mice and C57BL/6Ros-5cv (mdx^{5cv}) (X-linked muscular dystrophy) mice were obtained from The Jackson Laboratory. In the mdx^{5cv} allele, the dystrophin mRNA contains a 53-bp deletion of sequences from exon 10 (25). These animals display ≈10 times fewer dystrophin-positive revertant fibers than *mdx* mice due to their low frequency of mutation reversion (25, 26).

Construction of Lentiviral Transfer Vectors and Preparation of Viral Vectors. The transfer vectors pL-MSCV-eGFP, pL-MSCV-n*Lac*Z, and pL-MSCV-μdys were constructed by inserting either enhanced GFP (eGFP), nuclear-targeted *Lac*Z, or a human microdystrophin cDNA (ΔR4–R23/ΔCT; ref. 27) sequence into the polylinker of the pRRL-cPPT-CMV-X-PRE-SIN lentiviral transfer plasmid [kindly provided by W. Osborne (28)].

Isolation and Characterization of Muscle SP Cells. Mononuclear cells were isolated from skeletal muscle from 4- to 9-week-old C57BL/6N and mdx^{5cv} donor tissue as described (9, 29). Before HO342 staining, red cells were lysed (30). Primary myoblasts were resuspended at 10^6 cells per ml and stained with 12.5 $\mu g \cdot ml^{-1}$ HO342 in PBS-0.5% BSA for 60 min at 37°C. In parallel, 10^6 cells were stained with HO342 in the presence of 50 μM verapamil to set the gate for the isolation of SP cells by fluorescence-activated cell sorter (FACS, Becton Dickinson). This gate was independently set to analyze the percentage of SP cells in each sort of both genetic strains studied. To eliminate dead cells, 2 μg/ml propidium iodide was added to the cells before FACS analysis and sorting. For cell-lineage and myogenic marker analysis, see *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

Cell Culture and Lentiviral Infection. After FACS sorting, the cells were plated into 96-well collagen type I (Upstate Biotechnology, Lake Placid, NY) coated plates in proliferation medium: DMEM high glucose (CellGro, CellGenix, Freiburg, Germany) supplemented with 20% heat inactivated horse serum (GIBCO), 2.5% chicken embryo extract (Accurate Chemicals), mouse stem cell factor (25 ng/ml, R & D Systems), epidermal growth factor (100 ng/ml, SIGMA), and recombinant human fibroblast growth factor, and Basic (100 ng/ml, Promega). Cells were transduced during 3–6 h immediately after sorting with 75 μl of inoculums of L-MSCV-eGFP or L-MSCV-μdys lentiviral particles with a multiplicity of infection of 10. Cells were washed twice with PBS before transplantation. SP cell transduction was performed by using L-MSCV-eGFP vector and C2C12 transduction by using L-MSCV-nLacZ vector, respectively. eGFP-SP cells and proliferating C2C12 cells were cocultured for 48 h in proliferation media and stained with an anti-chicken Pax7 antibody (1/1,000) (DSHB, University of Iowa). eGFP-SP cells and differentiated C2C12 were cocultured for 48 h in fusion media (DMEM high glucose supplemented with 2% heat-inactivated horse serum) and stained with anti-human desmin antibody (1/100) (clone D33, DAKO). Equal numbers of eGFP-SP cells and nLacZ-C2C12 cells were cocultured in proliferation medium until confluence; then, fusion media were added. One week later, 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) staining coloration was performed (see *Supporting Materials and Methods*).

Muscle SP and Main Population (MP) Cell Transplantation. SP and MP cells were transduced after FACS analysis and sorting during 3– 6 h with 75-μl inoculums of L-MSCV-μdys or L-MSCV-eGFP lentiviral

