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Autologous Transplantation of Muscle-Derived CD133$^+$ Stem Cells in Duchenne Muscle Patients

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Duchenne muscular dystrophy (DMD) is a lethal X-linked recessive muscle disease due to defect on the gene encoding dystrophin. The lack of a functional dystrophin in muscles results in the fragility of the muscle fiber membrane with progressive muscle weakness and premature death. There is no cure for DMD and current treatment options focus primarily on respiratory assistance, comfort care, and delaying the loss of ambulation. Recent works support the idea that stem cells can contribute to muscle repair as well as to replenishment of the satellite cell pool. Here we tested the safety of autologous transplantation of muscle-derived CD133$^+$ cells in eight boys with Duchenne muscular dystrophy in a 7-month, double-blind phase I clinical trial. Stem cell safety was tested by measuring muscle strength and evaluating muscle structures with MRI and histological analysis. Timed cardiac and pulmonary function tests were secondary outcome measures. No local or systemic side effects were observed in all treated DMD patients. Treated patients had an increased ratio of capillary per muscle fibers with a switch from slow to fast myosin-positive myofibers.

Key words: Autologous stem cell transplantation; Muscular dystrophy; CD133

INTRODUCTION

The muscular dystrophies are a heterogeneous group of inherited disorders characterized by progressive muscle wasting and weakness. In Duchenne muscular dystrophy (DMD), which is due to lack of a functional dystrophin protein, skeletal and cardiac muscles are affected, leading to wheelchair dependency, respiratory failure, and premature death. Actually, the development of a clinical effective therapy for the muscular dystrophies remains a big challenge. However, as the final therapeutic goal would be the expression of a functional dystrophin in muscles, gene therapy presents itself as a potentially powerful treatment option. In this direction, a phase I clinical trial was assessed involving plasmid-mediated delivery of dystrophin cDNA to the muscle, but a low level of dystrophin expression was detected in dystrophic muscle fibers (23,24). Subsequent studies have indicated that the delivery of micro-dystrophin by adeno-associated viruses (AAV) to mdx mice can result in widespread transduction and improved muscle function (11,34). Nevertheless, it has yet to be determined if

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these minimal dystrophin proteins fully compensate for the lack of dystrophin in DMD patients.

Other strategies propose to use targeted corrective gene conversion methods; exon skipping is likely the most promising. More than 70% of DMD mutations are due to frame-shift deletions into the central rod domain of the dystrophin; this protein is well adapted for exon skipping application because in-frame removing of central spectrin-like repeats retains its functionality (19). In vivo exon skipping was previously achieved in mdx model through a gene therapy approach using an AAV vector to spread specific antisense oligonucleotides in dystrophic muscles (6,10). Even if the use of AAV vectors in DMD patients currently is envisaged, their use is principally limited by the high viral titers needed for clinical application and the immune response against the vector. New generations of viral vectors and increasing knowledge about various types of myogenic progenitors should be the basis for the next generation of trials, which may benefit from the design of combined gene and cell therapies. Patients with DMD could potentially be treated using their own myogenic progenitors that have been manipulated by gene therapy or exon skipping. In turn, these will need to be tested on patients independently from gene or cell therapy to verify the safety of these approaches for successive combined therapy.

In fact, from a safety perspective, questions remain about whether adult myogenic progenitors in the dystrophic muscle truly undergo functional integration and whether this may give rise to nonmuscle tissue formation, such as inflammatory cells or connective formation into grafted muscles. This would justify the planning of phase I clinical trials that could predict the further applications from ongoing experimental work. Thus, the identification of human myogenic progenitors might enhance the possibility to achieve an autologous cell therapy leading to the reexpression of a functional dystrophin in DMD skeletal muscles. Although myoblasts remain the cell type that retain the main myogenic activity in adult muscle (12,18), the clinical trials using myoblast transplantation were largely unsuccessful in terms of clinical benefit to the patient. A possible reason for these failures was related to an inadequate or a total lack of immunosuppressive treatment that allowed an immune reaction, with antibodies directed not only against donor cells but also against the wild-type dystrophin protein in the recipient patient (13,14,25,33). Further therapeutic trials involving immunosuppression in BMD (21) or DMD recipients (12,16,18) showed only a small beneficial effect with a transient expression of donor dystrophin and/or a small strength improvement in the transplanted muscles.

Myoblast transplantation was continued and optimized in few laboratories in preclinical studies and, recently, a novel clinical trial with multiple injections of partially matched donor cells and immune suppression showed reconstitution of up to 25% of dystrophin-positive fibers in the area of injection (28,29). However, myoblasts do not cross the muscle endothelium when delivered systemically and must be injected intramuscularly (22). The major problem still faced by this approach is the lack of dispersion of donor cells, which remain in the area of injection, making it difficult to reach an even distribution within the whole muscle. This feature alone makes their use in cell therapy protocols extremely difficult, at least with current technology.

Recent works support the idea that stem cells reach the site of muscle regeneration and contribute to muscle repair as well as replenish the satellite cell pool following arterial injection, suggesting that this technique is particularly suited for treating muscle dystrophy (7,8,27, 32). Moreover, from a clinical point of view, for diseases where there is little source of muscle precursors, such as DMD, adult stem cells represent a possible cellular target for the autologous transplantation of genetically corrected cells. The use of autologous $CD133^+$ bone marrow-derived cells to induce angiogenesis and restore myocardial tissue viability after infarction has already been shown to be beneficial (30). Moreover, stem cells positive for CD133 expression and negative for the hematopoietic and endothelial markers such as CD34, CD45, CD31, and KDR were also isolated from human skeletal muscle.

Although conclusive evidence is still lacking, there are many reports suggesting that muscle-derived stem cell (MDSC) may represent a population of progenitors lying in the interstitial spaces that originate from circulating bone marrow-derived cells and contribute to both muscle regeneration and to replenishment of the satellite cell pool (32). The aim of our study was to verify the safety of autologous transplantation of muscle-derived $CD133^+$ stem cells in muscular dystrophy and reasons are indicated in the following points. i) When approaching clinical trials of stem cells in muscular diseases, we should proceed cautiously because premature, inadequately designed clinical trials can substantially set back progress in an otherwise promising field of research. From a safety perspective, questions remain about whether adult stem cells in the dystrophic muscle truly undergo functional integration and whether this may give rise to nonmuscle tissue formation such as inflammatory cells or connective formation into grafted muscles. ii) Moreover, autologous transplantation of freshly isolated adult stem cells would represent a safe way to proof the principle of stem cell treatment for muscular dystrophy excluding the use of immunosuppressive drugs and minimizing the additional oncogenic risk and

or the transmission of infectious agents produced by extensive ex vivo proliferation. iii) Finally, the demonstration of the safety of the autologous transplantation of muscle-derived CD133+ stem cells will allow the authors to continue further study and address genetic engineering of engrafted cells for clinical gain.

