Short Communication

Adult Bone Marrow-Derived Stem Cells in Muscle Connective Tissue and Satellite Cell Niches

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Skeletal muscle includes satellite cells, which reside beneath the muscle fiber basal lamina and mainly represent committed myogenic precursor cells, and multipotent stem cells of unknown origin that are present in muscle connective tissue, express the stem cell markers Sca-1 and CD34, and can differentiate into different cell types. We tracked bone marrow (BM)-derived stem cells in both muscle connective tissue and satellite cell niches of irradiated mice transplanted with green fluorescent protein (GFP)-expressing BM cells. An increasing number of GFP⁺ mononucleated cells, located both inside and outside of the muscle fiber basal lamina, were observed 1, 3, and 6 months after transplantation. Sublaminal cells expressed unambiguous satellite cell markers (M-cadherin, Pax7, NCAM) and fused into scattered GFP⁺ muscle fibers. In muscle connective tissue there were GFP⁺ cells located close to blood vessels that expressed the Sca1 or CD34 stem-cell antigens. The rate of settlement of extra- and intralaminal compartments by BM-derived cells was compatible with the view that extralaminal cells constitute a reservoir of satellite cells. We conclude that both muscle satellite cells and stem cell marker-expressing cells located in muscle connective tissue can derive from BM in adulthood. (*Am J Pathol* 2004, 164:773–779)

In the late 1990s, adult bone marrow (BM) cells were shown to contain stem cells that can give rise to skeletal muscle fibers *in vivo*.^{1,2} BM cell derivation of muscle fibers was assessed by the expression of either a *lacZ*

transgene under the control of a muscle-specific promoter in normal mice¹ or dystrophin in *mdx* mice.² Therefore, induction of terminal myogenic differentiation was necessary to detect BM-derived cells, and, consistently, cells situated upstream from the myofiber in the muscle cell lineage could not be visualized. Now, this difficulty can be overcome by tracking BM-derived stem cells after BM transplantation with labeled cells from transgenic donor mice that constitutively express the green fluorescent protein (GFP).³

Evidence of a muscle stem-cell hierarchy came from the study of Pax7^{-/-} mice.⁴ The muscles of these mice contain a normal number of multipotent stem cells but lack so-called satellite cells. This points to at least two distinct compartments in the muscle stem cell hierarchy: 1) the compartment of satellite cells that are resident cells found in an anatomical niche located beneath the muscle fiber basal lamina; these extensively studied cells mainly express specific markers of committed muscle precursor cells, such as M-cadherin and Pax7, and ensure postnatal skeletal muscle growth and regeneration through a well-documented sequence of activation, proliferation, and fusion events;⁵ and 2) the recently described compartment of interstitial muscle stem cells,⁶ that are extralaminal,^{6,7} express the stem cell markers CD34^{6,7} and Sca1^{6,8,9} but not the markers of committed myogenic cells, and can differentiate into various cell constituents of the muscle tissue, eg, myogenic cells, endothelial cells, and adipocytes.^{6,7,10} Several authors have suggested that interstitial muscle stem cells may constitute a reservoir of satellite cells.^{6,8} Whether these cells reside and self-renew in muscle connective tissue from embryonic stages or can be recruited from BM during postnatal life remains undetermined.^{6,8}

In the present study, we tracked BM-derived stem cells in both muscle connective tissue and satellite cell niches

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in irradiated mice transplanted with GFP-expressing BM-cells. We used immunocytochemistry on muscle tissue sections for position markers (basal lamina antigens), a panel of myogenic cell markers, the stem cell antigens CD34 and Scal, and other cell-specific markers.

Materials and Methods

Mouse Strain

B6 (C57BL/6) mice were transplanted with BM-derived cells from B6Tg^{GFP} transgenic mice [C57BL/6 TgN-(actEGFP)Osb YO1] in which the GFP transgene is expressed under the control of a nontissue-specific promoter, chicken β -actin with cytomegalovirus enhancer, as a cytoplasmic protein.¹¹ In B6Tg^{GFP} mice, both BM cells and muscle fibers constitutively express GFP. Therefore, after BM transplantation, GFP may serve as an unambiguous marker for donor-derived cells in host muscle. B6 and B6Tg^{GFP} mice were housed in our level 2 biosafety animal facility and received food and water *ad libitum*. Before manipulations, animals were anesthetized using an intraperitoneal injection of chloral hydrate. This study was conducted in accordance with the European Community guidelines for animal care (Journal Officiel des Communautés Européennes, L358, December 18, 1986).