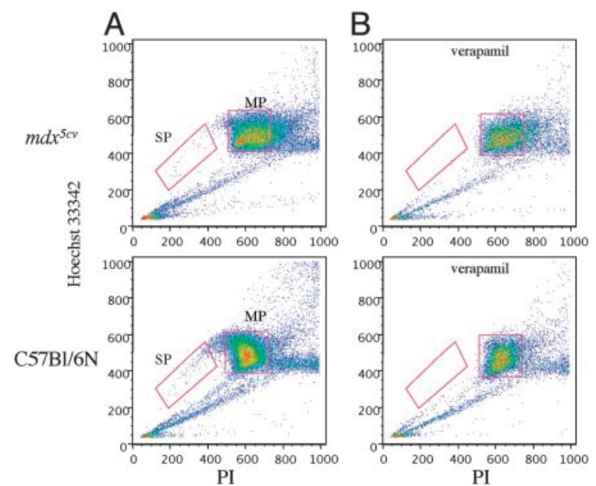

Fig. 1. FACS analysis of C57BL/6N SP cells and mdx^{5cv} SP cells. (*A*) Hoechst 33342 and propidium iodide profiles of SP and MP cells from skeletal muscle of mdx^{5cv} and C57BL/6N wild type mice. (*B*) Inhibition of Hoechst 33342 dye efflux by verapamil for mdx^{5cv} and C57BL/6N SP cells.

particles with a multiplicity of infection of 10. Before being injected into animals, cells were washed twice in PBS-0.5% BSA, resuspended in 200 μl of PBS, and intravenously transplanted into 3- to 5-week-old mdx^{5cv} or C57BL/6N recipients by means of the tail vein. All animal care was in accordance with institutional guidelines.

Culture of Single Muscle Fibers. Tibialis anterior from transplanted mice were dissected and torn into extensor digitorum longus-sized pieces. Tendons and membranes from the muscle were carefully removed. Muscles were cleaned in Hanks' balanced salt solution (HBSS, StemCell Technologies, Vancouver) twice and then incubated in a 24-well plate with 0.2% Collagenase type II (Worthington) for 40 min at 37°C. Digested muscles were resuspended in 0.2 ml of IMDM (CellGro) with 2% FBS and cultured in proliferation medium or Methocult M3434 (StemCell Technologies). Before staining, cultures were fixed for 20 min in 4% paraformaldehyde and washed twice in PBS. Anti-chicken Pax7 antibody (DSHB, University of Iowa), anti-human desmin antibody (clone D33, DAKO), and anti-rat myogenin (1/500) (clone F5D, DAKO) were used for immunohistochemistry analysis of cultured myofibers. Pax7, desmin, eGFP, and nLacZ-positive cells were visualized and counted under fluorescence and light microscope.

Immunoblot. For details on immunoblotting, see *Supporting Materials and Methods*.

Immunohistochemistry. Recipient animals were killed, and skeletal muscle was snap-frozen in cold isopentane and stored at −80°C. Immunohistochemistry was performed on tissue sections as described (31) (for details, see *Supporting Materials and Methods*).

Results

Culture and Proliferation of C57BL/6N and mdx^{5cv} Muscle SP Cells. Skeletal muscle isolated from mdx^{5cv} animals shows histopathological signs of degeneration and regeneration. Side population cells within the skeletal muscle of many different 4- to 9-week-old wild-type C57BL/6N and mdx^{5cv} mice were analyzed and sorted on different days by FACS (e.g., Fig. 1). To test differences in cell surface characteristics of these two SP populations, antibodies to different surface markers were used in the sorts. No statistically significant differences were detected between the two strains for these markers. Both strains showed that SP cells were mostly