MATERIALS AND METHODS

Cell Culture

Primary human muscle cells were obtained by enzymatic dissociation of biceps brachii muscle biopsies from 5–45-year-old donors and cultured as described previously (1). Mononuclear cells from muscle were then processed through a MACS magnetic separation column (Miltenyi Biotec) to obtain purified CD133+ cells. After selection an aliquot of the CD133+ cell fraction was analyzed to assess purity and the rest of cells were plated in the presence of a proliferation medium (PM) composed of DMEM/F-12 (1:1), 20% FBS, including HEPES buffer (5 mM), glucose (0.6%), sodium bicarbonate (3 mM), glutamine (2 mM), SCF (100 ng/ml; TEBU, Frankfurt, Germany), VEGF (50 ng/ml; TEBU), and LIF (20 ng/ml; R&D Systems, Inc). To determine the myogenic potential the CD133+-derived cells were exposed to differentiation medium consisted of Ham's F10 supplemented with 5% FBS, 10 ng/ml EGF, 10 ng/ml PDGF-BB, and antibiotics as described above. After 14 days of culture, enumeration of differentiated myotubes containing two or more nuclei (i.e., fusion index) and immunostaining with antibodies directed against slow myosin heavy chain (MyHCs) and desmin was assessed. To determine whether CD133+-derived cells exhibit stem cell characteristics of self-renewal, cells were plated at approximately 10 cells/cm^2, grown to 50–150 cells per colony, isolated with cloning cylinders, and transferred to separate wells. CD133+ cells isolated from normal and dystrophic muscles were also analyzed for their release of angiogenic factors on Angiokit (TCS CellWorks Ltd, Buckingham, UK) according to the manufacturer's protocols.

Characterization of Human CD133+ Cells Isolated From Muscle Tissues for FACS Analysis

The purity of muscle-derived CD133-selected cells was determined for each isolation experiment. We performed the flow cytometry count and the immunophenotyping of both muscle-derived and blood-derived CD133+ cells. Cell viability was determined using 7-amino-actinomycin D (7-AAD) viability probe. For four-color flow cytometric analysis, at least $10–20 \times 10^4$ cells from dissociated muscles or $30–80 \times 10^4$ cells from peripheral blood were incubated with the following monoclonal antibodies (mAbs): anti-CD133/2-phycoerythrin (PE) (Miltenyi, Biotec), anti-CD34-Allophycocyanin (APC) [Becton Dickinson (BD), Immunocytometry Systems, Mountain View, CA, USA], anti-CDw90 (Thy-1)-fluorescein-isothiocyanate (FITC, BD), anti-VEGF-R2 (KDR)-PE (R&D Systems, Inc.), anti-CD184 (CXCR4, fusin)-PE-Cy5 (BD), anti-CD45-FITC (BD), and anti-CD20-FITC (BD). For each mAb, an appropriate isotype-matched mouse immunoglobulin was used as control. After staining, performed at 4°C for 20 min, cell suspensions were washed in PBS containing 1% heat-inactivated FCS and 0.1% sodium azide. Cells were analyzed using a FACS Calibur flow cytometer and PAINT-a Gate software (BD). Each acquisition included at least 50,000-100,000 events. A light-scatter gate was set up to eliminate cell debris from the analysis. Subpopulations of CD133+ cells were analyzed on multiple double-fluorescence dot plots, activating a gate on cells with low side scatter and bright expression of CD133 antigen.

In Vitro Immunohistochemistry and PCR Analysis of Human Muscle-Derived CD133+ Cells

For immunocytochemistry, cells plated on lab-tek chamber slides (Life Technologies) were fixed in 70% ethanol in PBS for 1 min and permeabilized for 5 min with 0.5% Triton X-100 in PBS. Cells were than incubated with primary antibodies against the CD31 (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), VE-Cad (1:100; Chemicon International, CA, USA), CD34 (1:50; BD), VEGF/R2 (KDR)(1:20; Sigma), anti-human CD133/2 antibody (clone 293C3; Miltenyi Biotec), anti-α-smooth muscle actin (DAKO, Carpinteria, CA, USA), CD45 (1:100; BD), CD14 (1:50; Dako), CD11b (1:200; Dako), desmin (1:20; Sigma), M-cadherin (1:50; Nano Tools), fibronectin (1:150; Sigma), cytokeratin (1:150; Dako), vimentin (1:200; Santa Cruz), CD4 and CD8 (1:100; Santa Cruz), and CD14 (1:50, Santa Cruz) overnight at 4°C. After washing with PBS, cells were incubated with FITC-conjugated goat anti-mouse IgG for 1 h at room temperature and examined by epifluorescence microscopy. For quantitative analysis, after immunostaining, coverslips were counterstained with DAPI and anti-human nuclei (1:200; Sigma). Western blot conditions are specified in Torrente et al. (32). Briefly, the concentration of proteins was determined using the Lowry technique. Extracted proteins (100 μg) were separated on 6% polyacrylamide gels and electrotransferred onto nitrocellulose membranes (Biorad, Toronto Ont., Canada). To ensure that equivalent amounts of proteins were loaded for each sample, the membrane was stained with Ponceau S. (Sigma). Membranes were subsequently incubated with monoclonal antibodies directed against human MyHC (Sigma) and human M-cadherin and revealed using a commercially available chemiluminescence kit (Ultra ECL, Pierce, Rockford, IL, USA). Membranes were then exposed to BioMax

autoradiographic films (Kodak, Rochester, NY, USA), which were developed and scanned with a densitometer. Total RNA was extracted from muscles from injected mice by Trizol Reagent as indicated by the manufacturer's protocol (Gibco BRL, Life Technologies). First-strand cDNA was prepared by using Super Script First Strand Synthesis System for RT-PCR (Invitrogen, Life Technologies), starting from 2 μg total RNA with oligo $(dT)_{12–18}$ priming. For direct amplification of human markers, primers were specifically designed in the nonhomology region of human–mouse mRNA sequences as previously described (21). PCR was performed under the following conditions: 94°C for 5 min, and then 35 cycles at 94°C for 40 s, 68°C for 40 s, and 72°C for 1 min.

Clinical Protocol

This study was designed as a double-blind phase I clinical trial and was approved by the Ministry of Health and the Ethical Committee of the IRCCS Ospedale Maggiore Policlinico di Milano and the Ethical Committee of the IRCCS Eugenio Medea Bosisio Parini. After obtaining assent from patients and consent from parents of all participants, patients underwent two screening visits. If patients met all inclusion criteria, they were enrolled and randomly assigned to either stem cell group or sham group. Sample size was determined from 55 children who were assessed for eligibility and eight children were selected on the basis of the inclusion and exclusion criteria indicated in Table 1. Blinding was achieved according to the "double-dummy technique." Determination of whether a patient would be treated by stem cell (A case) or by saline solution alone (B case) was made by reference to a statistical series based on random sampling numbers drawn up by Professor Bresolin. The details of the series were unknown to any of the investigators or to the coordinator and were contained in a set of sealed envelopes bearing on the outside only the name of the hospital and a number. After acceptance of a patient the corresponding numbered envelope was opened at the central office; the card inside told if the patient was to be an A or B case, and this information was then given to the coordinator. Mean age of the enrolled children was 126.75 ± 21.28 months. At the muscular evaluation through the MRC scales, the muscular strength value at the upper limbs ranged from 3 to 4 proximally, and from 4 to 5 distally; at the lower limbs, proximal strength ranged from 3 to 4 and distal was 4. Blood tests did not show any signs of immunological deficits, inflammations, or endocrine dysfunctions. CK levels ranged from 4000 to 6000 U/L. None of the eight children presented cardiac dysfunction or respiratory failure. An ECG showed minimal abnormalities as reported in most of the DMD children (4) and echocardiography showed an ejection fraction >55–60% for all the children. The intelligence quotient was within the range typically shown in DMD children (5).

