

Identification of a novel population of muscle stem cells in mice: potential for muscle regeneration

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Three populations of myogenic cells were isolated from normal mouse skeletal muscle based on their adhesion characteristics and proliferation behaviors. Although two of these populations displayed satellite cell characteristics, a third population of long-time proliferating cells expressing hematopoietic stem cell markers was also identified. This third population comprises cells that retain their phenotype for more than 30 passages with normal karyotype and can differentiate into muscle, neural, and endothelial lineages both *in vitro* and *in vivo*. In contrast to the other two populations of myogenic cells, the trans-

plantation of the long-time proliferating cells improved the efficiency of muscle regeneration and dystrophin delivery to dystrophic muscle. The long-time proliferating cells' ability to proliferate *in vivo* for an extended period of time, combined with their strong capacity for self-renewal, their multipotent differentiation, and their immune-privileged behavior, reveals, at least in part, the basis for the improvement of cell transplantation. Our results suggest that this novel population of muscle-derived stem cells will significantly improve muscle cell-mediated therapies.

Introduction

Growth and repair of skeletal muscle in the postnatal stage are normally initiated by the activation of a population of mononucleated muscle precursors called satellite cells, which are located beneath the basement membrane of muscle fibers (Schultz et al., 1985; Bischoff, 1986). Based on their ability to repair injured or damaged muscle fibers in the postnatal stage, satellite cells were proposed as a population of muscle stem cells (Bischoff, 1986; Stockdale, 1990; Schultz and McCormick, 1994). The differential behavior of the satellite cells both *in vitro* and *in vivo* supports the evidence that satellite cells are heterogeneous in nature (Schultz and McCormick, 1994; Rantanen et al., 1995; Molnar et al., 1996). At least two populations of satellite cells, "fusing" and "nonfusing" satellite cells, have been isolated from skeletal muscle tissue of different species (Baroffio et al., 1996). A small population of satellite cells that divide more slowly than the main population has also been reported in mouse (Beauchamp et al., 1999) and rat muscle cell cultures (Schultz, 1996). By using a quantitative

marking technique and the dynamics of myoblast transplantation, it was found that the majority of transplanted muscle cells was eliminated shortly after transplantation and the newly regenerated myofibers in the host were formed by the progeny of a small percentage of the injected cells (Huard et al., 1994b; Beauchamp et al., 1999). This result suggests that there may be a unique subpopulation of stem cells within the satellite cell population. Similarly, previous studies have reported that some myoblasts become quiescent muscle precursors after implantation into the skeletal muscle of mdx mice, and persist for >1 yr after implantation (Morgan et al., 1994; Gussoni et al., 1997; Smith and Schofield, 1997). These results, taken together, suggest that satellite cells are heterogeneous and that a small subpopulation of these cells displays stem cell characteristics.

More substantial evidence of the existence of pluripotent stem cells in mouse skeletal muscle was recently reported. A side population (SP)* of cells was isolated from mouse muscle by

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*Abbreviations used in this paper: CNPase, 2',3'-cyclic nucleotide 3'-phosphohydrolase; EP, early preplate; EPa, activated early preplate; EPq, quiescent early preplate; GFP, green fluorescent protein; I, injected; LP, late preplate; MDSC, muscle-derived stem cells; MHC-1, major histocompatibility complex class 1; NGF, nerve growth factor; NI, noninjected; PE, phycoerythrin; PM, proliferation medium; pp1, pp2, and pp3, preplate 1, 2, and 3; SP, side population; TA, tibialis anterior; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor.

FACS[®], which previously was used to isolate hematopoietic stem cells (Gussoni et al., 1999). SP cells derived from both muscle and bone marrow were shown to be capable of differentiating into multiple lineages after intravenous transplantation into host mice (Gussoni et al., 1999). Jackson et al. (1999) also showed that a population of muscle-derived cells exhibited a remarkable capacity for hematopoietic differentiation after intravenous implantation into lethally irradiated mice. Importantly, a population of muscle cells derived from mice that were deficient in Pax7, a transcription factor expressed in satellite cells, exhibited a strong capacity for differentiation into the hematopoietic lineage but lacked the ability to differentiate into the myogenic lineage (Seale et al., 2000). This suggests that satellite cells and the muscle-derived SP cells displaying a hematopoietic potential are distinct cell populations. Additionally, recent studies have indicated that the progenitor cells isolated from bone marrow (Ferrari et al., 1998; Gussoni et al., 1999), the embryonic vasculature (De Angelis et al., 1999), the neuronal compartment (Clarke et al., 2000; Galli et al., 2000), and various mesenchymal tissues (Young et al., 2001a,b) can also differentiate into the myogenic lineage, suggesting that stem cells from other sources could contribute to muscle regeneration. Therefore, additional experiments are required to elucidate whether multiple types of muscle-derived stem cells (MDSC) exist and whether they display a differential ability to regenerate skeletal muscle.

Separation and characterization of muscle stem cells remain critical because of the cells' rarity (Schultz, 1996; Beauchamp et al., 1999; Gussoni et al., 1999; Lee et al., 2000) and the lack of specific markers to identify myogenic cells at very early stages of development (Dominov et al., 1998; Miller et al., 1999). The CD34 antigen, a transmembrane cell surface glycoprotein, has been identified and characterized as a hematopoietic stem cell marker in different species, including mice (Krause et al., 1994; Fennie et al., 1995; Morel et al., 1996). More recently, a population of CD34⁺ satellite cells was found in mouse skeletal muscle (Beauchamp et al., 2000; Lee et al., 2000), suggesting that the CD34 antigen is also expressed in some myogenic cells. If early myogenic cells display stem cell characteristics, they might also express similar surface antigens, as observed in murine hematopoietic stem cells. Sca-1, a protein expressed in murine hematopoietic stem cells (Nakauchi et al., 1999), may also be used as a muscle stem cell marker. The identification of the markers expressed in various types of muscle-derived cells will likely be helpful in delineating this small population of MDSC in mice.

We recently reported (Qu et al., 1998) the isolation (using the preplate technique) of a specific population of muscle-derived cells that could avoid early cell death after transplantation into skeletal muscle. We have also reported (Lee et al., 2000) the presence of two populations of myogenic cells, cells expressing m-cadherin and cells expressing CD34, in the skeletal muscle of normal mice, residing between the basal lamina and plasma membrane. Cloned cells isolated from the CD34⁺ population could differentiate into different lineages and, more importantly, could improve both muscle regeneration and bone healing in vivo (Lee et al., 2000). In the present study, three populations of muscle-derived cells were isolated from normal mice according to

their adhesion characteristics and proliferation behaviors: the early preplate cells (EP); the late preplate cells (LP); and a rare population of long-time proliferating cells that were derived from primary LP cultures (see Materials and methods). The levels of expression of stem cell and myogenic cell markers in these various populations of purified myogenic cells were investigated. The cell populations' ability to improve the efficiency of myoblast transplantation in dystrophin-deficient mdx mice was tested by investigating their capacity for self-renewal, long-time proliferation, multipotent differentiation, and immune-privileged behaviors. Our results suggest that the EP and LP cells are two populations of satellite cells, whereas the rare population of cells capable of high proliferation represents a novel type of pluripotent MDSC.

Results

Proliferation ability of MDSC and EP cells

We have only characterized the proliferation behavior of MDSC and EP cells, because most of the LP cells did not proliferate and died within 1–2 wk of culturing. These two populations of muscle-derived cells (EP and MDSC) displayed differences in cell culture proliferation behaviors. EP cells remained quiescent (EPq) during the first 1–3 d after initial seeding and then divided three to five times in proliferation medium (PM) (activated EP cells [EPa]), with an average division time of 12.6 h (\pm 3.9, SD; n = 3 separated cultures). Afterward, >80% of the EP cells in the culture fused into myotubes, even when cultivated in PM or at a low confluence (<30%). As mentioned above, the LP cells are distinguished from EP cells by their slow division and their lack of fusion. Due to the continuity of the preplate technique (see Materials and methods), we cannot exclude the possibility that some of the LP cells were isolated from a nonfusing progeny of EP cells, as previously described for satellite cells (Baroffio et al., 1996). The MDSC could divide for more than 30 passages in PM with an average division time of 15.1 h (\pm 2.3, SD; n = 3 separated cultures). In addition, we have observed that MDSC preserve their normal karyotype with culturing (30 passages). The normal diploid number of chromosomes ($2n$) seen in mice is 40. We randomly examined 28 cells in MDSC culture and found that the majority of cells counted (24/28) had a diploid number of 40, with three missing a single chromosome and one gaining one chromosome. The few aneuploid cells found within the MDSC are probably due to normal tissue culture artifact (Wenger et al., 1984; Barch, 1991).

The expression of stem cell and early myogenic cell markers in the populations of muscle-derived cells

Immunostaining to colocalize the stem cell antigen Sca-1, the myogenic antigen desmin, and Hoechst (which reveals nuclei) was performed on EP and MDSC cultures in vitro (Fig. 1, a and b). In the EP culture (Fig. 1 a), ~90% of the cells expressed desmin (green), and 30–40% of the cells coexpressed a heterogeneous, high level of Sca-1 (red, arrow). In the MDSC culture (Fig. 1 b), only 30–40% of the MDSC were desmin⁺ (green, arrow), whereas >90% expressed Sca-1 (red, arrowhead). To evaluate the relative percentages of CD34⁺ and Sca-1⁺ cells in