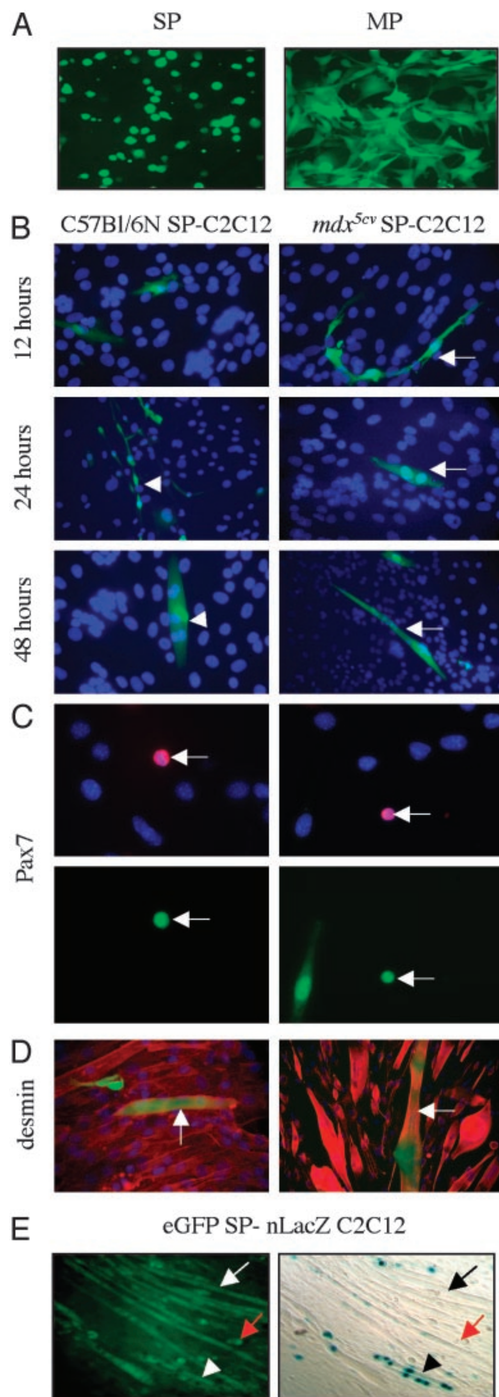

Fig. 2. eGFP-lentiviral transduction of muscle SP and MP cells and coculture with C2C12 cells. (*A*) eGFP expression by direct fluorescence microscopy of L-MSCV-eGFP-infected-SP and L-MSCV-eGFP-infected-MP cells. Results from the *mdx*^{5cv} transduction are presented, but no differences were found between the *mdx*^{5cv} and C57BL/6N mice. *Mdx*^{5cv} and C57BL/6N eGFP-SP cells were cocultured with differentiated C2C12-derived myotubes (*B*) or undifferentiated C2C12-derived myocytes (*C* and *D*) C2C12 cells. (*B*) A time course of coculture with nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue) and eGFP in green. The *mdx*^{5cv} SP cells were found aligned at 12 h and fused into multinucleated myotubes as early as 24 h (eGFP-SP arrow). The C57BL/6N SP cells were not found aligned until 24 h and did not fused into myotubes until 48 h postcoculture (eGFP-SP arrowhead). (*C*) Coculture of *mdx*^{5cv} and C57BL/6N eGFP-SP cells with C2C12 in proliferation media before confluence. Staining was done with Pax7 (Texas red) and DAPI (blue). Equal numbers of Pax7-eGFP-SP-derived cells were detected in both strains (arrows). (*D*) Four days after addition of confluence fusion medium, the cultures were stained with desmin (Texas red) and DAPI (blue) Desmin-positive-eGFP-SP-derived myotubes were detected in both strains (arrows). (*E*) Coculture of eGFP-SP cells with nLacZ marked C2C12 myogenic cells. Equal numbers of muscle eGFP-SP cells and nLacZ-C2C12 cells were cocultured. Multinucleated myotubes, which expressed eGFP but not nLacZ, were seen (black and white arrows), as well as multinucleated myotubes expressing both eGFP and nLacZ (black and white arrowheads). SP-derived cells not fused into multinucleated myofibers were also observed (red arrows).

positive for SCA-I (80–90%) and CD34 (65–80%) but did not express CD45 and c-kit (data not shown). The SP cells showed no evidence of Pax7 and desmin expression (Fig. 5, which is published as supporting information on the PNAS web site), thus distinguishing them from satellite cells and myoblasts. The same set of antibodies was used to assess cell surface marker expression in MP cells. Between 4% and 7% of these cells were positive for CD45, suggesting a possible hematopoietic origin, and served as a control for lack of CD45 expression on SP cells. The MP population was also found to be 14% Pax7-positive and 62% desmin-positive, reflecting the satellite cell and myoblast composition of this population, and serving as an antibody control for absence of these two myogenic markers on SP cells.

SP Transduction with Lentiviral Vectors. Lentiviral vectors have been proposed as one way to introduce dystrophin to diseased muscle (32). Systemic delivery of these viral vectors to muscle has been problematic. To determine whether self-inactivating lentiviral particles are capable of transducing muscle SP and MP cells, cells were transduced after FACS sorting of Hoechst-stained cells with L-MSCV-eGFP vectors by using a multiplicity of infection of 10. At 48 h posttransduction, almost 100% of the L-MSCV-eGFP-transduced cells, SP or MP, expressed eGFP under direct fluorescence microscopy (Fig. 2*A*). The expression of eGFP was stable in both cell types, with nearly all of the cells expressing eGFP 20 days postinfection (data not shown). After two washes in PBS posttransduction, supernatant of washed cells was used to infect new nontransduced cells, but no cells were found positive for eGFP, suggesting that there were no free virus particles left after the first transduction.

