

Human Umbilical Cord Blood Cells Differentiate into Muscle in *sjl* Muscular Dystrophy Mice

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ABSTRACT

Limb girdle muscular dystrophy type 2B form (LGMD-2B) and Miyoshi myopathy (MM) are both caused by mutations in the dysferlin (*dysf*) gene. In this study, we used dysferlin-deficient *sjl* mice as a mouse model to study cell therapy for LGMD-2B and MM. A single-blind study evaluated the therapeutic potential of human umbilical cord blood (HUCB) as a source of myogenic progenitor stem cells. Three groups of donor cells were used: unfractionated mononuclear HUCB cells, HUCB subfractionated to enrich for cells that were negative for lineage surface markers (LIN⁻) and substantially enriched for the CD34 surface marker (CD34⁺), and irradiated control spleen cells. We administered 1×10^6 donor cells to each animal intravenously and euthanized

them at different time points (1–12 weeks) after transplantation. All animals were immunosuppressed (FK506 and leflunomide) from the day before the injection until the time of euthanasia. Immunohistochemical analyses documented that a small number of human cells from the whole HUCB and LIN⁻CD34⁺-enriched HUCB subgroups engraft in the recipient muscle to express both dysferlin and human-specific dystrophin at 12 weeks after transplantation. We conclude that myogenic progenitor cells are present in the HUCB, that they can disseminate into muscle after intravenous administration, and that they are capable of myogenic differentiation in host muscle. *Stem Cells* 2004;22:981–993

INTRODUCTION

Progressive muscle wasting and weakness characterize muscular dystrophies. A subgroup of autosomal recessive dystrophies, the limb girdle muscular dystrophies (LGMDs), is characterized by weakness and wasting of muscles of the pelvic and shoulder girdle [1]. Another dystrophy, Miyoshi myopathy (MM), affects distal muscles at onset, with preferential early involvement of the gastrocnemius muscle. Mutations in a novel muscle gene, dysferlin, cause the type 2B

form of LGMD (LGMD-2B) and MM; dysferlin expression is reduced or absent in these patients [2, 3]. A 171-bp deletion in the murine *dysf* gene was detected in the *sjl* mice, with a corresponding reduction in dysferlin levels to 15% of normal [4]. Thus, the *sjl* mouse is a natural model of LGMD-2B/MM.

Muscle cells are formed developmentally by the fusion of multiple muscle precursor cells and are therefore multinucleated. Moreover, normal adult muscle retains a population

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of satellite cells that are capable of differentiating into myoblasts and fusing to regenerate muscle after injury. These observations led to the hypothesis that creation of chimeric muscle cells by introduction of wild-type, nondystrophic nuclei at the time of fusion could correct the deficiency of a protein in a dystrophic muscle cell. Although early studies of engrafting muscle precursor cells into dystrophic *mdx* host mouse muscle were promising [5], attempts to use human myoblasts to restore dystrophin expression in muscles of patients with Becker and Duchenne dystrophy were not successful [6–10] despite the long-term survival of some donor myoblasts [11]. One interpretation of these studies was that the donor myoblasts were at an inappropriate developmental stage to reproduce the behavior of satellite cells; conceivably, less-differentiated but muscle tissue-specific stem cells might engraft into host muscle more successfully. In the past 5 years, several studies have identified populations of undifferentiated cells within bone marrow that demonstrate properties of stem cells, including myogenic stem cells with the capacity to proliferate as myoblasts and participate in normal muscle regeneration [12, 13]. Hoechst-stained side populations of cells (SP cells) isolated from either bone marrow [14] or muscle [15] and a subpopulation of long-time proliferating cells [16] or Sca-1⁺/CD34⁺ muscle-derived cells [17] also engraft muscle and restore expression of a muscle-specific protein after local or systemic administration in recipient animals. These reports argue that systemic administration of muscle stem cells may provide an effective form of gene and protein replacement therapy for recessively inherited, loss-of-function muscular dystrophies.

If this therapy is to be clinically practical, one of many issues that must be addressed is the availability of appropriate numbers of muscle stem cells. Although xenogeneic transplantation strategies might be considered, it can also be argued that an additional constraint is that the cells should, optimally, be human in origin. Indeed, if human-derived donor stem cells can be obtained that immunomatch the recipient, it is conceivable that immune barriers to successful muscle cell therapy can be minimized. As an alternative to generating large numbers of immunomatched donor human muscle stem cells from sources such as donor biopsies, we have considered the possibility that human umbilical cord blood (HUCB) cells might be a renewable and noncontroversial source of stem cells containing myogenic precursors.

Since transplantation of HUCB cells was successfully performed for the first time in 1988 to treat Fanconi's anemia [18], there has been extensive experience in the use of HUCB cells to reconstitute the hematopoietic system in both humans [18–20] and mice [21, 22]. It is now apparent that the proliferative capacity of hematopoietic stem cells in cord blood is superior to that of cells in the marrow of blood from

adults [19]. Moreover, the use of HUCB reduces risk of graft-versus-host disease [20]. Some studies suggest that HUCB-derived cells can differentiate into nonhematopoietic cells. Thus, cells derived from HUCB can differentiate into neurons or glia both in vitro and in vivo [23, 24]. Mesenchymal precursor cells have also been obtained from HUCB [25]. Recent studies document that subpopulations of HUCB cells can transdifferentiate into hepatic, endothelial, or muscle cells [26, 27].

The present investigation was performed to test the concept that populations of HUCB cells can engraft into recipient dystrophic muscle cells after systemic administration, fuse, and become part of myofibers in muscle cells, thereby allowing expression of muscle proteins absent in the dystrophic host. We have tested this hypothesis using *sjl* mouse as a model of LGMD-2B/MM in which dysferlin is absent. In these studies, the end point for successful engraftment and myogenic differentiation of subsets of HUCB was the presence of dysferlin or, as a second marker of myogenicity, human-specific dystrophin in muscle that is normally devoid of these proteins.

MATERIALS AND METHODS

Cell Preparation

HUCB and control irradiated spleen cells were provided by ViaCell, Inc. (Cambridge, MA). Briefly, cryopreserved HUCB units were thawed and centrifuged. Pellets were resuspended in Dulbecco's phosphate-buffered saline (dPBS) (Invitrogen, Carlsbad, CA) and run on a Ficoll gradient. The leukocyte layer was removed, transferred to another conical tube, and washed twice with dPBS. Separation of lineage-negative (LIN⁻) UCB cells from lineage-positive (LIN⁺) UCB cells was performed using the StemSep™-based negative immunoselection system (StemCell Technologies, Vancouver, BC, Canada) to purge UCB cells that expressed surface markers of CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, and glycophorin A. Antibodies of these surface markers were cross-linked to magnetic dextran-coated iron particles using tetrameric antibody complexes. The cell suspension was then passed through a high-gradient magnetic column of stainless steel mesh. The magnetically labeled LIN⁺ cells were bound to the column, whereas the unlabeled LIN⁻ cells passed through and were collected as a highly enriched LIN⁻ cell preparation. An aliquot of the LIN⁻ HUCB cells was analyzed by flow cytometry to confirm the simultaneous enrichment of CD34 surface marker expression within this group (~50%–70% of LIN⁻ HUCB cells were CD34⁺). Control spleen cells were prepared from two NOD/*scid* mice, harvested aseptically, minced in dPBS, filtered (0.2 μm filter), and counted. These

cells were then irradiated with a Cesium 137 unit at 1,500 cGy, counted again, and kept in dPBS until transplantation.