Table 1. Patient Selection Criteria

Inclusion criteria
- Age at least 4 years old
- Diagnosis of Duchenne muscular dystrophy confirmed by: a) clinical evaluation with observation of proximal muscle weakness Gowers sign positive), pseudohypertrophy of the calves, lordotic and wide-based gait and stance; b) increase of the levels of muscular enzymes creatin kinase; c) muscle biopsy and dystrophin analyses (IF, WB, and molecular analysis) consistent with DMD)
- Preserved ability to ambulate at the time of the selection
- Adequate muscle strength and muscle bulk at the tibialis anterior

Exclusion criteria
- Patients not ambulant at the moment of the inclusion
- Onset symptoms before the age of 2–3 years old
- Severe cardiac and respiratory dysfunction
- Deficient immune system and/or autoimmune disease
- Presence of additional diseases (i.e., family history of epilepsy, cerebral palsy)
- Mental retardation (Intelligence Quotient through the Wechsler Intelligence scale above 70)
- Steroid therapy in the previous 6 months
- Psychological/psychiatric disorders

Autologous Transplantation of Muscle-Derived CD133$^+$ Stem Cells in DMD Patients

In preclinical studies, we excluded the tumorigenic potential of muscle-derived CD133 by transplantation in SCID and SCID/mdx mice (data not shown). The muscle-derived cells were obtained after enzymatic (Liberase HI) and mechanical dissociation of 1 g of muscle biopsy and grown in serum-free condition for 48 h in a quality controlled facility (Cell Factory, Milan, Italy). The presence of mycoplasma and bacteria in the cell culture was tested during this time of culture. After 48 h of culture the CD133$^+$ stem cells were isolated from the muscle-derived cells using the MACS method; 20,000 freshly isolated stem cells were resuspended in saline solution and 15-μl Hamilton syringes with 27-gauge needles were filled with this suspension. The appearance of the treatment (intramuscular transplantation) was identical to the subjects. Three parallel injections were done at 1 mm of interdistance in the middle of the left abductor digiti minimi (ADM) muscle either with cells (stem cell group A) or with saline solution (sham group B). For anesthesia, premedication was given with oral midazolam (0.40–0.60 mg/kg). Analgesia and sedation were achieved with fentanyl (total dose 25–75 γ) in repeated IV boluses during the initial biopsies.

The primary end point was percentage change in averaged maximal isometric voluntary contraction (MIVC) score of treated and untreated ADM muscles of all subjects. Secondary outcome measures included change in the MRI and histological analysis, cardiac and pulmonary function tests, and single fiber muscle force analysis. Outcome measures were performed by blinded clinical evaluators who were trained and shown to be reliable before study start-up. At 2, 4, and 6 months after the intramuscular transplantation follow-up studies comprehensive for muscular strength evaluation, neurophysiological analysis, cardiac evaluation, blood tests for antiviral screening (hepatitis A-B-C, HIV1 and 2, CMV, HSV), inflammatory markers, and immunological screening were performed.

Six months after transplantation each of the included patients had an MR evaluation of both untreated and treated ADM using a 1'5 TESLA unit (Avanto, Siemens, Germany). Slices (2–4 mm thick) were acquired in transaxial, coronal, and sagittal planes, using fast spin echo T2 weighted and 2D and 3D gradient echo T1 weighted sequences. Seven months after the injections, we analyzed the muscle biopsies of the treated and untreated ADM muscle of all patients. Muscle specimens and personal data from biopsied patients were labeled by progressive numeric code to make them anonymous to the investigators. Each histological, histochemical, and immunofluorescent reaction was tested on at least two serial sections. The ADM muscle has approximately 2.5 cm of longitudinal length and 0.8 cm of transversal length. These dimensions reflect the totality of included subjects. All muscle biopsies were taken from the side of injection (the central portion of the ventral part of the ADM muscle) and divided in three specimens of approximately 0.4×0.3 cm. The histology and immunhistochemical staining for fiber type distribution were performed on serial sections (8 μm thin) along the longitudinal axis of the first specimen (more than 300 muscle sections) and compared to the contralateral untreated muscle of the same subject.

Quantitative evaluation of necrotic and regenerating fibers and possible inflammatory cells will be made with a Leica microscope with imaging analyzer by four different examiners. Based on our unpublished preclinical results we speculated that transplanted CD133+ stem cells isolated with iron dyne beads could be visualized in muscle cryosections using Prussian blue staining, which recognizes iron particles (3). For the immunohistochemical staining, sections were incubated with the following monoclonal antibodies: anti-myosin heavy chain slow (Novocastra, 1:50), anti-myosin heavy chain fast (Novocastra, 1:30), anti-myosin heavy chain neonatal (Novocastra, 1:30), anti-desmin (DAKO), and anti-CD31 (BD, 1:50). The total number of muscular fibers, MyHC-positive fibers, and CD31+ vessels were quantified by counting them in six random fields. In order to characterize the effects induced by the injection of CD133+ cells we evaluated the expression of several growth factors by Western blot in treated and untreated muscle with anti-IGF-1 (1:100, Santa Cruz), anti-IGF-2 (1:50, Santa Cruz), anti-IGF-2R (1:200, Santa Cruz), and anti-VEGF-B (1:200, Santa Cruz) as previously described (21).

Evaluation of Muscle Function

The isometric force and the compound muscle action potential generated by the activation of the ADM muscle of both sides was measured before (*T*0) and 60 (*T*1), 120 (*T*2), and 180 days (*T*3) after either stem cell implant or saline solution injection.

Maximal Isometric Voluntary Contraction (MVC). The force generated by isometric muscle contraction was measured by a load cell (BC 302, DS Europe, Milan, Italy) connected to a DC-coupled amplifier Cambridge Cyberamp 1902 (Cambridge Electronic Design, Cambridge, UK). The force transducer was adapted through an adjustable metal support on the lateral surface of the second interphalangeal joint of the fifth finger of the hand in order to measure the strength of abduction. The metal support with the transducer was securely fixed to the hand through a specifically designed plastic splint. Patients before *T*0 practiced on the system in order to develop the maximum force. The force trace was displayed on a computer screen to provide participants with feedback, and force values were digitalized and stored on the computer disk for off-line analyses. In each subject, force at different time points, measured as the average value of the highest 20 recorded MVC, was expressed as percent of *T*0. In order to minimize the effect of learning and other variables linked to the repetition over time of experimental procedures we also expressed MVC for each observations (*T*1, *T*2, *T*3) as the difference between the left (treated) and right side (untreated) rate of change of MVC with respect to baseline (*T*0) (Δ%left −; right). A positive value means that the left treated side increased MCV relative to the right untreated side and vice versa. This parameter will be referred as "normalized MVC."

Statistical Analysis

The experimental data are expressed as the mean ± SD. Statistical analysis was performed by using a two-tailed Student *t*-test.

RESULTS

Identification and Characterization of CD133+ Cells Derived From the Normal and DMD Muscle

Cells were isolated from human muscle biopsies (age range 5–45 years old) of 2 cm^3 by proteolytic digestion.

