Human satellite cells have regenerative capacity and are genetically manipulable

Andreas Marg, …, Zsuzsanna Izsvák, Simone Spuler


Muscle satellite cells promote regeneration and could potentially improve gene delivery for treating muscular dystrophies. Human satellite cells are scarce; therefore, clinical investigation has been limited. We obtained muscle fiber fragments from skeletal muscle biopsy specimens from adult donors aged 20 to 80 years. Fiber fragments were manually dissected, cultured, and evaluated for expression of myogenesis regulator PAX7. PAX7+ satellite cells were activated and proliferated efficiently in culture. Independent of donor age, as few as 2 to 4 PAX7+ satellite cells gave rise to several thousand myoblasts. Transplantation of human muscle fiber fragments into irradiated muscle of immunodeficient mice resulted in robust engraftment, muscle regeneration, and proper homing of human PAX7+ satellite cells to the stem cell niche. Further, we determined that subjecting the human muscle fiber fragments to hypothermic treatment successfully enriches the cultures for PAX7+ cells and improves the efficacy of the transplantation and muscle regeneration. Finally, we successfully altered gene expression in cultured human PAX7+ satellite cells with Sleeping Beauty transposon–mediated nonviral gene transfer, highlighting the potential of this system for use in gene therapy. Together, these results demonstrate the ability to culture and manipulate a rare population of human tissue-specific stem cells and suggest that these PAX7+ satellite cells have potential to restore gene function in muscular dystrophies.

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Human satellite cells have regenerative capacity and are genetically manipulable

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Introduction

Satellite cells are the proper stem cells of the adult skeletal muscle. Like other stem cell types, these cells are maintained over very long time periods (1, 2). Despite their characterization as early as 1961, satellite cells have only been recognized recently as indispensable for muscle regeneration (3–5). Satellite cells are notoriously difficult to study due to their low abundance and their dispersed location in the specific stem cell niche, the space between basal lamina and sarcolemma of skeletal muscle. Recent animal experiments have allowed deeper insight into the function and regulation of satellite cells (2, 6–9). The studies indicated that satellite cells have an enormous potential for self-renewal and superb potential for muscle regeneration. However, expansion of human satellite cells for therapeutic purposes has not been successful. Therefore, therapy-oriented research has favored the use of other cell populations with myogenic potential, such as CD133+ cells, pericytes, inducible stem cells, mesoangioblasts, or PW1 cells (10–14). These cells are abundant, more accessible, and transverse blood vessels. However, compared with satellite cells, such alternative cell types have limited ability to form muscle stem cells (satellite cells) and to regenerate muscle tissue.

Satellite cells appear during development, and their differentiation and survival depends on the paired box proteins PAX3 and PAX7 (2). While PAX3 plays a key role in embryonic satellite cell development, PAX7 predominantly directs their postnatal survival (15, 16). PAX7 is expressed postnatally and is a reliable satellite cell marker (17). Additional markers like CD56 (NCAM), CD34, CXCR4, m-cadherin, α7-integrin, MET, syndecan-3, and syndecan-4 characterize murine muscle satellite cells (18–22), but their relevance for the identification of human satellite cells has not been established. After skeletal muscle injury, quiescent satellite cells become activated and either self renew or enter a differentiation program that culminates in fusion to the syncytial muscle fiber. Several factors and pathways drive this complex cascade, including myogenic regulatory factors (such as MYF5, MYOD, and myogenin) and signaling systems (such as WNT and NOTCH) as well as yet ill-defined factors provided by the extracellular matrix or systemic sources (reviewed in ref. 23).

Early transplantation studies demonstrated a promising contribution of mouse myoblasts to regeneration of host fibers in the mdx mouse model of Duchenne’s muscular dystrophy (24). Shortly thereafter, clinical trials were conducted and were based on the injections of cultured postnatal human myoblasts into muscle of patients with Duchenne’s muscular dystrophy. Unfortunately, transplanted myoblasts showed poor survival, low ability to migrate, and made little contribution to fiber regeneration (25–27). The aim of our study was to develop tools to render human muscle stem cells manageable for research on muscle regeneration and for gene therapy in muscular dystrophies and other muscle-wasting disorders.