Immunosuppression

Female *sjl* recipients (6 weeks old) were obtained from Jackson Laboratory (Bar Harbor, ME). The animals received a daily dose of 1 mg/kg FK506 (intraperitoneally) (Fujisawa, Osaka, Japan, <http://www.fujisawa.com>) together with 20 mg/kg leflunomide (by gavage) (Aventis Pharmaceuticals Inc., Kansas City, MO, <http://www.aventis.com>) from the day before cell injection until the time of euthanasia. The animal protocol used for this study was approved by Massachusetts General Hospital Subcommittee on Research Animal Care. All animal care was in accordance with institutional guidelines.

Transplantation

Whole, subfractionated HUCB cells and mouse control spleen cells were provided as coded preparations from ViaCell, Inc. *sjl* mice were divided into three groups ($n = 8$) for the transplantation experiments (groups A, B, and C). Each animal was injected with 1×10^6 of either HUCB or control spleen cells retro-orcularly at 7 weeks of age. All animals received isoflurane (light inhalation anesthesia) (Abbott Laboratories, North Chicago, IL) before the injection. Animals were analyzed blindly in these three groups (A, B, and C); the code for each of the cell groups was disclosed only after completion of the studies.

Muscle Samples and Slide Preparations

Two groups of muscles, gastrocnemius and quadriceps, were collected and snap-frozen from each treated *sjl* mouse during the time-course study. Two untreated *sjl* mice of the same age were euthanized, and muscle samples were collected at each time point as controls. Slides of 10 μm sections were prepared and stored at -80°C until ready for additional analysis.

Immunohistochemical Staining

Muscle sections were first blocked in 5% fetal bovine serum (FBS) (HyClone, Logan, UT) and 0.05% Tween 20 in PBS for 30 minutes at room temperature and then incubated with optimal amount of primary antibody for 1 hour. Sections were rinsed with PBS and incubated with secondary antibodies for 45 minutes. When coimmunostaining was performed, two rounds of staining (primary followed by secondary antibodies) were completed sequentially.

Primary antibodies used for the immunohistochemical staining were anti-dysferlin monoclonal antibody (undiluted) (Novocastra Laboratories Ltd., Newcastle upon Tyne, U.K.), a monoclonal antibody that is specific for human nuclear-mitotic apparatus protein (NuMA) (Oncogene Research Products, Boston, MA), used at 1:20, and a monoclonal anti-

body that reacts specifically with human dystrophin (Chemicon International, Temecula, CA), 1:5. Neither the NuMA nor the dystrophin antibodies cross-react with nuclear-mitotic apparatus protein or dystrophin protein of mouse origin. When dysferlin was coimmunostained with NuMA, the respective secondary antibodies were fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG) (Sigma, 1:250) and Cy3-conjugated goat anti-mouse IgG (Sigma, St. Louis) 1:300. When dysferlin was coimmunostained with dystrophin, the secondary antibodies were FITC-conjugated goat anti-mouse IgG (Sigma, 1:250) and tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG (Sigma, 1:100).

Sections of liver and kidney samples (10 μm) were also prepared from all three groups of animals at the 12-week post-transplantation time point and immunostained with the NuMA antibody to examine the dissemination rate of donor cells within these organs.

CD4 and CD8 Immunohistochemistry

Nonspecific binding sites on the 12-week post-transplantation gastrocnemius sections (10 μm) were blocked with 10% FBS in PBS for 30 minutes. The muscle sections were then incubated with a rat anti-CD4 antibody (clone H129.19; BD Pharmingen, San Diego, CA); 1:50 or a rat CD8a antibody (clone 53-6.7; BD Pharmingen; 1:50) for 1 hour. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in PBS for 10 minutes. The sections were incubated for 30 minutes with a biotin-conjugated goat anti-rat antibody (BD Pharmingen; 1:50), followed by a 30-minute incubation with streptavidin-peroxidase (BD Pharmingen, prediluted). Peroxidase activity was revealed with 3,3'-diaminobenzidine (BD Pharmingen; 1 drop DAB chromogen to 1 ml DAB buffer). Slides were counterstained with hematoxylin.

Microscopy

Slides were examined by an Eclipse E800 fluorescence microscope (Nikon Inc., Melville, NY), and photomicrographs were taken through a SPOT RT digital camera. Photomicrographs taken by the digital camera were processed using SPOT RT software v.3.2 (Diagnostic Instrument Inc., Sterling Heights, MI).

RESULTS

Specific Markers to Distinguish Human Origin Donor Cells

We used immunostaining with three different antibodies to determine whether HUCB cells survive and undergo myogenic differentiation within the *sjl* recipients after intravenous administration. The control experiments documented that anti-NuMA and anti-dystrophin antibodies bind specifically