As revealed by flow cytometry (Fig. 1), $CD133^+$ cells were present in both normal and dystrophic muscle-derived cells, although dystrophic muscle biopsies of young patients (5–14 years old) gave rise to a higher number of $CD133^+$ cells (>20 $CD133^+$cells/mg of muscle tissue) than muscle biopsies of healthy donors with similar range of ages (~5 $CD133^+$cells/mg of muscle tissue). On average $CD133^+$ cells represented 2% of the total dystrophic muscle-derived nucleated cells. The majority (more than 79%) of the dystrophic muscle-derived $CD133^+$ cells coexpressed CD34 and Thy-1 antigens (~56%). In contrast, less than 5% of the normal muscle-derived $CD133^+$ cells coexpressed the CD34 antigen, whereas ~53% of them coexpressed Thy-1. Less than 4% of the human dystrophic and normal muscle-derived $CD133^+$ cells were found to express CD45, the pan-leukocyte common antigen (Fig. 1), indicating their hematopoietic commitment. The normal and dystrophic $CD133^+$ muscle-derived cells proliferated in the presence of RPMI-1640 supplemented with 20% FBS, 2 mM glutamine, 200 U/l penicillin, 200 μg/L streptomycin, 5 mM HEPES. In this condition, cells were fed every 6–7 days and were maintained in a fully humidified atmosphere (37°C, 5% CO_2). At 80% confluence cells were dissociated to single cells by gently pipetting and cloned by limited dilution. Single cells were clonogenic, giving rise colonies (Fig. 1) with an efficiency of about 5%. The proliferation rate of muscle-derived $CD133^+$ stem cells was approximately 20 population doublings with a doubling time of approximately 36 h. We performed immunofluorescence staining, RT-PCR, and immunoblotting analysis to investigate whether the $CD133^+$ cells obtained from normal and dystrophic muscle tissues expressed myogenic markers and/or were capable of myogenic differentiation in vitro. RT-PCR with oligonucleotides specific for human Pax-7, Myf-5, MyoD, M-cadherin, MRF-4, and myogenin revealed transcripts for these human isoforms in both normal and dystrophic muscle-derived $CD133^+$ cells after 24 h of culture in the proliferation medium, suggesting a certain degree of myogenic commitment in cells from specimens (Fig. 1).

Detection of Injected Muscle-Derived $CD133^+$ Stem Cells in DMD Patients

Eight patients with Duchenne muscular dystrophy were included in this study and randomized into two groups. Stem cell group A ($n = 5$; subjects 03, 04, 05, 06, 07) and sham group B ($n = 3$; subjects 08, 09, 10). At *T*0, group A underwent stem cell implant in the left abductor digiti minimi muscles (ADM) and group B received saline solution injection into the same muscle. The patient and the experimenter were blind for the type of muscle inoculation. Muscle-derived cells were isolated from the tibialis anterior muscle of all included patients and plated in serum-free conditions. The microbiological and viral analysis of the supernatant of 24- and 48-h muscle cell culture were negative and excluded the presence of contaminant agents in all specimens. After 48 h of culture, the $CD133^+$ cells were enriched from cultured dystrophic muscle-derived cells using a magnetic cell sorting (MACS) (median purity ~88%; range 76–98%) and injected (2×10^4 cells) into the left ADM muscle of the same patient. In this study it was not possible to track the fate of injected cells using reporter genes or membrane dyes. However, $CD133^+$ stem cells isolated by iron dyne beads can be visualized by MRI at high-field strengths (26). Moreover, our preliminary findings demonstrate that $CD133^+$ stem cells isolated using the MACS method incorporate the iron dyne beads and can be visualized by Prussian blue staining in vitro (data not shown). The Prussian blue staining of muscle cryosections of scid/mdx mice transplanted with MACS isolated $CD133^+$ stem cells revealed injected cells as blue spots (data not shown). In these experiments, the number of Prussian blue-positive cells decreased throughout the time of transplantation, leading to few cells after 1 year of $CD133^+$ stem cell transplantation. No recognizable Prussian blue-positive cells in uninjected muscles were detected. A large number of Prussian blue-positive cells was also observed 24 h after the transplantation of $CD133^+$ stem cells with a high percentage of dead cells (more than 95%). However, in this case the staining was different in shape and not visible after 21 days of transplantation. It was therefore believed that isolated $CD133^+$ stem cells would not dilute the iron dyne beads from their membrane if they would enter a state of quiescent progenitor. In this perspective, injected, non-actively dividing cells can be detected using Prussian blue staining. We combined this histological staining and immunohistochemistry to exclude the detection of iron-positive macrophages and characterize the phenotype of injected cells. Prussian blue staining of the untreated muscles, which did not receive muscle-derived $CD133^+$ stem cells, showed no endogenous iron staining. Injected Prussian blue-positive cells were detected only in the treated ADM muscle sections of patients that received autologous muscle-derived $CD133^+$ stem cells. Prussian blue-positive cells were found around muscle fibers and muscle capillaries, which is indicative for the presence of few injected quiescent cells within ADM muscles 7 months after the injections (Fig. 2).

The Intramuscular Transplantation of Autologous $CD133^+$ Muscle-Derived Stem Cells in DMD Patients Is Safe

No systemic adverse effects were observed during the 7 months after autologous transplantation of muscle-derived $CD133^+$ cells. Neither local nor systemic signs

