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Article **Stem cells**

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Human circulating AC133+ stem cells restore dystrophin expression and ameliorate function in dystrophic skeletal muscle

Yvan Torrente,[1] Marzia Belicchi,[1] Maurilio Sampaolesi,[2] Federica Pisati,[1] Mirella Meregalli,[1] Giuseppe D'Antona,[3] Rossana Tonlorenzi,[2] Laura Porretti,[4] Manuela Gavina,[1] Kamel Mamchaoui,[5] Maria Antonietta Pellegrino,[3] Denis Furling,[5] Vincent Mouly,[5] Gillian S. Butler-Browne,[5] Roberto Bottinelli,[3] Giulio Cossu,[2] and Nereo Bresolin[1,6]

[1]Stem Cell Laboratory, Department of Neurological Science, Instituto di Ricovero e Cura a Carattere Scientifico Ospedale Maggiore Policlinico, Centro Dino Ferrari, University of Milan, Italy. [2]Stem Cell Research Institute, San Raffaele Hospital, Milan, Italy. [3]Department of Experimental Medicine, University of Pavia, Human Physiology Unit, Pavia, Italy. [4]Centro Trasfusionale e di Immunologia dei Trapianti, Ospedale Maggiore Policlinico, Milan, Italy. [5]Centre National de la Recherche Scientifique UMR 7000, Cytoskeleton and Development, Paris, France. [6]Instituto di Ricovero e Cura a Carattere Scientifico Eugenio Medea, Bosisio Parini, Italy.

Duchenne muscular dystrophy (DMD) is a common X-linked disease characterized by widespread muscle damage that invariably leads to paralysis and death. There is currently no therapy for this disease. Here we report that a subpopulation of circulating cells expressing AC133, a well-characterized marker of hematopoietic stem cells, also expresses early myogenic markers. Freshly isolated, circulating AC133+ cells were induced to undergo myogenesis when cocultured with myogenic cells or exposed to Wnt-producing cells in vitro and when delivered in vivo through the arterial circulation or directly into the muscles of transgenic *scid/mdx* mice (which allow survival of human cells). Injected cells also localized under the basal lamina of host muscle fibers and expressed satellite cell markers such as M-cadherin and MYF5. Furthermore, functional tests of injected muscles revealed a substantial recovery of force after treatment. As these cells can be isolated from the blood, manipulated in vitro, and delivered through the circulation, they represent a possible tool for future cell therapy applications in DMD disease or other muscular dystrophies.

Introduction

Adult skeletal muscle retains the ability to grow in response to increased workload and to repair or regenerate muscle fibers in response to damage. The capacity to generate new fibers resides in a population of mononucleated precursors, called satellite cells, which lie between the basal lamina and the sarcolemma of each myofiber (1). In healthy adult muscle, these cells remain in a nonproliferative, quiescent state. However, in response to stimuli such as trauma, satellite cells become activated, proliferate, and express myogenic markers. Ultimately, these cells either fuse with the existing muscle fibers or fuse together to form new myofibers, thus contributing to regeneration of damaged skeletal muscle; part of the satellite cell population returns to quiescence, thus maintaining a pool of progenitor cells (1, 2). In most myopathies, the clinical outcome is ultimately due to a failure of the myogenic satellite cells to maintain muscle regeneration after continuous degeneration-regeneration cycles (3–6). Satellite cells have been defined by morphological criteria (i.e., location between the basal lamina and the sarcolemma) and by the differential expression of markers such as M-cadherin (a marker of satellite cells) (7), c-Met (8), CD34, and MYF5 (9). It was believed that all myogenic cells in regenerating muscle were indeed satellite cells that in turn should originate from the dorsal domain of the somites (10, 11). This paradigm has been challenged by studies showing muscle regeneration by transplanted bone marrow cells (12, 13). It was also demonstrated that cells isolated from the embryonic dorsal aorta have a similar morphological appearance, express a number of markers in common with satellite cells, and likewise generate myogenic clones in vitro (14) or when transplanted into embryonic tissues (15). Furthermore, recent work from several laboratories supports the idea that bone marrow–derived cells, possibly hematopoietic or angioblastic in nature, can reach the site of muscle regeneration and contribute to muscle repair as well as to replenishment of the satellite cell pool (16, 17), whose origin solely from somites thus needs to be re-investigated.

We recently identified a subpopulation of human muscle–derived stem cells expressing the AC133 antigen that can differentiate into muscle, hematopoietic, and endothelial cell types when exposed to certain cytokines (Y. Torrente et al., unpublished observations). The AC133 is a newly identified 120-kDa glycosylated polypeptide expressed on a population of circulating human hematopoietic/endothelial progenitors (18–22). The function of AC133, which does not share homology with any previously described hematopoietic cell surface antigen, is not known. However, human-derived AC133+ cells can repopulate the bone marrow and differentiate into mature endothelial cells (22). We thus extended these observations by investigating the differentiation potential of human circulating AC133+ cells from normal blood

Nonstandard abbreviations used: CFU in culture (CFU-C); control (CTR); cross-sectional area (CSA); differentiation medium (DM); Duchenne muscular dystrophy (DMD); enhanced GFP (eGFP); force (Po); gastrocnemius (Gas); human dystrophin (Dys3); Iscove's modified Dulbecco's medium (IMDM); muscle-derived stem cell (MDSC); myosin heavy chain (MyHC); phycoerythrin (PE); proliferation medium (PM); quadriceps (Qd); sarcomere length (SL); soleus (Sol); stem cell factor (SCF); telomere repeat-amplification protocol (TRAP); tibialis anterior (TA); treated (TR).

Conflict of interest: The authors have declared that no conflict of interest exists.

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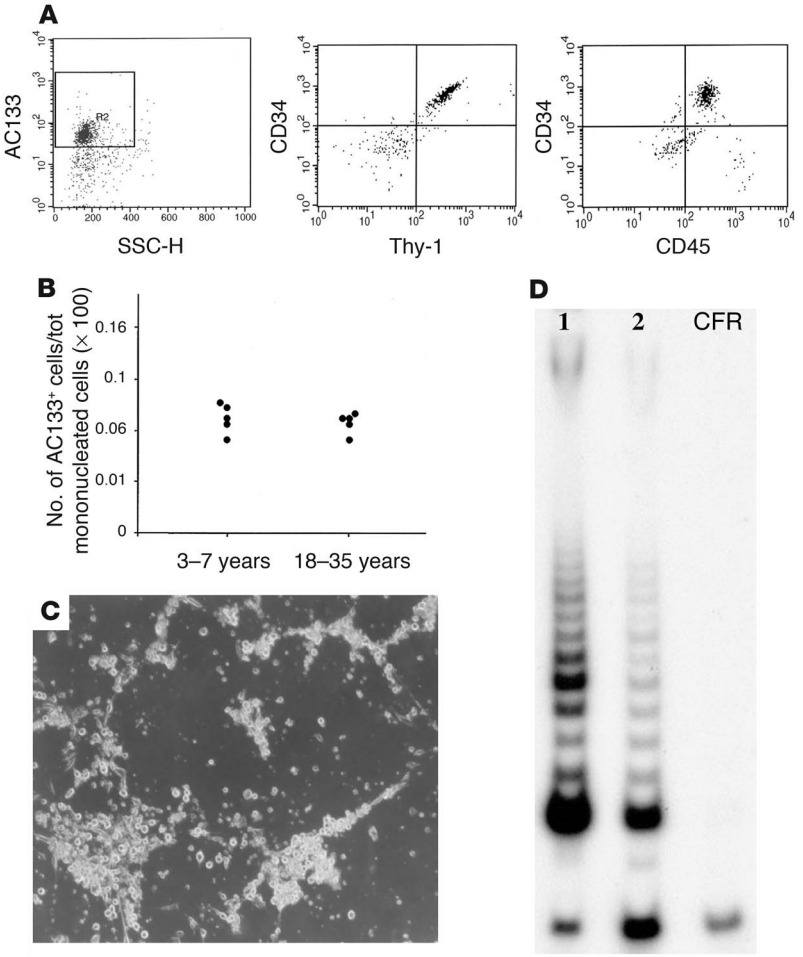

Figure 1
FACScan immunophenotyping of fractionated blood-derived cells. (**A**) After AC133 purification with MACS columns, the median purity of positively selected AC133$^+$ cells after magnetic cell sorting was about 90% (range, 85–95%) (left panel). SSC-H, side-scatter height. More than 92% of the AC133$^+$ cells isolated from human blood were CD34$^+$ and coexpressed Thy-1 (middle panel) and CD45 (third panel). (**B**) The percentage of AC133$^+$ cells isolated from the blood tissues at different ages. tot, total. (**C**) Single AC133$^+$ cells isolated from the blood give rise to small colonies. (**D**) Cell extracts from 10^4 blood-derived AC133$^+$ cells were analyzed by the TRAP assay (lane 1). Controls are a positive reaction (synthetic telomerase product) (lane 2) and a cell-free reaction (CFR).

samples. To this aim, we cocultured human circulating AC133$^+$ stem cells with mouse myoblasts or with Wnt-expressing cells and injected the cells into skeletal muscle tissues of *scid/mdx* dystrophic mice. Under these conditions, the human circulating AC133$^+$ cells formed myotubes in mixed cultures, participated in muscle regeneration, and also replenished the satellite cell compartment of the injected dystrophic muscles. Moreover, AC133$^+$ cells caused a significant amelioration of skeletal muscle structure and function when delivered to *scid/mdx* mice, a murine model of Duchenne muscular dystrophy (DMD) that tolerates human donor cells. Partial regeneration and consequent functional improvement in the muscles of DMD patients may be important in delaying the most severe symptoms of the disease. We speculate that transplantation of circulating AC133$^+$ stem cells could represent a future treatment for primary myopathies.

Results

Identification and characterization of AC133$^+$ cells derived from blood. Cells were isolated from human blood and characterized by flow cytometry (Figure 1). The AC133 selection of mononuclear cells showed a representative subpopulation of AC133$^+$ cells (0.06% of the total blood mononucleated cells) measured after flow cytometry with no interindividual differences between the specimens (3–35 years) (Figure 1B). It is important to note that more than 92% of the AC133$^+$ cells isolated from the human blood were CD34$^+$ and coexpressed the Thy-1 marker (95%). More than 97% of the selected blood-derived AC133$^+$ cells were CD45$^+$ with a lineage-negative phenotype (CD33$^-$CD38$^-$) (data not shown). The magnetic separation system and characterization results represent data collected from experiments performed using blood from ten separate isolation procedures.