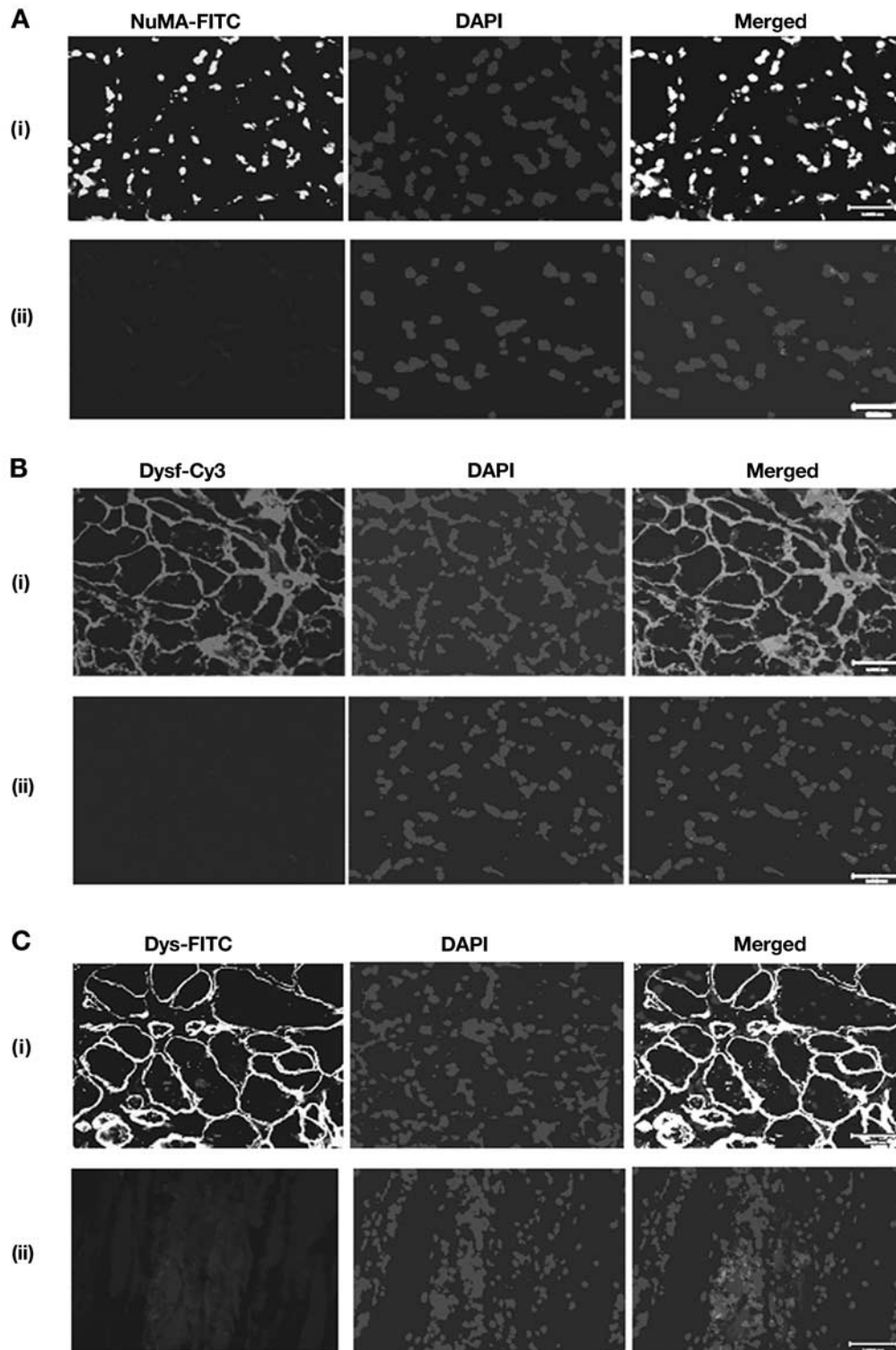


Figure 1. Specific markers identify human donor cells in recipient *sjl* muscle. **(A):** Immunostaining with human-specific anti-NuMA primary monoclonal antibody using an FITC-conjugated goat anti-mouse IgG second antibody. **(B):** Anti-Dysf immunostaining, detected with Cy3-conjugated goat anti-mouse IgG. **(C):** Immunostaining with human-specific anti-Dys monoclonal antibody, counterstained with FITC-conjugated goat anti-mouse IgG. All sections were counterstained with DAPI. In A, B, and C, the top row is human skeletal muscle (i); the bottom row is *sjl* mouse skeletal muscle (ii). Scale bar = 50 μ m. Abbreviations: Cy3, cyanine dye; DAPI, 4,6-diamidino-2-phenylindole; Dysf, dystrophin; Dysf, dysferlin; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G; NuMA, nuclear-mitotic apparatus protein.

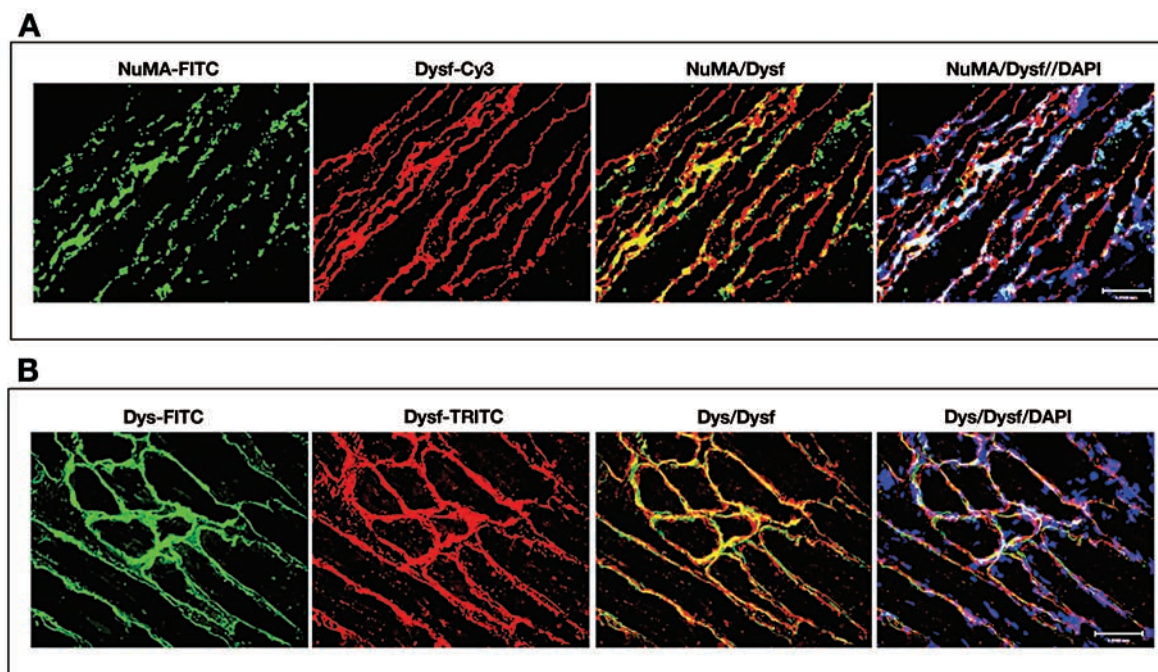


Figure 2. Dysf-positive fibers in human skeletal muscle are positive for NuMA and human Dys immunofluorescence. **(A):** Sequential staining with human-specific anti-NuMA antibody, FITC-conjugated goat anti-mouse Ig, anti-Dysf antibody, and Cy3-conjugated goat anti-mouse Ig. **(B):** Sequential staining with human-specific anti-Dys antibody, FITC-conjugated goat anti-mouse Ig, anti-Dysf antibody, and TRITC-conjugated goat anti-mouse Ig. All sections were counterstained with DAPI. Abbreviations: Cy3, cyanine dye; DAPI, 4,6-diamidino-2-phenylindole; Dysf, dysferlin; Dys, dystrophin; FITC, fluorescein isothiocyanate; NuMA, nuclear-mitotic apparatus protein; TRITC, tetramethylrhodamine isothiocyanate.

to human NuMA and dystrophin (Figs. 1A, 1C). The anti-dysferlin antibody interacts strongly with both human and murine dysferlin antigens, but, as expected, it does not detect dysferlin in dysferlin-deficient *sjl* mouse muscle (Fig. 1B). Other control experiments showed excellent costaining of NuMA and dysferlin proteins or dystrophin and dysferlin proteins (Figs. 2A, 2B). These three antibodies (NuMA, dysferlin, and dystrophin) thus clearly allow detection of engrafted, myogenic human donor cells and the restoration of dysferlin expression in the recipient animals.