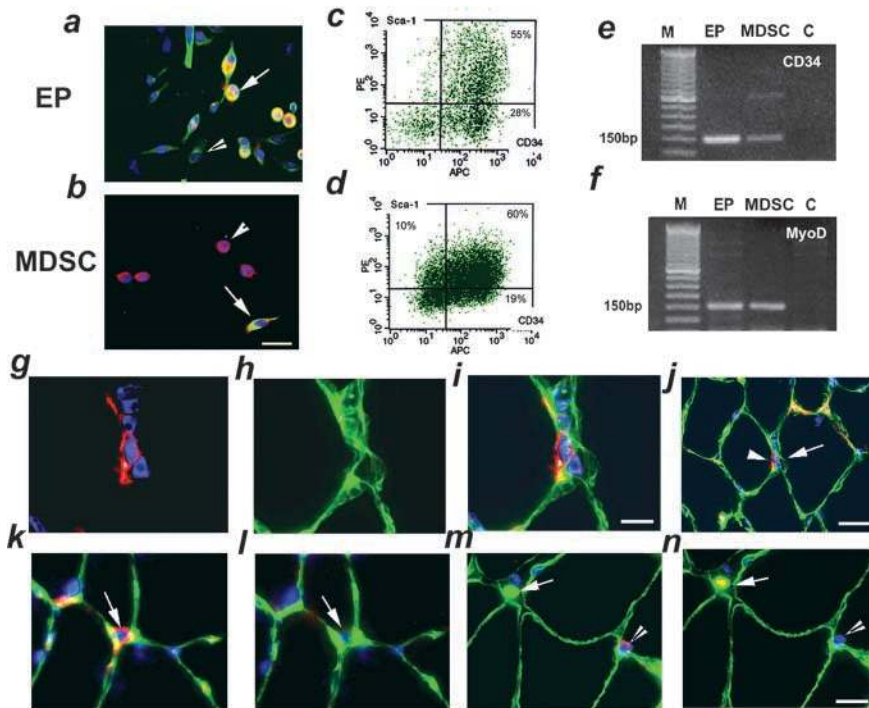


Figure 1. Phenotypes of EP cells and MDSC in cultures and in normal muscle sections. (a and b) Immunostaining revealed that in the EP culture (a), most of the cells are desmin⁺ (green, arrowhead), and some cells coexpressed a high level of Sca-1 (red, arrow). In contrast, many cells in the MDSC culture (b) expressed Sca-1 (red, arrowhead), but only 30–40% of these cells expressed desmin (green, arrow). (c and d) Flow cytometry showed that in the EP culture (c), 83% of the cells expressed CD34, and 55% of the cells coexpressed CD34/Sca-1. In the MDSC culture (d), 79% of the cells expressed CD34, and 60% of the cells coexpressed CD34/Sca-1. Unlike the EP culture (c), the MDSC culture (d) contained a population (10%) of cells that were Sca-1⁺ and CD34⁻. (e and f) RT-PCR for CD34 (e) and MyoD, an early stage marker of myogenesis (f), showed that the two populations (EP cells and MDSC) express both CD34 and MyoD. M, markers; C, control MDSC without reverse transcriptase. (i and j) Immunostaining of muscle cross sections prepared from normal mice showed that Sca-1-expressing cells

(g, red) were found beneath the basement membrane, as revealed by laminin staining (h–j, green). The arrowhead in j is showing the Sca-1-expressing cells, whereas the arrow is showing the basal lamina expressing laminin. The nuclei were revealed by Hoechst staining (g, i, and j). (k–m) Colocalization of m-cadherin (Cy5, red), Sca-1 (PE, red), laminin (FITC, green), and nuclei (blue) confirmed that the Sca-1-expressing cells (k, arrow) did not colocalize with m-cadherin⁺ cells (l, arrow). Similarly, the m-cadherin⁺ cells (m, arrowhead) did not colocalize with Sca-1-expressing cells (n, arrowhead). Notice that the capillaries are also Sca-1⁺ (m and n, arrows). Bars: (a, b, and k–n) 50 μ m; (g–i) 25 μ m; (j) 100 μ m.

the two cell populations, flow cytometry was also performed (Fig. 1, c and d). In the EP culture (Fig. 1 c), 83% of the cells expressed CD34, and 55% of the cells coexpressed Sca-1/CD34. In the MDSC culture (passage 10), 79% of the cells expressed CD34, and 60% of the cells coexpressed Sca-1/CD34

Table I. The phenotypes of EP, LP, and long-time proliferating cells (MDSC) investigated by immunocytochemistry, flow cytometry, and RT-PCR

Markers	EPq		EPa		LP		MDSC	
	Imm	Imm	Flow	RT	Imm	Imm	Flow	RT
CD34	-	+/-	+/-	+	+/-	+	+/-	+
Sca-1	-	+/-	+/-	ND	+/-	+	+/-	ND
c-Kit	ND	ND	-	ND	-	-	-	ND
CD45	ND	ND	-	ND	ND	ND	-	ND
Desmin	+	+	ND	ND	+/-	-/+	ND	ND
Bcl2	-	-	ND	+	ND	+	ND	+
MNF	ND	+	ND	+	ND	+	ND	+
MyoD	-/+	-/+	ND	+	+/-	ND	ND	+
Myogenin	+/-	+/-	ND	+	+/-	ND	ND	ND
m-cadherin	+/-	+	ND	ND	-/+	-	ND	ND

EP and LP cells were dissociated from the hindlimb muscle of normal newborn mice and separated by their adhesion characteristics (see Materials and methods for details). Two populations of EP cells were used here: the EP cells after 3 d of culturing (EPq, relatively quiescent) and the EP cells after 5 d of culturing (EPa, activated). The LP cells took 5–7 d to attach to flasks and were cultured for an additional 4 d. MDSC were isolated from a small population of long-time proliferating cells derived from the LP cells. In immunocytochemistry and flow cytometry, +, >95% of the cells were positive; +/-, 40–80% of the cells were positive; -/+, 10–30% of the cells were positive; -, <5% of the cells were positive. Flow, flow cytometry; Imm, immunocytochemistry; RT, RT-PCR.

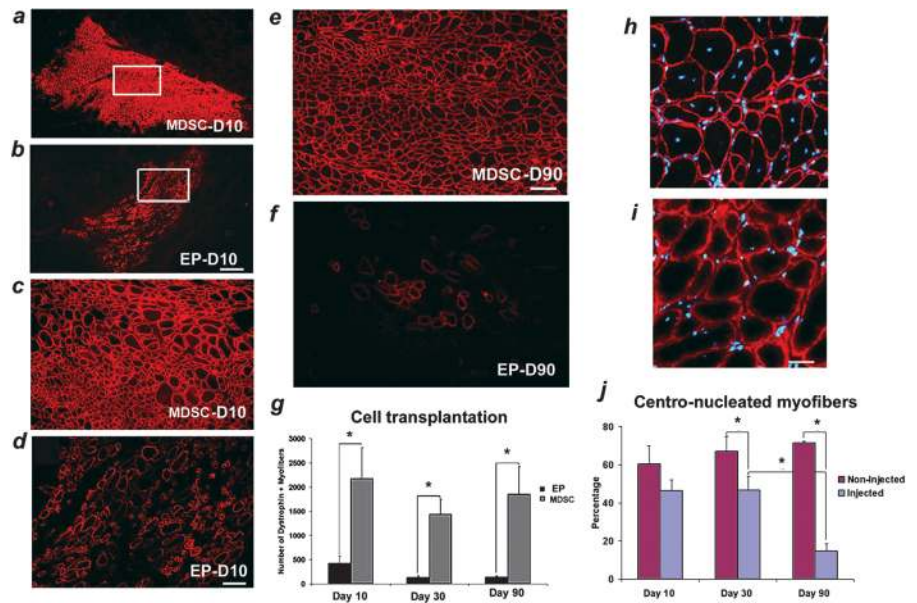
(Fig. 1 d). In contrast to the EP culture, the MDSC culture contained a population (10%) of cells that were Sca-1⁺ and CD34⁻. Finally, RT-PCR for CD34 (Fig. 1 e) and MyoD, an early myogenic marker (Fig. 1 f), further validated that both EP cells and MDSC expressed CD34 and MyoD.

The phenotypes of these different populations of muscle-derived cells determined by immunocytochemistry, RT-PCR, and flow cytometry are summarized in Table I. Two different populations of EP cells were characterized: relatively quiescent nondividing (EPq) cells and dividing (EPa) cells. The nondividing EP cells were a population that had been cultivated for 3 d from initial seeding, whereas the dividing EP cells had been cultivated for 5 d. The EPq cells displayed the phenotype desmin⁺, CD34⁻, Bcl2⁻, Sca-1⁻, MyoD^{-/+}, myogenin^{+/-}, and m-cadherin^{+/-}, whereas the EPa cells displayed the same characteristics for desmin, MyoD, myogenin, Bcl2, and m-cadherin, but were CD34^{+/-} and Sca-1^{+/-}. This result demonstrates that the cultivation of a similar population of cells for an extra 2 d may alter the expression pattern of myogenic and stem cell markers. The LP cells displayed the phenotype desmin^{+/-}, CD34^{+/-}, Sca-1^{+/-}, MyoD^{+/-}, myogenin^{+/-}, and m-cadherin^{-/+}, whereas the MDSC were desmin^{-/+}, CD34⁺, Bcl2⁺, Sca-1⁺, and m-cadherin⁻. All of the cell populations were found by flow cytometry to be negative for both c-Kit and CD45, eliminating their potential hematopoietic origin.

Although the two populations of proliferating myogenic cells (EP cells and MDSC) displayed similar patterns of expression of myogenic and stem cell markers, differences in the levels of Sca-1 and CD34 expression and the relative per-

Figure 2. Dystrophin expression in mdx skeletal muscle after MDSC and EP cell transplantation.

(a–g) Many dystrophin⁺ myofibers were detected in both MDSC-injected muscle (a) and the EP cell-injected muscle (b) at 10 d after injection; however, the number of dystrophin⁺ myofibers was significantly higher in the MDSC-injected muscle (g). Boxed regions in a and b are enlarged in c and d, and show that the MDSC-injected muscle contained many more small dystrophin⁺ myofibers (c) than the EP cell-injected muscle (d). Many dystrophin⁺ myofibers were still observed at 90 d after injection of MDSC (e), whereas very few were observed in EP cell-injected muscles (f). The number of dystrophin⁺ myofibers was significantly higher in the MDSC-injected muscle than in the EP cell-injected muscle at all time points analyzed (g; * $P < 0.01$, $n = 3$ –5 animals/group). (h–j) MDSC-injected muscle sections containing dystrophin staining (red) were also counterstained with Hoechst (blue) to reveal the location of nuclei. Many dystrophin⁺ myofibers were centronucleated at 30 d after implantation (h), but a significant decrease in the number of centronucleated myofibers occurred by 90 d after injection (i). We observed no difference in the percentage of centronucleated myofibers between injected and noninjected areas at 10 d after injection (j). However, at 30 and 90 d after transplantation, the number of centronucleated myofibers was significantly lower (* $P < 0.01$, $n = 3$ muscles/condition) at the injected site than at the noninjected site. Bars: (a and b) 500 μm ; (c and d) 50 μm ; (e and f) 150 μm ; (h and i) 25 μm .



centages of desmin-expressing cells were still observed between the populations. Unlike EP cells, most MDSC (>60%) do not express the myogenic marker desmin, and a small population (10%) of these cells is Sca-1⁺/CD34⁻; this latter characteristic also is found in primary hematopoietic stem cells (Nakauchi et al., 1999).