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

**PAX7+ satellite cells on freshly isolated human muscle fiber fragments.**

We obtained 69 different specimens of fresh muscle tissue from adult human subjects (age 20–80 years, 34 female and 35 male donors) after due approval and written, informed consent. Human muscle fiber fragments (HMFFs) were dissected manually, without enzymatic treatment (Figure 1 and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI63992DS1). We isolated and analyzed 884 fragments containing 1–8 fibers (Supplemental Table 1). We used various antigens previously characterized in mice to identify human satellite cells in freshly isolated HMFFs. Coimmunostaining for PAX7 and the neural cell adhesion molecule NCAM (CD56) (Figure 1B) reliably identified cells associated with the human fibers. The majority (80%) of these were double positive for PAX7 and NCAM, but we also observed similar proportions of cells staining either only for PAX7 or only for NCAM. Rarely, MYF5+/PAX7+ cells were found (Figure 1B). 2% to 4% of all nuclei in skeletal muscle fibers were PAX7+. We noted no major differences in the proportion of PAX7+ nuclei associated with fibers in different age groups. We also tested other markers, such as MET, CXCR4, and CD34. MET+ cells were abundant in a skeletal muscle biopsy specimen obtained from a 3-month-old child but were not present on muscle sections from adult donors. Occasionally, we identified CD34+ or CXCR4+ cells in adult muscle specimens or muscle fiber preparations, but these did not costain for PAX7 and were not located in the satellite cell niche. Therefore, these markers were not used further to identify satellite cells in humans (Supplemental Figure 2). We subsequently refer to PAX7+ cells as human satellite cells.

**Culture of HMFFs.**

We next tested whether satellite cells in HMFFs are viable and can be cultured. In cultured HMFFs, cells remained motile for up to 10 days (Supplemental Video 1). During the first days of culture, satellite cells remained associated with the fiber and did not grow out on the culture dish. Instead, they associated with the remaining scaffold of the fiber fragment and located inside the fiber, in which they were densely packed. After 5 to 10 days, the cells moved out of the fiber onto the culture dish and formed the first colonies.

During the time that PAX7+ cells remained associated with cultured fibers (5–10 days), their number increased considerably, and we estimated a 20- to 50-fold increase in quantity compared with that of freshly prepared fragments (Figure 2A). The majority of such cells were desmin+, and many were MYF5+/desmin+ (Figure 2, A and B), indicating that they correspond to activated satellite...
The fraction of nonmuscle cells (desmin− cells) in the colonies varied between 5% and 25% and, on rare occasions, was as high as 70%. However, the proportion was not higher in HMFF cultures from aged donors.

Growth factors and substrate rigidity are important for satellite cell maintenance and proliferation (28). Matrigel contains a mixture of matrix proteins and growth factors, and Matrigel-coated substrates are used frequently for cultures of mouse myogenic cells. We compared HMFFs cultured on plastic with or without Matrigel for 7 to 14 days and observed no obvious differences in the formation of colonies outside the fiber nor changes in the colony size (Supplemental Figure 3). Thus, the scaffold provided by the fibers themselves suffices to stimulate initial activation and proliferation of satellite cells, independent of external factors provided by Matrigel. Culture conditions without additives like Matrigel will be advantageous in experiments with cells used for retransplantations into patients.

Figure 2. Activated satellite cells proliferate within the HMFFs. (A) Marked proliferation of PAX7+ cells is observed in HMFFs, independent of the age of the donors. During the first 10 days of HMFF culture, approximately 80% of cells within the fiber fragment were PAX7+ (green: PAX7, red: desmin, blue: Hoechst dye). At day 21, the quantity of PAX7+ cells within the HMFFs is reduced to 10% of HMFF myoblasts. (B) Myoblasts within HMFF cultures are MYF5+ after 14 days (green: desmin, red: MYF5, blue: Hoechst). (C) The basal lamina (β2-laminin, green) ensheaths desmin− cells (red) in the HMFFs (14 days of culture). Scale bar: 50 μm. Illustrations in A and C depict HMFFs. MSC, muscle satellite cells.
The Journal of Clinical Investigation