Human Donor Cells Were Detected in *sjl* Mice Infused with Two Cell Types

We have divided the *sjl* mice into three groups, group A ($n = 8$), group B ($n = 8$), and group C ($n = 8$), corresponding to the transplantation with the following three different types of donor cells: whole HUCB, LIN⁻CD34⁺ subpopulation of HUCB, and irradiated spleen cells. Each mouse was injected with 1×10^6 HUCB cells or control spleen cells systemically. Two animals from each group (A, B, or C) were euthanized at each time point after the transplantation (1, 4, 8, or 12 weeks). Two skeletal muscles, gastrocnemius and quadriceps, were harvested from each animal for immunohisto-

chemical analyses. In this initial study, we did not evaluate time points after 12 weeks, because this was the longest point analyzed in previous studies of systemic delivery of muscle stem cells [12, 15, 28].

The first set of experiments analyzed the survival and engraftment levels of donor cells in the recipient animals. We used the human-specific antibody NuMA to distinguish the donor stem cells (human origin) from the host cells (mouse). Ten sections from each muscle (gastrocnemius and quadriceps) collected at the above four different time points were examined for NuMA immunofluorescent staining. This was readily detectable in animals infused with either the whole HUCB (group A) or the LIN⁻CD34⁺ HUCB (group B) and colocalized with nuclear staining by 4,6-diamidino-2-phenylindole (DAPI) (Figs. 3A, 3B). By contrast, no NuMA staining was seen from muscles in animals infused with the irradiated mouse spleen cells (group C).

Expression of Dysferlin within NuMA-Positive Muscle Cells

A small number of the NuMA-positive muscle cells, <1% per muscle section, expressed dysferlin in both group A and B animals (Fig. 4). Although NuMA immunofluorescent stain-

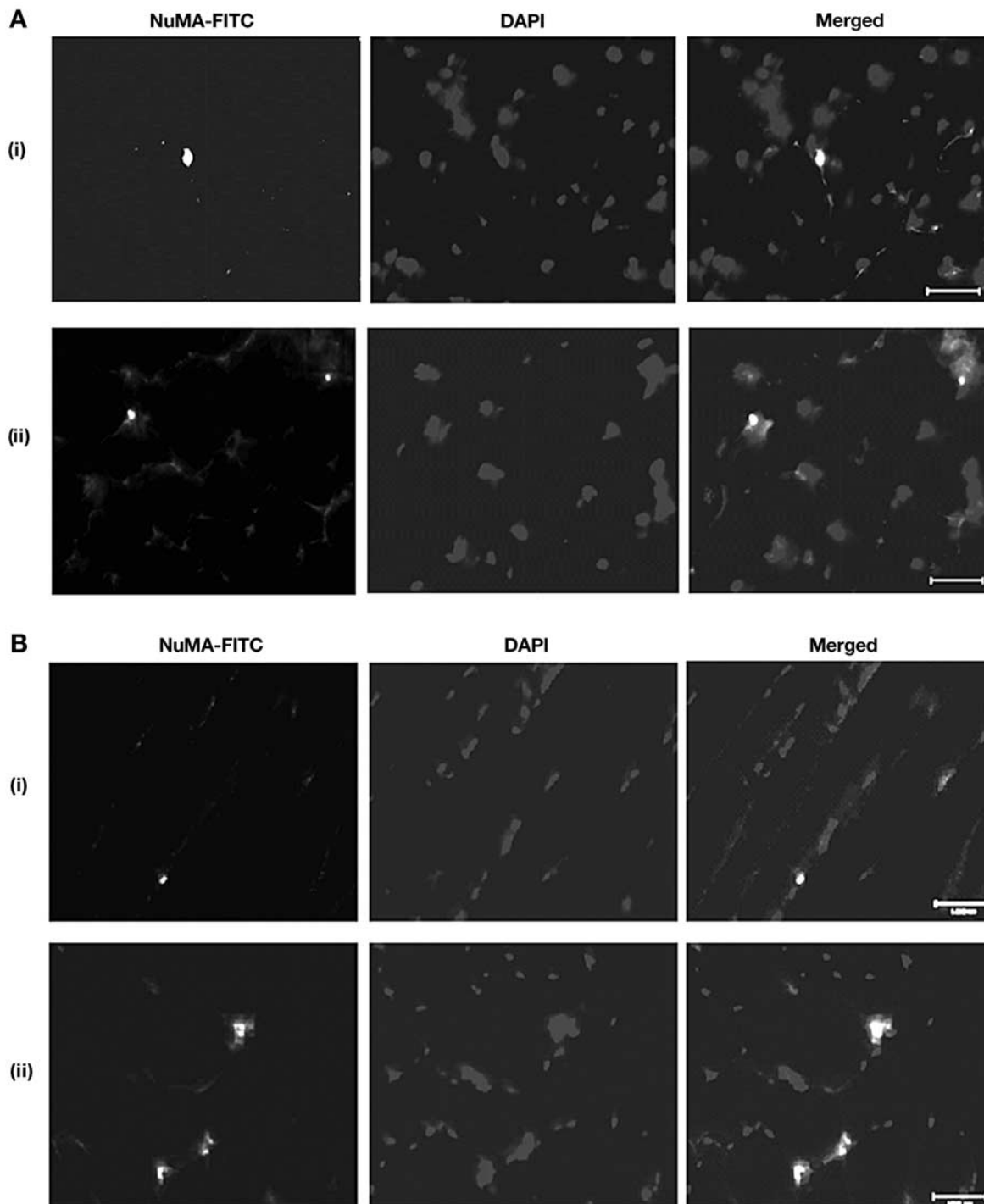


Figure 3. Human donor cells are detected in gastrocnemius muscle of *sjl* mice at 1 and 12 weeks after infusion of whole HUCB cells (A) or LIN⁻CD34^{+/-} HUCB cells (B). NuMA immunofluorescence is evident in *sjl* mouse muscle after administration of whole HUCB cells (A) or fractionated LIN⁻CD34^{+/-} HUCB cells (B). All sections were counterstained with DAPI. In both (A) and (B), the top row (i) is muscle examined at 1 week after HUCB infusion; the bottom row (ii) is muscle immunostained at 12 weeks after HUCB infusion. Scale bar = 25 μm. Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; HUCB, human umbilical cord blood; NuMA, nuclear-mitotic apparatus protein.