Clonogenic, self-renewal, and multipotency of AC133$^+$ cells from blood. To determine whether blood-derived AC133$^+$ cells exhibit the stem cell characteristic of self-renewal, we plated AC133$^+$ cells (5×10^4) freshly isolated from blood at a density of approximately 10 cells/cm^2 in wells of 96-well plates. To induce proliferation, we cultured blood-derived AC133$^+$ cells on a mitomycin C–inactivated mouse embryonic fibroblast feeder layer. Cells were fed every 6–7 days and were maintained in a fully humidified atmosphere (at 37°C and 5% CO2). At 80% confluence, cells were dissociated to single cells by gentle pipetting and were cloned by limiting dilution. Single cells replicated as typical stem cells, giving rise to small adherent colonies (Figure 1C); the cloning efficiency was 0.5%. When AC133$^+$ cells isolated from blood were assessed for telomerase activity with a telomerase repeat amplification protocol (TRAP) (23), they showed robust telomerase activity (Figure 1D). These data demonstrated the stem cell–like activity of the blood-derived AC133$^+$ cells. However, expansion in culture was limited and AC133$^+$ cells from blood arrested proliferation after several passages, suggesting that culture conditions still need to be optimized. To verify whether the human circulating AC133$^+$ cells contained hematopoietic capacity, we performed a CFU in culture (CFU-C) assay in methylcel-

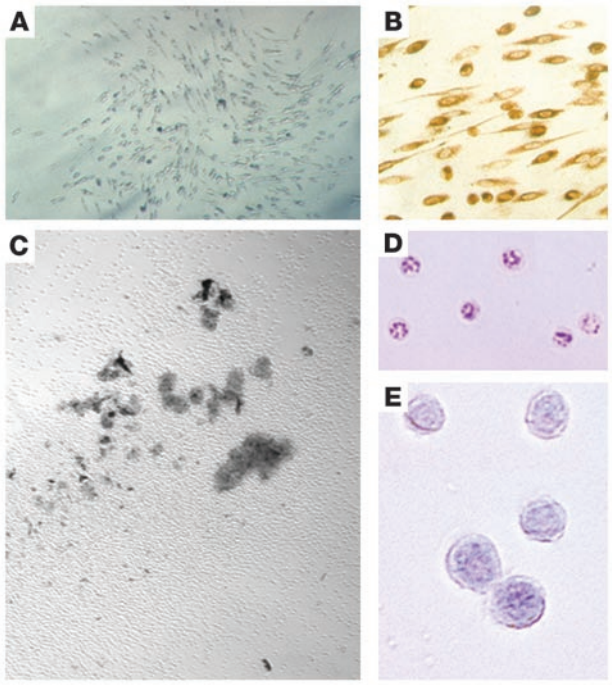

Figure 2
In vitro multipotentiality of blood-derived AC133$^+$ stem cells. (**A** and **B**) In vitro differentiation of human circulating AC133$^+$ cultured in endothelial medium for 9 weeks (**A**) was confirmed by immunoperoxidase staining for expression of the VE-cadherin endothelial marker (**B**). (**C**) Photomicrograph shows colonies of human circulating AC133$^+$ cells in methylcellulose under myeloid conditions. (**D** and **E**) The multimyeloid nature of the resulting cells (**C**) was confirmed by May-Grunwald-Giemsa staining, revealing predominantly granular polymorphonuclear cells (**D**) and immature myeloid morphologies (**E**).

lulose (24). In these conditions, cloned blood-derived AC133$^+$ cells formed several colonies (13%) (Figure 2) that displayed a multimyeloid potentiality (mainly blast cells, macrophages, and granular polymorphonuclear cells). The multimyeloid nature of the resulting cells was confirmed by May-Grunwald-Giemsa staining (Figure 2). Other groups (25–27) have shown that purified populations of AC133$^+$ cells have the capacity to differentiate into endothelial cells. On the basis of those results, we cultured the same clonal progeny of circulating AC133$^+$ cells in conditions that also support endothelial differentiation. Endothelial cells positive for CD31 and VE-cadherin were found in both early (after 5 days) and late (after 9 weeks) passages of nonadherent AC133$^+$ cells (Figure 2). All these data indicate the multipotential characteristics of the human circulating AC133$^+$ stem cells.

Expression of muscle markers in vitro by AC133$^+$ cells derived from blood tissues. We performed immunofluorescence staining, RT-PCR, and immunoblot analyses to investigate whether the AC133$^+$ cells obtained from blood tissues expressed myogenic markers and/or were capable of myogenic differentiation in vitro. RT-PCR with oligonucleotides specific for human PAX7, MYF5, MYOD,

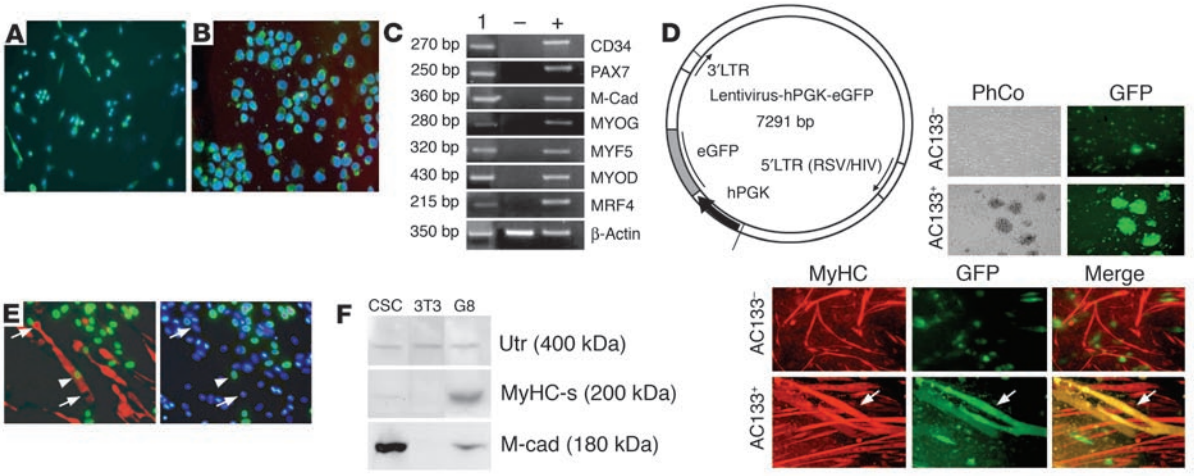

Figure 3
Myogenic differentiation of blood-derived AC133$^+$ cells in vitro. (**A** and **B**) In myogenic differentiation, AC133$^+$ cells from the blood do not form myotubes but remain as mononucleated, quiescent, desmin-positive cells (**A**) and M-cadherin–positive cells (**B**). (**C**) The early myogenic commitment (at 24 h of culture) was demonstrated by specific RT-PCR for (**C**) human markers performed on mRNA extracted from AC133$^+$ cells isolated from blood (lane 1), a C2C12 murine cell line (lane –), and a human cDNA library (lane +). (**D**) AC133$^+$ and AC133$^-$ cells derived from the blood were transduced with a third-generation lentivirus vector expressing GFP cDNA under the transcriptional control of the human phosphoglycerate kinase (PGK) promoter. GFP expression of the transduced cells is shown both in phase contrast (PhCo) and in fluorescence (GFP). Fluorescence analysis of transduced human circulating AC133$^+$ cells cocultured with uninfected C2C12 mouse myoblasts revealed several multinucleated, human slow MyHC–positive myotubes (arrows) that incorporated the GFP$^+$ cells (lower panel, bottom row). Moreover, transduced human blood–derived AC133$^-$ cells failed to differentiate into myosin-positive muscle cells when plated on the C2C12 feeder cells (lower panel, top row). (**E**) Double immunocytochemistry with antibodies against MyHC (red) and human lamin A/C (green) demonstrated the presence of human (arrowheads) and mouse (arrows) nuclei within myotubes, confirming the fusion of AC133$^+$ cells with the C2C12 murine myoblasts. Nuclei were stained blue with DAPI. (**F**) Immunoblot analysis of human MyHC (MyHC-s) and human M-cadherin (M-Cad) confirmed the myogenic differentiation of the circulating AC133$^+$ stem cells (CSC) cocultured for 14 days with C2C12 mouse myoblasts in low-serum fusion-promoting conditions. The 3T3 fibroblast and G8 myoblast cell lines were used as controls. The utrophin immunoblot (Utr) indicates that the same total protein concentration was present in all specimens.

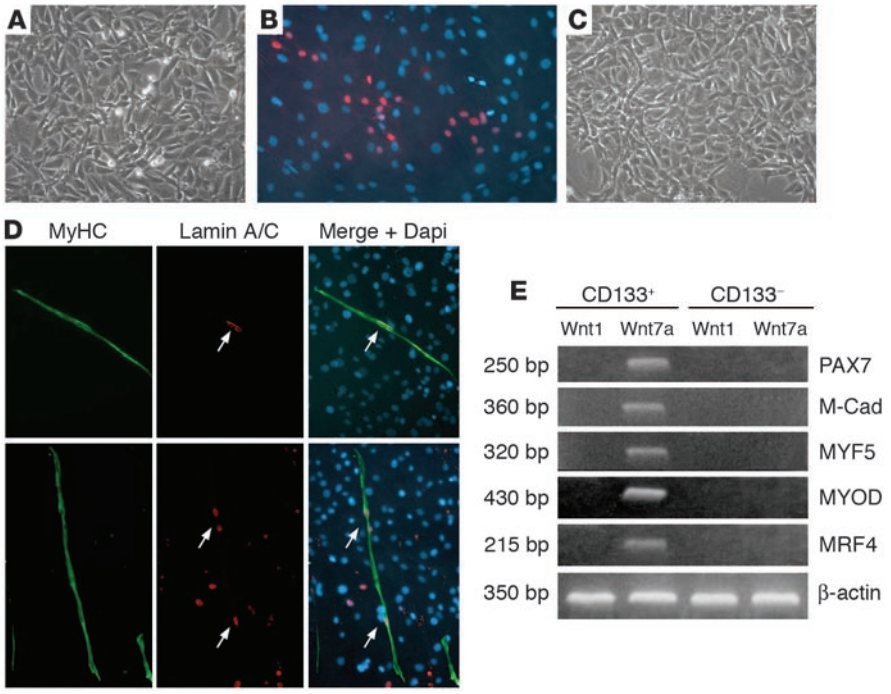

Figure 4
Role for Wnt signaling in myogenic differentiation of human circulating AC133+ stem cells cocultured with Wnt1- or Wnt7a-expressing fibroblasts. (**A**–**E**) Human circulating AC133+ cells were cocultured for 10 days with C3H10T1/2 fibroblasts expressing Wnt1 (**A**, phase contrast, and **B**) or Wnt7a (**C**, phase contrast, and **D**) and then were double-stained with polyclonal anti-MyHC (green) and monoclonal anti–human lamin A/C (red) (**B** and **D**). At this time in culture, only the AC133+ cells cocultured in the presence of Wnt7a differentiate into MyHC-positive myotubes with human nuclei (arrows in **D**) expressing either MRF4 or MYOD by RT-PCR (**E**). M-Cad, M-cadherin.

