Muscle Regeneration by Bone Marrow–Derived Myogenic Progenitors

Giuliana Ferrari, Gabriella Cusella–De Angelis, Marcello Coletta, Egle Paolucci, Anna Stornaiuolo, Giulio Cossu,* Fulvio Mavilio*

Growth and repair of skeletal muscle are normally mediated by the satellite cells that surround muscle fibers. In regenerating muscle, however, the number of myogenic precursors exceeds that of resident satellite cells, implying migration or recruitment of undifferentiated progenitors from other sources. Transplantation of genetically marked bone marrow into immunodeficient mice revealed that marrow-derived cells migrate into areas of induced muscle degeneration, undergo myogenic differentiation, and participate in the regeneration of the damaged fibers. Genetically modified, marrow-derived myogenic progenitors could potentially be used to target therapeutic genes to muscle tissue, providing an alternative strategy for treatment of muscular dystrophies.

In postnatal life, growth and repair of skeletal muscle fibers are mediated by a resident population of mononuclear myogenic precursors, the satellite cells. These cells, which are located between the sarcolemma and the basal lamina of the muscle fiber, divide at a slow rate to sustain both self-renewal and growth of differentiated tissue (*1*). In response to muscle injury, or in individuals with chronic degenerative myopathies, satellite cells divide and fuse to repair or replace the damaged fibers. However, the self-renewal potential of adult satellite cells is limited, decreases with age (*2*), and can be exhausted by a chronic regenerative process such as that characteristic of severe muscular dystrophies, in which most muscle tissue is eventually lost and is replaced by connective tissue (*3*).

The number of resident satellite cells in adult muscle is much smaller than the number of committed myogenic precursors that populate the muscle tissue soon after an injury (*4*). Several explanations for this apparent paradox have been proposed, from migration of satellite cells from adjacent fibers, or even neighboring muscles, to recruitment to myogenesis of resident nonmyogenic cells such as fibroblasts or mesenchymal progenitors (*5*). Bone marrow (BM) stroma–derived mesenchymal cells, which serve as long-lasting precursors for bone, cartilage, and lung parenchyma in mice (*6*), can differentiate into contractile myotubes under certain conditions in vitro (*7*). However, recruitment to myogenesis of stroma-derived cells has not been observed in vivo (*8*).

To investigate whether BM cells can convert to myogenesis in response to physiological stimuli, we chemically induced muscle regeneration in the tibialis anterior (TA) of 10 immunodeficient *scid/bg* mice (*9*). Unfractionated BM cells (10^6 per muscle) obtained from the C57/M*lacZ* transgenic mouse line (*10*), in which a *lacZ* gene encoding a nuclear β-galactosidase (β-Gal) is under the control of the muscle-specific myosin light chain 3F promoter (the MLC3F-*nlacZ* transgene), were then injected into the damaged muscles. Expression of this transgene is restricted to cardiac and skeletal muscle in adult mice (*11*), although it can be activated in other cell types on induction of myogenic differentiation (*12*). Satellite cells were obtained from the same transgenic mice (*13*) and injected (5×10^5 cells per muscle) as a control in the contralateral leg of all recipient animals. TA muscles were examined at various times after injection (5 days to 5 weeks) for the presence of β-Gal–positive nuclei (*14*).

Whole-mount histochemical staining of a TA muscle 2 weeks after injection of total BM (Fig. 1A) revealed fibers containing β-Gal$^+$ aligned nuclei similar to, although less numerous than, those observed in the contralateral leg injected with satellite cells (Fig. 1B). Transverse cryostat sections showed newly formed fibers with β-Gal$^+$ centrally localized nuclei in four out of six