Figure 3. Cell colonies originating from HMFF cultures contain satellite cells and retain normal proliferative and fusion capacity. (A) Four HMFFs after 14-day tissue culture on Matrigel (green: desmin, blue: Hoechst). Large colonies of desmin- cells are found as well as spontaneously fused multinucleated myotubes (top row). Scale bar: 200 μm (first and second image); 50 μm (third image). Approximately 50% of cells are PAX7+ satellite cells after 19 days in culture (red: PAX7, green: desmin, blue: Hoechst) (middle row). Scale bar: 20 μm. At 19 days of tissue culture, BrdU staining (red: BrdU, green: desmin, blue: Hoechst) confirms that myogenic cells are proliferating (bottom row). Scale bar: 20 μm. The asterisk indicates a dividing PAX7+ cell. (B) Sleeping Beauty transposon-mediated gene transfer of a Venus transgene (pT2-caggs-Venus). Venus expressed in satellite cells (top image). Venus+ cells form colonies outside the HMFF (middle and bottom images). Scale bar: 200 μm. The double asterisks indicate HMFF. (C) Cell colonies from HMFF cultures after 35 days of hypothermic treatment in serum-reduced medium (OptiMEM) and subsequent culture in SMGM at 37°C for 32 days (left). Solution A did not permit cells to survive hypothermic treatment (right). Scale bar: 200 μm. The double asterisks indicate HMFF. (D) Myogenic cells derived from HMFFs after 35 days of hypothermic treatment followed by 27 days (BrdU, PAX7, MYF5) and 48 days (MYOD, desmin) in SMGM at 37°C. Desmin stain demonstrates a pure myogenic population without contaminating fibroblasts. PAX7-desmin double stain after HMFFs were placed in 15 μM DAPT during hypothermic treatment (21 days). PAX7+ cells were abundant after 35 days at 37°C. Scale bar: 50 μm.

Sleeping Beauty–based gene transfer can be applied to HMFF satellite cells. We then tested whether gene delivery can be achieved in satellite cells in cultured HMFFs. Muscle fiber fragments were cultured for 2 days and then cotransfected with a Sleeping Beauty transposon-based vector encoding the fluorescent protein Venus (pT2-CAGGS-Venus) and a second expression plasmid encoding the CMV-SB100X transposase. Venus+ satellite cells were visible 1 day after transfection. After these cells had moved onto the culture dish, Venus+ myoblasts were present for up to 35 days (Figure 3B).

Satellite cells from HMFFs contribute to muscle regeneration after engraftment into mice. Next, we transplanted HMFFs into anterior tibialis muscles of NOG mice (n = 33). For transplantation, we used HMFFs cultured for 5 to 7 days, i.e., fibers containing activated satellite cells that had not yet left the fiber. We transplanted HMFFs from biopsy specimens obtained from 6 donors (aged 44–64 years) (Table 1, experimental groups A–D). When fibers were transplanted into nonirradiated muscle or into muscle irradiated with 9 Gy, few human nuclei were present 3 or 8 weeks after the transplantation, as assessed by the presence of human lamin A/C detected by an anti-human–specific lamin A/C antibody. Almost all human lamin A/C+ nuclei were located in the interstitium and not integrated in the muscle fibers (Figure 4). After focal irradiation of the muscle with 18 Gy, engraftment of human myoblasts was readily observed 3 or 7 weeks after transplantation (Figure 4). At the earlier time point, myotubes and myofibers containing human nuclei were detectable, but human nuclei were more abundant in muscle fibers 7 weeks after transplantation. Occasionally, murine and human nuclei were present in hybrid myofibers (Figure 5B). The size of the largest dissemination of human nuclei was 800 × 400 × 400 μm.