Coculture of SP Cells and C2C12 Cells. SP cells, when cultured, divide but tend not to differentiate into muscle or other apparent cell type, even at high-density platings. In the presence of primary or transformed myoblasts, muscle SP cells have been shown to differentiate into myoblasts and to contribute to myotubes (21). Using this coculture system, we tested whether SP cells transduced with the eGFP-expressing lentiviral vector could differentiate along with myogenic C2C12 cells and whether there were differences between normal and *mdx*^{5cv}-derived muscle SP cells. A time course of myotube formation was followed by immunohistochemistry for eGFP-multinucleated myotubes. At 12 h after coculture (Fig. 2*B*), the *mdx*^{5cv} eGFP-SP cells start to fuse, and it was not until 12 h later that C57BL/6N eGFP-SP cells began to fuse. eGFP-multinucleated myotube formation was complete at 24 h after culture with *mdx*^{5cv} eGFP-SP cells, but not until 48 h with C57BL/6N eGFP-SP cells (Fig. 2*B*). In both strains, between 17% and 22% of the eGFP-SP expressing cells had fused into multinucleated myotubes after 48 h. These results demonstrate a faster rate of progression through myogenesis for *mdx*^{5cv} SP cells, but not a major distinction from the normal SP population in the ability to form myotubes. During the coculture in proliferation media with C2C12 cells, 8% of the mononuclear eGFP-positive cells were found to be positive for Pax7 (Fig. 2*C*). After fusion, all myotubes were positive for desmin (Fig. 2*D*). The presence of Pax7-positive, eGFP-expressing mononuclear cells indicates that at least some of the SP cells could be precursor to satellite cells and follow a normal progression of muscle differentiation through Pax7 expression. The observa-

Table 1. eGFP-positive myofibers from tibialis anterior of eGFP-mdx^{5cv}-SP mdx^{5cv}-transplanted mice

Mice	Weeks after T*	Total	eGFP
1	4	123	2
2	4	151	1
3	8	145	4
4	9	198	2
5	10	102	3

After transplantation (T*), myofibers from tibialis anterior were isolated and cultured.

tions that cells differentiate more rapidly *in vitro* into the myogenic lineage are consistent with the hypothesis that muscle SP cells can participate in the permanent replacement of necrotic fibers in these disorders and that they are resident within muscle as a source of muscle precursor cells, but do not express known myogenic markers.

It was not clear whether muscle SP cells participated in the formation of myotubes together with C2C12 cells, or whether myotubes formed exclusively by fusion of C2C12 or muscle SP cells alone. To distinguish between these two possibilities, muscle SP cells from mdx^{5cv} mice were transduced with a lentivirus expressing GFP, and the C2C12 myoblasts were transduced with a lentivirus expressing nuclear LacZ. Equal numbers of transduced SP and C2C12 cells were cocultured together. After 1 week in fusion media, the cultures were stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal), and the nLacZ-eGFP expression was visualized under a fluorescence microscope (Fig. 2*E*). Among the eGFP-SP-derived multinucleated myotubes, $\approx$1/5 of them were expressing only eGFP and showed no evidence of detectable β-galactosidase (Fig. 2*E*). These results indicated that muscle SP cells can fuse with one another and differentiate into a multinucleated myotube without requiring a myoblast or myogenic nuclear program from a host cell. Most of the myotubes were expressing both eGFP and nLacZ markers, as expected, given the equal proportions of transduced cell types (Fig. 2*E*). Furthermore, there was no detection of multinucleated myotubes when muscle SP cells alone were cultured in differentiation media or with C2C12 supernatant (data not shown), suggesting that contact with a myogenic cell line and/or microenvironment in coculture is a critical condition for SP cells to undergo *in vitro* differentiation into myotubes. These observations demonstrate that, in the right environment, SP cells have the inherent potential to undergo the myogenic pathway on their own and can become myotubes without fusing with previously formed myotubes.