Based on these *in vitro* results, we hypothesized that the different populations of myogenic cells expressing these particular phenotypes might also be found in skeletal muscle *in vivo*. Because the quiescent EP cells were m-cadherin^{+/+}, CD34⁻, Sca-1⁻, and Bcl2⁻, whereas the MDSC were m-cadherin⁻, CD34⁺, Sca-1⁺, and Bcl2⁺, we investigated these markers by immunohistochemistry to detect the presence of these two types of myogenic cells in normal muscle sections. We already have reported a population of CD34⁺ and Bcl2⁺ cells beneath the basement membrane in normal skeletal muscle (Lee et al., 2000); in this study, we have further investigated whether Sca-1⁺ cells also could be located beneath the basal lamina of muscle fibers. We colocalized by immunohistochemistry Sca-1, laminin, and Hoechst/nuclei (Fig. 1, g–j). Sca-1 was revealed by Cy3 immunofluorescence (Fig. 1 g, red), whereas laminin was detected by FITC immunofluorescence (Fig. 1 h, green). As with CD34⁺ and Bcl2⁺ cells in normal muscle cross sections (Lee et al., 2000), the colocalization of Sca-1/laminin/nuclei (Hoechst/blue) revealed that the cells expressing Sca-1 were also located beneath the basement membrane (Fig. 1, i and j). Further staining to colocalize Sca-1 (phycoerythrin [PE], red), m-cadherin (Cy5, red), laminin (FITC, green), and nuclei (Hoechst, blue) confirmed that the Sca-1⁺ cells (Fig. 1 k, red, arrow) did not colocalize with m-cadherin (Fig. 1 l, arrow), and the m-cadherin-expressing cells (Fig. 1 m, arrowhead) did not colocalize with Sca-1 (Fig. 1 n, arrowhead). In muscle tissue, the majority of blood vessels and capillaries

were also Sca-1⁺, were often surrounded by basement membrane, and did not colocalize with Hoechst (Fig. 1, m and n, arrow). Some Sca-1⁺ cells were observed outside of the basement membrane (unpublished data), and they probably derived from circulating blood. We also counted the number of m-cadherin and Sca-1⁺ cells beneath the basal lamina in 15 gastrocnemius muscle sections from three normal mice. The ratio of Sca-1⁺ to m-cadherin⁺ cells was found to be less than 1:100 (4:600). These results support our previous findings (Lee et al., 2000), indicating the existence of distinct populations of muscle progenitor cells located beneath the basal lamina of normal myofibers.

Long-term restoration of dystrophin by MDSC transplantation

The purification of different populations of muscle-derived cells led us to investigate their role in muscle regeneration. Because both EP cells and MDSC showed the ability to proliferate in culture, an important criterion for cell transplantation, we focused our cell transplantation experiments on these two populations of cells. We transplanted the same number of EP cells and MDSC (3 – 4×10^5) from normal newborn mice (C57BL/6J) into the m. gastrocnemius of mdx mice (C57BL/10ScSn DMD^{mdx}/J; 6–8 wk old). We detected a large number of dystrophin⁺ myofibers in the MDSC-injected muscle (Fig. 2 a), in contrast to the EP cell-injected muscle (Fig. 2 b), at 10 d after transplantation. The MDSC-injected muscle contained many more small myofibers (Fig. 2 c) when compared with the EP cell-injected muscle (Fig. 2 d), suggesting that the injected MDSC may possess a higher proliferative ability *in vivo* than EP cells, as observed *in vitro*. Moreover, we observed many dystrophin⁺ myofibers at 30 d and 90 d (Fig. 2 e) after transplantation of the MDSC, but only a few dystrophin⁺ myofibers were de-

tected in EP cell-injected muscle at 30 d and 90 d after injection (Fig. 2 f). Despite injection of the same number of transplanted cells, the number of dystrophin⁺ myofibers was approximately five times higher in the MDSC-injected muscle than in the EP cell-injected muscle at 10 d after injection (2178.8 ± 628.5 , $n = 4$, in the MDSC-injected muscle vs. 420.4 ± 152.7 , $n = 5$, in the EP cell-injected muscle) (Fig. 2 g). At 30 and 90 d after transplantation, 10 times more dystrophin⁺ myofibers were found in MDSC-injected groups than in EP cell-injected groups (1435.8 ± 312.5 , $n = 4$, vs. 128.7 ± 37.8 , $n = 3$ at day 30; 1834.0 ± 489.9 vs. 139.0 ± 20.0 , $n = 3$ at day 90) (Fig. 2 g).

To further examine the effect of MDSC transplantation on the histology of the dystrophic muscle, we investigated the number of dystrophin⁺ myofibers that were centronucleated at 10, 30, and 90 d after transplantation. The percentage of centronucleated myofibers revealed by Hoechst (blue) was investigated in both the injected and noninjected areas of the transplanted muscles; 250–950 myofibers per site were analyzed (Fig. 2, h–j). The injected area was easily recognizable by the presence of numerous dystrophin⁺ myofibers. The percentage of centronucleated myofibers was not significantly different between the noninjected (NI) and injected (I) sites at 10 d after transplantation (Fig. 2 j), but a significant decrease in the percentage of centronucleated myofibers was observed between noninjected and injected areas at 30 d (NI, $67.1\% \pm 7.6$, vs. I, $46.5\% \pm 7.3$, $n = 4$) (Fig. 2, h and i) and 90 d after injection (NI, $71.3\% \pm 1.1$, vs. I, $16.3\% \pm 4.1$, $n = 3$) (Fig. 2, i and j). These results suggest that the delivery of dystrophin via transplantation of MDSC can restore, at least in part, the histology of dystrophic muscle for up to 3 mo after transplantation.

The unique ability of the novel population of MDSC to improve both the extent of muscle regeneration and the persistence of dystrophin delivery within dystrophic mice when compared with EP cells led us to investigate the mechanism by which these cells improve cell transplantation. We therefore tested (a) the self-renewal and multipotent differentiation ability of these muscle-derived cells and (b) the cells' immune-privileged behavior after transplantation *in vivo*.

The self-renewal ability of MDSC *in vitro* and *in vivo*

To examine the self-renewal ability of MDSC, we first investigated whether the high percentages of CD34⁺ and Sca-1⁺ cells in the MDSC population could be maintained for a period of time with culturing *in vitro* (10 and 30 passages) and whether this phenotype could be preserved between cloned and subcloned MDSC. High percentages of CD34⁺ and Sca-1⁺ cells in the MDSC culture (at passage 30) were observed by flow cytometry; 77% of the cells were CD34⁺, 57% were CD34⁺/Sca-1⁺, and 8% were CD34⁻/Sca-1⁺ (Fig. 3 a), which is very similar to the MDSC at passage 10 (Fig. 1 d). Moreover, these similarities also were observed in the MDSC clone cells (unpublished data), of which 73% were CD34⁺, 62% were CD34⁺/Sca-1⁺, and 11% were CD34⁻/Sca-1⁺, as well as in some subclones of MDSC cloned cells (Fig. 3 b), of which 91% were CD34⁺, 59% were CD34⁺/Sca-1⁺, and 5% were CD34⁻/Sca-1⁺. These results indicate that MDSC have the capacity for self-renewal *in vitro*.

The self-renewal capacity of MDSC *in vivo* was tested further by transducing both MDSC and EP cells with a retrovi-

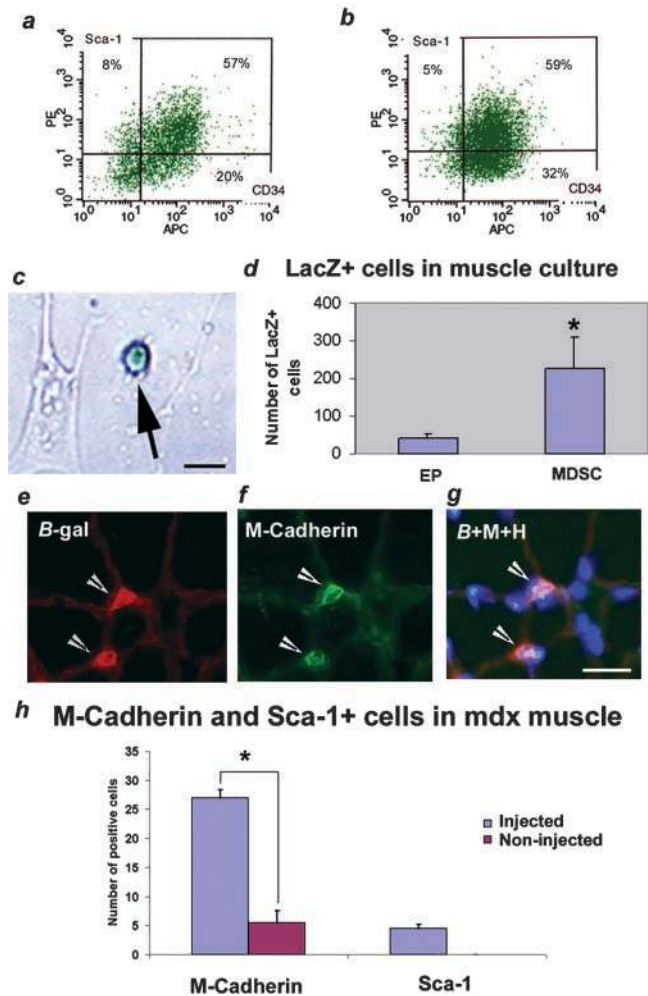


Figure 3. Self-renewal ability of MDSC and EP cells. (a and b) Flow cytometric analysis of CD34 and Sca-1 expression of MDSC (at passage 30) (a) and subcloned MDSC (b). Of the MDSC at passage 30, 77% were CD34⁺, 57% were CD34⁺/Sca-1⁺, and 8% were CD34⁻/Sca-1⁺, which is very similar to observations of the MDSC at passage 10 (Fig. 2 d). A subclone culture displayed a similar pattern of phenotypes: 91% of the cells were CD34⁺; 59% were CD34⁺/Sca-1⁺; and 5% were CD34⁻/Sca-1⁺ (b). (c and d) EP cells and MDSC were transduced with a retrovirus encoding for the *lacZ* reporter gene and injected into mdx hindlimb muscle. *LacZ*⁺ muscle-derived cells (c, arrow) were isolated from the injected dystrophic muscles 30 d after transplantation. Up to five times more *lacZ*⁺ cells were observed in the cultures prepared from the MDSC-injected muscle than in those prepared from the EP cell-injected muscle (d; * $P < 0.05$, $n = 3$ cultures). (e–g) To reveal the donor-derived satellite cells, some MDSC-injected muscles were sectioned, and β -galactosidase (red) was colocalized with m-cadherin (green) and Hoechst (blue) by immunohistochemistry. β -Galactosidase⁺ cells (e, arrowheads) expressing m-cadherin (f, arrowheads), which colocalized with nuclei (g, arrowheads; triple exposure), were detected in transplanted muscle. (h) We further performed immunohistochemistry to evaluate the number of m-cadherin⁺ and Sca-1⁺ cells in MDSC-injected muscle at 90 d after injection. The number of m-cadherin⁺ and Sca-1⁺ cells was higher (* $P < 0.01$, $n = 3$ muscles/experiment) in the injected site (dystrophin⁺ myofibers) than in the noninjected site (dystrophin⁻ myofibers). (Panel g) B, β -galactosidase staining; M, m-cadherin staining; H, Hoechst staining. Bars: (c) 25 μ m; (e–g) 50 μ m.