BM Transplantation

Briefly, donor BM cells were obtained by flushing femurs of B6Tg^{GFP} mice with Dulbecco's modified Eagle's medium (Invitrogen, Paisley, UK), and washed twice in cold phosphate-buffered saline (PBS, Invitrogen). Retro-orbital injection of 3 to 5×10^7 BM cells in 0.1 ml of mouse serum and PBS (1:1), was done in 9.0-Gy-irradiated, 4-week-old B6 mice (⁶⁰Co γ -rays within 1 day before BM transplantation). After transplantation, mice received 10 mg/kg/day ciprofloxacin for 4 weeks to prevent infection during the aplastic phase.

Flow Cytometry Analysis

To quantify the amount of engraftment, the peripheral blood mononuclear cells of transplanted mice were analyzed by flow cytometry using a XL cytometer (Beckman-Coulter, Hialeah, FL) before sacrifice, ie, at 1, 3, and 6 months after transplantation. Leukocytes were gated on, and GFP fluorescence was measured under the fluorescein isothiocyanate channel. All analyses and quantitation were performed using the System II software from Beckman-Coulter.

Tissue Preparation

Paraformaldehyde fixation was used to retain GFP within cells, rapid loss of the GFP signal being observed in fresh-frozen sections. At sacrifice time mice were anesthetized and sequentially transcardially perfused with PBS and buffered 4% paraformaldehyde. Whole muscle

groups were then gently removed, postfixed in 4% paraformaldehyde for 2 hours, and soaked in 10% sucrose in PBS for 2 hours and then in 30% sucrose overnight at 4°C. Whole muscle samples were snap-frozen in embedding medium (Tissue-Tek; Sakura) and serial 7- μ m-thick cryosections were performed. All sections were then coverslipped with Vectashield mounting medium for fluorescence (Vector Laboratories, Burlingame, CA) with or without a nuclear counterstaining by 4,6-diamidino-2-phenylindole. Images were captured on a Zeiss Axiophot microscope (Carl Zeiss Inc., Germany) with an Orca ER digital camera (Hamamatsu Photonics, Japan) using Simple PCI (C-Imaging, Compix Inc.) software.

Immunohistochemistry

In all these experiments, muscle sections were gently trypsinized for 10 minutes at 37°C using commercial trypsin-ethylenediaminetetraacetic acid (Invitrogen) for antigen retrieval. Samples were blocked for 20 minutes in PBS/20% fetal calf serum/0.3% Triton X-100 (Sigma-Aldrich, St. Louis, MO). Primary antibodies were incubated with the sections at 37°C for 1 hour. Three 15-minute washings in PBS were performed between each incubation. Mouse monoclonal antibodies were used at the following concentrations: anti-NCAM (1:100; BD Pharmingen, San Diego, CA), anti-M-cadherin (1:100; NanoTools, Teningen, Germany), anti-Pax7 (1:100; Developmental Studies Hybridoma Bank), anti-laminin-1 (1:100, Sigma-Aldrich), anti-laminin-2 (1:100; Novocastra, New Castle Upon Tyne, UK) with M.O.M. kit (Vector Laboratories) allowing the use of mouse monoclonal antibodies for mouse tissues. The secondary antibody used was tetramethyl-rhodamine-isothiocyanate-conjugated goat anti-mouse (1:200, Jackson Laboratory, Bar Harbor, ME). We also used biotinylated rat anti-mouse antibodies to CD34 (1:50), Scal (1:50), CD11b (1:100), and CD45 (1:50) (BD Pharmingen) revealed by tetramethyl-rhodamine-isothiocyanate-conjugated streptavidin (1:400, Vector Laboratories).

Statistical Analysis

Unpaired Student's *t*-test was used for all statistical analyses (GraphPad-InStat software).

Results

Nine B6 mice transplanted with GFP⁺ BM cells were included in the study. All had a proportion of GFP⁺ peripheral blood mononucleated cells >95% as compared to donor values.

