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 mdx5cv 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 mdx5cv 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 mdx5cv 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 mdx5cv mice revealed differences in the potential to differentiate into skeletal muscle in vitro. The mdx5cv 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 mdx5cv 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<sup>6N</sup> 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-5ev (mdx<sup>6N</sup>) (X-linked muscular dystrophy) mice were obtained from The Jackson Laboratory. In the mdx<sup>6N</sup> 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-nLacZ, and pL-MSCV-µdys were constructed by inserting either enhanced GFP (eGFP), nuclear-targeted LacZ, 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<sup>6N</sup> donor tissue as described (9, 29). Before HO342 staining, red cells were lysed (30). Primary myoblasts were resuspended at 10<sup>6</sup> cells per ml and stained with 12.5 µg/ml HO342 in PBS-0.5% BSA for 60 min at 37°C. In parallel, 10<sup>6</sup> 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% high glucose (CellGro, CellGenix, Freiburg, Germany) supplemented with 20% heat inactivated horse serum (GIBCO), 2.5% mouse embryonic 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. 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<sup>6N</sup> 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 was 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<sup>6N</sup> Muscle SP Cells.** Skeletal muscle isolated from mdx<sup>6N</sup> animals shows histopathological signs of degeneration and regeneraation. Side population cells within the skeletal muscle of many different 4- to 9-week-old wild-type C57BL/6N and mdx<sup>6N</sup> 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...
positive for SCA-1 (80–90%) and CD34 (65–80%) but did not express CD45 and e-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<sup>scv</sup>-derived muscle SP cells. At 12 h after coculture (Fig. 2 B), the mdx<sup>scv</sup>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<sup>scv</sup>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<sup>scv</sup> 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-
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<sup>Scv</sup> 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-galactoside (X-gal), and the nLacZ-eGFP expression was visualized under a fluorescence microscope (Fig. 2 E). Among the eGFP-SP-derived multinucleated myotubes, ~1/5 of them were expressing only eGFP and showed no evidence of detectable β-galactosidase (Fig. 2E). 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. 2E). 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<sup>Scv</sup> 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<sup>Scv</sup> muscle were transduced with lentiviruses expressing eGFP. After transduction, 15,000 mdx<sup>Scv</sup> muscle eGFP-SP or eGFP-MP cells were injected into five mdx<sup>Scv</sup> 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 transplanted 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–Ac). Myofibers group together, and form new multinucleated myotubes (Fig. 3 Bd). Single myofibers from the transplanted mdx<sup>Scv</sup> tibialis anterior muscle, both expressing eGFP and not expressing eGFP (example given in Fig. 3Bb) 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. 3Bb). At day

Table 1. eGFP-positive myofibers from tibialis anterior of eGFP-mdx<sup>Scv</sup>-SP mdx<sup>Scv</sup>-transplanted mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Weeks after T*</th>
<th>Total</th>
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<tr>
<td>1</td>
<td>4</td>
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<td>2</td>
<td>4</td>
<td>151</td>
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<td>3</td>
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<td>5</td>
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After transplantation (T*), myofibers from tibialis anterior were isolated and cultured.
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 mdx5cv muscle also seem to be “primed” to differentiate into muscle more rapidly than muscle SP cells derived from normal muscle. These properties of mdx5cv muscle SP cells are likely the result of the recruitment 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 progenitor 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 mdx5cv muscle also seem to be “primed” to differentiate into muscle more rapidly than muscle SP cells derived from normal muscle. These properties of mdx5cv muscle SP cells are likely the result of the recruitment 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 progenitor cells.
nonirradiated i.v. injection of as few as 15,000 SP cells into immunocompetent, muscle SP-derived satellite cells were detected when transplanted animals were cultured, they were found to regenerate and were detected on single myofibers. When fibers through the various steps of myogenesis to become mature muscle muscle formation with primary myoblasts, were able to contribute to the formation of satellite cells, yet still have normal levels of muscle SP cells

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 basalm lamina, much like satellite cells. 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 donor-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 mdxSCV mice was performed. This recruitment was observed only when dystrophic recipients were used, not normal dystrophy patients using intramuscular injections of normal myoblasts, were shown to traffic from the circulation and take up residence in the basal lamina, much like satellite myotubes.

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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