Transplantation of Lentivirus-Transduced Muscle SP Cells into mdx^{5cv} Mice. Muscle SP cells from normal mice have been shown to fuse into host myofibers and express mouse dystrophin at low frequency when transplanted via tail vein into lethally irradiated *mdx* mice (9). Recent work has shown that the damage resulting from the dystrophic phenotype is sufficient to recruit skin SP cells from the circulation after tail vein injections (33). Therefore, the systemic delivery of muscle SP cells to damaged muscle makes them an attractive candidate for autologous transplantation of *ex vivo* genetically modified muscle SP cells in nonirradiated dystrophic mice. To test this hypothesis, MP and SP cells isolated from mdx^{5cv} muscle were transduced with lentiviruses expressing eGFP. After transduction, 15,000 mdx^{5cv} muscle eGFP-SP or eGFP-MP cells were injected into five mdx^{5cv} mice each. Two mice were killed 4 weeks after transplantation, and the other three mice at 8, 9, and 10 weeks, respectively. In all five mice transplanted with eGFP-SP cells, eGFP positive myofibers were found (Table 1). However, no eGFP-positive fibers were detected in the eGFP-MP-transplanted mice (not shown). Thus, the muscle SP cells can be used to systemically deliver virally expressed genes, and these cells can contribute to muscle regeneration *in vivo* in nonirradiated dystrophic animals whereas an equal number of MP transduced cells cannot.

Single myofibers from the tibialis anterior of the transplanted mice were cultured *in vitro* to better document the contribution of muscle SP cells to the repair of damaged muscle and to determine whether SP cells fused together and/or to existing fibers are committed to the myogenic pathway. In single myofiber cultures, the fibers seem to shed proliferating cells that migrate to the periphery of the culture (Fig. 3 *Aa* and *Ab*), group together, and form new multinucleated myotubes (Fig. 3 *Ac* and *Ad*). Single myofibers from the transplanted mdx^{5cv} tibialis anterior muscle, both expressing eGFP and not expressing eGFP (example given in Fig. 3*B*) were cultured. The relative proportions of eGFP single myofibers are tabulated in Table 1. Both types of fibers yielded eGFP-Pax7-positive cells after 2 days in culture (Fig. 3*Bb*). At day