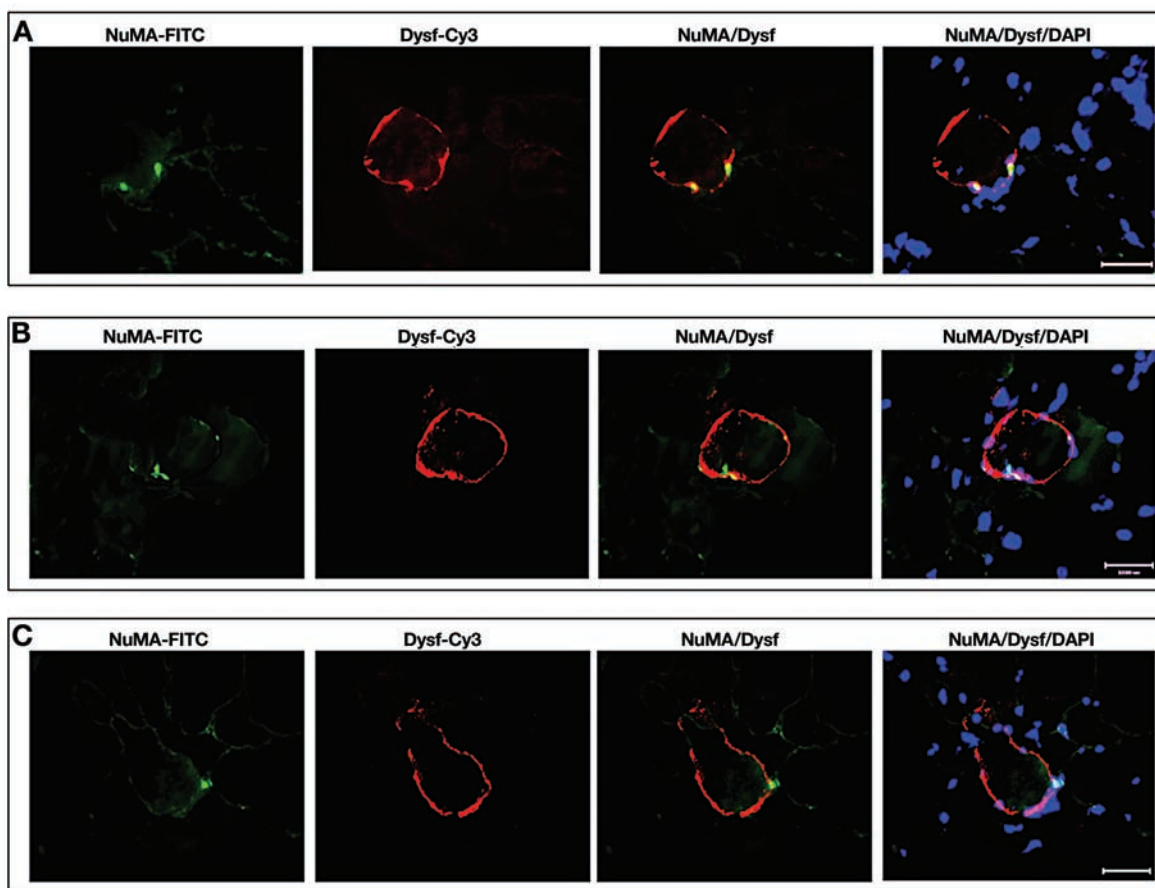


Figure 4. Dysf is detected in NuMA-positive muscle cells from *sjl* mice 12 weeks after infusion of whole and fractionated LIN⁻CD34^{+/+} HUCB cells. **(A):** Coimmunostaining for NuMA and Dysf in *sjl* muscle after infusion of unfractionated HUCB cells. **(B, C):** Coimmunofluorescent staining for NuMA and Dysf in *sjl* muscle after administration of LIN⁻CD34^{+/+} HUCB cells. All sections were counterstained with DAPI. **(A, B):** Gastrocnemius muscle. **(C):** Quadriceps muscle. Scale bar = 25 μ m. Abbreviations: Cy3, cyanine dye; DAPI, 4,6-diamidino-2-phenylindole; Dysf, dysferlin; Dys, dystrophin; FITC, fluorescein isothiocyanate; HUCB, human umbilical cord blood; NuMA, nuclear-mitotic apparatus protein.

ing was readily detected from all postinfusion muscle samples (1, 4, 8, and 12 weeks) in both group A and B animals, dysferlin expression was only detected at 12 weeks after transplantation (Fig. 4).

Muscle-Specific Protein Is Synthesized by Human Donor Cells 12 Weeks after Infusion

To exclude the possibility that the dysferlin-positive fibers are endogenous *sjl* muscle fibers that have undergone spontaneous mutational reversion that permits expression of mouse dysferlin, we have repeated the immunohistochemical analyses to survey for fibers that are positive for both human-specific dystrophin and dysferlin. In these studies, colocalization of dystrophin and dysferlin was evident at 12 weeks after transplantation (Fig. 5). The positive immunostaining for human-specific dystrophin clearly designates these muscle cells as human. Thus, these dystrophin-positive fibers

constitute additional evidence that a subpopulation of the HUCB cells is capable of undergoing myogenesis.

Semi-Quantitative Analysis of HUCB Donor Cell Engraftment and Restoration of Dysferlin Expression in *sjl* Recipients

Table 1 summarizes our results. It is clear that at 12 weeks after transplantation, there are detectable cells and myofibers of human origin specifically in those *sjl* mice that received preparations either of whole HUCB or LIN⁻CD34^{+/+} HUCB. Because no dysferlin or human dystrophin was detected at weeks 1, 4, and 8, Table 1 only summarizes the 12-week data.

As indicated, quantitation of immunopositivity in multiple sections revealed that the numbers of immunopositive cells (NuMA, dysferlin, and dystrophin) were very small. However, approximately half of the NuMA-positive cells were associated with dysferlin staining. Moreover, every cell