Hypothermic treatment of HMFFs enriched cultures for myogenic cells and improved transplantation efficacy. We next tested whether HMFFs used as a source for satellite cells are storable, which would be advantageous for autologous transplantation and gene repair experiments. Glucose-containing buffered salt solution (Solution A) did not permit survival of muscle stem cells. However, when we kept HMFFs at 4°C in medium with low serum (OptiMEM) (hypothermic treatment) without oxygenation for as long as 35 days and subsequently cultured them at 37°C and 21% oxygen (experimental design III in Figure 1A and Figure 3C), we detected satellite cell colonies outgrowing the fiber. Hypothermic treatment resulted in delayed formation of colonies on the culture dishes, i.e., the first colonies were observed after 2 weeks, and some colonies appeared only after 6 weeks of HMFF culture. Remarkably, colonies from HMFFs subjected to hypothermic treatment contained 100% myogenic cells (desmin+ cell) (Figure 3D). Thus, nonmyogenic cells like fibroblasts did not survive prolonged storage at hypothermic conditions. Although the colonies only consisted of myogenic cells, they were heterogeneous, containing PAX7+ cells (between 20% and 60%), cells that express MYF5 and MYOD, and frequently fusing myotubes (Figure 3D). After BrdU labeling, colonies from HMFFs subjected to hypothermic treatment and freshly obtained HMFFs displayed similar labeling indices.

Freshly isolated HMFFs always gave rise to colonies, but, after hypothermic treatment, outgrowth was more variable and myoblasts grew only from 50% of HMFFs. Colony formation after hypothermia and hypoxia did not correlate with donor age. Hypoxia is known to activate NOTCH signaling, and NOTCH is known to be important for satellite cell maintenance (29–31). The presence of DAPT, a γ-secretase and NOTCH signaling inhibitor, during hypothermic treatment did not interfere with survival of satellite cells, and we observed similar colony sizes and numbers of PAX7+ cells in HMFF cultures regardless of the presence or absence of the inhibitor during storage at 4°C (Figure 3D).

We then tested whether HMFFs after hypothermic/hypoxic treatment could be transplanted successfully. HMFFs were kept at 4°C for 3 weeks, placed into 37°C tissue culture for 2 weeks, and

Table 1. Transplantation of HMFFs into NOG mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Donor</th>
<th>Pretreatment of HMFFs</th>
<th>Successful transplantsations</th>
<th>Irradiation (Gy)</th>
<th>Human cells after 3 weeks</th>
<th>Human cells after 7 to 8 weeks</th>
<th>Multinuclear human fibers, PAX7+ cells</th>
</tr>
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<tr>
<td>A</td>
<td>44 f, 47 f</td>
<td>No</td>
<td>9/10</td>
<td>No</td>
<td>5–150</td>
<td>5–150</td>
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<tr>
<td>B</td>
<td>53 m</td>
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<tr>
<td>C</td>
<td>53 m, 64 f</td>
<td>No</td>
<td>8/10</td>
<td>18</td>
<td>5–300</td>
<td>5–300</td>
<td>Few</td>
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<tr>
<td>D</td>
<td>62 m, 64 m</td>
<td>ht for 3 weeks</td>
<td>6/10</td>
<td>18</td>
<td>5–150</td>
<td>n.t.</td>
<td>Frequent</td>
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*Age in years and gender; #transplants with human cells per total number of transplanted tibialis anterior muscles; ¹number of human nuclei per cryosection. f, female; m, male; n.t., not tested; ht, hypothermic treatment.
then transplanted into mouse tibialis anterior muscles. Analysis 3 weeks after transplantation revealed that the transplants had participated in muscle regeneration more efficiently than HMFFs without hypothermic treatment. Muscle fibers in the transplants contained more and multiple human nuclei (Figure 4 and Figure 5A). Further, human lamin A/C+ cells that coexpressed PAX7 were detected in a satellite cell position below the lamina (Figure 5B). A quarter of the human nuclei were PAX7+ in one section of one transplant. Only very few human nuclei were detected in the interstitium. Lack of nonmyogenic cells like fibroblasts might account for this difference.

**Discussion**

We investigated a long-term tissue culture model to define properties and growth characteristics of human satellite cells that remained associated with muscle fibers (HMFF culture). We provide a detailed characterization, beginning with the quiescent satellite cell located in the stem cell niche, their subsequent activation, migration, and differentiation. We show that enrichment and expansion of satellite cells is feasible, that cultured human fiber fragments can be transplanted and contribute to muscle regeneration, and that they generate human satellite cells that home correctly to the satellite cell niche. Further, using transposon-mediated gene insertion into the genome (Sleeping Beauty System), gene delivery was accomplished in satellite cells in cultured fibers.