rus carrying the *lacZ* reporter gene and injecting them into the m. gastrocnemius of mdx mice. *LacZ*⁺ muscle-derived cells were isolated from the EP cell- (unpublished data) and

MDSC-injected muscles (Fig. 3 c) of dystrophic mice at 1 mo after transplantation. The number of *lacZ*⁺ cells was counted in each culture in 20 fields at 200 \times magnification (three animals per group). The number of *lacZ*⁺ cells derived from the MDSC-injected muscles was significantly higher than the number of *lacZ*⁺ cells derived from the EP cell-injected muscles (226.3 ± 81.0 vs. 43.0 ± 8.7 , $n = 3$, $P < 0.05$) (Fig. 3 d). This suggests that transplantation of both EP cells and MDSC resulted in the formation of donor-derived muscle progenitors, but that the MDSC were more efficient than the EP cells at generating muscle progenitors in the transplanted mdx muscles. To further confirm the ability of MDSC to replenish muscle progenitors, some of the MDSC-injected muscles were sectioned and stained with β -galactosidase and m-cadherin by immunocytochemistry (Fig. 3, e–g). The β -galactosidase⁺ cells (Fig. 3 e, red) expressing m-cadherin (Fig. 3 f, green) were indeed colocalized within the injected muscles at 3 mo after transplantation (Fig. 3 g), demonstrating that the MDSC contributed to the replenishment of the satellite cell compartment in the injected skeletal muscle.

Finally, the number of m-cadherin⁺ and Sca-1⁺ cells in the injected areas was monitored at 90 d after injection and compared with the noninjected areas. Sca-1⁺ cells lying beneath the basal lamina were determined by colocalizing Sca-1, laminin, and nuclei. The injected sites were revealed by dystrophin⁺ myofiber; the numbers of Sca-1⁺ and m-cadherin⁺ cells in five fields of the injected and noninjected areas were monitored at 200 \times magnification. Interestingly, the injected areas contained five times more m-cadherin⁺ cells than the noninjected areas (I, 26.7 ± 1.2 , vs. NI, 5.3 ± 1.5 , $P < 0.05$, $n = 3$ per group) (Fig. 3 h). Similarly, Sca-1⁺ cells were found in the injected areas, whereas no Sca-1⁺ cells could be detected in the noninjected areas analyzed (5.0 ± 1.0 vs. 0, $n = 3$). These results provide further evidence that the injected MDSC contribute to the replenishment of myogenic progenitors (i.e., both m-cadherin- and Sca-1-expressing cells) in the transplanted muscles.

Immune-privileged behavior of MDSC in transplanted muscle

To investigate whether the improved transplantation capacity of the MDSC was related to a potential immune-privileged behavior of the injected cells, immunohistochemical staining to reveal CD4 and CD8 T lymphocytes was performed on muscles injected with EP cells and MDSC. By 10 d after injection of the EP cells, both CD4 (Fig. 4 b, arrowhead) and CD8 (Fig. 4 c, arrowheads) lymphocytes had infiltrated the injected site, which was revealed by the detection of dystrophin⁺ myofibers and green fluorescent microspheres (Fig. 4 a) in the transplanted muscles ($n = 4$). We incubated the fluorescent microspheres with the cells before injection in order to follow their fate after transplantation. At 30 d after injection, we still observed some CD4⁺ cells (Fig. 4 d, arrowhead) in the muscles ($n = 4$) into which EP cells were transplanted; consequently, we observed a dramatic decrease in the number of dystrophin⁺ myofibers (Fig. 4 e). In contrast, at 30 and 90 d after injection, we detected CD4⁺ and CD8⁺ cells in only half of the MDSC-injected muscles (3:6). More importantly, we observed a complete absence of CD4⁺ and CD8⁺ cells in the injected area of

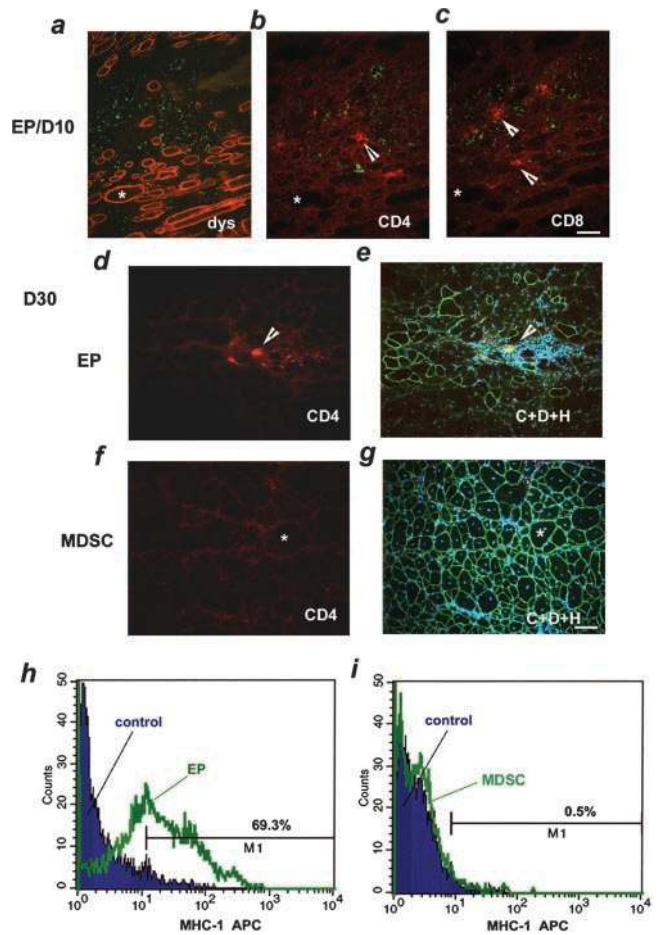


Figure 4. Detection of CD4 and CD8 lymphocytes in transplanted muscles and characterization of MHC-1 expression on MDSC and EP cells. (a–c) Immunostaining for dystrophin (dys; a), CD4 (b), and CD8 (c) cells was performed in muscle serial sections prepared from EP cell-injected muscles. By 10 d after injection (EP/D10), we detected both CD4 (b, arrowhead) and CD8 (c, arrowheads) lymphocytes in the injected area, as revealed by the green beads and the presence of numerous dystrophin⁺ myofibers (a–c). Stars in a–c indicate the same muscle fiber in serial muscle sections. (d–g) We also performed immunostaining to colocalize CD4 (red) and dystrophin (green) in MDSC- and EP cell-injected muscles, which were counterstained with Hoechst (blue) at 30 d after injection (D30). We observed some CD4⁺ cells in the EP cell-transplanted areas (d, arrowhead) in mdx muscles. In these areas, we also observed a dramatic decrease in the number of dystrophin⁺ myofibers (e). In contrast, we detected an absence of CD4⁺ cells in the MDSC-injected muscle (f) despite the presence of a large number of dystrophin⁺ myofibers at 30 d after transplantation (g). In e and g, the letters C, D, and H represent the colocalization of CD4, dystrophin, and Hoechst. The stars in f and g show the same myofibers. (h and i) We analyzed the percentage of MHC-1-expressing cells on the MDSC and EP cell populations by flow cytometry. The MDSC were almost completely negative (0.5%) for the MHC-1 (i), whereas 69.3% of the EP cells were positive for MHC-1 (h). Bars, 100 μ m.

some MDSC-injected muscles, despite the presence of a large number of dystrophin⁺ myofibers at 1 mo after injection (Fig. 4, f and g).

We quantitated the number of infiltrating lymphocytes in the EP cell- and MDSC-injected muscle and compared the results among the different groups. The number of CD4

and CD8 lymphocytes was monitored in four muscle sections of each injected animal at 30 d after injection. We observed that the number of CD4 and CD8 lymphocytes was significantly lower in the MDSC-injected muscle than in the EP cell-transplanted muscles (CD4, 10.0 ± 3.7 in MDSC, $n = 4$, vs. 48 ± 13.3 in EP, $n = 3$; CD8, 5 ± 3.5 in MDSC, $n = 4$, vs. 48.7 ± 13.3 in EP, $n = 3$). There was a significantly lower number of CD4 and CD8 lymphocytes within the injected MDSC than within the EP cells.

In an attempt to elucidate the mechanism by which the MDSC display this immune-privileged behavior, the MDSC and EP cells were tested for the expression of the major histocompatibility complex class 1 (MHC-1), the antigens that are mainly responsible for rejection (Hall et al., 1984; Milton and Fabre, 1985; Mason and Morris, 1986). It has been observed that the MHC-1 is strongly expressed in myoblasts (Roy et al., 1991) and that the MHC-1 expression on target cells is a prerequisite for antigen-specific T cell-mediated cytotoxicity (Emslie-Smith et al., 1989). Therefore, the expression of MHC-1 on the MDSC and EP cells was determined by flow cytometry. Surprisingly, the MDSC were almost completely negative (0.5%) for the MHC-1 (Fig. 4 i), whereas 69.3% of the EP cells were found to be positive for the MHC-1 (Fig. 4 h). These results suggest that the improved transplantation capacity of the MDSC may be attributed to their inability to trigger infiltration of activated lymphocytes because of their low expression of MHC-1, which would eventually play a role in the immune rejection of the transplanted cells.

The influence of growth factors on the MDSC

To investigate whether the MDSC could differentiate into other lineages, the MDSC and two MDSC subclones (msc1 and msc3) were stimulated with nerve growth factor (NGF) for 5 d, and their expression of 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNPase) was analyzed by immunocytochemistry. CNPase is a myelin-associated enzyme that is a constituent of both the oligodendrocyte progenitor cells in the central nervous system and Schwann cells in the peripheral nervous system (Sprinkle et al., 1985). The number of CNPase⁺ cells was counted at 200 \times magnification in five fields, and a total of 452–1020 cells were counted for each condition. An absence of CNPase-expressing cells was observed in the MDSC culture before stimulation (Fig. 5 a). However, 1.1% of the cells became CNPase⁺ when incubated in NGF-supplemented medium (10 ng/ml) for 5 d (Fig. 5, b and c). Interestingly, CNPase⁺ cells were detected in msc1 and msc3 cultures without NGF stimulation. However, after stimulation with NGF, the number of CNPase⁺ cells increased by 0.7% in the msc3 culture and by 2.1% in the msc1 culture (Fig. 5 c).