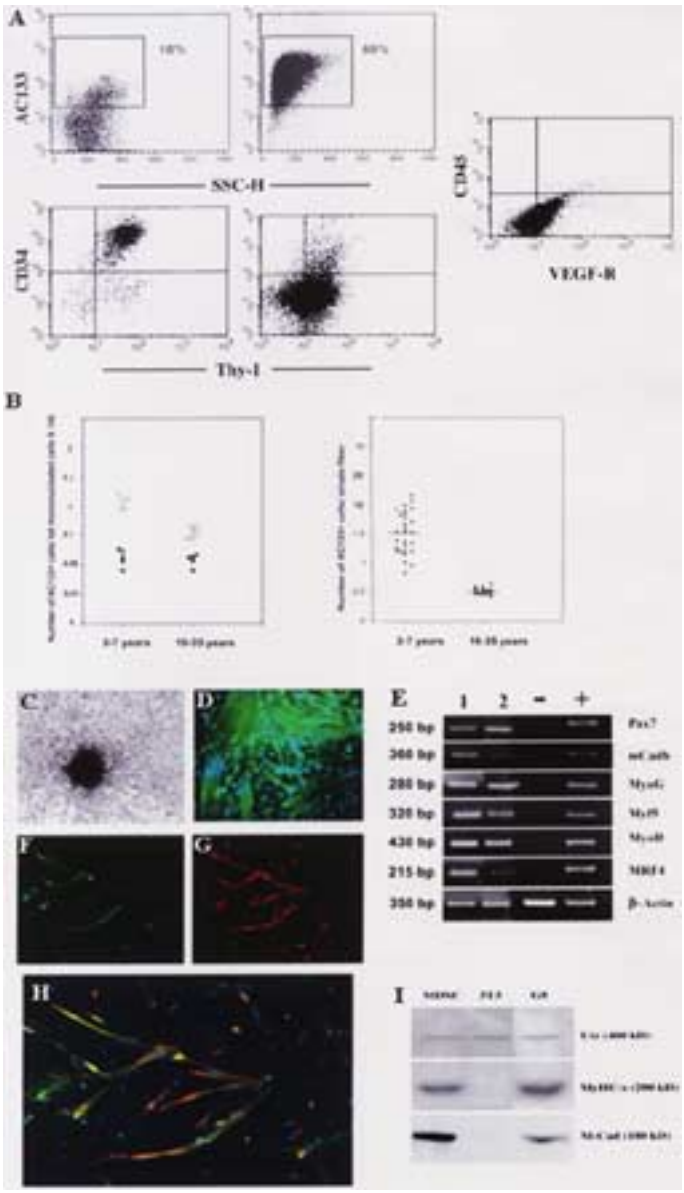

Figure 1. (A) FACscan immunophenotyping of the unfractionated and fractionated muscle-derived cells. The unfractionated muscle-derived cells (MDCs) obtained by whole muscle dissociation were tested for CD133, CD34, and Thy-1 antigen expression. $CD133^+$ cells (18%) were present within the MDCs (first screen in first row), whereas after MACS selection the purity of $CD133^+$ was 88% (second screen in the first row). The SSG-H shows the side scatter of the cells. The majority (more than 79%) of the dystrophic muscle-derived $CD133^+$ cells coexpressed CD34 and Thy-1 antigens (~56%)(first screen in second row). In contrast, less than 5% of the normal muscle-derived $CD133^+$ cells coexpressed the CD34 antigen, whereas ~53% of them coexpressed Thy-1 (second screen in second row). Less than 4% of the human dystrophic and normal muscle-derived $CD133^+$ cells were found to express CD45, the pan-leukocyte common antigen and the VEGF-receptor (KDR). (B) The percentage of $CD133^+$ cells isolated from muscle tissues at different ages. The number of $CD133^+$ cells is expressed per single fiber. Characterization of self-renewal potential and myogenic differentiation of muscle-derived $CD133^+$ cells. Colonies from single cells within the $CD133^+$ cells isolated from the muscle (C) were obtained in proliferative conditions described in the Materials and Methods. In myogenic differentiation several $CD133^+$ cells from the muscle formed SMA-positive cells (D). The myogenic commitment was demonstrated by specific RT-PCR (E) for human markers performed on mRNA extracted from $CD133^+$ cells isolated from normal muscle (lane 1), DMD muscle (lane 2), C2C12 murine cell line (lane –), and human cDNA library (lane +). When $CD133^+$ cells isolated from muscle tissues were induced to differentiate by serum deprivation we obtained a large number of desmin-positive (F) and MyHC-positive (G) myotubes. A merge of (F) and (G) is showed in (H). (I) Immunoblotting analysis with slow myosin heavy chain (MyHC) and M-cadherin also showed that the $CD133^+$ cells isolated from the human muscle (MSCs) expressed myogenic markers after 14 days of culture in differentiation medium. 3T3 fibroblast and G8 myoblast cell lines were used as control. Utrophin immunoblotting indicated that the same total protein concentrations were present in all specimens.

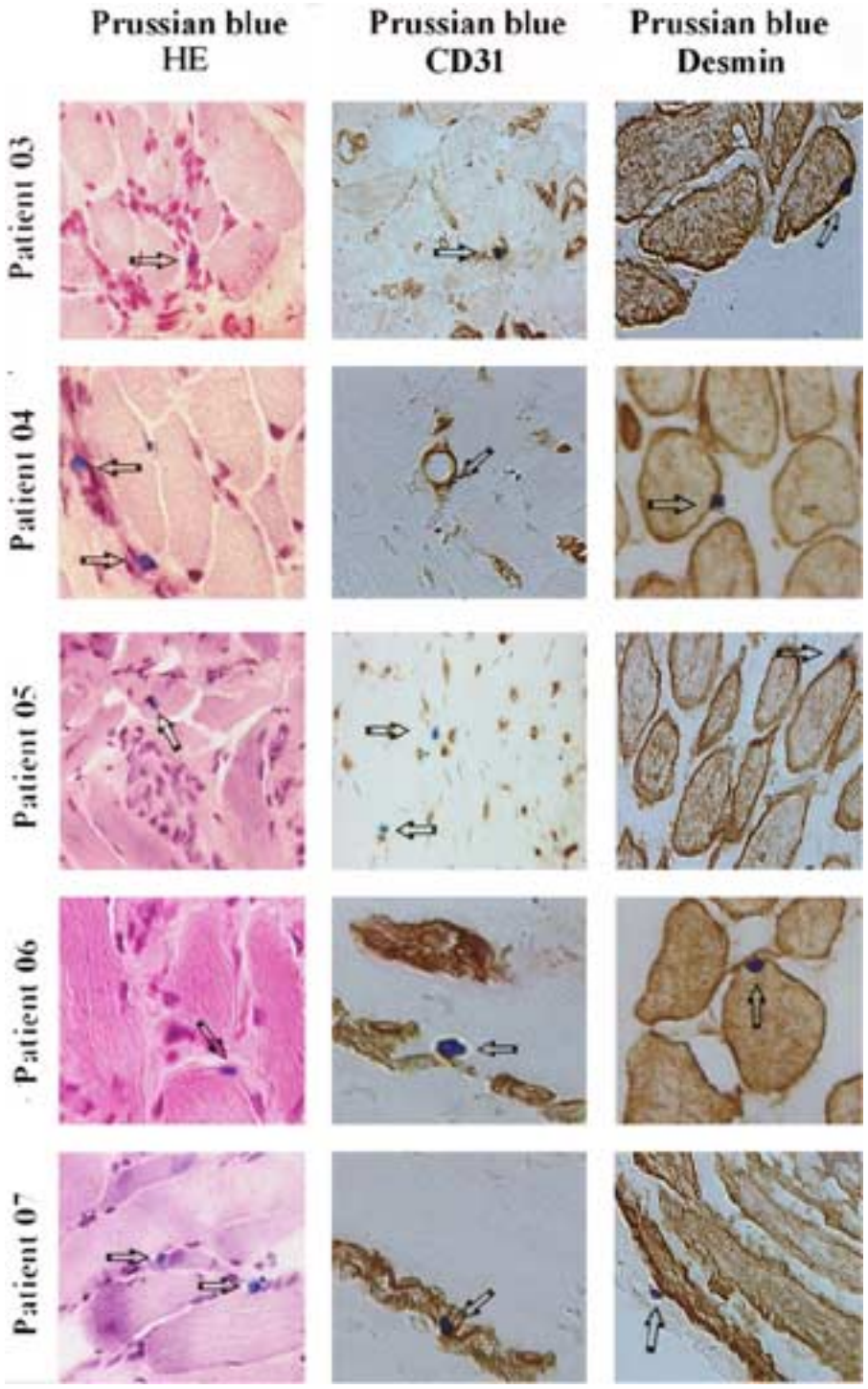

Figure 2. Localization of autologous injected muscle-derived CD133$^+$ cells around muscle fibers and vascular structures after their intramuscular transplantation. Prussian blue analysis of the MDA stem cell-injected muscles revealed Prussian blue-positive cells (arrows) coexpressing CD31 in the vessels of the interstitial spaces and desmin inside of the basal lamina.

of inflammation were reported: no fever, no pain, no modification in biological parameters such as C-reactive protein, CK levels, and white blood cell count. The MRI analysis of all treated ADM muscles excluded the presence of structural abnormalities 6 months after transplantation (Fig. 3). As demonstrated by muscle strength analysis, the procedure did not alter the function of the injected ADM muscles. At the body muscular evaluation through MRC scale patients of both CD133-treated and sham groups showed a moderate decrease of muscular strength. This may be simply related to the natural course of the disease. The cardiac evaluation did not show any alteration in the dimensions and cardiac function.