M-cadherin, MRF4, and myogenin revealed the appearance of transcripts for these human genes encoding these molecules in blood-derived AC133+ cells after 24 hours of culture in proliferation medium (PM), suggesting a certain degree of myogenic commitment in this cell population (Figure 3C). After 14 days in low-serum "fusion-promoting" conditions, blood-derived AC133+ cells did not fuse into multinucleated myosin-positive myotubes and remained as mononuclear cells positive for desmin and M-cadherin (Figure 3, A and B) but negative for myosin. To test whether myogenesis may be induced by coculture, we transduced AC133+ and AC133− blood-derived cells with the hPGK-GFP vector, which efficiently expressed enhanced GFP (eGFP) protein in more than 90% of the cell population (Figure 3D). Transduced human circulating AC133+ cells differentiated into myotubes coexpressing GFP and myosin heavy chain (MyHC) when cocultured with uninfected C2C12 mouse myoblasts (0.3% ± 0.04% of the total GFP+ cells; Figure 3D). Moreover, MyHC+ myotubes coexpressed human lamin A/C, confirming the fusion of the human AC133+ cells with the C2C12 murine myoblasts (Figure 3E). After 14 days of coculture in differentiation medium (DM), the blood-derived AC133+ cells expressed human slow MyHCs at a low level and human M-cadherin at a very high level, as revealed by immunoblot analysis (Figure 3F). Transduced human blood–derived AC133− cells failed to differentiate into myosin- or M-cadherin–positive muscle cells even when plated on a feeder of C2C12 cells (Figure 3D). It has been recently shown that Wnt proteins activate myogenesis in mouse side population cells in vitro. In order to investigate a possible role for Wnt signaling in regulating myogenesis of human circulating AC133+ cells, we utilized a coculture approach based on the stimulation of these cells with Wnt1 and Wnt7a, as described previously (28). In these experiments, human circulating AC133+ and AC133− cells were cocultured on Wnt1-

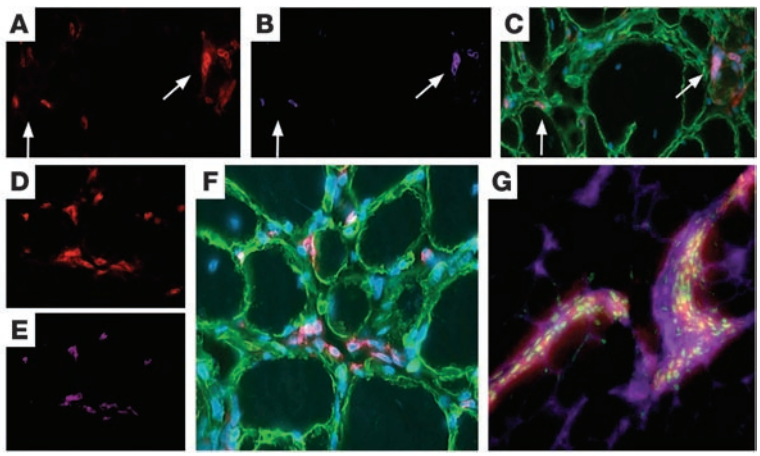

Figure 5
Localization of human endothelial cells in the interstitial space between muscle fibers and into vascular structures after intramuscular transplantation of circulating AC133+ cells. (**A**–**C**) Immunofluorescence analysis of dystrophic TA muscle injected with blood-derived AC133+ cells 21 days before revealed human nuclear lamin A/C–positive cells (arrows in **A**, **B**, and **C**) coexpressing CD31 (**B**) in the interstitial spaces between muscle fibers (**C**, merge of **A** and **B** with laminin in green). The location outside of the basal lamina of the human nuclear laminin A/C (**D**) and VE-cadherin (**E**) double-positive cells confirmed that several injected AC133+ cells expressed endothelial markers (**F**, merge of **D** and **E** with laminin in green). Examination of vascular structures of injected dystrophic muscles disclosed incorporation of human injected cells expressing endothelial markers. **G** shows the localization of human nuclear lamin A/C–positive (green) cells incorporated into vascular structures stained with antibodies to the CD31 (red) and VE-cadherin (magenta) markers.

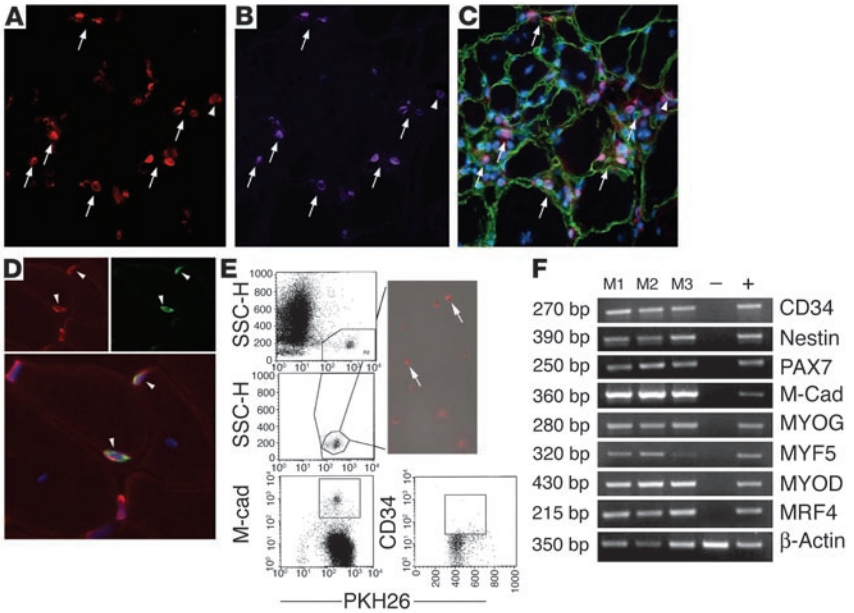

Figure 6
Contribution of circulating AC133$^+$ stem cells to the replenishment of the satellite cell pool in dystrophic muscles. (**A**–**C**) After injection into dystrophic muscle, a few human nuclear lamin A/C–positive cells were distributed near the capillary network (arrows) and regenerating myofibers, identified by a centrally located nucleus (blue after DAPI staining). Double-staining with antibodies against human nuclear lamin A/C (**A**) and M-cadherin (**B**) shows that some donor cells express markers typical of satellite cells both outside and underneath (arrowheads) the basal lamina (identified by laminin staining in green). These cells appear pink in the merged image (**C**) of **A** and **B**. (**D**) Note the presence of several donor lamin A/C (red) cells and M-cadherin (green) double-positive cells (arrowheads) with nuclei localized at the periphery of the fibers under the sarcolemma. (**E**) We injected a PKH26-labeled AC133 population intramuscularly and sorted PKH26 cells from muscle-derived cells of three *scid/mdx* mice injected 21 days before. The gated area shown in the top left screen of **E** corresponds to the sorted population of PKH26-labeled injected cells (inset) that had been stained with anti–M-cadherin (5% of the total PKH26-sorted cells) and resulted in mostly CD34$^-$ cells. Few of these cells expressed bright fluorescence such as that of nondividing cells (arrows in inset of **E**). (**F**) Expression of endothelial and myogenic markers within three injected TA muscles (M1, M2, and M3) 45 days after injection of AC133$^+$ cells isolated from the blood was also demonstrated by RT-PCR. +, positive control; –, negative control.

or Wnt7a-expressing C3H10T1/2 fibroblasts. Figure 4 shows several myotubes double-positive for MyHC and human lamin A/C (0.6% ± 0.05% of the total human lamin A/C–positive cells; Figure 4A) derived from the circulating AC133$^+$ cells grown for 10 days on Wnt7a-expressing C3H10T1/2 fibroblasts. In contrast, no myogenic cells were detected in cultures of human circulating AC133$^+$ cells grown on C3H10T1/2 fibroblasts expressing Wnt1 (Figure 4B). In addition, RT-PCR analysis showed expression of MYF5, MYOD, MRF4, and M-cadherin only in human circulating AC133$^+$ cells cultured for 10 days in the presence of Wnt7a (Figure 4E). In contrast, human blood–derived AC133$^-$ cells failed to differentiate into myogenic cells in the presence of Wnt proteins (data not shown). These data support the evidence that the human circulating stem cells expressing myogenic markers were confined to the AC133$^+$ cell population.

Engrafted blood-derived AC133$^+$ cells can differentiate into myofibers and satellite cells after in vivo transplantation. Xenogeneic transplantation studies were performed using the *scid/mdx* mouse model. In these experiments, we investigated whether human progenitors isolated from the blood can exhibit in vivo myogenic properties after implantation into the muscle of adult recipients. AC133-derived blood cells were injected into the tibialis anterior (TA) muscle of 15 *scid/mdx* mice and, at various intervals after grafting, sections were stained with human-specific antibodies. We counted the donor human lamin A/C–positive cells in all the serial sections of injected TA muscles. Muscles of injected animals contained a few mononucleated human lamin A/C–positive cells (red in Figure 5, A and D) in the interstitial spaces that coexpressed CD31 (7.7% ± 1.2% of the total human lamin A/C–positive cells per section; $n = 5$) and VE-cadherin (9.7% ± 0.1% of the total human lamin A/C–positive cells per section; $n = 5$), markers of endothelial cells (29) (purple in Figure 5, B and E). A small number of these human lamin A/C–positive cells appeared to be incorporated into vessels of the injected area (7% ± 2% of human lamin A/C–positive cells per cross-section; Figure 5G). We also investigated whether circulating AC133-derived cells could be located underneath the basal lamina of host fibers and to this aim we colocalized by immunohistochemistry M-cadherin, laminin, human lamin A/C, and Hoechst nuclei. A small population of the human lamin A/C–positive donor cells (red) expressed the M-cadherin (revealed in purple by Cy5-labeled secondary antibody) (Figure 6, A and B) (6% ± 1.1% of total human lamin A/C–positive donor cells per section). These cells were identified underneath the basal lamina (laminin was detected by FITC immunofluorescence in green) of injected skeletal muscle closely juxtaposed against skeletal muscle fibers and accounted for 1.5% of the total sublaminal nuclei per muscle sections (Figure 6, C, and confocal images in D). To test the characteristics of the donor satellite cells, we injected human PKH26-labeled AC133$^+$ cells derived from the blood into the TA muscles of three *scid/mdx* mice and quantified the percentage of cells expressing M-cadherin after sorting PKH26 cells from muscle-derived cells 14 days after the injection. Because of the considerable loss of PKH26 intensity that occurred in proliferating cells during the time elapsed after injection, we adjusted the gates for the sorting using an aliquot of blood-derived AC133$^+$ cells that had been labeled with PKH26 after they had passed through the FACS apparatus. Within the established gates, a low percentage of the sorted cells (5%) were still PKH26^{bright} (Figure 6, inset in E) and expressed M-cadherin, suggesting a quiescent, nondividing state. In addition, a large majority (> 90%) of the sorted injected cells were CD34$^-$CD45$^-$ (Figure 6E). These results strongly suggest that intramuscularly transplanted blood-derived AC133$^+$ cells are able to give rise to interstitial endothelial cells and to satellite cells.