A similar method was used to analyze the expression of von Willebrand factor (vWF), an endothelial cell marker (Mukai et al., 1980), in the MDSC after stimulation with vascular endothelial growth factor (VEGF). vWF⁺ cells were detected in the MDSC culture without VEGF stimulation, but their number increased in the presence of this growth factor (by 16% with 15 ng/ml and by 21% with 25 ng/ml) (Fig. 5 d). At the same time, the number of desmin⁺ cells decreased in the MDSC culture when stimulated with

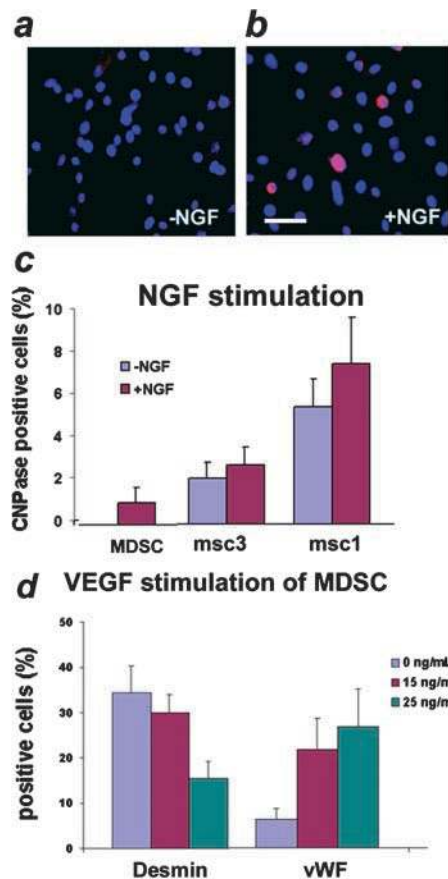


Figure 5. **Multipotent differentiation of MDSC in vitro.** (a–c) Immunostaining was performed on MDSC and the subclones of MDSC with or without stimulation by NGF. MDSC without stimulation did not express CNPase (a), whereas some cells became CNPase⁺ in the presence of NGF-supplemented (10 ng/ml) medium for 5 d (b). Hoechst staining showed the total number of cells in culture (a and b). Interestingly, we detected CNPase⁺ cells in two of the subclone cultures, msc1 and msc3, before NGF stimulation (c). However, the percentage of CNPase⁺ cells was increased in all cell populations when incubated with NGF-supplemented medium for 5 d (c). (d) We also detected vWF⁺ MDSC without VEGF stimulation, and the percentage of vWF⁺ cells increased in the presence of VEGF-supplemented medium (15 and 25 ng/ml). We also observed that the number of desmin⁺ cells decreased after stimulation with VEGF. Bar, 50 μ m.

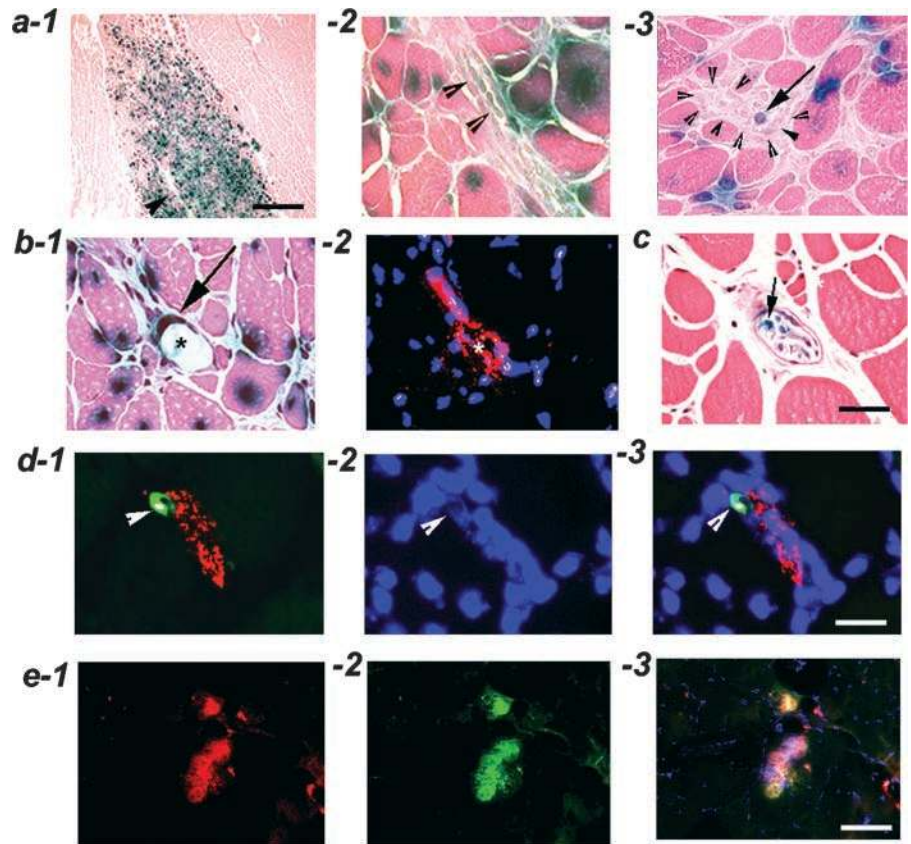
VEGF (Fig. 5 d). This is in accordance with our recent observation that a subclone of MDSC (mc13), when cultivated in medium supplemented with rhBMP-2 (an osteogenic factor) for 6 d (Lee et al., 2000), displayed a significant decrease in the number of desmin⁺ cells while acquiring the ability to express alkaline phosphatase (a preosteoblast marker).

Multipotent differentiation of MDSC in vivo

To further validate these in vitro findings, cloned MDSC devoid of NGF or VEGF stimulation were genetically engineered to express the *lacZ* reporter gene (nuclear location), and were injected into the m. gastrocnemius of mdx mice. By 10 d after injection, a large number of myofibers containing *lacZ*⁺ nuclei was observed in the injected dystrophic muscle (Fig. 6 a-1). Additionally, some of the *lacZ*⁺ nuclei were found in vascular-like (Fig. 6 a-2, ar-

Figure 6. Multipotent differentiation of MDSC in the injected skeletal muscle in vivo.

10 (a) and 25 d (b–e) after transplantation. (a-1–a-3) MDSC that were genetically engineered to express the *lacZ* reporter gene (nuclear localization) were injected into the m. gastrocnemius of mdx mice. At 10 d after injection, we detected many *lacZ*⁺ cells in the transplanted muscle (a-1). In the injected area, a vessel-like structure containing *lacZ*⁺ nuclei was also found (a-2, arrowheads). Some peripheral nerve-like structures (a-3, arrowheads) with *lacZ*⁺ nuclei (a-3, arrow) were also found in the injected site. (b) At 25 d after transplantation, we observed some *lacZ*⁺ nuclei (b-1, arrow) in the endothelium of well-differentiated blood vessels (b-1, star), which was confirmed by vWF staining in adjacent sections (b-2, star). (c) *LacZ*⁺ nuclei (arrow) were also found in well-differentiated peripheral nerves in the injected skeletal muscle at 25 d after transplantation. (d) Colocalization of β -galactosidase, vWF, and Hoechst by immunocytochemistry revealed β -galactosidase⁺ nuclei (d-1, green, arrowhead) in vWF⁺ structure (d-1, red) and costained with Hoechst (d-2 and d-3, arrowhead). (e) After transplantation of MDSC isolated from GFP transgenic mice, some peripheral nerve structures expressing CNPase immunoreactivity, a Schwann cell marker (e-1, red), were colocalized with GFP-expressing cells (e-2) and Hoechst (e-3) in the transplanted mdx TA muscle. Triple exposure of CNPase, GFP, and Hoechst (blue) is shown in e-3. Bars: (a-1) 500 μ m; (b, c, and e) 50 μ m; (d) 25 μ m.



rowheads) and peripheral nerve-like structures (Fig. 6 a-3, arrow). Arrowheads show the border of the nerve-like structure (Fig. 6 a-3). By 25 d after transplantation, *lacZ*⁺ nuclei were found in the endothelium of well-differentiated blood vessels (Fig. 6 b-1, arrow), as defined by both morphology and vWF immunostaining (Fig. 6 b-2). *LacZ*⁺ nuclei were also found in the Schwann cells of well-differentiated peripheral nerve (Fig. 6 c, arrow) in transplanted muscle at 25 d after implantation. To confirm the nature of these various differentiated cell types, colocalization of β -galactosidase/vWF/Hoechst by immunocytochemistry was performed on muscle section prepared from MDSC-injected muscles at 25 d after transplantation (Fig. 6, d-1–d-3). β -Galactosidase⁺ nuclei (green fluorescence) were observed in vWF⁺ (red fluorescence) structure (Fig. 6 d-1), and costained with Hoechst (Fig. 6, d-2 and d-3). Moreover, after transplantation of green fluorescent protein (GFP)-expressing MDSC (Fig. 6, e-1–e-3), some peripheral nerve structures displaying CNPase immunoreactivity (Fig. 6 e-1) were colocalized with GFP-expressing cells (Fig. 6 e-2) and Hoechst (Fig. 6 e-3) at 25 d after transplantation. These results demonstrate that MDSC differentiate into both endothelial and peripheral nerve cells after implantation in mdx skeletal muscle. The ability of the MDSC to differentiate into blood vessels and neural lineages may contribute to the regeneration of functional muscle tissue with adequate neurogenic and vascular supplies.

Discussion

Three populations of muscle-derived cells, EP cells, LP cells, and MDSC, were isolated in the present study according to their adhesion characteristics and proliferation behaviors. EP and LP cells represent two populations of satellite cells based on their patterns of myogenic marker expression and their behavior in vitro. However, the MDSC displayed unique characteristics that are usually associated with noncommitted progenitor cells. First, the detection of similar phenotypes in MDSC, as well as in cloned and subcloned MDSC, and the maintenance of the MDSC phenotype for a long period of time in vitro (30 passages) indicate that the MDSC are capable of self-renewal in vitro. Importantly, these highly proliferating cells can be expanded in vitro for over 30 passages while preserving a normal karyotype and are incapable of developing tumors in immunodeficient scid mice. Second, the ability of the MDSC to replenish myogenic progenitors (m-cadherin- and Sca-1-expressing donor-derived cells) within the injected dystrophic muscle suggests that the MDSC also are capable of self-renewal in vivo. Third, the ability of the MDSC to highly proliferate in vitro and to contribute to a persistent restoration of dystrophin⁺ myofibers within the transplanted muscle, in which a large number of small regenerating myofibers can be observed for up to 90 d after injection, demonstrates that the MDSC possess a high capacity for long-term proliferation in vitro and in vivo. Fourth, the detection of donor-derived cells in myofi-

bers, peripheral nerves, and blood vessels within the MDSC-injected muscle provides evidence of the multipotent nature of MDSC *in vivo*, as observed *in vitro*, when appropriately stimulated with growth factors. Based on this evidence, the MDSC were confirmed as a novel population of muscle stem cells with the capacities for high self-renewal, long-term proliferation, and multipotent differentiation.

