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Biological Progression from Adult Bone Marrow to Mononucleate Muscle Stem Cell to Multinucleate Muscle Fiber in Response to Injury

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Summary

Adult bone marrow-derived cells (BMDC) are shown to contribute to muscle tissue in a step-wise biological progression. Following irradiation-induced damage, transplanted GFP-labeled BMDC become satellite cells: membrane-ensheathed mononucleate muscle stem cells. Following a subsequent exercise-induced damage, GFP-labeled multinucleate myofibers are detected. Isolated GFP-labeled satellite cells are heritably myogenic. They express three characteristic muscle markers, are karyotypically diploid, and form clones that can fuse into multinucleate cells in culture or into myofibers after injection into mouse muscles. These results suggest that two temporally distinct injury-related signals first induce BMDC to occupy the muscle stem cell niche and then to help regenerate mature muscle fibers. The stress-induced progression of BMDC to muscle satellite cell to muscle fiber results in a contribution to as many as 3.5% of muscle fibers and is due to developmental plasticity in response to environmental cues.

Introduction

In the past three years, numerous reports have demonstrated that following a marrow transplant, bone marrow-derived cells (BMDC) are present in diverse tissues in mice, where they express characteristic tissue-specific proteins. These tissues include heart, epithelium, liver, skeletal muscle, and brain (Bittner et al., 1999; Brazelton et al., 2000; Ferrari et al., 1998, 2001; Fukada et al., 2002; Gussoni et al., 1999; Jackson et al., 2001; Krause et al., 2001; Lagasse et al., 2000; Mezey et al., 2000; Orlic et al., 2001). Tissue-specific stem cells, such as hepatic oval cells and hematopoietic stem cells, have long been recognized as fulfilling a function in replenishing damaged liver and blood, respectively. Remarkably, the recent results suggest that repair of tissues may also derive from other cells that are not tissue-specific stem cells. Even in adulthood, cells within the bone marrow appear to be capable of unexpected differentiation into a variety of tissue types, not only in mice, but also in humans (Korbling et al., 2002; Quaini et al., 2002). Whether these changes in cell function are the result of a random and rare event or result from a biological process remains to be determined, as the frequencies of these events has generally been very low. Here we address this question, taking advantage of properties unique to muscle as well as a genetic marker of transplanted BMDC, GFP, to track their fate following lethal irradiation and reconstitution of the blood.

To determine whether the observed contribution of BMDC to tissues is biologically relevant, we examined whether BMDC could become mononucleate diploid heritable stem cells en route to becoming multinucleate differentiated myofibers. Specifically, we tested the hypothesis that in mice, a progression from adult bone marrow to adult muscle fibers occurs via a tissue-specific stem cell intermediate, the quiescent muscle satellite cell. Tissue-specific stem cells occupy niches, microenvironments that instruct and support stem cell self-renewal, proliferation, and differentiation (Schofield, 1978), providing specific cellular neighbors, signaling molecules, and extracellular matrix components (Spradling et al., 2001; Watt and Hogan, 2000). It is well known that in response to a stress-inducing injury, endogenous satellite cells contribute to mature muscle fibers at a relatively high frequency (Grounds, 1999) and that injected muscle cell precursors can replace endogenous satellite cells ablated by γ irradiation (Blaveri et al., 1999). Although tissue-specific stem cells reside within a niche, not all niches are readily accessible and may therefore be difficult to study. By contrast, the muscle satellite cell is a particularly attractive choice for addressing the hypothesis that bone marrow-derived regeneration of nonhematopoietic tissues occurs as a progression through a tissue-specific stem cell. As a result, muscle stem cells, known as satellite cells, are well defined anatomically and biochemically, both in vivo and in vitro (Cornelison and Wold, 1997; Mauro, 1961; Zammit and Beauchamp, 2001), and are ideal for this purpose.

In this report, we demonstrate that following bone marrow transplantation, cells from the bone marrow respond to two temporally distinct biological cues. First, irradiation-induced damage, which leads to ablation of endogenous satellite cells in the muscle stem cell niche, resulted in occupancy of this niche by BMDC. Second, subsequent exercise-induced damage caused BMDC satellite cells to participate in the regeneration of multinucleate muscle fibers at a frequency (3.5%) significantly greater than previously reported for any bone marrow to muscle conversion. The bone marrow-derived cells became heritably myogenic. As satellite cells they expressed muscle-specific proteins in vivo and in vitro and exhibited self-renewal in tissue culture, giving rise to proliferative clones of myoblasts. These myoblast progeny could differentiate to form myotubes in culture or fuse with host myofibers following injection into muscle tissues of mice. Clones derived from single BMDC myoblasts expressed GFP as well as the muscle markers desmin, Myf-5, cMet-R, and α7-integrin, on a par with control primary myoblasts. Together, these data demonstrate that in adult mice, bone marrow-derived cells give rise to tissue-specific karyotypically diploid stem cells, muscle satellite cells, both anatomically and