Moreover, to obtain expandable human satellite myogenic clones from transplanted muscles, we explanted the TA muscles injected 45 days before. After 7 days of culture, approximately 5% (mini-

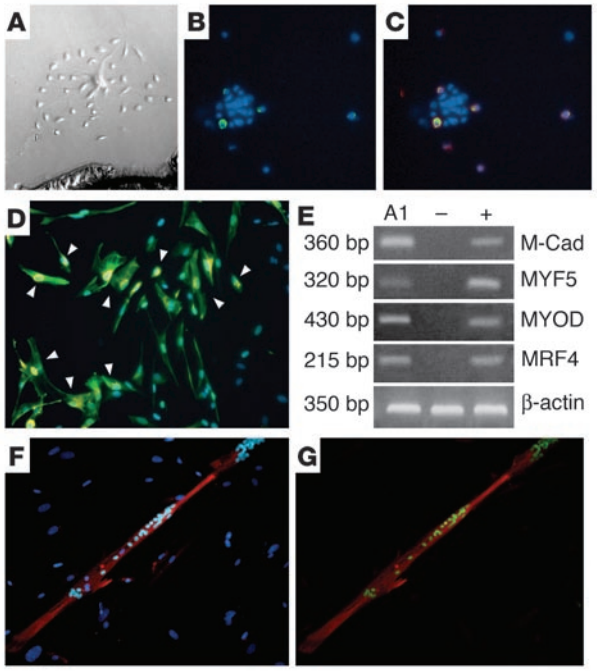

Figure 7
Characterization and clonal isolation of muscle-derived cells that emerged from the explants of TA muscles injected with human circulating AC133$^+$ cells. (**A**–**C**) Cells from transplanted muscle explants were cultivated in vitro and analyzed after 7 days (**A**, phase contrast) for coexpression of M-cadherin (**B**) and human lamin A/C (**C**). (**D**) Double immunocytochemistry with antibodies against desmin (green) and human lamin A/C (red) demonstrated the presence of human myogenic cells (arrowheads) after 6 weeks of explant culture. For cloning, single cells within the explant-derived cells were plated in proliferative conditions as described in Methods. (**E**) Human myogenic clones obtained from clone A1 express several specific myogenic human markers, as assessed by RT-PCR. The C2C12 murine cell line (lane –) and a human cDNA library (lane +) were used as controls. (**F** and **G**) A1-derived cells differentiated well into myotubes coexpressing the late myogenic marker MyHC (red in **F**) and human MYOD (green in **G**).

mum value, 2% and maximum value, 7%) of the cells that migrated out of the explants were human cells and expressed M-cadherin (Figure 7, A–C). After 6 weeks, 90% of these human cells were found to express desmin (Figure 7D). Clonal analysis of cells obtained from explants at this time revealed that after 7 days in DM, the majority of the total clones (36 of 48) did not fuse but remained mononucleated cells expressing neither desmin nor myogenin. However, the remaining 12 clones expressed desmin and fused into myotubes. Of these clones, only 2 (called A1 and A2) expressed the human lamin A/C antigen. These human clones possess myogenic potential, as shown by RT-PCR for human M-cadherin, MYF5, MYOD, and MRF4 expression, and differentiated well into myotubes coexpressing MyHCs and human MYOD (Figure 7, E–G).

To confirm the in vivo ability of AC133$^+$ cells isolated from the blood to differentiate into human muscle fibers, we performed RT-PCR to analyze human M-cadherin, MYF5, myogenin, PAX7, MYOD, and MRF4 mRNA in muscles from *scid/mdx* mice that had been injected intramuscularly with AC133$^+$ cells. M-cadherin and PAX7 are known to be expressed in both quiescent satellite cells and myoblasts in adult muscle (29). Human M-cadherin, MYF5, and PAX7 were detected in the injected TA muscles, suggesting that blood-derived AC133$^+$ stem cells may differentiate in vivo into satellite cells (Figure 6F). Moreover, human-specific MYOD and MRF4 mRNAs were also detected in the injected TA muscles (Figure 6F). No human transcripts were detected in the muscles of control (not injected) *scid/mdx* mice. More importantly, the circulating AC133$^+$ stem cells had also fused into regenerating and mature myofibers expressing human dystrophin at 21, 45, and 60 days after intramuscular transplantation (Table 1). The number of human dystrophin–positive myofibers per muscle maximal cross-sectional area (CSA) after intramuscular transplantation increased to 574.7 ± 120.2 at 60 days after injection from 89.2 ± 25 at 21 days after injection (Table 1), suggesting a constant recruitment of donor cells in the regenerating dystrophic muscle. The injected human lamin A/C–positive cells were counted in five nonadjacent cross-sections of the intramuscularly injected TA, and the length of the positive area was about 800 μm. At 21 days after intramuscular injection, injected (Hoechst-positive) human lamin A/C–positive cells within dystrophic muscle were detected at an average number of 33 ± 7 per section ($n = 5$). The average number of the injected human lamin A/C–positive cells was significantly reduced ($P < 0.001$; Table 1) after 45 days

Table 1
Blood-derived AC133$^+$ cells yield human dystrophin–positive myofibers within injected dystrophic muscles

	Time after transplantation					
	21 days		45 days		60 days	
	i.m.	i.a.	i.m.	i.a.	i.m.	i.a.
No. 1 Lamin A/C$^+$ cells	31 ± 5^A	5 ± 1	4 ± 1	21 ± 9^A	3 ± 2	2 ± 1
No. 1 Dys$^+$ fibers	66 ± 57^A	13 ± 9	135 ± 78^A	10 ± 4	570 ± 200^A	3 ± 1
No. 1 Dys/LamA/C$^+$ fibers	48 ± 61^A	5 ± 7	117 ± 69^A	4 ± 7	495 ± 89^A	2 ± 1
No. 2 Lamin A/C$^+$ cells	30 ± 9	25 ± 11	9 ± 5	2 ± 1	7 ± 1	5 ± 3
No. 2 Dys$^+$ fibers	125 ± 45^A	9 ± 4	190 ± 70^A	3 ± 2	1118 ± 145^A	23 ± 15
No. 2 Dys/LamA/C$^+$ fibers	106 ± 73^A	4 ± 9	155 ± 83^A	2 ± 4	992 ± 75^A	2 ± 1
No. 3 Lamin A/C$^+$ cells	35 ± 10^A	3 ± 1	3 ± 2	4 ± 2	4 ± 1	4 ± 3
No. 3 Dys$^+$ fibers	75 ± 32^A	5 ± 1	166 ± 71^A	6 ± 5	807 ± 106^A	6 ± 2
No. 3 Dys/LamA/C$^+$ fibers	58 ± 41^A	2 ± 4	125 ± 56^A	3 ± 6	698 ± 55^A	3 ± 6
No. 4 Lamin A/C$^+$ cells	29 ± 5	10 ± 1	7 ± 4	6 ± 2	6 ± 5	9 ± 3
No. 4 Dys$^+$ fibers	102 ± 56^A	4 ± 3	187 ± 91^A	5 ± 3	719 ± 350^A	8 ± 4
No. 4 Dys/LamA/C$^+$ fibers	83 ± 48^A	2 ± 6	144 ± 37^A	3 ± 5	582 ± 95^A	2 ± 6
No. 5 Lamin A/C$^+$ cells	35 ± 7^A	2 ± 1	10 ± 6	3 ± 1	4 ± 2	15 ± 1^A
No. 5 Dys$^+$ fibers	89 ± 15^A	3 ± 1	102 ± 7^A	8 ± 6	662 ± 19^A	5 ± 1
No. 5 Dys/LamA/C$^+$ fibers	65 ± 77^A	2 ± 9	95 ± 71^A	7 ± 3	592 ± 41^A	4 ± 7

Results are expressed as number of human lamin A/C–positive (Lamin A/C$^+$), human dystrophin-positive (Dys$^+$), and human Dys/lamin A/C–positive (Dys/LamA/C$^+$) cells per section of muscle tissue (± SD) in a longitudinal dimension of about 800 μm. i.m. intramuscular injection; i.a. intra-arterial injection. $^AP < 0.01$.