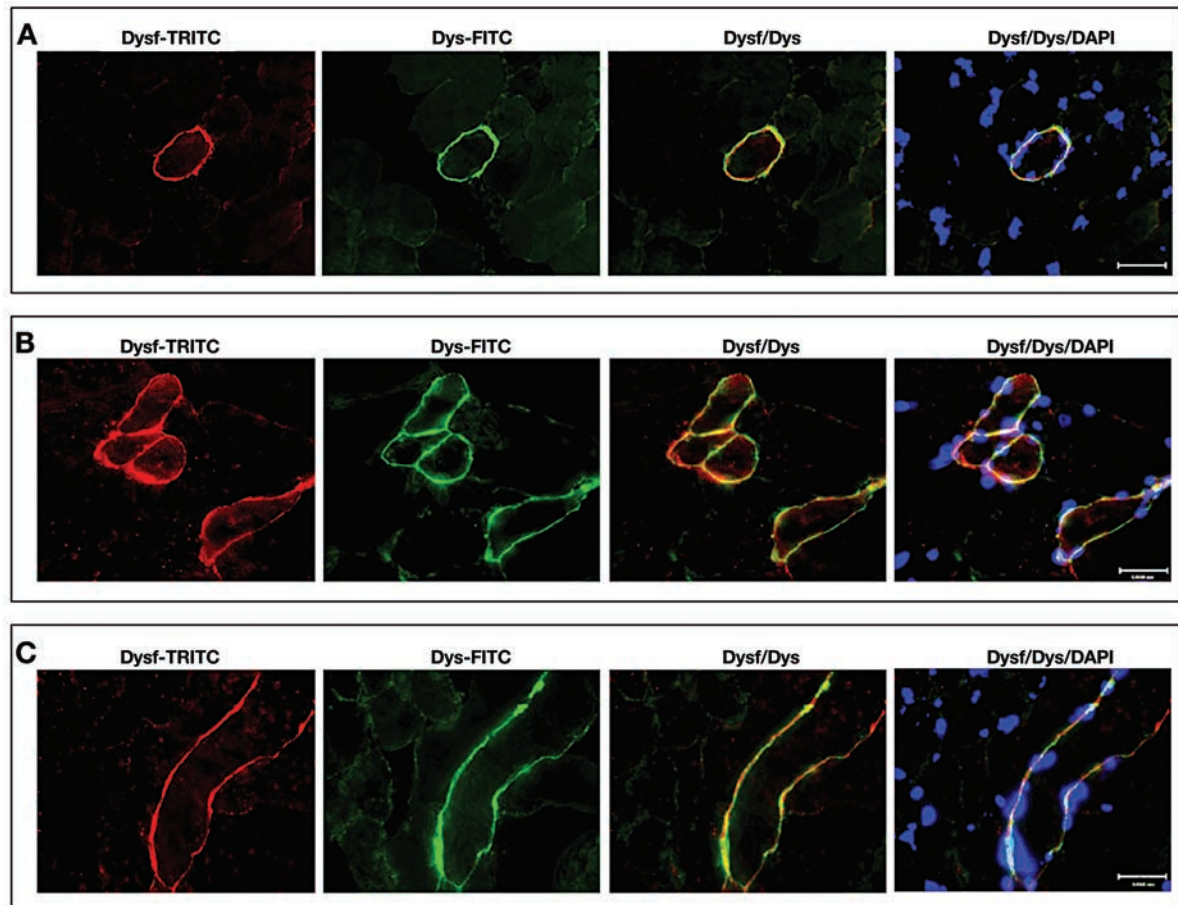


Figure 5. Human-specific Dys and Dysf are detected in *sjl* mice 12 weeks after infusion of unfractionated and LIN-CD34^{+/-} HUCB cells. **(A):** Coimmunofluorescent staining for Dysf and human-specific Dys after administration of unfractionated HUCB. **(B, C):** Coimmunofluorescent staining for Dys and human-specific Dys after infusions of LIN-CD34^{+/-} HUCB cells. All sections were counterstained with DAPI. **(A, B):** Gastrocnemius muscle. **(C):** Quadriceps muscle. Scale bar = 25 μ m. Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; Dysf, dysferlin; Dys, dystrophin; FITC, fluorescein isothiocyanate; HUCB, human umbilical cord blood; TRITC, tetramethylrhodamine isothiocyanate.

that expressed dysferlin also expressed human dystrophin and reciprocally. Both human-specific markers (NuMA and dystrophin) and the dysferlin immunostaining were negative from all 10 sections examined from animals infused with the mouse spleen cell preparation. It was our impression that the selected LIN-CD34^{+/-} cells were more favorable for the myogenic differentiation, in part because dysferlin-positive cells of LIN-CD34^{+/-} origin were slightly more frequent and because they sometimes appeared in clusters, as if they had undergone local proliferation. It is clear that additional studies will be needed to confirm this observation, because the number of surviving and differentiated HUCB cells is small.

Human Donor Cells Were Detected in Liver and Kidney of *sjl* Mice Infused with Two Cell Types

Because intravenous administration results in a more systemic distribution of donor cells within the recipient animals,

various organs were also collected and analyzed to account for the possibility of donor cell engraftment within organs other than the skeletal muscle in these animals.

Four nonserial sections from both liver and kidney were examined for NuMA immunofluorescent staining. Once again, this was readily detectable in animals infused with the whole HUCB (group A) or the LIN-CD34^{+/-}-enriched HUCB (group B) and colocalized with nuclear staining by DAPI (Fig. 6). No NuMA staining was seen from livers and kidneys collected from animals infused with the irradiated mouse spleen cells (group C).

Low Levels of T-Lymphocyte Infiltration Were Detected in FK506/Leflunomide-Treated *sjl* Mice

Assessing the infiltration of immune cells in the muscles of the injected animals allows us to evaluate the immune rejection against transplanted cells. Only one muscle (gastroc-

Table 1. Semiquantification of human umbilical cord blood (HUCB) cells in *sjl* recipient skeletal muscle

| I. NuMA versus dysferlin staining | | | | |
|--|--|---------------|--------------------------------------|--------------|
| Group | # of sections (+) (<i>n</i> = 10) | | # of cells (+) in 10 sections | |
| | NUMA (+) | Dysferlin (+) | NUMA (+) | Dysferlin(+) |
| Gastrocnemius | | | | |
| A | 7 | 2 | 15 | 3 |
| B | 5 | 3 | 11 | 7 |
| C | 0 | 0 | 0 | 0 |
| Quadriceps | | | | |
| A | 6 | 1 | 13 | 2 |
| B | 5 | 2 | 12 | 6 |
| C | 0 | 0 | 0 | 0 |

| II. Dysferlin versus dystrophin staining | | | | |
|---|--|----------------|--------------------------------------|---------------|
| Group | # of sections (+) (<i>n</i> = 10) | | # of cells (+) in 10 sections | |
| | Dysferlin (+) | Dystrophin (+) | Dysferlin(+) | Dystrophin(+) |
| Gastrocnemius | | | | |
| A | 0 | 0 | 0 | 0 |
| B | 3 | 3 | 7 | 7 |
| Quadriceps | | | | |
| A | 2 | 2 | 3 | 3 |
| B | 3 | 3 | 8 | 8 |

Ten gastrocnemius or quadriceps muscle sections were examined from two animals of each group for either NuMA/dysferlin or dysferlin/dystrophin double staining. Numbers indicate the average of positive sections (total number of sections, *n* = 10) and the average of positive cells (from those 10 sections) from both animals in the same group. The total number of muscle fibers for each section is estimated at 3,000–5,000 (ImageTool software, NIH).

Group A: unfractionated HUCB; group B: Lin⁻CD34^{+/-} HUCB cells; group C, irradiated mouse spleen cells.

Abbreviations: NuMA, nuclear-mitotic apparatus protein.

mius) from the 12-week time point of both animals from all three groups was examined for CD4 or CD8 immunoperoxidase staining. A total of three nonserial sections were analyzed, and an average of ~30 to 100 CD4⁺ or CD8⁺ cells per section were seen from all of the animals that were immunosuppressed with both FK506 and leflunomide (Fig. 7).