Based on the relative number of cells isolated in the different fractions of muscle-derived cells (EP, LP, and MDSC), EP cells represent the main population of myogenic cells derived from skeletal muscle. More than 90% of the EP cells express the myogenic marker desmin and markers of the later stages of myogenesis, such as m-cadherin and myogenin (Miller et al., 1999), suggesting that EP cells represent a late myogenic precursor. The EP cells exhibited a very fast but limited ability to proliferate, as well as a strong capacity to differentiate into myotubes. These EP cell characteristics are similar to previous findings in satellite cells (Bischoff, 1986; Baroffio et al., 1996; Schultz, 1996). A main population of m-cadherin⁺ satellite cells was also detected in normal muscle sections beneath the basement membrane, indicating that the EP cells were derived from the common satellite cell population. Surprisingly, although the transplanted EP cells were highly purified and 95% of them expressed desmin, they still displayed a limited capacity to regenerate skeletal muscle after cell implantation. We believe that the poor ability of the EP cells to proliferate and to self-renew in the regenerating mdx muscles explained, at least in part, their limited regeneration capabilities *in vivo*.

The LP population represents ~1% of satellite cells, and their function in skeletal muscle remains unclear because they displayed a very limited capacity to proliferate and differentiate. In a preliminary experiment, transplantation of quiescent LP cells that were cultured for only 1–2 d did not show the same efficiency of muscle regeneration at 30 d after transplantation, when compared with the MDSC. This is intriguing because the primitive MDSC were isolated from the LP cultures (see Materials and methods). It is possible that the primitive MDSC within LP cultures need specific unknown conditions to be activated before they become effective in contributing to muscle regeneration. Therefore, activation of primitive MDSC by appropriate growth factors in LP cultures may play a major role in the activation process and should be investigated in future experiments.

Difficulty was encountered when evaluating the relative number of cells in the MDSC populations within the muscle cell culture. Culturing LP cells at high confluence (60–70% confluence) often resulted in large numbers of differentiated cells with typical fibroblast morphology. The fibroblast-like cells may come from either the LP culture or from the differentiation of MDSC daughter cells, which often grow faster than their parent cells. When the LP cells were cultured at low confluence (<30%), most of the LP cells died within 1–2 wk, and only rare primary MDSC clones appeared. According to our findings in muscle cultures prepared from newborn mice, the ratio of the three populations of myogenic cells is as follows: 10⁵ relatively quiescent EP cells contained 10² relatively quiescent LP cells, in which one MDSC clone could be obtained.

Additionally, it is difficult to investigate the source of MDSC, considering their relatively low number in skeletal muscle. Given recent studies in which pluripotent bone marrow stem cells (Ferrari et al., 1998; Gussoni et al., 1999), blood vessel progenitors (De Angelis et al., 1999), neural stem cells (Clarke et al., 2000; Galli et al., 2000), and mesenchymal stem cells isolated from interstitial connective tissue (Young et al., 2001a,b) have all shown the capacity to differentiate into muscle lineage, these MDSC can be derived from various sources. We found that MDSC express the hematopoietic stem cell markers CD34 and Sca-1, as well as neural and endothelial markers (when appropriately stimulated). Various features suggest, however, that the MDSC derived from skeletal myofibers. These features include (a) the potential of MDSC to differentiate into skeletal muscle lineage both *in vitro* and *in vivo*, a potential which is even higher than that of satellite cells; (b) their ability to spontaneously express myogenic markers, such as desmin and MyoD, without the requirement of myogenic stimulation; and (c) their similarity in phenotype to a subpopulation of cells that has been identified within the basal lamina of myofibers, i.e., Sca-1⁺/m-cadherin⁻ (Fig. 1, g–l), as well as CD34⁺ and Bcl2⁺ (Lee et al., 2000).

Although >95% of cells in expanded MDSC cultures expressed desmin, an early stage myogenic marker, only 30–40% of MDSC at earlier passages (10–12 passages) were found to be desmin⁺. It is reasonable to speculate that the primitive MDSC do not express desmin. Therefore, the primary MDSC may be characterized phenotypically by myogenic lineage⁻, CD34^{+/-}, and Sca-1⁺. In the transplantation experiment, long-term engraftments were observed in the muscle injected with the MDSC (myogenic lineage^{-/+}, CD34^{+/-}, Sca-1⁺), but not in the muscle injected with the EP cells (myogenic lineage⁺, CD34⁺, Sca-1^{+/-}). In contrast to the EP culture, a small population (10%) of MDSC is Sca-1⁺/CD34⁻. It is very interesting that murine hematopoietic stem cells with long-term reconstituting capability are found to be in the hematopoietic lineage⁻, CD34^{low/-}, Sca-1⁺ populations (Osawa et al., 1996; Sato et al., 1999). It is possible that different organ-specific stem cells may share some similarities in terms of surface antigen expression. The identification of surface antigens specific to muscle stem cells will help to enhance the rapid isolation of this minority of long-time proliferating stem cells from muscle, as this would result in a more efficient isolation technique. Indeed, we recently demonstrated that the use of magnetic cell sorting to rapidly isolate and transplant phenotypically pure Sca-1⁺ myogenic cells (CD45⁻) resulted in the regeneration of skeletal muscle and restoration of dystrophin expression in host mdx mice (Jankowski et al., 2001).

An important point of the current investigation is the demonstration of the functional ability of this specific population of MDSC to regenerate dystrophic muscle (mdx) after transplantation. Transplantation of normal muscle precursors for treatment of inherited myopathies, such as Duchenne muscular dystrophy, has been widely studied (Partridge et al., 1989; Partridge, 1991; Morgan et al., 1990, 1993, 1994; Miller et al., 1997; Qu and Huard, 2000). The results from animal and human clinical trials have suggested that although myoblast transplantation is feasible (Partridge, 1991; Morgan et al., 1993;

Miller et al., 1997), it is rather inefficient (Huard et al., 1992a,b; Karpati et al., 1992; Tremblay et al., 1993; Mendell et al., 1995; Miller et al., 1997). The low survival rate, poor spreading of transplanted cells, and immunorejection of the donor cells are still major problems facing myoblast transplantation (Huard et al., 1994a,b; Fan et al., 1996; Guerette et al., 1997; Beauchamp et al., 1999; Hodgetts et al., 2000; Skuk et al., 2000; Smythe et al., 2000). In animal experiments, immunodeficient animals and/or immune-suppressive regimens (Huard et al., 1992a, 1994a; Morgan et al., 1993, 1994; Kinoshita et al., 1994; Pavlath et al., 1994), preirradiation of the injected muscle (Morgan et al., 1993), and myonecrotic agents (Huard et al., 1994a; Vilquin et al., 1995) have been used extensively to improve the success of this technique. Although these approaches may be used to improve the restoration of dystrophin in mdx mice, the success of this technique remains rather limited and largely clinically impractical.

In the present study, the cell transplantation was successful from 10 to 90 d after injection of the MDSC into mdx muscle. The number of dystrophin⁺ myofibers observed at 10 d after injection (2,178 myofibers) was not significantly different from the number of dystrophin⁺ myofibers observed at 90 d (1,851 myofibers) after transplantation. Interestingly, the mdx mice used as recipients in this experiment were not immunosuppressed, and the injected muscles were not preirradiated or injured with a myonecrotic agent. The injection of MDSC resulted in 10 times more dystrophin⁺ myofibers in the injected muscle than did the transplantation of EP cells (1,435 vs. 136 fibers) by 30 d after transplantation, although the same number of cells ($3\text{--}4 \times 10^5$) was injected in both groups. These results illustrate that the MDSC display an improved transplantation capacity in skeletal muscle when compared with the EP cells. Therefore, we have attempted to elucidate the mechanism by which the MDSC display improved transplantation capacity by characterizing their self-renewal ability, along with their immune-privileged behavior and their multipotent differentiation, after implantation in skeletal muscle.

Unlike the MDSC, the transplanted EP cells exhibited a dramatic decrease in the number of dystrophin⁺ myofibers from day 10 to day 30. In view of the previous report that dystrophin is antigenic in dystrophin-deficient mice (Ohtsuka et al., 1998) and also the reports in which serum antibody formation has been found to be triggered by donor myoblasts (Huard et al., 1992a; Tremblay et al., 1993; Vilquin et al., 1995), we have investigated the immune rejection against the injected cells. Detection of CD4 and CD8 T lymphocytes in all EP-injected muscles at 10 and 30 d after injection provides evidence to support the immune rejection of the EP cells. Surprisingly, an absence of infiltration with CD4 or CD8 T-lymphocytes was observed in many of the MDSC-injected muscles at 30 and 90 d after transplantation, despite the large numbers of dystrophin⁺ myofibers found in the injected muscles. These results suggest that MDSC are less immunogenic than EP cells. The lack of expression of MHC-1 by the MDSC further supports this contention. These latter results suggest that the improved transplantation capacity of MDSC may be attributed to their inability to trigger an infiltration of activated lymphocytes, which would participate in the immune rejection of the transplanted cells.

We also have observed that the MDSC can self-renew both in vitro and in the injected skeletal muscle in vivo. Indeed, MDSC can be expanded in vitro for up to 30 passages while maintaining their phenotype in terms of both myogenic and stem cell marker expression profiles. In addition, subclones of MDSC display a phenotype almost identical to the MDSC clone from which they were derived; this fact provides evidence of the in vitro self-renewing capability of these cells. Finally, five times more *lacZ*⁺ cells were detected in the muscle cultures derived from MDSC-injected muscle, when compared with the EP cell-injected muscle. Moreover, the detection of Sca-1⁺ and m-cadherin⁺ cells in MDSC-injected muscle suggests that MDSC give rise to myogenic precursor cells more effectively than do EP cells. The high self-renewal ability displayed by MDSC both in vitro and in vivo may also contribute to the persistent dystrophin delivery into mdx mice.