Simple expansion of satellite cells in culture results in rapid differentiation, and the cell population with self-renewal and regenerative potential is rapidly lost (32). Others have used suspensions of cultured human primary satellite cells (myoblasts) in transplantation experiments; however, such transplanted cells survive poorly and do not contribute to fibers. We show here that cultured and transplanted HMFFs can fuse to muscle fibers and participate in the endogenous stem cell pool, indicating that fiber culture overcomes the previous limitations of cultured human primary satellite cells (myoblasts).

Recent work has provided important insight into satellite cell biology, revealing molecular mechanisms that might help to expand and maintain such stem cells. For instance, culture of murine satellite cells on soft hydrogel substrates rather than rigid plastic enriches cultures for a self-renewing stem cell population with improved regenerative efficacy (28). The HMFF culture system introduced here provides a 3-dimensional environment with a soft matrix and shows that satellite cells in vivo also use damaged fibers as scaffolds during regeneration.

In addition to satellite cells, HMFFs contain other cell types like fibroblasts. In accordance, colonies from freshly cultured HMFFs also contain nonmyogenic cells, but such nonmyogenic cells are not longer observed when HMFFs are stored for prolonged periods at 4°C prior to culture. Quiescent stem cells survive under extreme conditions, for instance, in postmortem tissue (33) or under hypoxic microenvironments (reviewed in ref. 29). Metabolic requirements of quiescent satellite cells appear distinct from those of activated stem cells or from other cell types, such as fibroblasts. The long-term survival of satellite cells and their enrichment will be advantageous in cell replacement or gene therapy approaches, as they imply the option of storing the muscle fibers for several weeks.

We found that gene transfer can be applied successfully to cultured satellite cells, which are associated with HMFFs. We documented the delivery of a Venus cDNA gene using the Sleeping Beauty transposon system. Transposons can be considered to
represent natural gene delivery vehicles. Transposon-based tools are versatile and can be used in gene therapy (34, 35). SB100X-mediated gene transfer allows stable, long-term gene expression in various primary cells and stem cells and functions over a broad range of cDNA size. Thus, transposon-based gene transfer might be suitable for deliver transgenes that encode very large muscle proteins, such as the full-length dysferlin or dystrophin, into cells that are transplanted into skeletal muscle.

Local irradiation was a prerequisite for successful engraftment and for participation of transplanted cells in muscle regeneration. Endogenous stem cells in the healthy muscles of the recipients are damaged by this procedure, indicating that the transplanted cells only contribute to regeneration when endogenous stem cells are impaired. In contrast to other protocols, apart from a small muscle incisure, into which the HMFF fragments were deposited, no additional damage, like freeze trauma or cardiotoxin, was applied. Future experiments will be required to assess whether multiple small injuries combined with HMFF transplantation will suffice to efficiently deliver cells into larger muscle groups. It is noteworthy that in the most efficient transplants observed, every fiber in the damaged and regenerating area contained a human nucleus. Restoration of 10% gene activity could be sufficient for therapy in muscular dystrophies, indicating that the participation of transplanted cells observed here might already be beneficial.

Human satellite cells are scarce and can currently not be isolated by FACS. Indeed, the low number of satellite cells in HMFFs would not allow sorting, even if a suitable surface marker were available. Cultured HMFFs containing satellite cells might provide starting material for autologous cell transplantation for treatment of muscular dystrophies, provided that efficient gene delivery can be achieved. We observed that HMFFs obtained from all adult subjects analyzed here contained human satellite cells that could be expanded in culture. Autologous satellite cells provide an advance over nonautologous cell sources (for instance, satellite cells obtained from human cadavers, ref. 33), circumventing difficulties associated with obtaining tissue or the life-long requirement of immunosuppressant after allotransplantation.