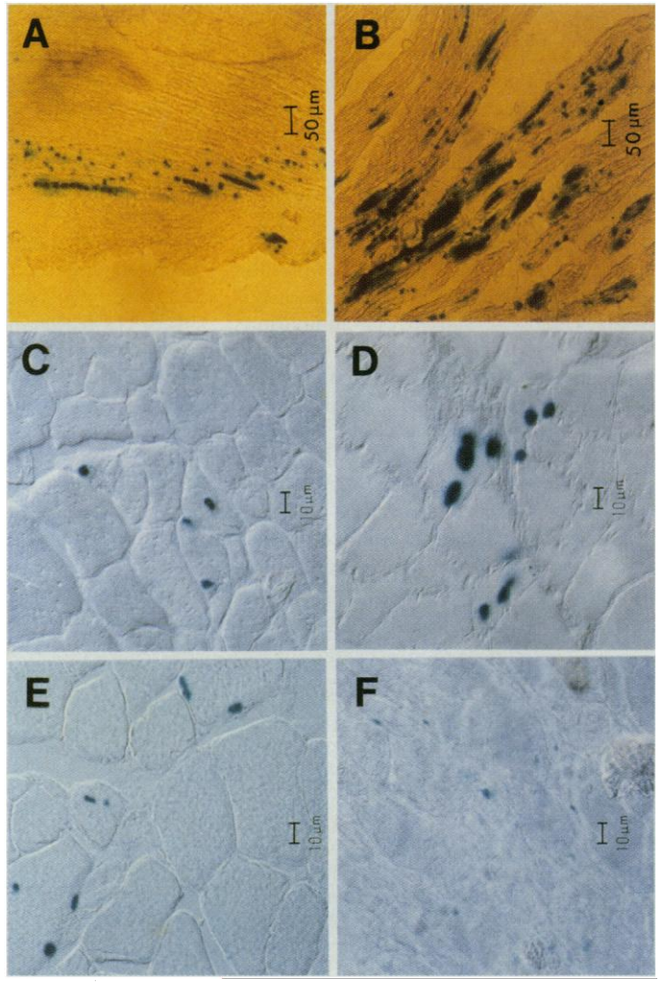

Fig. 1. Analysis of nuclear *lacZ* expression in whole-mount dissected fibers (**A** and **B**) or cryostat sections (**C** through **F**) of regenerating TA muscles of *scid/bg* mice. Mice were injected with unfractionated (A and C), adherent (E), or nonadherent (F) BM cells, or with control satellite cells (B and D), from C57/M*lacZ* transgenic mice. (A and B) Bright field; scale bars, 50 μm. (C through F) Nomarski optics; scale bars, 10 μm.

G. Ferrari, E. Paolucci, A. Stornaiuolo, F. Mavilio, H. San Raffaele–Telethon Institute for Gene Therapy (TIGET), 20132 Milan, Italy.
G. Cusella–De Angelis, Istituto di Anatomia Umana Normale, University of Pavia, 27100 Pavia, Italy.
M. Coletta and G. Cossu, Dipartimento di Istologia ed Embriologia Medica, University of Rome La Sapienza, 00161 Rome, Italy.

*To whom correspondence should be addressed. E-mail: cossu@axrma.uniroma1.it (G.C.) or mavilio@tigem.it (F.M.).

mice at 2 to 5 weeks after injection of BM cells (Fig. 1C). No staining was observed in two mice analyzed after 5 days and one after 10 days. Conversely, centrally localized, β-Gal$^+$ nuclei were observed in all muscles injected with satellite cells, as early as 5 days after injection (Fig. 1D) (*15*).

In a second series of experiments, BM from C57/M*lacZ* mice was fractionated in vitro into adherent and nonadherent components (*16*). These components were then separately injected into the regenerating TA muscles of 15 *scid/bg* mice, and the mice were analyzed after 1, 2, and 6 weeks. β-Gal$^+$ nuclei were observed in six mice injected with adherent cells and in three injected with the nonadherent fraction at 2 to 6 weeks after injection (Fig. 1, E and F). Activation of the MLC3F-*nlacZ* transgene in BM or blood cells was never observed in a nonmuscle environment in vitro or in vivo (for example, in inflammatory cells elicited by the intraperitoneal injection of thioglycollate). Thus, a population of cells within the BM entered a myogenic differentiation pathway when exposed to a regenerating muscle environment and actively participated in the formation of new muscle fibers.