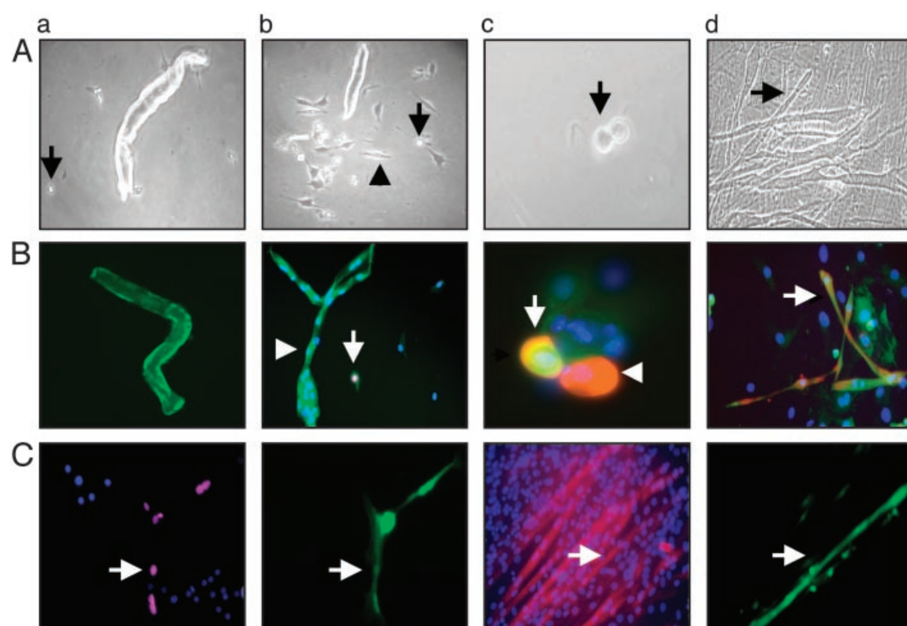

Fig. 3. Culture of single myofibers from transplanted animals. (*A*) Light microscopy of an example of single myofiber culture. (*Aa*) Day 1, satellite cells (arrow) migrate to the periphery of the fiber. (*Ab*) Week 1, in culture satellite cells (arrow) differentiate into myoblasts (arrowhead). (*Ac*) Myocytes are detected during the first week in culture (arrow). (*Ad*) Days 10–15, new multinucleated myotubes appear (arrow). (*B*) Culture of single myofibers from eGFP-SP transplanted mice. (*Ba*) Day 1, culture of an eGFP single myofiber from a transplanted mdx^{5cv}. (*Bb*) Day 2, detection of an eGFP multinucleated (DAPI in blue) myofiber (arrowhead) and an eGFP-Pax7-positive (Texas red)-SP-derived cell (arrow). (*Bc*) Day 5, detection of an eGFP-desmin-positive (Texas red)-SP derived myocyte (arrow) and a desmin-positive only (Texas red) myocyte (arrowhead). (*Bd*) Day 10, presence of an eGFP-demin-positive (Texas red)-SP-derived multinucleated (DAPI in blue) myotube (arrow). (*C*) Culture of single myofibers from eGFP-SP-transplanted mice. (*Ca* and *Cb*) Day 15, a myogenin-positive (Texas red plus DAPI) also expressing eGFP in a multinucleated myotube (arrow). (*Cc* and *Cd*) Day 15, desmin-positive myofibers (Texas red), which also express eGFP (arrow). Pictures are at low magnification (×20), except *Ac* and *Bc* (×40.)

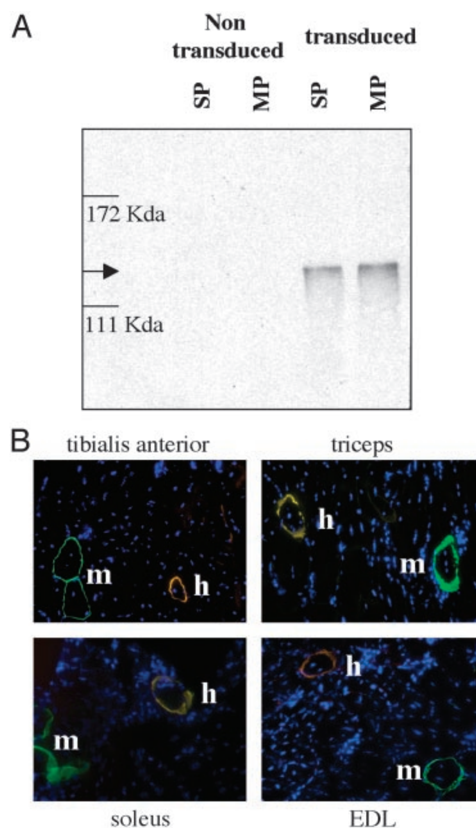

Fig. 4. Transduction and transplantation of muscle SP and MP cells with microdystrophin-expressing lentivirus. (*A*) Detection of a 137-kDa band (human microdystrophin) by immunoblot analysis of L-MSCV-μdys-infected-SP and L-MSCV-μdys-infected-MP cells extracts. (*B*) After sorting, 15,000 *mdx*^{5cv} muscle SP or MP cells were transduced with microdystrophin lentiviral vectors and then intravenously transplanted into C57BL/6N or *mdx*^{5cv} mice. Detection of mouse (m) dystrophin-positive revertant fibers (FITC stained green) and human (h) dystrophin (merge FITC and Texas red) in four different skeletal muscles from μdys-SP *mdx*^{5cv}-transplanted mice with an anti-mouse dystrophin (which also recognizes human dystrophin) and a specific anti-human dystrophin antibody 4 weeks after transplantation, respectively. Nuclei were stained with DAPI (blue). (Magnification, ×20.)

5, there were eGFP-desmin-positive mononuclear cells that resembled myocytes (Fig. 3*Bc*), and at 10 days there were clearly desmin-positive, GFP-positive myotubes (Fig. 3*Bd*). These eGFP-positive myotubes were also expressing myogenin (Fig. 3 *Ca* and *Cb*). Finally, at day 15, eGFP-positive and -negative myotubes aligned, forming mature desmin-positive myotubes (Fig. 3 *Cc* and *Cd*). Thus, the eGFP-marked SP cells, which were Pax7 negative before transplantation (Fig. 5), when injected intravenously in *mdx*^{5cv} mice were able to exit the circulation and contribute to the repair of dystrophic muscle after a myogenic program. These cells had the ability to differentiate into Pax7-positive precursor cells, become desmin-positive myocytes, and then fuse to form myogenin-expressing myotubes. These results reconfirm the myogenic potential of SP cells *in vivo* and *in vitro* and demonstrate that there is a high likelihood that they are a resident precursor source for satellite cells.