We also investigated whether the injected cells can differentiate into blood vessels and nerve cells with the potential to contribute to the regeneration of functional muscle tissue with adequate vascular and nerve supplies. Indeed, our in vitro results suggest that the stimulation of the MDSC with NGF and VEGF increased the number of CNPase- and vWF-expressing cells, respectively. More importantly, in the in vivo experiments, although the MDSC were not stimulated or transduced with NGF and VEGF, the injected cells could still differentiate into endothelial and peripheral nerve lineages. Based on these results, we believe that the release of environmental stimuli (e.g., growth factors) within the dystrophic muscle triggers the differentiation of the MDSC toward other lineages. Such results suggest that multipotent differentiation of MDSC may contribute to the regeneration of functional muscle tissue in transplanted mdx mice. The ability of the MDSC to restore the function of the dystrophic mdx muscle is important and will be investigated further. It was also found that primary MDSC (passage 10) showed much higher potential than later passages (after 30 passages) to differentiate into muscle and other lineages. In extended cultures (after 40 passages), some of the MDSC gradually lost their ability to regenerate muscle fibers or differentiate into other lineages, suggesting that the mechanism that governs the differentiation of multipotent muscle stem cells toward a specific lineage is yet to be determined. Further investigation of the gene expression program, as well as the proper culture condition by which the proliferation and differentiation of multipotent stem cells could be controlled, is paramount for the development of muscle stem cell transplantation for Duchenne muscular dystrophy patients.

In summary, three populations of myogenic cells have been isolated by a modified version of the preplate technique. The EP and LP cells are derived from the main population of satellite cells and are a type of committed muscle precursor. The MDSC, derived from the subpopulation of LP cells, are characterized by myogenic lineage^{-/+}, CD34^{+/-}, Sca-1⁺, c-Kit⁻, and CD45⁻. The use of MDSC can circumvent hurdles facing myoblast transfer therapy and, consequently, improve the efficiency of muscle regeneration and dystrophin delivery to dystrophic muscle. The unique features of the MDSC, including (a) their long-time proliferating ability, (b) their strong self-renewal, (c) their multipotent differentiation, and (d) their immune-privileged be-

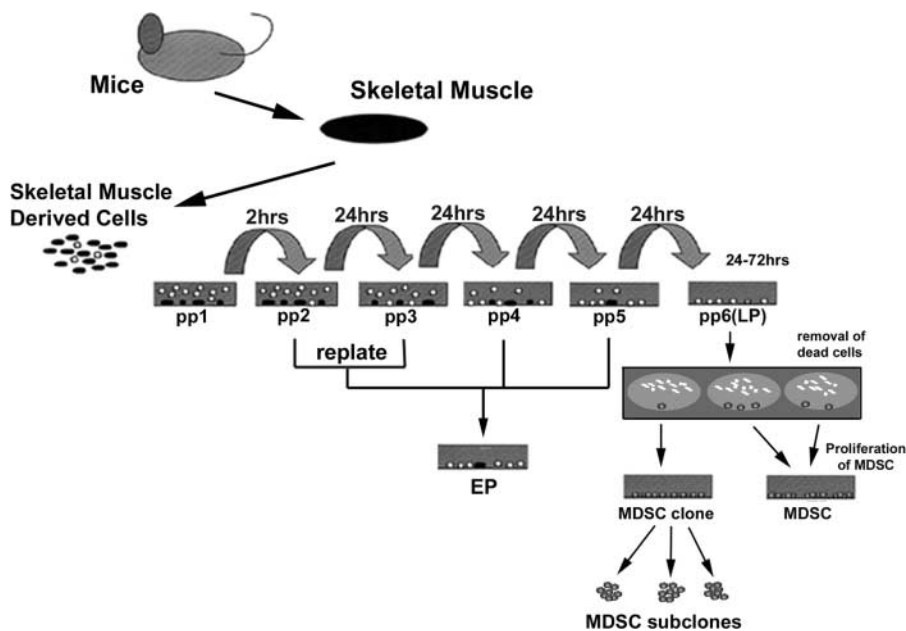


Figure 7. Isolation of three populations of muscle-derived cells. Muscle-derived cells were enzymatically dissociated from neonatal mouse skeletal muscle and separated by their adhesion characteristics to collagen-coated flasks (modified preplate technique). After the enzymatic dissociation, the muscle cell extract was resuspended in PM and preplated on collagen-coated flasks. Different populations of muscle-derived cells were isolated based on adhesion characteristics. pp1 represented a population of primary fibroblasts that adhered in the first 2 h after isolation; subsequent preplates, containing a mixture of myogenic and nonmyogenic cells, were obtained at 24-h intervals (pp2–6). The nonmyogenic cells in pp2 and pp3 were removed from the cultures by replating the cells, and the resulting enriched pp2 and pp3 desmin⁺ cells were combined with pp4 and pp5 cells and were termed the EP population.

Cells in the pp6 cell population took an additional 24–72 h to attach to collagen-coated dishes after transfer from pp5 and were termed LP cells. Most of the LP cells died during the first 1–2 wk of the cultivation period, with very few of the adherent surviving cells proliferating and forming clonal colonies. The surviving clones are called MDSC. We also isolated subclones from a single clone of MDSC, as shown in the flow chart.

havior after implantation, at least partially elucidate the basis of the improved transplantation capacity of this novel population of MDSC.

Materials and methods

Animals

Normal mice (C57 BL/6J) and mdx mice (C57BL/10ScSn DMD^{mdx/J}) used in this experiment were purchased from Jackson ImmunoResearch Laboratories. All animal protocols used for these experiments were approved by the Children's Hospital of Pittsburgh's IACUC committee (protocol nos. 2/00 and 7/00).

Preparation of different populations of muscle-derived cell cultures

Primary muscle cultures were prepared from newborn (3–5 d) normal mice, and the MDSC were purified from the primary culture using a modified version of a previously described preplate technique (Richler and Yaffe, 1970; Rando and Blau, 1994; Qu et al., 1998; Qu and Huard, 2000). The hindlimb muscles of neonatal mice were removed, and the bones were dissected. The muscle was then minced into a coarse slurry using scalpels. The muscle tissue was enzymatically dissociated at 37°C in 0.2% collagenase-type XI (Sigma-Aldrich) for 1 h, and then centrifuged at 3,500 rpm for 5 min. The cells were collected, incubated in dispase (GIBCO BRL), prepared as 2.4 units/1 ml HBSS (GIBCO BRL), for 45 min, and then incubated for 30 min in 0.1% trypsin-EDTA (GIBCO BRL) diluted in HBSS. After the enzymatic dissociation, the muscle cells were centrifuged and resuspended in proliferation medium (PM). PM consists of DME containing 10% horse serum, 10% FBS, 0.5% chick embryo extract, and 1% penicillin–streptomycin (all reagents purchased from GIBCO BRL). Different populations of muscle-derived cells were isolated based on their adhesion characteristics. A flow chart for the isolation of muscle-derived cells (EP, LP, and MDSC) is shown in Fig. 7. The muscle cells were plated on collagen-coated flasks (collagen type 1; Sigma-Aldrich) for 2 h (preplate 1 [pp1]). The nonadherent cells were then transferred to other flasks (pp2), and the adherent cells in pp1 were discarded. It has been reported that the cells that rapidly attach are highly fibroblastic in nature (Richler and Yaffe, 1970; Rando and Blau, 1994; Qu et al., 1998; Qu and Huard, 2000). After 24 h, the floating cells in pp2 were collected, centrifuged, and plated on new flasks (pp3). These procedures were repeated at 24-h intervals until serial preplates (pp4–6) were obtained. All cell populations (pp2–6) were maintained in PM with daily changes. Based on the previous report (Qu et al., 1998), 30–40% of the

cells in pp2 and pp3 are known to be nonmyogenic, whereas up to 95% desmin⁺ cells can be found in pp4 and pp5. To further purify the desmin⁺ cells within pp2 and pp3, the cells were subsequently trypsinized and replated in fresh collagen-coated flasks using a previously described protocol (Qu and Huard, 2000). After 30 min, the supernatant containing mostly nonadherent cells (>90% of which were shown to be desmin-expressing cells) were plated in new flasks. These nonadherent cells from pp2 and pp3 were combined with the cells from pp4 and pp5 and used in the transplantation experiment as the EP population. To isolate MDSC, pp6 cells (also termed LP cells) were plated in collagen-coated 12-well dishes at 50–100 cells/well (Fig. 7). Though most of the LP cells died within 1–2 wk of culturing, the MDSC formed clonal colonies during the first 2 wk of culturing. The MDSC take 5–7 d to attach to collagen-coated flasks, and require ~1–2 wk to develop clones, from 1 to ~300–500 cells. The doubling time of these cells is ~24–36 h at the early stage of culturing, which means that these cells divided eight to nine times in the first 2 wk of culturing. The wells containing multiple MDSC colonies were trypsinized (0.25% trypsin) and the detached cells were transferred into a collagen-coated 6-well dish. The MDSC were then grown in PM to ~30% confluence and subsequently passaged. The well containing a single colony was selected and these cells were called MDSC clones. These MDSC clones were expanded and used to isolate subclones via a protocol previously described (Lee et al., 2000). The MDSC cloned and subcloned cells were also expanded in PM as described above. We have also isolated MDSC, by using the same technique as described above, from the skeletal muscle of GFP transgenic mice (C57BL/6-TgN[ACTbEGFP]) (Okabe et al., 1997).

Cell cycle duration

The EP cells and MDSC were plated in 24-well collagen-coated plates at a density of 450 cells/well in PM. Using the CytoWorks platform, visible imaging was obtained for individual cells. This system uses a biobox incubator mounted to the stage of a Nikon microscope, which is linked to a CCD camera (Automated Cell Technologies, Inc.). Stage movement was computer controlled, allowing for images of each view field to be acquired at 10-min intervals for 5 d. For each population, 100 cells were selected and tracked. The division time of each cell was determined by direct observation of the cells through the CytoWorks platform as previously described (Deasy et al., 2002). The initiation of cell division was marked at the time when two daughter cells were formed, and these cells were subsequently followed until their respective division. The lapsed time between those two division events was recorded as the length of the cell division cycle.

Immunocytochemistry on cultures

The primary antibodies used in this study were mouse anti-desmin (1:200; D-1033; Sigma-Aldrich), rabbit anti-mouse m-cadherin (1:50; obtained from A. Wernig), rabbit anti-mouse Bcl2 (1:1,000; 15616E; BD PharMingen), biotin anti-Sca-1 (1:200; BD PharMingen), biotin anti-mouse CD34 (1:200; 09432D; BD PharMingen), mouse anti-myogenin (1:100; 65121A; BD PharMingen), and mouse anti-MyoD (1:200; 13941A; BD PharMingen). EP cell, LP cell, and MDSC (cloned and subcloned) cultures were fixed and stained as previously described (Lee et al., 2000).