To test whether myogenic progenitors could be physiologically recruited from BM and access a site of muscle regeneration from the peripheral circulation, we transplanted genetically marked BM cells from the C57/M*lacZ* line (H-2^b) into 12 irradiated *scid/bg* mice (H-2^d) (*17*). Five weeks after BM transplantation, muscle regeneration was induced in both TA muscles of nine surviving mice (*18*). All mice were examined 1, 2, and 3 weeks after induction of muscle damage for reconstitution of both the immune and nonimmune components of the hematopoietic system by analysis of the morphology and phenotype of BM, spleen, and peripheral blood cells. Flow cytometric analysis of peripheral blood nucleated cells revealed that all transplanted animals possessed a circulating lymphocyte population; such a population was virtually absent in *scid/bg* animals (Fig. 2A). CD4 and CD8 marker analysis confirmed that mature lymphocytes were present in a proportion (34.0 ± 2.9 and 15.2 ± 1.9%, respectively) similar to that of normal donors (Fig. 2B). Analysis of the H-2^b (donor) haplotype in the total nucleated cell population of engrafted *scid/bg* mice revealed that all such cells were donor-derived (Fig. 2C). Virtually complete chimerism (80 to 90%) was also apparent in BM and spleen cells (*19*).

Regeneration was analyzed histochemically in the TA muscles of all transplanted mice. Transverse cryostat sections showed regenerating fibers containing β-Gal$^+$ nuclei in five of six reconstituted animals analyzed at 2 and 3 weeks after induction of muscle injury (Fig. 3, B through D) (*20*). Hoechst nuclear staining showed that β-Gal$^+$ nuclei were present both in immature centrally nucleated fibers (Fig. 3, B and C) and in more mature peripherally nucleated fibers (Fig. 3D). Blue nuclei were observed in none of the three mice analyzed after 1 week, when TA muscles showed an early regeneration pattern characterized by marked infiltration of mononuclear cells and a majority of small newly formed myofibers (Fig. 3A). In rare instances, β-Gal$^+$ nuclei were apparent in mononuclear cells infiltrating the areas of muscle regeneration or in a peripheral position within a centrally nucleated fiber (Fig. 3, E and F).

Our data indicate the existence of BM-derived myogenic progenitors that can migrate into a degenerating muscle, participate in the regeneration process, and give rise to fully differentiated muscle fibers. These cells appear to be recruited by long-range, possibly inflammatory, signals originating from the degenerating tissue, and they appear to access the damaged muscle from the circulation, together with granulocytes and macrophages. The kinetics of differentiation of BM-derived progenitors differ from those for differentiation of committed adult myogenic precursors. Injected satellite cells fused into muscle fibers within 5 days, whereas β-Gal$^+$ nuclei of BM origin were not detected in regenerating fibers before 2 weeks after induction of muscle damage. This observation, together with the observed clustering of β-Gal$^+$ nuclei in regenerated fibers, may suggest that BM-derived progenitors undergo a longer, and possibly multistep, differentiation process, which may comprise migration, cell division, commitment to the myogenic lineage, and eventual terminal maturation and fusion.

The origin of the BM-derived myogenic cells, as well as their physiological role in the homeostasis of muscle tissue, are not known. It is possible that these cells originate from multipotent, mesenchymal stem cells in the BM stroma that have been shown, by similar transplantation experiments, to give rise to bone, cartilage, and connective tissue (6). Whether or not myogenic cells are derived from the same mesenchymal component, our experiments suggest that the BM could serve as a reservoir of progenitors for muscle tissue, and that, under conditions of extended damage, these progenitors might expand or maintain the pool of resident, more differentiated, muscle-forming precursors.