Systemic Delivery of Human Dystrophin into Host Myofibers of *mdx*^{5cv} Mice. Lentiviral vectors can accommodate short inserts of cDNA. Recently a microdystrophin cDNA (μdys) was constructed and cloned into these vectors (L-MSCV-μdys), and these viruses were used to transduce *mdx*^{5cv} muscle SP and MP cells. A 137-kDa band was detected (expected size for μdys) in immunoblots of L-MSCV-μdys-infected SP and MP cell extracts, by using an antibody against dystrophin (Fig. 4*A*). *mdx*^{5cv} muscle μdys-SP cells (15,000) were injected into five *mdx*^{5cv} and three C57BL/6N mice. In addition, five *mdx*^{5cv} mice were transplanted with 15,000 μdys-MP cells. Four and eight weeks after transplantation, mice were killed, and sections from different muscles were analyzed by using a mouse monoclonal antibody (Mab1690), which was developed against human dystrophin. Human dystrophin expression was detected in all μdys-SP *mdx*^{5cv}-transplanted mice and only in these mice (Fig. 6, which is published as supporting information on the PNAS web site). There was no evidence of μdys expression in the muscles of animals transplanted with μdys-MP cells nor was there expression in the muscles of normal mice transplanted with μdys-SP cells (data not shown). The results support the hypothesis that disease-damaged muscle could attract muscle SP cells from the circulation. Also, the SP cells were more efficient at homing to and fusing into host myofibers than MP cells because there was no expression in the MP-transplanted animals where equal numbers of cells were transplanted.

To determine that μdys-positive myofibers were not an artifact due to an antibody crossreactivity with dystrophin revertant fibers in the dystrophic mice, muscle sections were incubated with two different antibodies, one designed to recognize both mouse and human dystrophin (Ab6-10) and the second antibody reported to detect only human dystrophin (Mab1690). The Ab6-10 and Mab1690 antibodies were detected by using an FITC (stained green) and Texas red secondary antibody, respectively. Immunohistochemical analysis of the *mdx*^{5cv} mice transplanted with μdys-SP cells revealed the presence of both mouse (FITC, stained green) and human (FITC plus Texas red) dystrophin-expressing myofibers. Mouse revertant fibers were seen as green because they were positive for the Ab6-10 antibody only whereas fibers expressing human dystrophin were seen as orange because they were positive for both antibodies (merge of FITC and Texas red) (Fig. 4*B*). Equal low levels of human μdys expression (<1% of the myofibers) were found in the soleus, tibialis anterior, extensor digitorum longus, quadriceps, triceps, and diaphragm (Figs. 6 and 4*B*). No expression was detected in cardiac muscle. All *mdx*^{5cv} animals transplanted with μdys-SP cells showed similar low levels of human microdystrophin expression. To rule out the possibility that genetically modified-SP cells fuse only into dystrophin-positive revertant fibers, revertant fibers were counted from four *mdx*^{5cv}-nontransplanted mice. Between 0.1 and 0.2% of *mdx*^{5cv}-nontransplanted mice fibers were dystrophin positive (revertant fibers) compared with 1% in genetically modified-SP cells transplanted mice. These results indicated that SP cells can be used as a delivery vehicle, by means of the circulation, of human dystrophin for the autologous transplantation of muscle stem cells.