Autologous transplantation of HMFFs after correction of the disease-causing mutation may not overcome all known disadvantages of myoblast transplants, such as lack of direct access to specific muscle groups (e.g., diaphragm) or lack of alternative delivery routes that are precluded by the inability of the delivered cells to

Figure 5. Characterization of HMFFs, after and without prior hypothermic treatment, transplanted into 18 Gy irradiated tibialis anterior muscle. (A) Nuclei positive for human lamin A/C are readily detected in regenerating muscle fibers after transplantation of freshly prepared HMFFs (yellow: lamin A/C, red: desmin). Interstitial nuclei are also present in some abundance. After hypothermic treatment, muscle fibers containing multiple human nuclei were identified frequently. Interstitial human nuclei were not present when HMFFs were transplanted after hypothermic treatment. Scale bar: 10 μm (first column); 50 μm (second column); 20 μm (third and fourth columns). (B) Occasional fibers display both human and murine nuclei (arrows), indicating mouse/man chimeras (7 weeks after transplantation). PAX7+ human cells are found in their anatomical niche (3 weeks after transplantation). Scale bar: 10 μm.
migrate across vessels. Indeed, we observed limited migration of human cells from the insertion site after muscle transplantsations of HMFFs. This state of affairs is similar to the restricted dispersal of transplanted cells described in previous experiments that used different sources of satellite cells/myoblasts and transplantation protocols. It is possible that local application of autologous human satellite cells combined with intravenous application of other cell types, such as CD133+ cells that possess the capacity for transvesSEL migration, will provide a route to more efficient therapies of muscular dystrophies.

Methods

Patients and materials. HMFFs were prepared from muscle specimens obtained during hip surgery from individuals without neuromuscular disorders or for diagnostic purposes (ethical approval EA1/203/08, Charité). In total, 1,156 fiber bundles from 69 subjects were analyzed. Donor age ranged from 20 to 80 years; 34 were men and 35 were women. Analysis of frozen sections included an additional biopsy specimen (3 months of age) taken for diagnostic purposes after permission.

Preparation of single HMFF cultures. Immediately after biopsy, the muscle specimen was transferred into Solution A, which contains 30 mM HEPES, 130 mM NaCl, 3 mM KCl, 10 mM D-glucose, and 3.2 μM Phenol red (pH 7.6). Dissection of single fiber fragments was performed mechanically using miniature forceps under a stereomicroscope (Leica Microsystems). Secondary bundles were dissected by cutting the perimysial fascia, followed by the dissection of primary bundles in the same way. Primary bundles were carefully loosened to obtain single fibers. Single fiber fragments of a length of 2 to 3 mm were further mechanically relieved of connective tissue. HMFFs were either placed on Matrigel-coated (BD Biosciences) coverslips or cultured in regular plastic dishes. HMFFs were cultured in Skeletal Muscle Growth Medium (SMGM, PromoCell) supplemented with 10% FCS, glutamax, and gentamicin and cultured in a humidified atmosphere containing 5% CO2 at 37°C. For characterization of fresh single fibers, isolated fiber fragments were fixed in 4% PBS-buffered formaldehyde immediately after mechanical dissection. Staining of freshly prepared muscle fibers was initiated within 45 minutes after the biopsy was taken. HMFF cultures were set up within 2 hours after the biopsy procedure or subjected to hypothermic treatment. In hypothermic treatment, muscle specimens were placed in Solution A or in serum-reduced optimized minimal essential medium (OptiMEM, PromoCell) and deposited at 4°C for up to 35 days. The stored muscle was not specifically aerated.

Sleeping Beauty-based gene transfer to HMFFs. Gene delivery studies were performed using the hyperactive Sleeping Beauty transposase SB100X (32). Cotransfection of HMFFs with a Sleeping Beauty transposon-based vector encoding the fluorescent protein Venus (pT2-CAGGS-Venus) and an expression plasmid for CMV-SB100X transposase was performed 2 days after isolation in SMGM. 400 ng pT2-CAGGS-Venus vector and 20 ng SB100X were diluted in 75 μl jetPRIME buffer (Polyplus-transfection SA) mixed with 2 μl jetPRIME reagent (Polyplus-transfection SA), incubated for 15 minutes at room temperature, and added to fiber cultures. Twelve hours after transfection, the medium was replaced by fresh SMGM. Analyses were performed between 1 and 35 days after transfection.