The existence of circulating myogenic progenitors has implications for cell or gene therapy for inherited muscle disorders. Efficient delivery to diseased muscles of genetically modified myoblasts, or even of viral vectors containing therapeutic genes, is one of the major hurdles currently limiting both ex vivo and in vivo approaches (*21*). Despite some anecdotal observations, it is generally accepted that satellite cells taken from skeletal muscle cannot colonize muscle tissue if delivered from the circulation (*22*). The availability

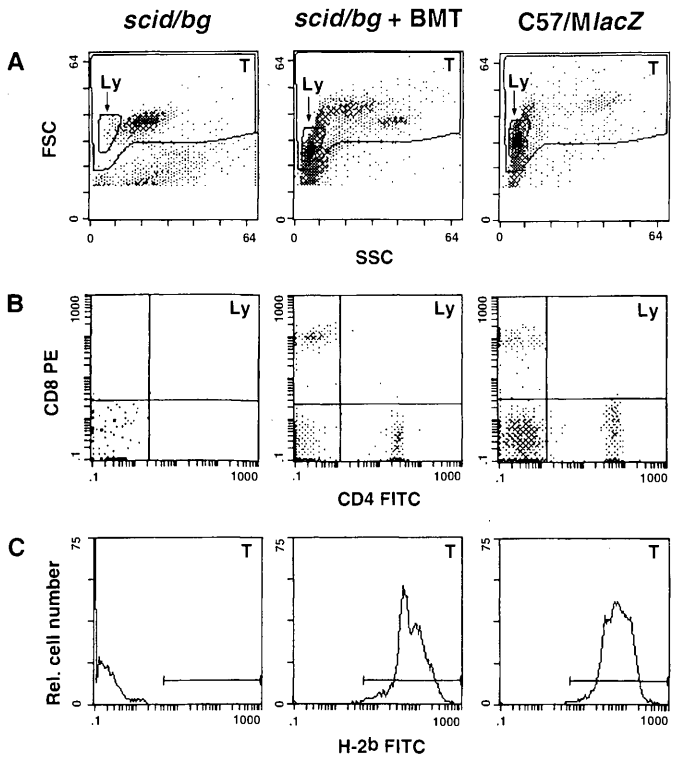

Fig. 2. Flow cytometric analysis of peripheral blood nucleated cells from a *scid/bg* mouse 8 weeks after transplantation with BM from C57/M*lacZ* mice (*scid/bg* + BMT). (**A**) Forward (FSC) and side (SSC) scatter plots. The gated lymphocyte (Ly) and total (T) cell populations are shown. (**B**) Lymphocyte population stained with fluorescein isothiocyanate (FITC)–conjugated antibodies to CD4 (*x* axis) and phycoerythrin (PE)–conjugated antibodies to CD8 (*y* axis). (**C**) Total cell population stained with FITC-conjugated antibodies to H-2K^{b}D^b. Log-scale fluorescence is shown on both axes in (B), and on the *x* axis in (C). Analysis of cells from untransplanted *scid/bg* mice and immunocompetent, donor C57/M*lacZ* mice is shown in the left and right panels, respectively. Rel., relative.