Morphometric and Phenotypic Modifications After Intramuscular Transplantation of Autologous Muscle-Derived CD133+ Stem Cells in DMD Patients

Seven months after intramuscular transplantation of autologous muscle-derived CD133+ cells we did not observed significant morphological difference between treated and untreated ADM muscles of groups A and B. All muscle biopsies showed a clear dystrophic pattern. Quantitative evaluation of necrotic fibers showed similar percentage of necrosis in all treated and untreated ADM muscles of both groups. Several interstitial CD68+ macrophages were present in all specimens, in particular near or around necrotic fibers. Dystrophin-positive revertant fibers were absent in most specimens with one exception of ADM muscles of patient 05 (less than 0.1% of total fibers per section). No granulomatous lesions or perivascular cell infiltrates were observed. Antibodies against CD4, CD8, B cells (CD19), and MHC-I were negative in all biopsies. In all patients, the myofiber density did not significantly differ between treated (410 ± 110 myofibers per mm^2>) and untreated ADM muscles (498 ± 108 myofibers per mm^2) of both groups. The range percentage of slow MyHC-positive myofibers in all untreated ADM muscles was about 62–74%, indicating a type I predominance. In the sham-treated ADM muscles, there was no modification of this range of percentage (Fig. 4). However, in the stem cell-treated ADM muscles of one of the patients (patient 03) the percentage of slow-type myofibers was significantly decreased (Student's *t*-test, $p < 0.001$) with a significant increase of the percentage of the fast-type myofibers in three patients (patients 04, 05, 06) (Student's *t*-test, $p < 0.001$) (Fig. 4). The increased proportion of fast-type myofibers was also correlated to an increased number of neonatal myofibers in the treated muscle of patient 06 (Student's *t*-test, $p < 0.001$).

The capillary to fiber ratio (number of microvessels per 100 myofibers) did not differ significantly in the ADM-treated muscles of the sham group and in all untreated muscles of both groups (Fig. 5). Surprisingly, the capillary to fiber ratio markedly increased in the treated ADM muscles in 4 of 5 patients of the stem cell group (patients 03, 04, 05, 06) (Fig. 5). Interestingly, the treated ADM muscles of patient 03 and 06 showed a significant increase of the number of capillaries per fast–slow and neonatal myofibers (Fig. 5) in comparison to the untreated collateral ADM muscles. The mean diameter of the microvessels in the treated ADM muscles of these two patients was significantly larger (5.9 ± 0.5 μm) than in untreated ones (3.5 ± 0.7 μm) ($p = 0.01$) (Fig. 5). To investigate directly whether VEGF might affect the growth of the muscle capillaries in treated versus untreated muscles we characterized by Western blot the section lysates of all patients. We did not detect the expression of isoforms VEGF-A and C in any specimen, whereas we found the expression of VEGF-B in both treated and untreated ADM muscle sections of patients 03 and 06 from the stem cell group and of patient 07 from the sham group (Fig. 5). No significantly difference was seen in the number of regenerating fibers and in the percentage of muscle area occupied by connective tissues (endomysial fibrosis) in all muscle specimens. We also explored the expression of IGF-1 and IGF-2 in all specimens and we found that IGF-1 was expressed at detectable levels in the treated but not in control untreated ADM muscles of patients 03, 04, and 06 from the stem cell group, whereas no expression in the rest of specimens was found (data not shown). However, IGF-2 was only expressed in both treated and untreated muscles of patient 03 (data not shown).

Muscle Strength Effects of Autologous Intramuscular Transplantation of CD133+ Muscle-Derived Stem Cells in DMD Patients

Before the treatment at the basal evaluation there was no significant difference between the right and the left ADM (left: 154 ± 13.97 g; right: 139.7 ± 14.41 g). The muscle force of ADM, estimated at each time point as the difference of percentage of the basal force between the treated and untreated side, did not differ significantly between groups A and B at any time point (Fig. 6). Individual patients had a more heterogeneous behavior. In group A subject 03 showed a positive "normalized MVC" at all time point (*T*1, 75.74%; *T*2, 101.29%; *T*3, 85.32%). Subject 06 improved nonsignificantly at *T*0 and significantly at *T*1 (13.81%) and *T*3 (24.88%). Conversely, three patients (04, 05, 07) had negative values (i.e., worsened their force). In the control group all the subjects had negative force values at different times except for patient 09 at *T*3 (5.63%) and patient 10 at *T*2 (3.53%). In conclusion, group statistics failed to demonstrate any difference between treated and untreated muscle of all patients.

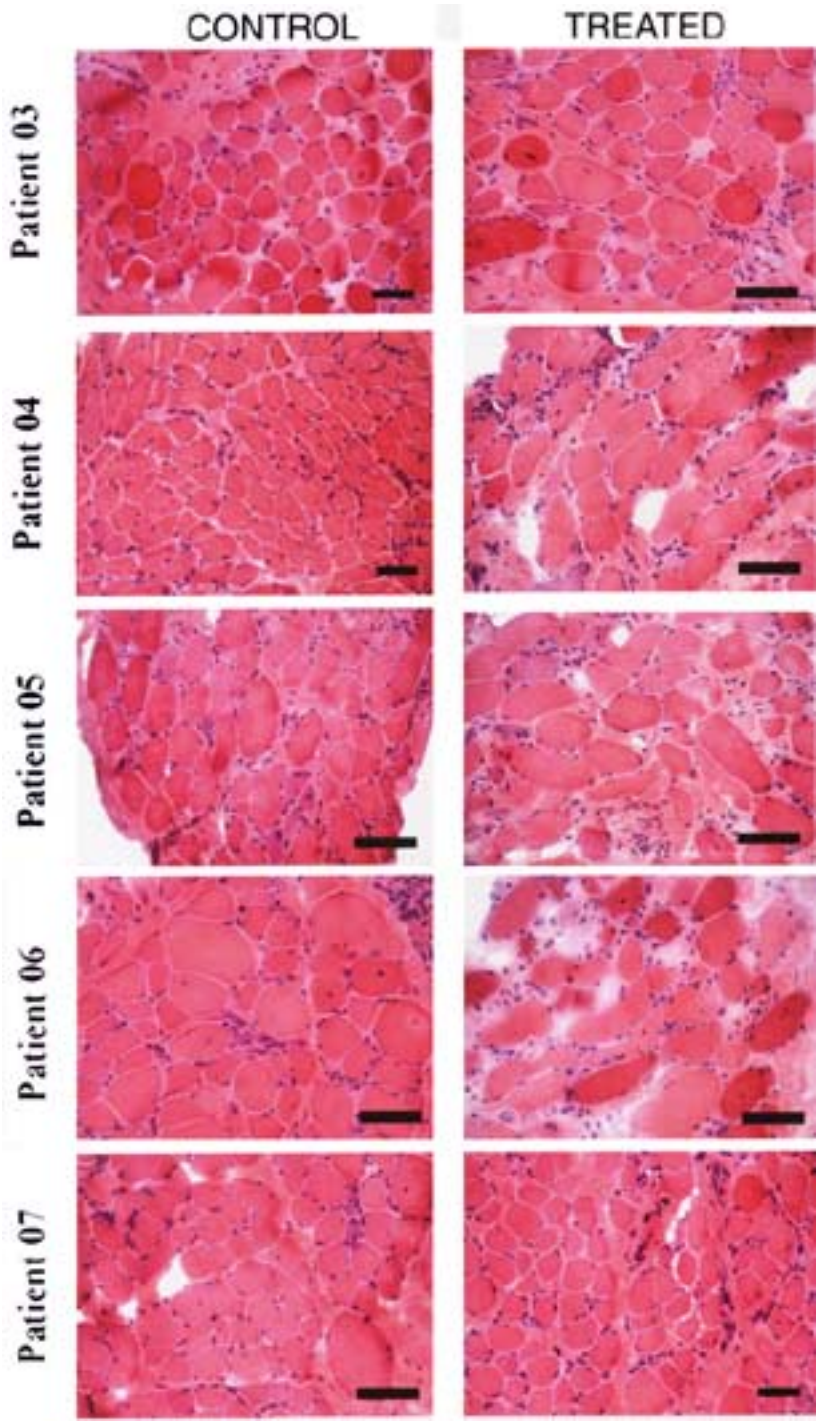

Figure 3. No local adverse effects were observed during 7 months after autologous transplantation of muscle-derived CD133$^+$ cells. H&E staining demonstrated the absence of signs of inflammation or tissue abnormalities in the ADM muscles of all stem cell-treated patients. The MRI analysis of all treated ADM muscles excluded the presence of structural abnormalities. Scale bars: 50 μm.