RNA analyses

RT-PCR analyses of EP and MDSC cultures were performed as previously described (Lee et al., 2000). Total RNA was isolated using TRIzol reagent (Life Technologies). Reverse transcription was performed using SuperScript™ First-Strand Synthesis System (Life Technologies) according to the manufacturer's instructions. PCR primer sequences and reaction parameters are from references as follows: Bcl2 (Dominov et al., 1998); CD34 (Lee et al., 2000); myogenin and MyoD (Rohwedel et al., 1995); and MNF (Yang et al., 1997). PCR product sizes were analyzed on agarose gel. The expected product sizes were Bcl-2, 480 bp; CD34, 147 bp; myogenin, 86 bp; MyoD, 147 bp; and MNF, 305 bp. Genomic DNA contamination was excluded by (a) primers spanning an intron and (b) reverse transcription reactions without reverse transcriptase.

Flow cytometry

The percentages of CD34⁺ and Sca-1⁺ cells in the MDSC and EP cultures were analyzed by flow cytometry as recently described (Jankowski et al., 2001). Cultured cells were trypsinized, spun, washed in cold PBS (Dulbecco phosphate-buffered salt solution 1×; Mediatech) containing 0.5% BSA (ICN Biomedicals), and counted. The cells were then divided into equal aliquots and spun to a pellet. A 1:10 mouse serum (Sigma-Aldrich) in PBS solution and Fc Block (rat anti-mouse CD16/CD32; BD PharMingen) was used to resuspend each pellet, and the suspensions were incubated for 10 min on ice. Optimal amounts of both direct and biotin-conjugated rat anti-mouse monoclonal antibodies (c-kit, CD34, Sca-1, and CD45; all from BD PharMingen) were predetermined and added directly to each tube for 30 min. A similar protocol was used to separately analyze MDSC and EP cells for MHC-1 expression using a biotin-conjugated H-2k^b antibody (BD PharMingen). Streptavidin-allophycocyanin conjugate was added to tubes containing cells labeled with biotinylated antibodies for 20 min. Just before analysis, 7-amino-actinomycin D (Via-Probe; BD PharMingen) was added to each tube for dead cell exclusion. Live cell events were collected and analyzed on a FACSCalibur® (Becton Dickinson) flow cytometer using Cell Quest software.

Stimulation of MDSC with growth factors

MDSC and two subclones of the MDSC clones (msc1 and msc3) were stimulated with NGF (10 ng/ml; Sigma-Aldrich) or VEGF (15 and 25 ng/ml; Sigma-Aldrich) for 5 d. Cells were plated at 20–30% confluence on collagen-coated flasks. PM containing growth factors was added to the wells, and PM without NGF and VEGF was added to the control wells. Immunocytochemistry was performed to examine the expression of CNPase and vWF. For the CNPase immunostaining, the primary antibody used was a mouse anti-CNPase (1:400; Sigma-Aldrich), and the secondary antibody was an anti-mouse conjugated to Cy3 (1:200; Sigma-Aldrich). For vWF immunostaining, the primary antibody was a rabbit anti-vWF (1:400; Dako), and the secondary antibody was an anti-rabbit-conjugated Cy3 (1:200; Sigma-Aldrich). Desmin expression was also monitored in both the treated and untreated wells using the protocol described above.

Cell transplantation

MDSC and EP cells were transplanted as previously described (Qu et al., 1998; Qu and Huard, 2000). In brief, 3–4 × 10⁵ viable cells suspended in 20 μl of HBSS were transplanted (single-point injection) in the gastrocnemius muscle of mdx mice (C57BL/10ScSn DMD^{mdx/j}). The mdx mice used as recipients in this experiment were not immunosuppressed, and the injected muscles were not preirradiated or injured with a myonecrotic agent before transplantation. EP cells were cultivated for only 5 d after the initial seeding, whereas the MDSC were at passages 10–12 at the time of transplantation. The animals were killed at 10, 30, and 90 d after injection. The injected muscles were isolated and then frozen in 2-methylbutane precooled in liquid nitrogen.

EP and MDSC cultures also were transduced with a retrovirus carrying the gene encoding for β-galactosidase (nuclear localization) for 24 h before transplantation, using a protocol previously described (Lee et al., 2000). We injected 5 × 10⁵ cells into each gastrocnemius muscle. By 30 d after injection, the animals were killed; some of the injected muscles were frozen, sectioned, and stained for *lacZ* expression (histochemistry),

whereas others were used to prepare muscle cell cultures using a protocol similar to that described above (Fig. 7). The GFP-expressing MDSC (1 × 10⁵ cells for each population) were injected into the tibialis anterior (TA) muscles of mdx mice (9 wk old). By 25 d after injection, the animals were killed; TA muscles were fixed with 1% paraformaldehyde, frozen, and sectioned for immunohistochemistry.

Immunohistochemical staining of muscle sections: normal muscle

Normal muscle samples were obtained from 4-wk-old mice and frozen in 2-methylbutane precooled with liquid nitrogen. Serial cryosections of 10 μm in thickness were prepared from the frozen muscles. The sections were fixed with cold acetone (−20°C) for 2 min, preincubated in 5% horse serum diluted with PBS for 30 min, and then rinsed thoroughly with PBS. The following is a list of stainings performed on these muscle sections. Control stainings without primary and secondary antibodies were also performed.

Colocalization of Sca-1 and laminin. The sections were incubated with a biotin anti-mouse Sca-1 antibody (1:200 dilution) for 2 h and streptavidin-conjugated Cy3 (1:200) for 90 min. The sections were then incubated with rabbit anti-mouse laminin (1:100) for 1 h and anti-rabbit IgG-conjugated fluorescein for 1 h (1:100). The nuclei were revealed by Hoechst 33342 staining.

Colocalization of Sca-1, m-cadherin, and laminin. After preincubation, the sections were incubated with PE-conjugated anti-mouse Sca-1 (1:150; BD PharMingen) for 2 h, rabbit anti-m-cadherin (1:50) for 3 h, biotinylated anti-rabbit IgG (1:100; Vector Laboratories) for 90 min, and FluoroLink™ CyTM5-labeled streptavidin (1:1,000, Amersham Pharmacia Biotech) for 90 min, respectively. The sections were then incubated with rabbit anti-mouse laminin (1:100) for 30 min and anti-rabbit IgG-conjugated fluorescein (1:200) for 30 min. The nuclei were revealed by Hoechst 33342 staining.

Immunohistochemical staining of muscle sections: transplanted mdx muscle

Dystrophin staining. The staining was performed on muscle sections as previously described (Qu and Huard, 2000), and the sections were counterstained with Hoechst to visualize the locations of the nuclei.

Colocalization of m-cadherin and dystrophin. The sections were incubated with a rabbit anti-m-cadherin antibody (1:50) for 2 h and anti-rabbit IgG-conjugated fluorescein for 90 min. The sections were then stained for dystrophin and counterstained with Hoechst using the same protocol described above.

CD4 and CD8 staining. Sections were incubated with rat anti-mouse CD4 or CD8 (1:400; BD PharMingen) for 2 h and then with biotinylated anti-rat IgG (1:200; Vector Laboratories) and streptavidin-conjugated Cy3 (1:200; Sigma-Aldrich) for 90 min. The number of CD4 and CD8 lymphocytes was also monitored and compared between the EP- and MDSC-injected muscles ($n = 4$). Some of the sections were also stained for dystrophin (see "Dystrophin staining" above).

vWF/β-galactosidase staining. Muscle sections were incubated with mouse anti-β-galactosidase (1:100; Chemicon), and the next steps were performed in accordance with the instructions of the MOM kit manufacturers (FMK-2201; Vector Laboratories). The sections were then incubated with rabbit anti-vWF (1:400; Dako) followed by a 1-h incubation with an anti-rabbit IgG-conjugated fluorescein (1:100). Finally, the sections were stained with Hoechst as described above.

CNPase staining. The TA muscle sections (injected with GFP-expressing MDSC) were incubated with mouse anti-CNPase (1:400; Sigma-Aldrich), and the next steps were performed in accordance with the MOM kit manufacturer's instructions, except for using streptavidin-conjugated Cy3 to replace fluorescein-conjugated avidin. The sections were consequently stained with Hoechst as described above.

Histochemistry

LacZ staining for culture. The muscle cells isolated from the EP- and MDSC-injected muscles were fixed in 1% glutaraldehyde and incubated overnight with X-gal substrate at 37°C.

LacZ staining for muscle sections. The sections were fixed in 1% glutaraldehyde, incubated overnight with X-gal substrate at 37°C, and then counterstained with eosin (Sanes et al., 1986).

Karyotype analysis of MDSC

Cytogenetic analysis of the MDSC was performed using a technique previously described (Barch, 1991; Lee et al., 2000). Cells were grown to ~40% confluency in a T75 collagenated flask in DME (GIBCO BRL) supplemented with 10% horse serum (GIBCO BRL), 10% FBS (GIBCO BRL), 0.5% chick embryo extract (GIBCO BRL), and 1% Pen-Strep solution (GIBCO BRL). Colcemid solution (GIBCO BRL) was added to the flask at a

final concentration of 0.05 $\mu\text{g/ml}$ and allowed to incubate for 3 h at 37°C. Cells were then harvested by adding 0.1% trypsin-EDTA to the cells until they lifted. Cells were spun down (3,200 rpm, 5 min), resuspended in 5 ml 0.75 M KCl, and incubated for 10 min at 37°C. Next, 3 ml of Carnoy's fixative (3:1 methanol to glacial acetic acid) was added to the solution, which was vigorously pipetted. The cells were then pelleted as before. Fresh fixative was added and cells were again pelleted. This rinsing and pelleting was performed three additional times. Cells were then dropped on glass slides and allowed to dry overnight at 55°C. Metaphase cells were GTG banded following a protocol outlined by Invitrogen. 28 metaphase cells were then counted for modal number using a magnification of 630 \times . Images for karyotype analysis were captured using AKS software (Image-netics, Inc.).

Statistical analysis

The data in this manuscript are presented as means \pm SD. Means were compared using an unpaired *t* test for the number of dystrophin⁺ myofibers between EP cell- and MDSC-transplanted groups. A paired *t* test was used to compare the numbers of centronucleated myofibers, m-cadherin⁺ cells, and Sca-1⁺ cells between injected and noninjected areas in transplanted mdx muscle.

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