Abundant GFP⁺ mononuclear cells appeared in muscle tissue after transplantation (Figure 1; A to D). Their number increased from 15.9 per 100 muscle fibers at 1 month to 26.4 at 6 months ($P < 0.03$) in cross sections of the tibialis anterior muscle (Table 1). Labeling with anti-laminin 1 or 2 antibodies showed GFP⁺ mononucleated

Figure 1. Detection of GFP⁺ BM donor-derived cells in skeletal muscle of transplant recipient mice. Representative cross and longitudinal 7- μ m sections of a fixed tibialis anterior muscle from a GFP⁺ BM recipient mouse analyzed by fluorescent microscopy 3 months after transplantation. **A:** GFP⁺ (green) spindle-shaped perivascular cells that do not express CD45 are shown. **B:** Merge image of GFP⁺ cells and laminin 1 (red) showing BM-derived cells in both interstitial connective tissue and sublaminal satellite cell niche (arrow). L1 refers to laminin 1. The areas where myofibers take place are labeled by asterisks. **C** and **D:** Longitudinal and cross sections of GFP⁺ muscle fibers. Peripheral myonuclei are seen in **D**. The longitudinal section shows two adjacent GFP⁺ muscle fibers, one with a homogenous sarcoplasmic expression of GFP and one with a heterogeneous expression suggesting distinct nuclear cytoplasmic domains because of the recent fusion of donor-derived cells with the recipient muscle fiber (**C**). Nuclei are labeled with 4,6-diamidino-2-phenylindole (blue). Scale bar, 10 μ m.

cells both inside and outside of the muscle fiber basal lamina (Figure 1B).

The proportion of GFP⁺ cells found in a sublaminal satellite cell niche increased with time (Table 1), 4.1% of GFP⁺ cells being sublaminal at 1 month, 9.4% at 3 months, and 14.4% at 6 months (all $P < 0.003$). These cells consistently expressed M-cadherin (Figure 2; A to

D), a specific satellite cell marker,⁵ GFP⁺ M-cadherin⁺ cells accounting for 0.9%, 1.7%, and 2.6% of M-cadherin⁺ cells at 1, 3, and 6 months, respectively (1 versus 6 months; $P < 0.02$). Moreover, some GFP⁺ cells distinctly expressed Pax7, a nuclear factor essential for the specification of muscle stem cells into sublaminal satellite cells⁴ (Figure 2; E to H), and NCAM (leu19) a marker

Table 1. Quantitation of GFP⁺ Mononuclear Cells and Myofibers in Bone Marrow Transplant Recipient Mice

(A) Mononuclear cells quantified in cross-sections of <i>tibialis anterior</i> muscle									
Time after transplant	Animals (n)	Myofibers evaluated (n)	GFP+ cells (for 100 myofibers)	GFP+ CD11b+/GFP+ cells (%)	GFP+ satellite cells/GFP+ cells (%)	GFP+ M-cad+/M-cad+ cells (%)	GFP+ CD34+/CD34+ cells (%)	GFP+ Sca1+/Sca1+ cells (%)	GFP+ vWF+/vWF+ cells (%)
1 month	3	970–5100	15.9 $\pm$ 1.6	37.0 $\pm$ 9.4	4.1 $\pm$ 1.0	0.9 $\pm$ 0.1	0.8 $\pm$ 0.3	0.0 $\pm$ 0.0	0.1 $\pm$ 0.2
3 months	3	800–4165	17.0 $\pm$ 3.5	35.3 $\pm$ 5.8	9.4 $\pm$ 0.9	1.7 $\pm$ 0.2	1.7 $\pm$ 0.2	0.8 $\pm$ 0.1	0.2 $\pm$ 0.1
6 months	3	830–1255	26.4 $\pm$ 5.0	33.4 $\pm$ 2.8	14.4 $\pm$ 2.3	2.6 $\pm$ 0.8	4.1 $\pm$ 0.4	1.3 $\pm$ 0.3	0.2 $\pm$ 0.1

(B) Myofibers quantified in cross-sections of various muscles							
Time after transplant	Animals (n)	Myofibers evaluated	Tibialis anterior muscle (%)	Rectus abdominalis muscle (%)	Soleus muscle (%)	Gastrocnemius muscle (%)	Diaphragm
1 month	3	5100	0.0 $\pm$ 0.0	—	—	—	—
3 months	3	4165	0.5 $\pm$ 0.4	—	—	—	—
6 months	3	752–1255	1.3 $\pm$ 0.8	2.6 $\pm$ 0.2	1.6 $\pm$ 0.6	1.9 $\pm$ 0.9	1.5 $\pm$ 1.0

Figure 2. Detection of GFP⁺ satellite cells exhibiting co-localization of definitive markers. Representative cross sections of fixed tibialis anterior muscles 6 months after transplantation showing co-expression of GFP and a panel of satellite cell antigens, including M-cadherin (**A–D**), the nuclear transcription factor Pax7 (**E–H**), and NCAM (**I–L**). Nuclei are labeled with 4,6-diamidino-2-phenylindole (blue). Each image in **D**, **H**, and **L** corresponds to an increasing of background to make myofibers more apparent. Scale bar, 10 μ m.