Discussion

Animals and human patients with muscular dystrophy are characterized by a progressive muscle myofiber regenerative/degenerative process, which results in an inflammatory environment rich in cytokines and cell-signaling molecules. Mononuclear muscle precursor cells attempt to repair these damaged muscles by constantly replacing the necrotic fibers. The cycle of regeneration-degeneration of muscle fibers is much more frequent in animals with muscular dystrophy than in wild-type. Normally, it is thought that the cell that is used for this repair is a muscle progenitor cell or satellite cell committed to the myogenic lineage. Here, we show that a subpopulation of lineage uncommitted cells called muscle SP cells participate in this repair. Muscle SP cells derived from *mdx*^{5cv} muscle also seem to be "primed" to differentiate into muscle more rapidly than muscle SP cells derived from normal muscle. These properties of *mdx*^{5cv} muscle SP cells are likely the result of the turnover of cells in regenerating muscle and the signals that initiate these events. The SP cells are likely to contribute to this repair process, and may serve as a stem cell reservoir for muscle progen-

itors without being committed to the muscle differentiation pathway. When recruited into the repair process, they seem to be primed to fuse into new myofibers.

Separation of the SP muscle cell lineage from that of satellite cells was first documented in Pax7-deficient mice, which lack myogenic satellite cells, yet still have normal levels of muscle SP cells (22). Pax7 expression was not required for myogenic differentiation of muscle SP cells. Pax7-deficient muscle SP cells, when cocultured with primary myoblasts, were able to contribute to the formation of myotubes in culture (21). Here, cocultured muscle SP cells marked with eGFP fused with a myogenic cell line C2C12 and did so in some cases through the Pax7 lineage (Fig. 2). After fusion into multinucleated myogenic cells, eGFP-marked cells were positive for desmin (Fig. 2). Consequently, muscle SP cells can contribute to muscle formation *in vitro*. In some instances, muscle SP cells differentiate into Pax7-positive satellite cells and then progress through the myogenic lineage into mature multinucleated myotubes.

Muscle SP cells expressing Myf5 promoter-driven LacZ were injected intramuscularly into the regenerating TA muscle of adult *scid/bg* immunodeficient mice treated with cardiotoxin. The SP-derived cells were found within the basal lamina, much like satellite cells (21). Similar to previous intramuscular injections, in our study, SP cells, when delivered intravenously *in vivo*, were able to follow through the various steps of myogenesis to become mature muscle myotubes. The SP cells were found to directly contribute to muscle regeneration and were detected on single myofibers. When fibers from these transplanted animals were cultured, they were found to support a population of SP-derived cells that expressed Pax7 and subsequently fused to produce desmin-positive myotubes (Fig. 3). Interestingly, muscle SP-derived satellite cells were detected when i.v. injection of as few as 15,000 SP cells into immunocompetent, nonirradiated mdx^{5cv} mice was performed. This recruitment was observed only when dystrophic recipients were used, not normal recipients, suggesting that factors from damaged muscle are necessary to attract these cells from the vasculature and further contribute to muscle repair.

The delivery of dystrophin into *mdx* or Duchenne muscular dystrophy patients using intramuscular injections of normal myoblasts, including satellite cells, has had somewhat disappointing results (4, 34–38). The major flaw of these intramuscular injections was the lack of systemic delivery of dystrophin normal myoblasts to other noninjected muscles. Moreover, in some cases, dystrophin expression in the new myotubes that formed was not documented to be of donor origin, reflecting the possibility of revertant fibers (38). The SP cells transduced with lentiviruses expressing μdys represent a way to test the systemic delivery of human dystrophin to muscle and to document the recruitment of these cells to muscle. Numerous antibodies directed against dystrophin exist, but few are species specific. The 12B2 antibody is specific for human dystrophin and does not detect mouse dystrophin. Transplanted μdys-SP cells were shown to traffic from the circulation and take up residence within host muscles. Using a pan-antibody, which detects both mouse and human dystrophin, coupled with a human-specific one, we were able to unambiguously document the expression of human dystrophin in the transplanted muscle. Given that there were more dystrophin-negative fibers in the transplanted animals, it is likely that the donor cells formed new muscle fibers or fused to existing dystrophin-negative fibers. The expression of dystrophin was low in the transplanted animals but significant. It is possible that dissection of the signals that recruit these cells from the circulation could improve the efficiency of recruitment. Recent encouraging work using intraarterial delivery of a different stem cell population (39) reported donor-cell engraftment levels approaching 50% in transplanted host muscle.

The fact that lentiviral vectors can efficiently transduce a muscle stem cell and those cells can contribute to the repair of damaged muscle has substantial implications for therapy of muscular dystrophy. Any major improvement in the engraftment of those cells could lead to human trials, and these trials could be accomplished by using autologous cells.

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