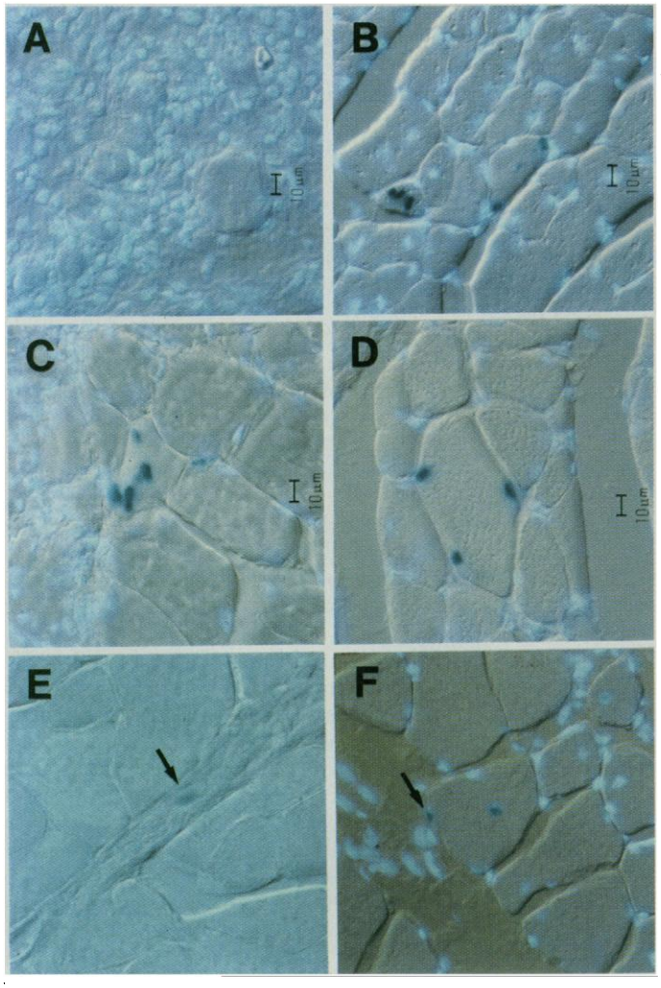

Fig. 3. Analysis of nuclear *lacZ* expression in cryostat sections of regenerating TA muscles from *scid/bg* mice transplanted with BM from C57/M*lacZ* mice. Muscles were analyzed 1 week (**A**), 2 weeks (**B**), and 3 weeks (**C** and **D**) after cardiotoxin injection. (**E**) A mononuclear cell with nuclear β-Gal staining (arrow) 2 weeks after cardiotoxin injection. (**F**) A peripheral β-Gal$^+$ nucleus (arrow) within a centrally nucleated regenerating fiber. Nomarski optics; scale bars, 10 μm. (A) through (D) and (F) also show Hoechst fluorescent nuclear staining, revealing areas of early (centralized nuclei) or more advanced (peripheral nuclei) fiber regeneration.

of a cell population that could be engineered and then systemically delivered to a large number of muscles might aid in the development of a cell-mediated replacement therapy. In our experiments, BM-derived progenitors contributed only minimally to muscle regeneration. However, resident satellite cells are healthy in *scid/bg* mice and are unaffected by the low-dose radiation administered before BM transplantation. The situation might be substantially different in a dystrophic background characterized by chronic muscle degeneration, in which genetically corrected BM-derived cells could progressively replace the exhausted pool of satellite cells. The therapeutic potential of transplanted BM cells awaits further verification in such a model.