typically expressed by satellite cells and myoblasts (Figure 2; I to L). In addition to an increasing number of GFP⁺ muscle satellite cells, scattered GFP⁺ muscle fibers appeared at 3 and 6 months after transplantation (Figure 1, C and D). At 6 months, 1.3% of fibers in the tibialis anterior muscle were GFP⁺. A similar percentage was observed in the four other evaluated muscle groups (Table 1). Serial cross-sections and longitudinal sections showed that GFP was either homogeneously expressed along the whole muscle fiber length (up to 1500 μ m) or, occasionally, as distinct nuclear cytoplasmic domains (Figure 1C). Unlike muscle fibers undergoing regeneration after necrosis, most GFP⁺ muscle fibers had peripheral nuclei. These findings were consistent with fusion of GFP⁺ satellite cells to pre-existing fibers.

In addition to the well-defined GFP⁺ satellite cells and muscle fibers, GFP⁺ mononucleated cells were found in muscle interstitial tissue. Approximately one third of GFP⁺ cells expressed the monocyte/macrophage lineage marker CD11b at all time points after transplantation (Table 1). In addition, GFP⁺ mononucleated cells expressing Scal or CD34 were detected in the interstitial connective tissue (Figure 3; A to F), at perivascular and endomysial sites known to host multipotent muscle stem cells.^{6,8}

The GFP⁺ Scal⁺ cells significantly increased in number with time ($P < 0.05$) but remained less numerous than GFP⁺ M-cadherin⁺ cells at all time points (Table 1, Figure 4). GFP⁺ Scal⁺ cells were usually detected in the vicinity of large and small vessels, as previously described,⁸ and were not found within satellite cell niches (Figure 3; A to C). GFP⁺ CD34⁺ cells were detected throughout muscle connective tissue (Figure 3; D to F). Although CD34 was normally expressed by small vessel endothelial cells, no GFP⁺ CD34⁺ cell lining a distinct lumen or enclosed by a laminin 1-positive vascular basal lamina was detected. Consistently, a very small number of GFP⁺ cells expressed the von Willebrand factor endothelial cell marker (0.1 to 0.2% at the different time points). Although sublaminal CD34⁺ cells were occasionally seen, GFP⁺ CD34⁺ cells without basal lamina material interposed between the plasmalemma and the underlying muscle fiber were rarely detected after double staining with anti-laminin antibodies (1 of 2664 GFP⁺ cells). GFP⁺ CD34⁺ cells were more abundant than GFP⁺ Scal⁺ cells and less abundant than GFP⁺ M-cadherin⁺ cells, at all time points (Figure 4).

Interstitial GFP⁺ cells expressing CD45⁺, the exclusive marker of the hematopoietic lineage, were rarely

Figure 3. Characterization of interstitial stem cells originating from donor BM. Representative sections of fixed tibialis anterior muscles 6 months after transplantation showing co-expression of GFP and the stem cell antigens *Sca1* and CD34, and hematopoietic lineage marker CD45. **A to C:** A donor-derived *Sca1*⁺ cell is located in interstitial connective tissue close to a muscle fiber. Nuclei are labeled with 4,6-diamidino-2-phenylindole (blue). **D to F:** Two donor-derived CD34⁺ cells are located in muscle connective tissue in a perivascular area. The vessel is a medium-sized artery as assessed by the presence of an autofluorescent elastic lamina observed in all wavelengths. **D to F:** GFP⁺ cells are located outside the vascular basal lamina stained with an anti-laminin 1 antibody (L1, blue). **G to I:** The rarely detected GFP⁺ cells expressing CD45 were rounded like mononuclear blood cells. Scale bar, 10 μ m.

detected and appeared rounded like mononuclear leukocytes (Figure 3; G to I). Although spindle-shaped interstitial GFP⁺ cells could express CD34 (Figure 3; G to I), they did not express CD45 (Figure 1A).

Discussion

In this study we observed an increasing number of BM-derived GFP⁺ mononucleated cells in skeletal muscle at 1, 3, and 6 months after transplantation, including satellite cells, characterized by both sublaminal location and expression of the unambiguous markers of committed

myogenic cells, and interstitial cells expressing the stem cell antigens Sca-1 and CD34. These BM-derived cells were able to fuse as assessed by the formation of an increasing number of GFP⁺ muscle fibers.