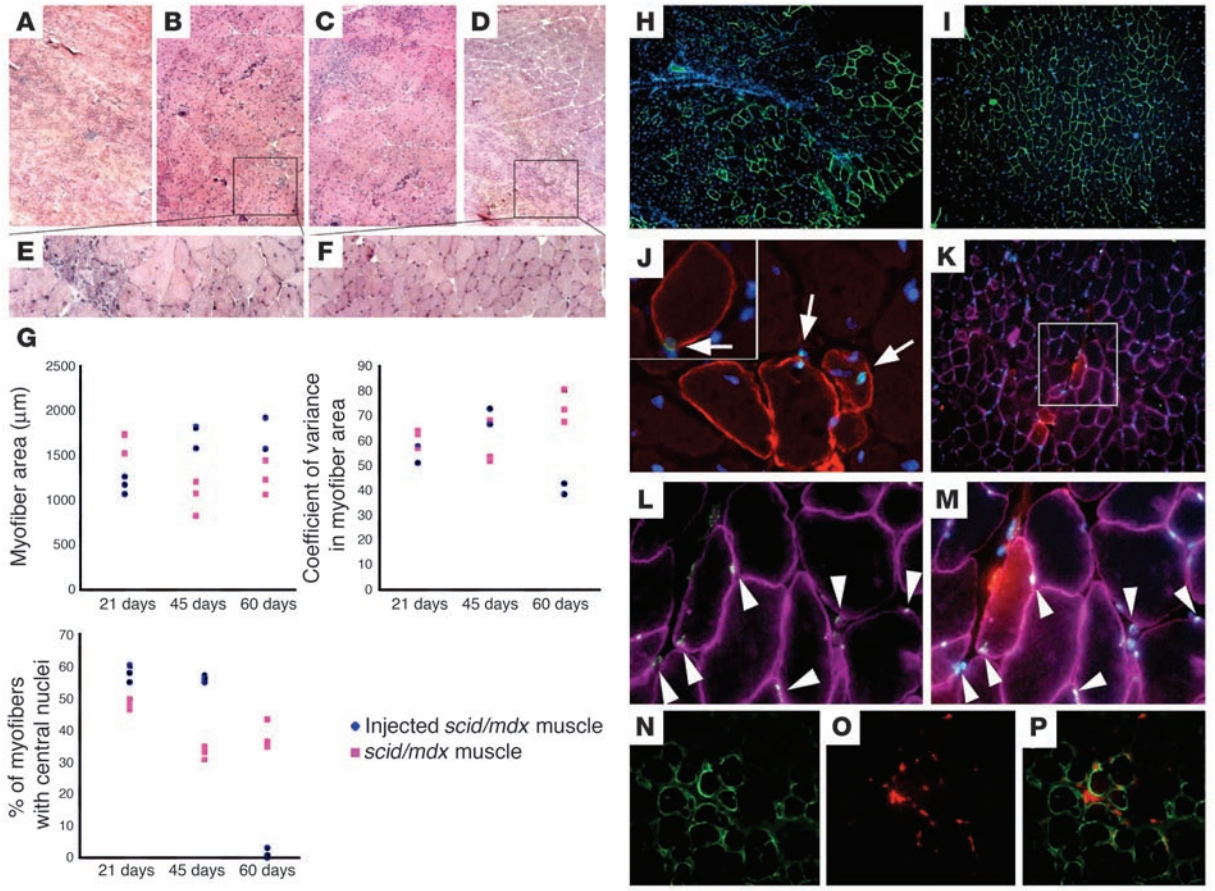

Figure 8
Rescue of dystrophic muscle after intramuscular and intra-arterial transplantation of AC133$^+$ cells derived from blood. (**A**) The *scid/mdx* muscles showed classic signs of muscular dystrophy. (**B** and **C**) The muscles of *scid/mdx* animals showed a considerable decrease in necrosis and central nucleated myofibers 21 (**B**) and 45 (**C**) days after intramuscular transplantation. (**D**) After 60 days, the myofibers generated by intramuscular transplantation of blood-derived AC133$^+$ cells were larger in both CSA and diameter, with peripheral nuclei. (**E** and **F**) Higher magnification of the inset in **B** and **D** is shown in **E** and **F**. Morphometric analysis of H&E–stained muscle sections from injected and uninjected *scid/mdx* mice during aging was also performed (**G**). The number of human dystrophin–positive myofibers 60 days after intramuscular transplantation (**I**) was higher than in muscle injected 21 days before analysis (**H**). (**J**) Staining with anti–human dystrophin (red) confirmed that several Dys3$^+$ myofibers expressed the anti–human nuclear lamin A/C antigen (green; arrows) 21 days after intramuscular transplantation of cloned AC133$^+$-derived cells (**J**). Human Dys3$^+$ myofibers (purple) with human lamin A/C nuclei (green) were present in dystrophic muscles after intra-arterial transplantation of the AC133$^+$ cells (**K**). Higher magnification of the inset in **K** is shown in **L** (arrowheads indicate the human lamin A/C–positive nuclei within Dys3$^+$ myofibers) and **M** with DAPI (blue) and PKH26 (red). Human Dys3$^+$ myofibers (**N**) were detected near mononucleated human lamin A/C–positive cells after intra-arterial delivery (**O**). A merge of **N** and **O** is shown in **P**.

(7 ± 3 cells per section; $n = 5$) and 60 days (5 ± 1 cells per section; $n = 5$), suggesting that mononuclear cells had been recruited into muscle fibers. Immunohistochemical examination of serial frozen sections of intramuscular injected muscles allowed us to correlate the changes in dystrophic muscle tissue characteristics with the number of human dystrophin myofibers and donor-derived human lamin A/C–positive cells (Figure 8).

To analyze whether injected AC133$^+$ cells may revert at least in part to the dystrophic phenotype, we performed morphometric analysis of hematoxylin and eosin–stained (H&E–stained) muscle sections from intramuscularly injected and uninjected *scid/mdx* mice. The uninjected *scid/mdx* muscles showed classic signs of muscular dystrophy, including hypertrophic fibers, small regenerating fibers, necrosis, centrally located nuclei, and occasional fibrosis (Figure 8A). Analysis of injected muscles of *scid/mdx* animals ($n = 5$ for each time point) showed a considerable decrease in these characteristics of dystrophic muscles in mice as old as 4 months (Figure 8, B–D). The myofibers generated by the blood-derived AC133$^+$ cells were larger in both CSA and diameter; however, the coefficient of variance in myofiber diameter was normal (250 or less; see ref. 30) into the injected muscle area and was abnormal in the uninjected *scid/mdx* muscles examined (more than 500; see ref. 31). Another indicator of ongoing regeneration in muscular dystrophy is an increase in the percentage of centrally located myofiber nuclei. High concentrations of centrally located nuclei (25%) were observed in all *scid/mdx* muscles examined, and these levels were significantly reduced in injected muscles as the mice aged (near 7%) (Figure 8G). To conclusively demonstrate that the newly formed muscle fibers were indeed derived from AC133$^+$ cells, we cloned the AC133-enriched population and selected three large clones that we pooled and injected into

the TA muscles of dystrophic *scid/mdx* mice. Before injection, an aliquot of the population was stained with anti-AC133 and was shown to be more than 98% positive for the antigen. Three weeks after the injection, we identified nuclei positive for donor human lamin A/C (3 ± 1 nuclei per section; $n = 5$) within clustered human dystrophin–positive myofibers (11 ± 5 per section; $n = 5$) (Figure 8J).

Intra-arterial delivery of human AC133⁺ cells. To determine whether blood-derived AC133⁺ cells are also capable of adhesion to muscle blood vessels, migration, engraftment, and myogenic differentiation after intravascular injection, we also injected *scid/mdx* mice intra-arterially with these cells. The injected human lamin A/C–positive mononuclear cells were present within the quadriceps (Qd), gastrocnemius (Gas), soleus (Sol), and TA muscles of the legs of animals injected intra-arterially, and the number was unchanged at 21, 45, and 60 days after injection (9 ± 4 cells per section; $n = 9$) (Table 1). Several areas of the Qd, Gas, Sol, and TA muscles expressed human dystrophin, whereas the protein was completely absent from muscles of the untreated legs. In this experiment, the number of human dystrophin–positive myofibers was more variable after between 21 and 60 days and showed intra-individual differences within injected dystrophic animals (Table 1). The highest number of dystrophin-positive fibers (23 ± 15) was detected within muscles of animal 2 after 60 days of transplantation (Figure 8, K–P). In all injected muscles, many of the human dystrophin–positive fibers expressed human lamin A/C (Figure 8, L and M). As observed in the muscles directly injected with circulating stem cells, few cells coexpressing the human lamin A/C and VE-cadherin antigens were found in vessels near areas of regeneration in the TA, Qd, and Sol muscles after intra-arterial injection. Indeed, cells double-positive for human lamin A/C and M-cadherin were found underneath the fiber basal lamina and near the human dystrophin–positive myofibers, suggesting that circulating AC133⁺ stem cells had differentiated into satellite and muscle cells after intra-arterial injection (data not shown).

Intramuscular injection of blood AC133-derived cells is followed by functional recovery of single muscle fibers in dystrophic mice. To assess whether the improvement in the signs of muscular dystrophy indicated by morphometric analysis produced sustained recovery of muscle function, we studied the physiology of TA muscles. Of the skeletal muscles studied in *mdx* mice, only the diaphragm shows consistent functional alterations (32, 33), whereas contradictory results have been obtained in comparisons of limb muscles from *mdx* and control mice (34–36). One of the reasons for such contradictory findings might be that in vitro analysis of whole muscles is not a sensitive approach for demonstrating impairment of function even when clear deterioration of animal mobility occurs, such as in α-sarcoglycan–null mice (37). To reliably assess skeletal muscle function, we performed in vitro analysis of single muscle fibers dissected from TA muscles. Right and left TA muscles of four 2-month-old *scid/mdx* mice were injected intramuscularly with 5×10^4 blood-derived AC133⁺ cells and mice were sacrificed at 8 months of age. A large population ($n = 369$) of single muscle fibers was dissected from the eight injected TA (*scid/mdx* treated [TR]) muscles, from eight uninjected TA muscles of *scid/mdx* mice ($n = 4$), and from four TA muscles of C57 control (CTR) mice ($n = 2$). Expression of human dystrophin and human δ-sarcoglycan in the TA *scid/mdx* TR muscles used for functional analysis was found by Western blot analysis (Figure 9D). As expected on the basis of previous findings (38), TA muscles expressed only two of the four adult mice MyHC isoforms, MyHC-2B and MyHC-2X. No human MyHC isoforms were found in *scid/mdx* TR muscles by SDS-PAGE (Figure 9E). As in all muscles, the majority of single fibers used for functional analysis contained MyHC-2B (76% in *scid/mdx* and 71% in *scid/mdx* TR), few fibers contained MyHC-2X (3% and 7%, respectively), and many coexpressed MyHC-2B and MyHC-2X (20% and 21%, respectively); to enable comparison of

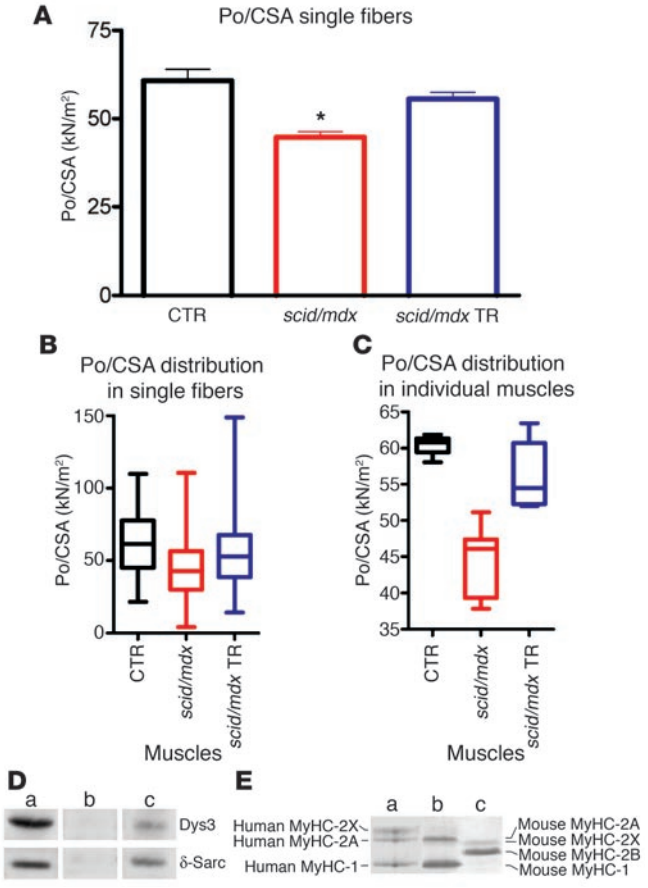

Figure 9

In vitro analysis of specific force (Po/CSA) of type 2B single muscle fibers from both TA muscles of C57 control (CTR), *scid/mdx*, and *scid/mdx* TR mice. (**A**) Mean Po/CSA values (± SEM) of single muscle fibers from CTR ($n = 50$), *scid/mdx* ($n = 163$), and *scid/mdx* TR ($n = 156$) mice. Single fibers from *scid/mdx* mice were significantly weaker (*) than single fibers from CTR and *scid/mdx* TR mice ($P < 0.0001$). (**B**) Box-and-whiskers plot showing the distribution of mean Po/CSA values of single fibers and individual muscles (**C**) within CTR, *scid/mdx* and *scid/mdx* TR muscles. The median is indicated by the line in the middle of each box, the 25th percentile by the bottom of each box, the 75th percentile by the top of each box, and the maximum and minimum values by the vertical lines. (**D**) Immunoblot analysis of human δ-sarcoglycan (δ-Sarc) and of human dystrophin obtained from *scid/mdx* TR mice. The first lane (a) of each immunoblot corresponds to a homogenate of human vastus lateralis muscle. Each second lane (b) corresponds to the homogenate of the TA muscle of a *scid/mdx* mouse, and each third lane (c) corresponds to the injected TA muscle of a *scid/mdx* TR mouse. (**E**) Identification of MyHC isoforms. Lane a, muscle sample from a human vastus lateralis muscle. Lane b, muscle sample from the soleus muscle of a C57 mouse. Lane c, muscle sample from a TA muscle of a *scid/mdx* TR mouse. The area of migration of human or mouse MyHC isoforms are indicated on the left and on the right, respectively.