DISCUSSION

Using the human-specific anti-NuMA antibody, we have established the presence of human donor HUCB cells in the muscles of recipient *sjl* mice several weeks after the intravenous infusion of HUCB (Fig. 3). The expression of dysferlin within NuMA-positive muscle cells provides strong support for the hypothesis that a subpopulation of HUCB has myogenic potential (Fig. 4). Critical to interpreting these data is the longstanding observation that a small percentage of dystrophin-positive muscle fibers are detected in older, dystrophin-deficient *mdx* mice as a consequence of postna-

tal, somatic cell mutations in the dystrophin gene that restore an open reading frame in the dystrophin gene [29]. Although no background dysferlin staining was detected from our uninjected *sjl* animals (Fig. 1), we decided to additionally analyze the injected host muscle using a human-specific anti-dystrophin antibody, because a low expression level of dysferlin in *sjl* mice (~15% of normal) was observed by Bittner et al. [4] when using Western immunoblotting technique. After documenting that it does indeed show human-specific binding (Figs. 1B, 2B), we confirmed that the host *sjl* mice that had received either whole HUCB or LIN⁻CD34^{+/-} HUCB had fibers that expressed both dysferlin and human dystrophin at 12 weeks after transplantation (Fig. 5).

Recently, it has been demonstrated that some stem cell studies purporting to show transdifferentiation may, in fact, reflect a process of fusion of donor stem cells with host cells [30–34]. This raises the important caveat that it is difficult to verify that true stem cell transdifferentiation occurs. Two

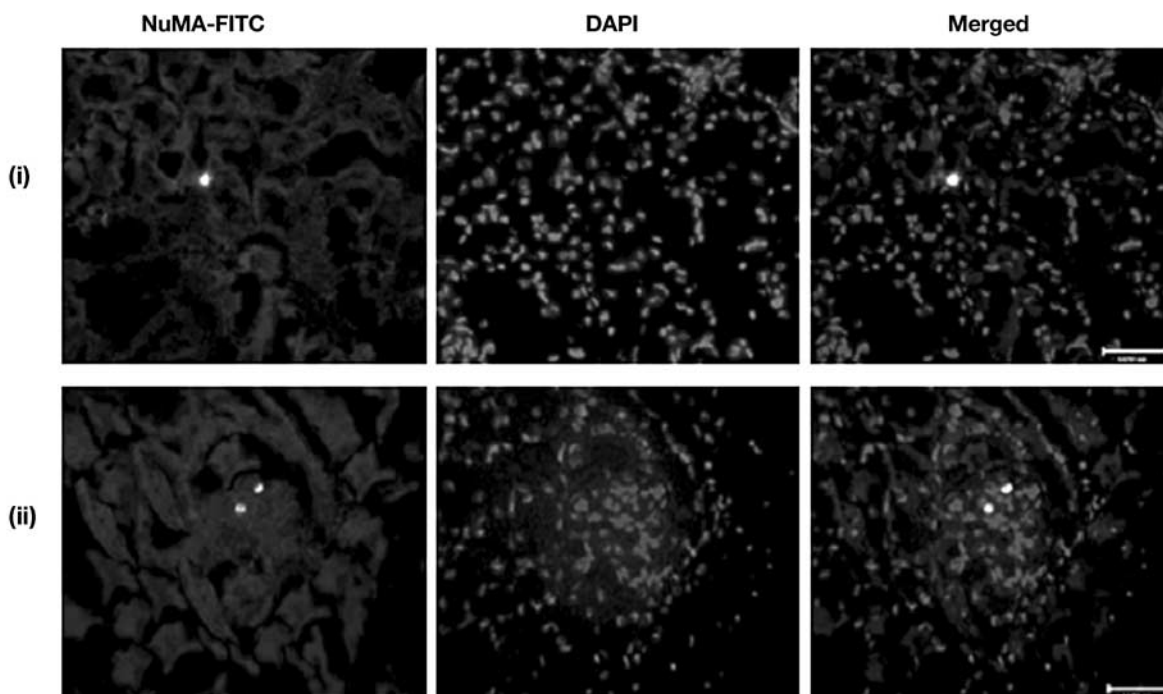
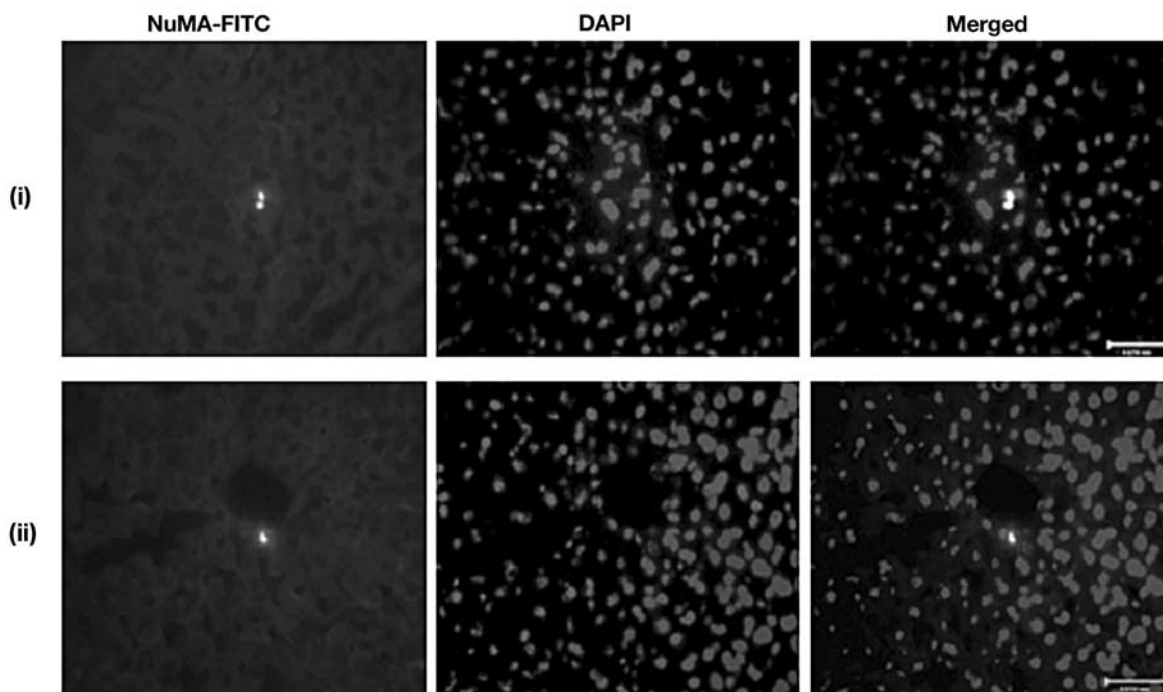
A Kidney**B Liver**

Figure 6. Human donor cells are detected in liver and kidney of *sjl* mice at 12 weeks after infusion. NuMA immunofluorescence is evident in *sjl* mouse kidney (**A**) or liver (**B**) after administration of either whole HUCB cell (i) or fractionated LIN⁻CD34⁺ HUCB cells (ii). All sections are counterstained with DAPI. In both (**A**) and (**B**), the samples were collected 12 weeks after HUCB cell infusion. Scale bar = 25 μ m. Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; HUCB, human umbilical cord blood; NuMA, nuclear-mitotic apparatus protein.