While this study was in progress, two articles have reported the possible BM-derivation of the muscle satellite cells using GFP⁺ BM transplantation procedures.^{12,13} GFP⁺ satellite cells could not be identified by specific markers,¹² or only expressed certain satellite cell markers such as c-met, α 7-integrin, and Myf-5.¹³ We report herein that BM-derived satellite cells express the well-known markers M-Cad, Pax7, and NCAM.⁵ Moreover,

Figure 4. Respective numbers of GFP⁺ cells exhibiting co-localization of different markers per whole tibialis anterior muscle cross-section at various time points after transplantation. Three animals were analyzed at each time point. Results are expressed as mean $\pm$ SEM. M-Cadherin (M-Cad) was used as a satellite cell marker, *Sca1* and CD34 as stem cell markers, and von Willebrand factor (vWF) as an endothelial cell marker.

previous studies used teased muscle fiber preparations, a procedure implying removal of connective tissue, thus precluding analysis of muscle interstitial cells. We extend previous observations on teased fiber preparations by demonstrating that stem cell marker-expressing cells found in connective tissue can derive from BM in adulthood.

Sca1, an early marker of murine hematopoietic stem cells¹⁴, has been previously found expressed by mononuclear cells in skeletal muscle tissue.⁸ Such *Sca1*⁺ cells likely constitute one phenotype of muscle multipotent stem cells,⁸ called side population (SP) cells based on their ability to efflux the fluorescent dye Hoechst 33342.^{2,15} As in the present study, they are usually observed in the vicinity of endomysial vessels⁸ but virtually never in subliminal location.¹⁶ Muscle *Sca1*⁺ cells have been subdivided into CD45⁺ hematopoietic cells and CD45⁻ cells that contain the bulk of the myogenic activity.¹⁷ Skeletal muscle *Sca1*⁺ cells also include both CD34⁻ and CD34⁺ subpopulations.¹⁸

Distribution of CD34⁺ cells in muscle is more controversial than that of *Sca1*⁺ cells.⁶ Indeed, CD34 expression by satellite cells was repeatedly reported,^{8,19,20} but could not be substantiated by immunoelectron microscopy.⁶ Because CD34 is a versatile marker expressed on *in vivo* and *in vitro* activation,^{21,22} it is possible that the *in vivo* fixation we used, as did Tamaki and colleagues,⁶ accounted for the relatively low number of CD34⁺ satellite

cells observed in our study as compared to teased fiber studies.^{8,19}

As in previous studies, BM transplantation was performed in mice irradiated at 9 to 10 Gy,^{12,13} a dose inducing a 66% decrease of the muscle satellite cell number.¹³ It is, therefore, likely that muscle irradiation behaves as a conditioning procedure, emptying satellite cell niches presumably favoring their subsequent occupancy by BM-derived cells. Our experiments were conducted in standard, nonexercise-enriched, conditions. Such conditions are associated with a rather low rate of GFP⁺ muscle fiber formation, whereas chronic exercise can induce a marked increase of GFP⁺ muscle fiber density.¹³ In this setting, the higher settlement rate of GFP⁺ M-cadherin⁺ (satellite) cells as compared to GFP⁺ *Sca1*⁺ (interstitial) cells likely reflected an imbalance between cell translocation from the interstitial compartment and the slow output toward muscle fiber formation. Therefore, the remarkable accumulation of BM-derived cells into satellite cell niches indirectly supports the view that extralaminar cells constitute a reservoir of satellite cells.⁶

GFP⁺ muscle fiber formation likely resulted from fusion of BM-derived cells with existing fibers, according to a phenomenon known as myonuclear accretion.²³ Unlike other tissues, such as the central nervous system, where stem cells can misleadingly fuse with differentiated cells to form tetraploid cells of unknown significance,²⁴ BM-derived stem cells likely achieved true myogenic differentiation in skeletal muscle, as suggested by the diploid status of GFP⁺ satellite cells.¹³ The BM cell subset that may give rise to muscle fibers, ie, hematopoietic stem cells or mesenchymal stem cells or both, remains controversial.²⁵

It is too early to decide if BM transplantation will prove useful in the treatment of patients with muscular dystrophy in the future.²⁶ Dystrophin restoration in *mdx* mice after allogeneic BM transplantation^{2,12} remains far below the levels needed to provide clinical benefits in Duchenne muscular dystrophy. It could be expected, however, that further studies aimed at characterizing the pathways for the homing of stem cells would aid in the improvement of the muscle settlement by BM cells.^{27,28} In addition to the fascinating new insights it provides on renewal of the skeletal muscle tissue, transplantation of GFP⁺ BM-derived cells constitutes an appropriate procedure to conduct such studies.

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