a sufficient number of fibers of the same type, analysis focused on type 2B fibers only. Maximum specific force (Po/CSA) was determined and fibers were thereafter identified on the basis of their MyHC isoform composition determined by electrophoresis in denaturating conditions (SDS-PAGE). A significant loss (–25%, $P < 0.0001$) of Po/CSA was observed in fibers from *scid/mdx* muscles (mean Po/CSA, 46.00 ± 20.00 kN/m^2; $n = 163$) compared with the Po/CSA of fibers from CTR muscles (60.76 ± 22.70 kN/m^2; $n = 50$), and a significant recovery (± 20%, $P < 0.0001$) was observed in fibers from *scid/mdx* TR muscles (56.56 ± 24.36 kN/m^2; $n = 158$) (Figure 9A). Although fibers from injected muscles were somewhat weaker than fibers from control muscles, no statistically significant difference was observed, suggesting almost complete recovery toward normal values in the treated fibers. The variability of Po/CSA of individual fibers was larger in *scid/mdx* and *scid/mdx* TR muscles than in CTR muscles (Figure 9B). As for individual muscle-to-muscle variation, the mean Po/CSA of single fibers of individual muscles showed a larger range of variability in *scid/mdx* and in *scid/mdx* TR muscles than in CTR muscles (Figure 9C). However, (a) the loss of force in *scid/mdx* and the recovery of force in *scid/mdx* TR muscles involved most fibers, that is, the 25th and 75th percentiles and the median were lower in *scid/mdx* mice (32.23, 56.87, and 42.79, respectively) than in CTR mice (44.87, 77.59, and 61.44, respectively) and significantly recovered in *scid/mdx* TR mice (38.11, 68.04, and 52.79, respectively) (Figure 9B), and (b) the ranges of variability of mean Po/CSA of single fibers of individual *scid/mdx* muscles overlapped only slightly with the ranges of variability of single muscle fibers of individual CTR and *scid/mdx* TR muscles (Figure 9C), strengthening the observation of a substantial recovery of force after treatment.

Discussion

Recent studies have demonstrated that circulating bone marrow–derived cells are able to contribute to muscle regeneration (12, 13, 39). Conversely, adult stem cells expressing CD45 (a lineage-restricted pan-hematopoietic marker) appear to represent a resident population of muscle-derived stem cells (MDSCs) with myogenic potential (40). It is likely that these progenitors are recruited for muscle regeneration from a pool of circulating hematopoietic stem cells. In our analysis, we found that AC133 was expressed on a subset of circulating stem cells with myogenic potential. Based on previous studies with blood-derived AC133$^+$ cells (41, 42), we hypothesized that the AC133-derived cells act as a multipotent population. In our in vitro conditions, these cloned AC133-derived stem cells were capable of commitment to more than one lineage (hemopoietic and endothelial), given the right environmental cues. Moreover, these cells expressed several myogenic markers such as MYF5, M-cadherin, and PAX7. However, the blood-derived AC133$^+$ cells did not form myogenic cells spontaneously when myogenesis was induced in vitro but remained morphologically undifferentiated and maintained expression of CD34 and M-cadherin. In contrast, when AC133$^+$ cells derived from the blood were cultured in the presence of a feeder layer of mouse myogenic cells, they fused, forming MyHC-expressing heterozygous-specific human/murine myotubes. As different Wnt's are necessary and sufficient for the myogenic specification of dorsal somite (15) and adult stem cells during muscle regeneration (40), we asked whether the myogenic differentiation of the blood-derived AC133$^+$ cells may be induced by specific Wnt's. To examine this, we cocultured the human circulating AC133$^+$ cells with fibroblasts expressing Wnt1 and Wnt7a, which preferentially activate MYF5 and MYOD, respectively (15). In this report we have established a role for Wnt7a but not for Wnt1 in the induction of myogenesis in human circulating AC133$^+$ cells. The frequency of this phenomenon was low, suggesting that the in vitro conditions may be suboptimal and/or other molecules, yet to be identified, cooperate with Wnt7a. This will require further studies. No myogenic cells were derived from the blood-derived AC133$^-$ cells, even in coculture experiments. Thus, AC133 expression may represent an important tool for the isolation of progenitors that can be induced to myogenic differentiation. For this reason we investigated the ability of circulating human AC133$^+$ cells to restore dystrophin expression and eventually regenerate the satellite cell pool in dystrophic mouse muscles after intramuscular or intra-arterial delivery. Human AC133$^+$ cells colonized the mouse muscle, forming hybrid regenerated fibers expressing human dystrophin. Although the number of injected human lamin A/C–positive cells decreased between 21 and 60 days after transplantation, we observed a large increase in the expression of human dystrophin (up to 10% human dystrophin–positive [Dys3-positive] fibers) in dystrophic muscles injected intramuscularly, suggesting that blood-derived AC133$^+$ cells are recruited into the myogenic pathway. The myogenic potential of the blood-derived AC133$^+$ cells was also confirmed by the expression of human dystrophin after intramuscular transplantation of cloned AC133$^+$-derived cells. However, the reduction in in vivo myogenic differentiation we observed suggests an intrinsic difference between the clonal population and the whole population of blood-derived AC133$^+$ cells. It is possible that the latter contains other cell types that promote in vivo myogenesis of the AC133$^+$ cells. The long-term data on the recovery of specific force in single muscle fibers after intramuscular injection of blood-derived AC133$^+$ cells are consistent with recruitment of AC133$^+$ cells to myogenesis and strongly suggest that such a phenomenon not only ameliorates morphology but also can significantly restore skeletal muscle function toward normal. These data reinforce previous suggestions that dystrophin-positive myofibers can provide functional support to neighboring dystrophin-negative fibers (43). Alternatively, injected donor cells may produce soluble factors that increase survival/function of dystrophic fibers. Our results show also that human blood–derived AC133$^+$ cells differentiate into satellite cells when transplanted into dystrophic skeletal muscle, the identity of which was suggested by their location beneath the basal lamina, distribution along freshly isolated fibers, and coexpression of M-cadherin (7). Moreover, when reisolated from the injected muscle, donor PKH26-labeled AC133$^+$ cells showed a low expression of M-cadherin but an absence of CD34 after flow cytometry. It is important to note that coexpression of CD34 defines the majority of blood-derived AC133$^+$ cells that may represent a more primitive stem cell–like population responsible for progressive replenishment of the satellite cell pool. Clonal populations of human muscle cells isolated from the explants of injected muscles display satellite cell markers with high myogenic differentiation capacity, suggesting the in vivo muscle commitment of these stem cells. These results confirm and extend the data from the Rudnicki and Blau laboratories (16, 17) and, in addition, they provide a new method for investigation of the satellite cell activity of human stem cells after in vivo transplantation. Furthermore, using RT-PCR of injected muscles from *scid/mdx* mice, we were also able to show expression of human MYF5, PAX7, MYOD,

and M-cadherin, indicating that blood-derived AC133$^+$ cells participate actively in muscle regeneration and contribute to the replenishment of the pool of satellite cells. Moreover, a few of the blood-derived AC133$^+$ donor cells were also incorporated into the blood vessels. In these vessels, human lamin A/C–positive nuclei were identified in cells that coexpressed the CD31 or VE-cadherin endothelial markers, suggesting the in vivo endothelial potential of circulating AC133$^+$ cells after transplantation in dystrophic muscle. These data confirmed the in vitro evidence of the myogenic and endothelial potency of circulating AC133$^+$ cells.

We also investigated the ability of the circulating AC133$^+$ cells to migrate from the bloodstream to muscles after intra-arterial injection into *scid/mdx* mice. Several human lamin A/C–positive cells were detected in all hindlimb skeletal muscles analyzed after intra-arterial injection. At that time, foci of donor human circulating AC133-derived cells were located mainly at the periphery of fibers, where they expressed M-cadherin and were near regenerating and peripherally nucleated mature fibers expressing human dystrophin (<1%). Moreover, several human dystrophin–positive fibers showed the presence of lamin A/C–positive nuclei underneath the fiber basal lamina. We also showed that human donor cells were detected in several vessels near areas of regeneration, where they expressed human VE-cadherin and CD31. These results confirmed the data obtained after intramuscular injection and showed that human blood–derived AC133$^+$ cells can migrate within muscle tissues and differentiate into endothelial, satellite cells, and human dystrophin–positive muscle fibers. In contrast to results obtained by intramuscular injections, we observed a decrease in the total number of donor human lamin A/C–positive cells within muscles treated by intra-arterial injection. The lower efficiency of intra-arterial versus intramuscular injection may be explained by the low expression of endothelial adhesive proteins on the surface of immature progenitors, which may impair the adhesion and migration to dystrophic muscle and extravasation of circulating stem cells. Furthermore, it is possible that within the AC133$^+$ population, only those cells that have already progressed toward the myeloid lineage succeed in crossing the endothelium. In such a case, these cells may have a lower self-renewal ability than their more undifferentiated counterparts, and this may explain their progressive reduction in number. Finally, the possibility of species differences in the proteins required for human circulating cells to adhere and cross mouse endothelium may contribute to the lower colonization observed in this case, compared with similarly injected mouse mesoangioblasts (44). Work is currently in progress to improve the expression of adhesion molecules of human circulating AC133$^+$ stem cells by using combinations of cytokines in vitro.