Immunohistochemistry. For immunohistochemical staining, muscle cryosections (6 μm) were fixed in 3.7% formaldehyde. HMFFs and cultured myoblasts were permeabilized in 0.2% Triton X-100 for 10 minutes and incubated in 1% BSA/PBS for 45 minutes; frozen sections were incubated in 5% normal goat serum/PBS for 45 minutes. Primary antibodies were used as described in Supplemental Table 2 and incubated for 4 to 16 hours at 4°C. After washing, samples were incubated with secondary antibodies (Cy3-conjugated anti-rabbit, Cy3-conjugated anti-rat, Alexa Fluor 488–conjugated anti-mouse antibodies, each 1:1,000) and Hoechst 33342 (1:1,000) for 45 minutes in 1% BSA/PBS at room temperature. Alternatively, UEA (1:100, Vector Labs) was used for staining of endothelial layers of capillaries. For PAX7 staining, muscle sections were incubated in preheated antigen-unmasking Vector buffer (80°C, Vector Labs) for 15 minutes, fixed in Zamboni fixative (PFA/picric acid, pH 7.3) for 20 minutes, and, after additional washing, incubated in blocking buffer (10% horse serum, 0.5% blocking reagent [PerkinElmer], 0.1% Triton X-100 in PBS). Subsequently, sections were incubated with anti-PAX7 hybridoma supernatant for 12 hours. Sections were washed in PBS/0.1% Triton X-100 and incubated with fluorescein-conjugated secondary antibodies for 30 minutes. Samples were imaged with a Leica DMi 6000 (Leica Microsystems) or a Zeiss LSM 700 confocal microscope (Carl Zeiss MicroImaging GmbH). Images were composed and edited in Photoshop CS (Adobe) or CorelDRAW 15. 3-dimensional visualization of z-stacks was performed with Amira (Visage Imaging). As a rule, all modifications were applied to the whole image. Time-lapse microscopy was performed with a Leica DMI 6000 in a humidified atmosphere at 37°C and 5% CO2. All antibodies and dilutions are listed in Supplemental Table 2.

Focal irradiation of tibialis anterior muscle. Female 6-week-old NOD. Cg-Prkdcscid Il2rgtm1Sug/JicTac mice (referred to as NOG mice herein) (Taconic) were anesthetized with ketamine-xylazine in PBS (9 mg/ml ketamine, 1.2 mg/ml xylazine) with a dose of 160 μl/20 g administered by intraperitoneal injection. To provide a reproducible position for the mice, an acrylic glass block with surface fiducial markers was constructed. For the calculation of digitally reconstructed radiographs (DRRs) as well as for treatment planning, a computed tomography (CT) scan, with 0.75-mm slice thickness, was conducted with a dedicated CT scanner (Siemens Emotion). This CT data set served as an exemplary basis for calculation of the DRRs, for tracking, and for radiation dose distribution, assuming all legs and muscles of the selected mice were similar. The CT volume data set was used for target delineation of the murine body as organ at risk. The prescription dose was 18 Gy, as earlier proposed by Boldrin et al. (36). Because of the inherent inhomogeneity of dose distribution, a maximum of 19.5 Gy was accepted in the targeted leg, with the 90% isodose line completely enclosing the target region. All mice were treated with a single irradiation fraction of 18 Gy using the image-guided robotic system (CyberKnife Radiosurgery System, Accuray Inc.) (Supplemental Figure 4). The mice were placed in the customized acrylic mold with attached fiducial markers. The fiducial markers were used for superimposing the live positioning images during the treatment procedure with previously calculated radiograms. The 3-dimensional correction values were used by the robotic treatment couch. The treatment procedure itself could be conducted within a 5-minute time span.

Transplantation of HMFFs into murine tibialis anterior muscle. Mice were placed under anesthesia using ketamine-xylazine (9 mg/ml ketamine, 1.2 mg/ml xylazine) with a dose of 160 μl/20 g. Anterior tibial muscles were cut longitudinally, and HMFFs (4–8 muscle fibers) were placed inside the muscle parallel to the orientation of the murine fibers. The tibialis anterior muscles were closed without sutures. The overlying skin was closed using polyglycolic acid threads (MARLIN

The Journal of Clinical Investigation

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TECHNICAL ADVANCE

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The service of the transplants, tibialis anterior muscle was cut horizontally in the middle, and every tenth section into the upper and lower part of the muscle was stained for human lamin A/C and desmin.

Study approval. The internal review board of Charité approved the study, and written, informed consent was obtained from all participants (EA1/203/08 and EA2/041/10). Animal studies were performed under license G0016/13.