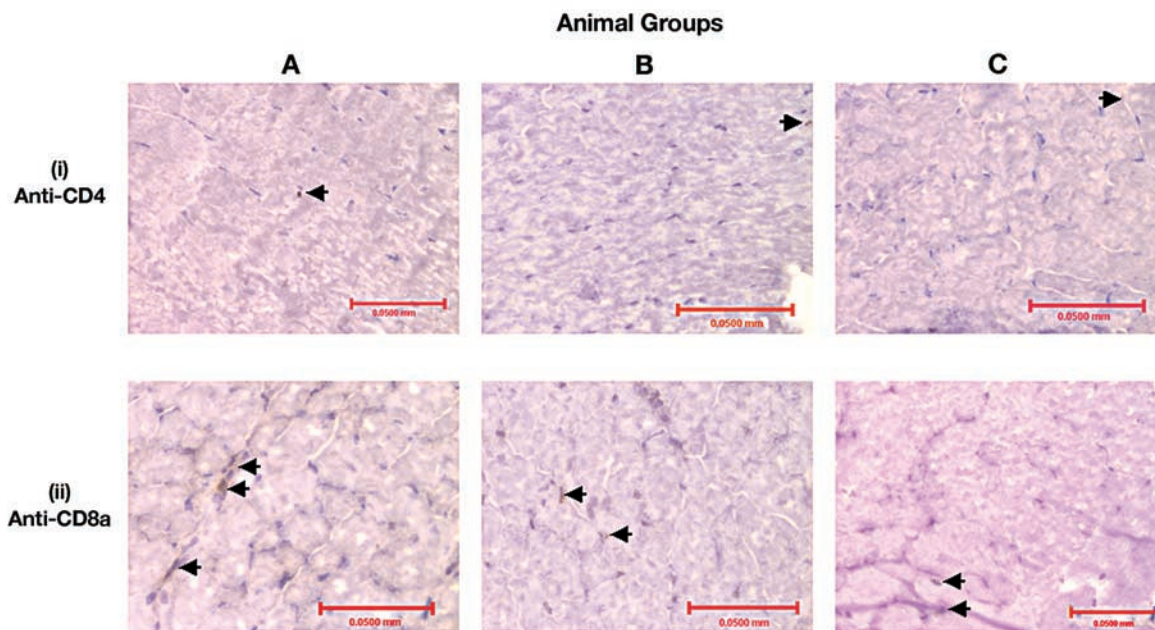


Figure 7. T-lymphocyte infiltration is present in gastrocnemius samples collected from immunosuppressed *sjl* mice 12 weeks after infusion. Immunoperoxidase staining for the presence of immune cells in gastrocnemius samples collected from group A (A), group B (B), or group C (C) animals. Row (i) shows immunostaining with anti-CD4 antibody. CD4⁺ cells are indicated by arrowheads. Row (ii) shows immunostaining with anti-CD8a antibody. CD8⁺ cells are indicated by arrowheads. All sections are counterstained with hematoxylin. Scale bar = 50 μ m.

points are noted in this context regarding our study. First, it is highly likely that donor HUCB cells were incorporated into host muscle by the process of fusion. It is well established that multinucleated myofibers form by fusion of mononuclear cells and that this fusion event can produce chimeric myotubes with nuclei originating from more than one source [12, 15, 28]. Furthermore, chimerization of muscle cells is documented in dystrophic muscle after implantation of myoblasts [11, 35], differentiated muscle cells [36], bone marrow [28, 37], or mesoangioblasts [38]. In our study, we demonstrate that host muscle expresses human-specific dystrophin at 12 weeks after HUCB administration. Moreover, some nuclei in the same cells are positive for the human-specific NuMA antigen and therefore must be derived from the HUCB. In our view, this finding clearly showed that the HUCB nuclei are capable of acquiring a muscle phenotype leading to expression of human muscle proteins. Taken together, these data convincingly demonstrate that the donor HUCB cells are capable of undergoing myogenic differentiation to express human muscle proteins in mouse muscle cells.

Our studies show that not all cells are capable of achieving fusion and myogenic differentiation. In all experiments, we have included irradiated mouse spleen cells as control (group C). None of the recipient muscles displayed evidence

of dysferlin immunostaining. This observation argues that the process of myogenic differentiation from HUCB cells is not nonspecific. We do not know what range of cell types can reproduce the properties of HUCB described here; this will remain a subject of subsequent investigations.

Recently, Pesce et al. [27] reported that HUCB cells can undergo myoendothelial differentiation both in vitro and after intramuscular implantation in vivo. Our studies confirm and extend that observation, emphasizing the additional points that a subfraction of HUCB cells can potentially be disseminated widely throughout the musculature by intravenous delivery and these cells can engraft to undergo myogenic differentiation, restoring expression of a protein that is absent from host dystrophic muscle.

This study raises the possibility that HUCB might ultimately have a role in cell therapy of degenerative disorders of muscle such as the muscular dystrophies. Possible advantages of HUCB cells as myogenic precursor cells include both the accessibility and the noncontroversial nature of this cell type. In addition, several clinical studies have also shown that HUCB recipients are less susceptible to graft-versus-host disease compared with bone marrow cell recipients [19, 20]. Another point is the fact that we have observed myogenic cells within the host muscle after infusing cells system-

ically; this finding is consistent with the earlier reports that myogenic stem cells (SP cells) can migrate into the muscle of *mdx* mice and thereby replace dystrophin [15]. Although we are aware of these theoretical advantages to the potential use of HUCB in cell therapy, a critical practical issue at this juncture is the very low frequency of the myogenic differentiation event in our recipient *sjl* mice transplantation. Although the reasons for this low percentage of uptake are not yet clear, we surmise that this reflects at least three factors: a low percentage of stem-like cells in the HUCB preparation; clearance of most intravenously administered cells by the other organs, such as liver and kidney (Fig. 6); and incomplete immunosuppression and clearance of the HUCB cells by the mouse immune system (Fig. 7).

We are hopeful that forthcoming experiments will distinguish these alternate mechanisms and that it will be possible to augment the levels of engraftment, survival, and myogenic differentiation of the myogenic precursor cells within the HUCB through a variety of methodological improvements, including, possibly, intra-arterial infusions of cells [17, 38, 39] and muscle pretreatment [40, 41].

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