Several studies have suggested the existence of muscle stem cells that express CD34, adhere slowly to the substrate in vitro after muscle enzymatic digestion, and can give rise to myogenic and endothelial (24, 45), adipogenic or osteogenic (46), and hematopoietic lineages (47–49). Although conclusive evidence is still missing, there are many reports suggesting that MDSCs may represent a population of progenitors lying in the interstitial spaces (29) that originate from blood-borne, bone marrow–derived cells and may contribute to both muscle regeneration and to replenishment of the satellite cell pool. The precise nature of this circulating muscle stem cell remains to be defined. Recent work suggests that satellite cells may derive at least in part from angioblastic precursors associated with embryonic vasculature and that multipotent stem cells are associated with the vasculature in adult tissues (12, 50). Expression of AC133 transcripts in hematopoietic adult tissues has been attributed to a hematopoietic/endothelial progenitor with hemangioblastic properties (18). Thus, the similarity between MDSCs in the mouse and the circulating multipotent AC133$^+$ stem cells that we have identified in human blood is remarkable. This in turn suggests two considerations: (a) circulating progenitors with myogenic potential may counteract the pathological changes induced by muscular dystrophy, and the late phases of the disease may be in part due to their exhaustion or to inaccessibility of the sclerotic dystrophic muscle; or (b) circulating AC133$^+$ stem cells from patients may be amenable to ex vivo manipulation, even though current methods for expansion of these cell populations in vitro still need to be optimized. Further studies of the multipotent AC133$^+$ stem cells will be essential for identifying the signals governing their definitive myogenic differentiation and to improve transplantation efficiency in DMD.

Methods

Isolation of human AC133$^+$ cells from the blood. Blood was obtained from 34 healthy volunteer subjects (3–55 years of age) after informed consent was obtained, according to the guidelines of the Committee on the Use of Human Subjects in Research of the Policlinico Hospital of Milan (Milan, Italy). Samples of human blood were diluted 1:3 in Iscove's modified Dulbecco's medium (IMDM) (Gibco; Invitrogen Life Technologies, Grand Island, New York, USA). Mononuclear cells were collected by centrifugation (Ficoll-Hypaque; Pharmacia Biotech, Uppsala, Sweden), incubated with AC133 monoclonal antibody–conjugated with paramagnetic MicroBeads (AC133 Isolation Kit; Miltenyi Biotech, Bergisch-Gladbach, Germany), washed, and processed through a MACS magnetic separation column (Miltenyi Biotech) to obtain purified AC133$^+$ cells. After selection, an aliquot of the AC133$^+$ cell fraction was analyzed for assessment of purity.

Characterization of human blood–derived AC133$^+$ cells for FACS analysis. The purity of AC133-selected cells was determined for each isolation experiment. For three-color flow cytometry, 5×10^4 cells were incubated with anti-AC133–phycoerythrin (anti-AC133–PE) (Miltenyi Biotech), anti-CD34–PE (BD Biosciences — Pharmingen, San Diego, California, USA), anti–CDw90 (Thy-1)–fluorescein isothiocyanate (FITC; BD Biosciences — Pharmingen), anti–VEGFR (KDR)–PC5 (BD Biosciences — Pharmingen), anti-CD45–FITC (BD Biosciences — Immunocytometry Systems, Mountain View, California, USA). Isotype-matched mouse immunoglobulin served as a control. After each incubation performed at 4°C for 20 minutes, cells were washed in PBS containing 1% heat-inactivated FCS and 0.1% sodium azide. Cells were analyzed with a FACSCalibur flow cytometer and CellQuest software (BD Biosciences — Immunocytometry Systems). Each analysis included at least 5,000–10,000 events. A light-scatter gate was set up to eliminate cell debris from the analysis. The percentage of AC133$^+$ cells present in the sample was assessed after correction for the percentage of cells reactive with the isotype control. With isotype controls for PE and FITC, gates for phenotypic analysis of CD34$^+$ cells were set so that the lower left panel on the resulting dot plots contained at least 98% of the total cells analyzed.

Cell culture. AC133$^+$ cells isolated from blood tissue were plated in Falcon six-well tissue culture plates (Falcon Labware, Oxnard, California, USA) at a density of 10^5 cells per well in the presence of PM, composed of DMEM/F-12 (1:1) and 20% FBS, plus

HEPES buffer (5 mM), glucose (0.6%), sodium bicarbonate (3 mM), and glutamine (2 mM). The following cytokines were added to the PM: stem cell factor (SCF), 100 ng/ml (Tebu-Bio, Frankfurt, Germany); VEGF, 50 ng/ml (Tebu-Bio); and leukemia inhibiting factor, 20 ng/ml (R&D Systems Inc., Minneapolis, Minnesota, USA). Cells were passaged every 8 days. For determination of myogenic potential, AC133$^+$-derived cells were maintained in PM as they reached 60–70% confluence. At this time, cells were exposed to DM, which consisted of Ham's F10 medium supplemented with 5% FBS, 10 ng/ml EGF, 10 ng/ml PDGF-B, and antibiotics as described above.

Coculture experiments. For coculture experiments, C2C12 murine myoblasts or Wnt-expressing 10T1/2 fibroblasts (28) were mixed at a ratio of 5:1 with human AC133$^+$ or AC133$^-$ cells derived from blood. Human AC133$^+$ and AC133$^-$ cells derived from blood were transduced with a third-generation lentivirus vector expressing eGFP cDNA as a reporter gene and were cocultured with C2C12 murine myoblasts (15). The cocultures were maintained in PM for 3 days and then were switched to DM for differentiation experiments. After 14 days of culture, the percentage of differentiated myotubes containing two or more nuclei (i.e., the fusion index) was assessed. Differentiated muscle cells were detected by immunostaining with antibodies directed against slow MyHCs and human lamin A/C.

Self-renewal and pluripotentiality of human blood–derived AC133$^+$ cells. To determine whether circulating AC133$^+$ cells exhibit the stem cell characteristic of self-renewal, we plated AC133$^+$-derived cells at a density of approximately 10 cells/cm^2, grew them to a density of 50–150 cells per colony, isolated them with cloning cylinders, and transferred them to separate wells on a mouse embryonic fibroblast feeder layer inactivated by mitomycin C (10 ng/ml). These conditions promoted the proliferation of blood-derived AC133$^+$ cells in the presence of RPMI 1640 medium supplemented with 20% FBS, 2 mM glutamine, 200 U/l penicillin, 200 μg/l streptomycin, and 5 mM HEPES. The activity of clonal AC133$^+$-derived cells was monitored in different in vitro conditions.

The ability of the cloned human circulating AC133$^+$ to undergo differentiation into hematopoietic lineages was tested with methylcellulose culture using a method described previously (24). In our experiments, 1 ml of culture medium included about 10^3 cells, 1.2% methylcellulose (Fisher Scientific, Norcross, Georgia, USA), and IMDM containing 15% FBS, 1% deionized bovine serum albumin (Sigma-Aldrich, St. Louis, Missouri, USA), 0.1 mmol/l 2-mercaptoethanol (Eastman Organic Chemicals, Rochester, New York, USA), 150 ng/ml recombinant mouse SCF (Sigma-Aldrich), and 10% (volume/volume) X63 IL-3–conditioned medium. The mixture was incubated in 35-mm non–tissue culture dishes (Falcon Labware) in a humidified atmosphere with 5% CO_2 at 37°C. The colonies were counted under an inverted microscope after 8 days of culture. Colony types were confirmed by being lifted from the semisolid medium on day 8 of culture, and Cytospin preparations (Shandon Southern Instruments Inc., Sewickley, Pennsylvania, USA) were stained with May-Grunwald-Giemsa. For induction of differentiation in endothelial cells, cloned AC133$^+$-derived cells were cultured as described previously (51) in endothelial growth medium (M199; Gibco BRL; Invitrogen Life Technologies) supplemented with 20% FBS (HyClone Laboratories, Logan, Utah, USA), VEGF (10 ng/ml, Sigma-Aldrich), FGF (5 ng/ml; human recombinant basic FGF; Sigma-Aldrich), heparin (5 U/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml). These cells were placed on 12-well plates coated with 0.2% gelatin and were incubated at 37°C in a humidified environment with 5% CO_2. This process resulted in attachment of cells consisting mostly of monocytes or mature endothelial colonies on the well plates. Nonadherent cells were transferred after 4 to 5 days to other wells coated with 0.2% gelatin and grown in endothelial growth medium. Endothelial colonies were identified with primary mouse antibodies against human VE-cadherin (BD Biosciences — Immunocytometry Systems) and CD31 (Chemicon International, Temecula, California, USA) used at a dilution of 1:100 and were visualized with an HRP-coupled secondary antibody (BioRad Laboratories, Toronto, Ontario, Canada) in PBS containing 0.6 mg/ml diaminobezidine (Sigma-Aldrich). Human umbilical vein endothelial cells were used as a positive control for VE-cadherin staining.

Telomerase assays. The telomerase activity of blood-derived AC133$^+$ cells was determined using the TRAP (23) with the TRAPeze telomerase detection kit (Oncor, Gaithersberg, Maryland, USA).

In vitro immunohistochemistry and PCR analysis of human blood–derived AC133$^+$ cells. For immunocytochemistry, cells plated on Lab-Tek chamber slides (Life Technologies) were fixed in ethanol 70% in PBS for 1 minute and were permeabilized for 5 minutes with 0.5% Triton X-100 in PBS. Cells were than incubated overnight at 4°C with primary antibodies against CD31 (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, California, USA), VE-cadherin (1:100 dilution; Chemicon International), CD34 (1:50 dilution; BD Biosciences — Immunocytometry Systems), VEGF/R2 (1:20 dilution; Sigma-Aldrich), α–smooth muscle actin (DAKO, Carpinteria, California, USA), CD45 (1:100 dilution; BD Biosciences — Immunocytometry Systems), CD14 (1:50 dilution; DAKO), CD11b (1:200 dilution; DAKO), desmin (1:20 dilution; Sigma-Aldrich), human MYOD (1:50 dilution; BD Biosciences — Pharmingen), M-cadherin (1:50 dilution; Nanotools, Munich, Germany), fibronectin (1:150 dilution; Sigma-Aldrich), cytokeratin (1:150 dilution; DAKO), vimentin (1:200 dilution; Santa Cruz Biotechnology), CD4 and CD8 (1:100 dilution; Santa Cruz Biotechnology), and CD14 (1:50 dilution, Santa Cruz Biotechnology). After being washed with PBS, cells were incubated with FITC-conjugated goat anti-mouse IgG for 1 hour at room temperature and were examined by epifluorescence microscopy. For quantitative analysis, after immunostaining, coverslips were counterstained with 4,6-diamidino-2-phenylindole and anti–human nuclear lamin A/C (1:200 dilution; Novocastra Laboratories, Newcastle, United Kingdom). Western blot conditions are specified in ref. 24. Briefly, 100 μg of extracted proteins were separated by 6% polyacrylamide gel electrophoresis and were electrotransferred onto nitrocellulose membranes (BioRad Laboratories). Membranes were subsequently incubated with monoclonal antibodies directed against human MyHC (Sigma-Aldrich) and human M-cadherin, and the proteins bound to antibody were revealed using a commercially available chemiluminescence kit (Ultra ECL; Pierce, Rockford, Illinois, USA). Membranes were then exposed to BioMax autoradiography films (Kodak, Rochester, New York, USA), which were developed and scanned with a densitometer. Total RNA was extracted from cells or muscles from injected mice by TrizolReagent as indicated by the manufacturer's protocol (Gibco; Invitrogen Life Technologies). First-strand cDNA was prepared with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies), starting with 2 μg total RNA and oligo(dT)$_{12–18}$ priming. For direct amplification of human markers, primers were specifically designed in nonhomology regions of human-mouse mRNA