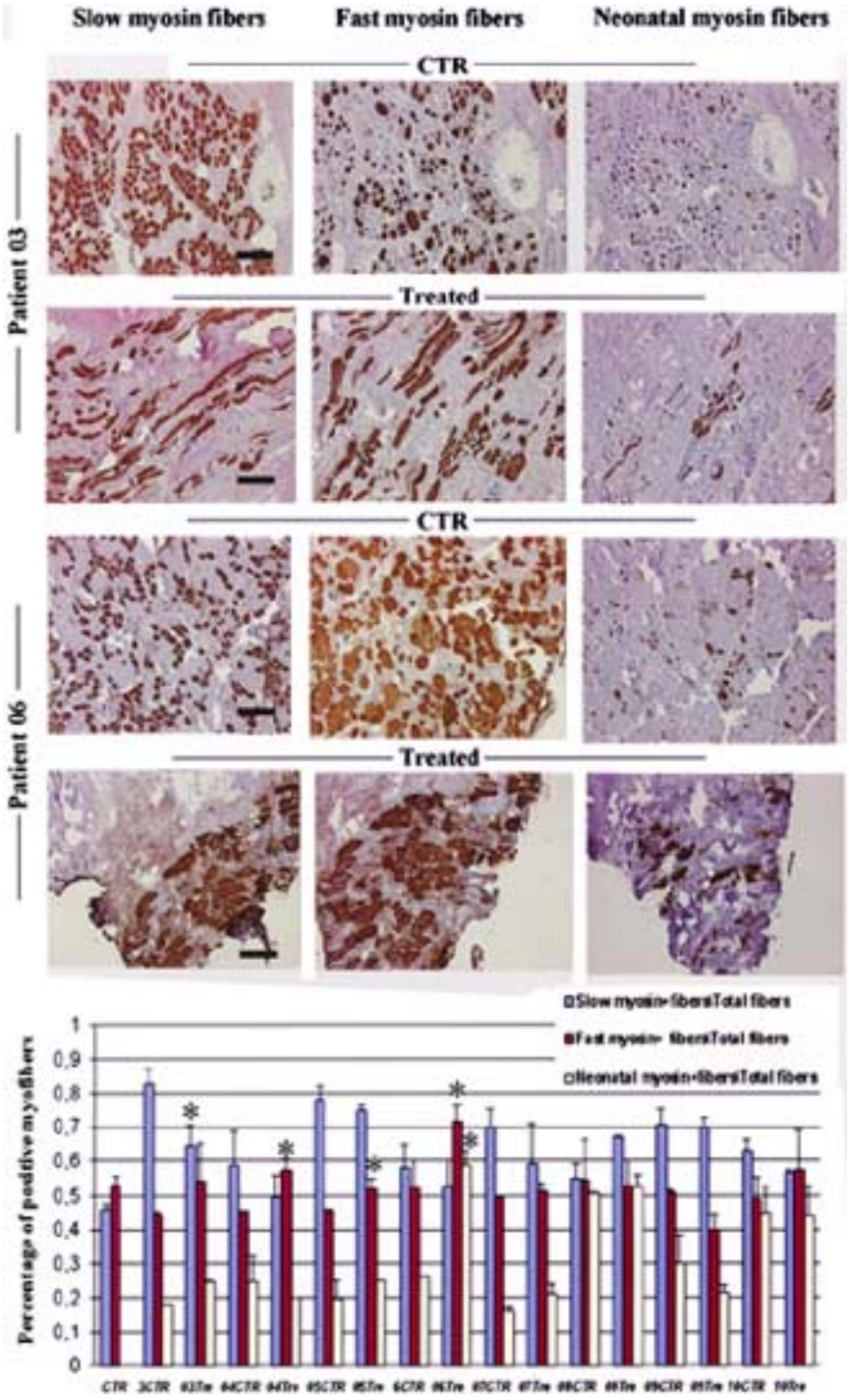

Figure 4. Characterization of the MyHC isoform myofibers after autologous transplantation of muscle-derived CD133+ stem cells. In the stem cell-treated ADM muscles the percentage of slow-type myofibers was significantly decreased only in 1 of 5 patients (patient 03) (Student's *t*-test, $p < 0.001$) with a significant increase of the percentage of the fast-type myofibers in 3 of 5 patients (patients 04, 05, 06) (Student's *t*-test, $p < 0.001$). The increased proportion of fast-type myofibers was also correlated to an increased number of neonatal myofibers in the treated muscle of patient 06 (Student's *t*-test, $p < 0.001$). However, the range percentage of slow MyHC-positive myofibers in the untreated ADM muscles indicate a type I predominance. In the sham group, there was no modification of this range of percentage. Statistical analysis (Student's two-tailed *t*-test) proved the difference between control and treated ADM muscles ($*p < 0.001$). The first columns of the histogram correspond to the percentage of the slow, fast, and neonatal myosin myofibers of the ADM muscle biopsy of a 12-year-old normal subject. Scale bars: 100 μm.