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9. The *scid/bg* mouse strain combines the characteristics of *scid* (severe combined immunodeficiency) animals, which lack functional B and T cells, with those of *beige* (*bg*) animals, which have intrinsically low natural killer (NK) cell activity. The *scid/bg* mice show a more stable SCID phenotype and are, in general, better recipients for allograft transplantation [A. M. Krensky, *Nature Biotechnol.* **15**, 720 (1997)]. Seven- to 9-week-old C.B-17 *scid/bg* mice were obtained from Charles River (Calco, Italy) and maintained under specific pathogen-free conditions. Mice were anesthetized and regeneration was induced in TA muscles by injection of 25 μl of 1 mM cardiotoxin (Latoxan, Rosans, France). Animal experimentation protocols were approved by the HSR Institutional Animal Care and Use Committee.
10. The C57/M*lacZ* strain was obtained by crossing the MLC3F-*nlacZ*-E transgenic line (*11*) with C57/BL6 inbred mice (Charles River), followed by three generations of backcrossing to the C57/BL6 strain. Segregation of the transgene was monitored by whole-mount 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining [J. R. Sanes, J. L. R. Rubenstein, J. F. Nicolas, *EMBO J.* **5**, 3133 (1986)] of skinned tail sections. BM was obtained by flushing femurs of 4- to 7-week-old C57/M*lacZ* mice and was injected [10^6 cells in 25 μl of phosphate-buffered saline (PBS)] into regenerating TA muscles of *scid/bg* mice 24 hours after cardiotoxin injection.
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13. Satellite cells were isolated from skeletal muscle of 4- to 6-week-old C57/M*lacZ* mice by digestion with dispase and collagenase [G. Salvatori *et al.*, *Hum. Gene Ther.* **4**, 713 (1993)], and were then plated on plastic petri dishes and maintained for 1 to 2 hours at 37°C in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Hyclone). Nonadherent cells were collected, resuspended in PBS, and injected (5×10^5 cells in 25 μl) into regenerating TA muscles of *scid/bg* mice 24 hours after cardiotoxin injection.
14. TA muscles were removed, fixed in 4% paraformaldehyde, embedded in ornithyne carbamoyl transferase, frozen in liquid nitrogen–cooled isopentane, and cut on a cryostat into 15- to 20-μm serial sections. β-Gal activity was assayed in whole-mount preparations or in cryostat sections by X-Gal staining (*10*).
15. TA muscles were sectioned entirely, and β-Gal$^+$ nuclei were counted in all sections of muscles injected with BM cells and in one of every five sections of muscles injected with satellite cells. The number of positive nuclei ranged from 100 to 500 per muscle in mice injected with total BM. Recovery of satellite cells (number of β-Gal$^+$ nuclei divided by the total number of injected cells) ranged from 0.5 to 8.5%.
16. C57/M*lacZ* BM cells were plated in DMEM supplemented with 12.5% FBS and 12.5% horse serum (Hyclone) in petri dishes and incubated at 37°C under humidified 5% CO_2 for 24 hours. Nonadherent (10^6) and adherent (5×10^5) cells were collected, resuspended in 25 μl of PBS, and injected separately into regenerating TA muscles of *scid/bg* mice 24 hours after cardiotoxin injection.
17. Seven-week-old *scid/bg* (H-2^d) mice were subjected to sublethal irradiation (2.5 to 3.0 gray) with an x-ray source. Four to 5 hours later, unfractionated BM cells (15×10^6 to 20×10^6) from 6- to 7-week-old C57/M*lacZ* (H-2^b) donors were injected into the tail vein of the irradiated animals. Animals were maintained under specific pathogen-free conditions throughout the procedure. The *scid/bg* mutants were chosen as a model for BM transplantation because of a lack of a suitable co-isogenic host and because (i) immunodeficient mice are fully reconstituted after low-dose irradiation [G. M. Fulop and R. A. Phillips, *J. Immunol.* **136**, 4438 (1986)]; (ii) graft-versus-host disease is not observed in *scid* mice, avoiding the necessity of any pretransplant manipulation, such as T cell depletion, of BM cells; and (iii) *bg* mice are severely deficient in the NK cell component, thus overcoming the problem of NK cell–mediated allograft rejection [W. J. Murphy, V. Kumar, M. Bennett, *J. Exp. Med.* **165**, 1212 (1987)].
18. Two mice did not survive radiation treatment, and one animal was killed after 4 weeks to monitor engraftment.
19. Analysis of the size and cellularity of the thymus and spleen, usually reduced to one-tenth of normal in *scid/bg* animals, analysis of Giemsa-stained cytocentrifuged cell preparations, and flow cytometric analysis of BM, thymus, and spleen cells were used as additional criteria to confirm engraftment in central and peripheral hematopoietic organs.
20. Muscles were sectioned entirely, and all sections were scored for the presence of β-Gal$^+$ nuclei. Positive muscles contained 50 to 100 blue nuclei, clustered in <1% of the regenerated fibers.
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