Table 2
mRNA sequences of primers for human markers

Gene	FW/RV sequence 5′→3′	PCR fragment
MRF4 (MYF6)	GATCCCACCGACCCTTCCTGG	215 bp
	GAGGCTAGACCTAAGCCACTCGC	
Myogenin	GTCTTCCAAGCCGGGCATCCTTG	280 bp
	GAGCTGGGGCATACACGAGGGG	
MYOD	CGATATACCAGGTGCTCTGAGGG	430 bp
	GGGTGGGTTACGGTTACACCTGC	
MYF5	CCAGGCTTATCTATCATGTGCTATG	320 bp
	GTTAAGCATTGCAACAAGCTACCC	
PAX7	GTACGGCCAGAGTGAGTGCCTG	245 bp
	CTGTTGGAGCCATAGTACGGAAG	
M-cadherin	CCCGTGCGGGTTGGAGTACGG	360 bp
	CACTGGTAGGAGGCAAAGGTGGG	
Nestin	GGAGTGGAAAATAAGGATGAGGCAG	390 bp
	CTGGCTCAGCTCCCGCAGCAG	
CD34	GACACTGTGGACTTGGTCACCAG	270 bp
	GAGGAGGAAGCCATGGAGATCAG	
β-Actin	TGGCACCACACCTTCTACAATGAG	350 bp
	CCGTGGTGGTGAAGCTGTAGCC	

FW/RV, forward (top line) and reverse (bottom line).

sequences (see Table 2). PCR was performed with the following conditions: 94°C for 5 minutes, then 35 cycles at 94°C for 40 seconds, 68°C for 40 seconds, and 72°C for 1 minute.

Mice. In order to transplant human stem cells in dystrophic *mdx* (52) mice, we created an immune-incompetent host by crossing *mdx* mice with *scid* mice that lack both T and B lymphocytes (53). Blood samples of *scid/mdx* mice were characterized by flow cytometry and PCR analysis, and only CD4/CD8/B220–depleted mice were selected for transplantation studies. In cytofluorimetric analyses, mononucleated cells from the blood were stained with CD4, CD8, and CD45 markers. The presence of the *mdx* point mutation in the *scid* genome was tested by PCR analysis of blood tissue as described previously (41). The sense PCR primer has been designed in order to introduce a mutation that creates a new site for the restriction enzyme *Mae*III in the sequence of the normal dystrophin gene but not in the mutated gene of *mdx* mice (42). In this manner, it was possible to recognize the heterozygous and homozygous *mdx* phenotypes. PCR amplification of genomic DNA obtained from *scid/mdx* showed the presence of 150- and 50-bp bands, confirming the homozygous *mdx* phenotype (52).

Transplantation of human blood–derived AC133⁺ cells into dystrophic muscle and sorting of labeled, injected cells. Two-month-old *scid/mdx* mice were anesthetized with ketamine hydrochloride (80 mg/kg) and xylazine (10 mg/kg). Human AC133⁺ cells isolated from the blood were injected (20×10^3 cells in 7 μl of PBS) into the TA muscle ($n = 15$). Three injections were performed and the needle was left in position for approximately 3 minutes and then was gradually withdrawn, and the skin overlying the muscle injection site was sealed with surgical glue. Intra-arterial delivery of 5×10^5 cells of human AC133⁺ cells isolated from blood was also performed in nine *scid/mdx* mice as described previously (41). In all treated animals, muscle degeneration-regeneration was induced 24 hours before transplantation by intense swimming exercise (41). Then, 21, 45, and 60 days later, muscle tissues were removed, frozen in liquid nitrogen–cooled isopentane, and cut into serial sections on a cryostat. Grafted cells were also labeled with PKH26 as described previously (54). In a group of five 2-month-old dystrophic *scid/mdx* mice, we also evaluated the in vivo myogenic potential of 20×10^3 cloned AC133⁺-derived cells 21 days after their transplantation into the right TA. As described above, the muscle damage in these animals was induced by swimming exercise 24 hours before transplantation. Flow cytometry of an aliquot of the injected population showed that more than 98% of the cells were positive for AC133.

For histochemistry of tissue sections, samples were frozen in liquid nitrogen–cooled isopentane and were sectioned on a cryostat. Serial sections 8 μm in thickness were counted at 80-μm intervals and were examined by immunohistochemical analysis and with H&E staining. Detection of human dystrophin was performed with monoclonal antibodies directed against the 60-kDa carboxy-terminal fragment of dystrophin (NCL-Dys3; Novocastra Laboratories) as described previously (41). Monoclonal anti–mouse laminin (Chemicon International) was used at a dilution of 1:100. To improve the cadherin staining, we used anti–M-cadherin (1:50 dilution) with 10 mM Ca^{2+}. Nonetheless, given that PKH26 labeling is diluted in proliferating cells, we evaluated whether blood-derived AC133⁺ cells labeled with PKH26 were still detected using a sorting procedure in a group of three *scid/mdx* mice 14 days after intramuscular transplantation. In this experiment, injected muscles were gently dissociated by enzymatic and mechanical procedures as described in Methods, and mononucleated muscle-derived cells were sorted for the presence of the cell surface dye PKH26 with FACSVantage cell sorting (BD Biosciences — Immunocytometry Systems). Expression of M-cadherin and CD34 proteins was assessed by labeling of portions of positively selected fractions with PE-conjugated anti-CD34 (RAM34; BD Biosciences — Pharmingen) and Alexa-conjugated M-cadherin anti-mouse (Sigma-Aldrich). The effect of blood-derived AC133⁺ on host muscle regeneration was evaluated by calculation of the mean of the diameter of myofibers, the coefficient of variance of the diameter, and the percentage of regenerative myofibers from acquired digital images using ImageQuest software with a DMIR2 microscope (Leica, Brussels, Belgium). Images were also captured with the Leica TCS SP2 confocal system (Leica).

Explant cultures of injected muscles. For explant experiments, the TA muscle was excised from ten *scid/mdx* mice injected with human circulating AC133⁺ cells 45 days before and was finely minced in the presence of 0.2% collagenase type IV. Then, 25–50 explants were plated into noncoated Petri dishes (diameter, 60 mm) in 2 ml of growth medium consisting of DMEM containing 0.1 g/l gentamicin and 20% FCS (Gibco; Invitrogen Life Technologies). Serum that would optimize the growth capacity of human myoblasts was selected. After mononucleated cells had migrated out from the explants (between 5 and 7 days), the cells were removed by trypsinization (1.5% trypsin and 0.04% EDTA) and were replated at a density of 1.8×10^3 cells/cm² in 5 ml of the same growth medium in 60-mm dishes. This culture medium was changed three times a week. At the time of cell isolation, all cell populations were considered to be at one mean population doubling. At between five and ten divisions after their isolation, they were plated at clonal density (6 cells/cm²). When individual clones contained about 100–200 cells, growth medium was replaced by differentiation medium and cells were analyzed for expression of desmin, MyHC, and human lamin A/C.

CSA and Po of single fibers after transplantation. The experimental approach and the solutions used to determine the CSA and Po of isolated muscle fibers have been described previously in detail (55,

56). "Skinned" fibers (that is, fibers whose plasma membrane is removed) were used. As it is difficult to dissect intact fibers from skeletal muscles of small mammals, "skinned" fibers have been widely used to study the contractile properties of muscle fibers from small mammals and humans and have proven to be very reliable specimens for this kind of analysis (57, 58). "Skinned" fibers lacking plasma membranes were maintained in a relaxed state in solutions that did not contain calcium (relaxing solution) and were activated by exposure first to preactivating solution and then to a solution containing the activating ion Ca^{2+} (activating solution). The solutions used, skinning (5 mM EGTA; pCa 9.0), relaxing (5 mM EGTA; pCa 9.0), preactivating (EGTA 0.5 mM; pCa 9.0), and activating (EGTA 5 mM; pCa 4.5), were prepared as described previously (55, 56). After the sacrifice of the animal, muscle bundles to be used for single-fiber analysis were pinned at the bottom of a Petri dish covered with Sylgard (Dow Corning, Midland, Michigan, USA) and filled with a 1:1 mixture (volume/volume) of skinning solution and glycerol. The Petri dish was stored at –20°C for up to 3 weeks. On the day of the experiment, a bundle was transferred to a dish containing skinning solution maintained at 10°C and, under a stereomicrosope (magnification, ×10 to ×60; Wild M3; Leica), single fibers were dissected manually. Fibers were chemically skinned by exposure to skinning solution plus 1% Triton X-100 for 1 hour. Light aluminium clips were attached to both ends of fiber segments (0.5–1.0 mm in length), which were then transferred to the experimental setup for mechanical measurements. The experimental setup (41, 42) enabled determination of CSA and sarcomere length (SL) at a magnification of ×320, and of Po (55, 56). For Po determination, fiber segments were maximally activated (pCa 4.5) at 12°C and optimal SL for force developing (2.5 μm) for 40–60 seconds. Activation was repeated at least three times and mean maximum force was calculated. At the end of the mechanical experiment, the fiber was removed from the setup and placed in sample solution (59) for subsequent electrophoretic analysis of MyHC isoform composition. MyHC isoform analysis was performed on 8% polyacrylamide slab gels after denaturation in SDS (SDS-PAGE) and enabled fiber typing (55, 56). In the MyHC region, four bands corresponding to the four adult MyHC isoforms (MyHC-1, MyHC-2A, MyHC-2X, and MyHC-2B) can be separated. On the basis of their MyHC isoform content, single fibers from mice can be classified as four pure fiber types (types 1, 2A, 2X, and 2B) and three hybrid fiber types (types 1-2A, 2AX, and 2XB) (55). As the majority of fibers (71–76%) used for functional analysis were found to contain only MyHC-2B, the comparison focused on type 2B fibers. For Western blot analyses, 100 μg of protein extracted from human vastus lateralis, TA of *scid/mdx* mice, and TA of *scid/mdx* TR mice was separated by 6% polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose membranes (BioRad Laboratories) and was incubated with monoclonal antibodies directed against either human dystrophin (NCL-Dys3; Novocastra Laboratories) or human -sarcoglycan (NCL-d-SARC; Novocastra Laboratories) and revealed with a commercially available chemiluminescence kit (Ultra ECL; Pierce).

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Address correspondence to: Yvan Torrente, Department of Neurological Science, University of Milan, Padiglione Ponti, Ospedale Policlinico, via Francesco Sforza 35, 20122 Milan, Italy. Phone: 39-02-55033874; Fax: 39-02-50320430; E-mail: torrenteyvan@hotmail.com.

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