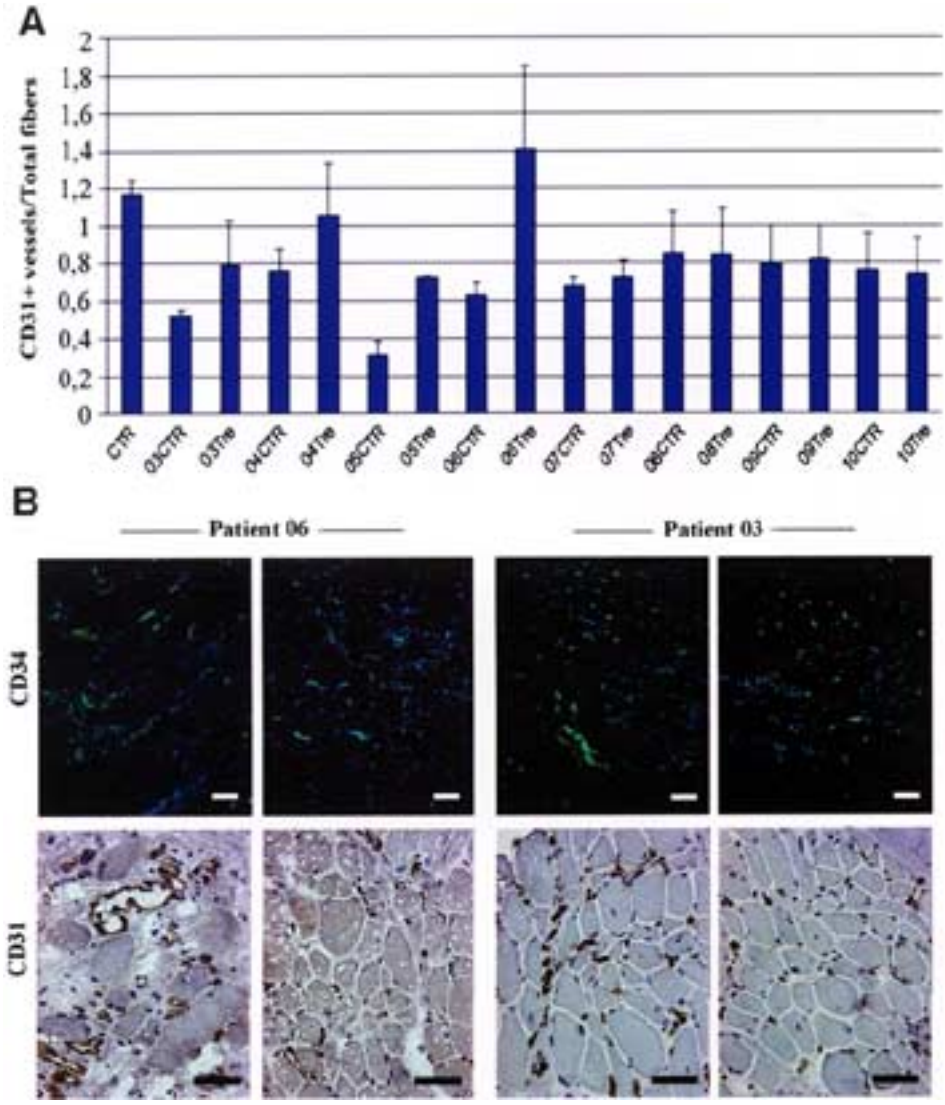

Figure 5. Characterization of ADM muscle vessels of all patients showed a significant increase of the number of CD31+ capillaries in patients 03 and 06 in comparison to the untreated collateral ADM muscles (A). The first column of the histogram corresponds to the number of microvessels per 100 myofibers of the ADM muscle biopsy of a 12-year-old normal subject. We also found an increase of CD31+ and CD34+ vessels in both treated ADM muscle sections of patients 03 and 06 from the stem cell group (B). Scale bars: 50 μm.

DISCUSSION

In this study we have investigated the safety of autologous transplantation of muscle-derived CD133+ stem cells in muscular dystrophy. We observed that CD133+ cells from muscle well differentiate into multinucleated myotubes that readily express MyHCs and several myogenic markers' mRNA, such as Myf-5 and MRF-4. For this reason we consider the muscle-derived CD133+ cells as a class of myogenic progenitors and investigated their safety following transplantation in DMD muscles. Our data show for the first time that intramuscular transplantation of muscle-derived CD133+ stem cell in DMD muscle patients is a safe procedure and is feasible. In fact, we found an excellent safety profile for CD133+ stem cells in all injected patients. Moreover, the ability to combine Prussian blue stain and immunohistochemistry on the same section allowed us to detect injected autologous stem cells in the dystrophic muscle environment. This method provided the demonstration that few injected CD133+ stem cells remain as mononucleated cells around vessels and muscle fibers.

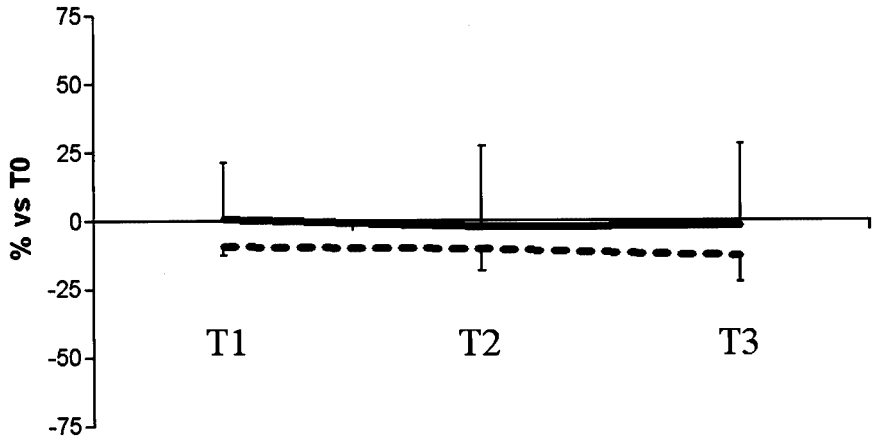

Figure 6. Muscle strength effects of autologous intramuscular transplantation of CD133+ muscle-derived stem cells in DMD patients. Maximal voluntary force of ADM in patients treated with stem cells (solid line) and with saline solution (dashed line). *x* axis: time after treatment (*T*1 60 days, *T*2 120 days, *T*3 180 days); *y* axis: normalized MVC expressed as percent of force at *T*0 (see Materials and Methods). Error bars are standard error. Note that the normalized MVC is almost the same in the two groups.

Of the five DMD patients treated with stem cells we analyzed, four patients showed an increased number of capillaries per muscle fiber and two of them expressed a change in the ratio of slow-to-fast myosin myofibers. Although the increased number of capillaries observed into the treated ADM muscles could be explained by the angiogenic potential of the injected CD133+ stem cells, as also previously demonstrated within the infarcted myocardium (31), it is not clear how these cells could promote the switch of slow-to-fast muscle fiber type. Muscle fiber type specification is dependent on innervation because early stages of development and motor innervation is essential for the maturation of the muscle fibers during postnatal development and for the maintenance of fiber type specificity throughout life (15). Moreover, several other factors, including the calcium/calmodulin-dependent enzymes such as calcineurin and CaMK, seem to be involved in the regulation of muscle fiber types (35). However, muscle growth and fiber specification are also modulated by growth factors and hormones during development and in the adult. Several reports demonstrated the role of IGF-1 as paracrine/autocrine factor implicated in the shift of myofibers towards a fast-glycolytic phenotype (9,17,20). IGF-1 expression in muscle has been found to induce muscle hypertrophy in young animals and prevent muscle atrophy and loss of the fastest, most powerful type 2B fiber types seen in old muscles (2). It is interesting to note that muscle-derived CD133+ stem cells express high levels of IGF-1 (our unpublished observations) and similar high levels of IGF-1 were observed in transplanted ADM muscles (data not shown).

These findings suggest that muscle-derived CD133+ stem cells may promote the muscle fiber switch to a fast muscle gene program via IGF-1 release. Therefore, it is unlikely that fiber type transformation induced after autologous transplantation of muscle-derived CD133+ stem cells is the result of a single factor but it seems more likely that the switch in muscle gene regulation requires still unknown intermediate steps, possibly involving different mechanisms. We believe the present data represent the first step for future clinical trials for DMD based on the autologous transplantation of engineered stem cells and need at least four potential improvements: i) to isolate cells from an easily accessible anatomical site such the blood; ii) to expand them in vitro without the loss of stem cell properties; iii) to efficiently transduce them with viral vectors that promote the expression of dystrophin by exon skipping; and iv) to deliver them to diseased muscle through arterial circulatory routes. To achieve such aims we requires a better understanding of the biological mechanism regulating the stem cell behavior in the human muscle structures in order to gain the route of the stem cell therapy for DMD.

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