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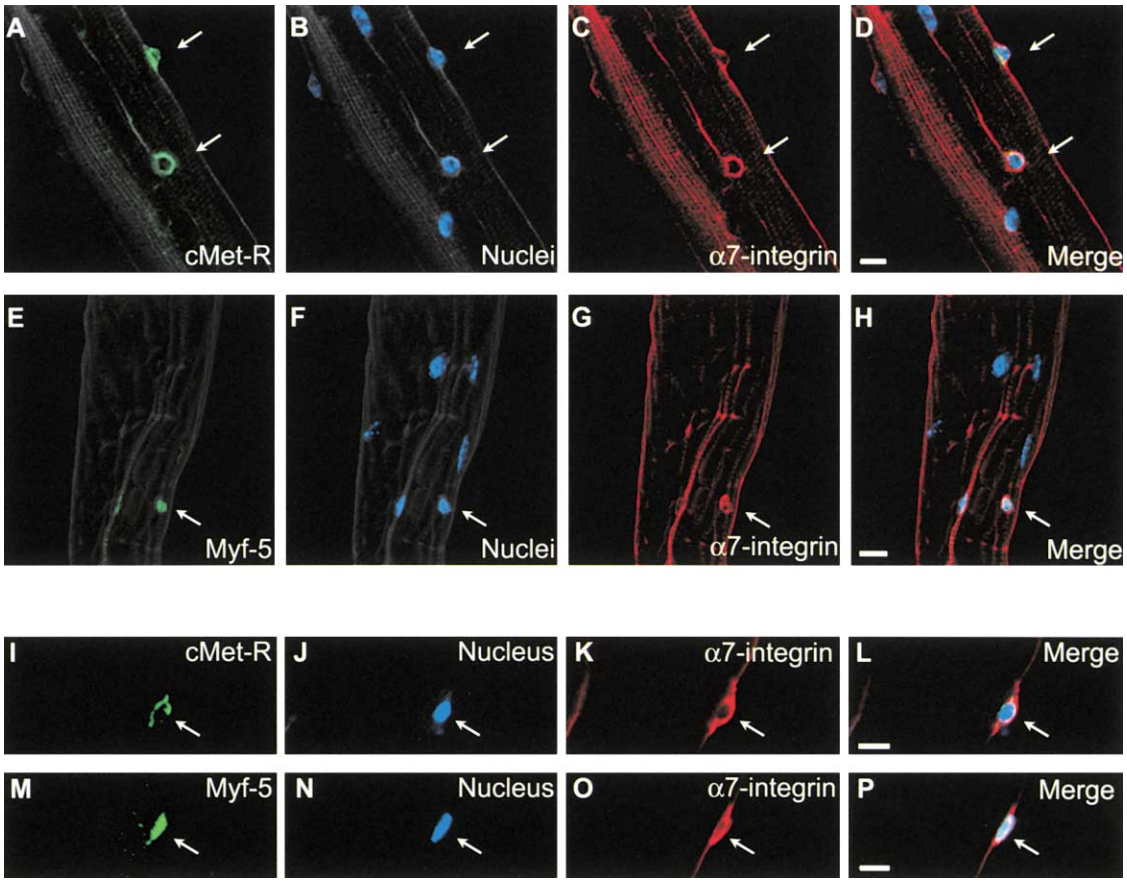

Figure 1. Characterization of Muscle Satellite Cells, Tissue-Specific Stem Cells, on Isolated Single Myofibers

Shown here are representative samples from five experiments. 3D reconstructions were compiled from 20 optical sections of single myofibers collected with a laser scanning confocal microscope. A subset of the total satellite cells are indicated in this field by arrows to demonstrate that satellite cells are circumscribed by α7-integrin on the membrane (C) that surrounds ToPro-stained nuclei (B), on which the tyrosine kinase receptor cMet-R is evident (A), as shown in the merged image (D). Together, these markers outline a discrete boundary between the muscle-specific stem cells (satellite cells) juxtaposed to each myofiber. α7-integrin is present on satellite cell and myofiber membranes showing the relationship of the two compartments, whereas cMet-R is specific to satellite cell membranes. In another 3D reconstruction, a satellite cell nucleus in this field (arrow) (F) is expressing the transcription factor Myf-5 (E) and α7-integrin (G) as shown in the merged image (H). A single 1.5 μm optical section shows cMet-R (I) circumscribing the nucleus (J) of a satellite cell (arrow) clearly surrounded by α7-integrin (K), with the merged image (L) demonstrating a clear boundary between satellite cell and myofiber. A second 1.5 μm optical section shows a different satellite cell (arrow) staining for Myf-5 (M), with nucleus (N), α7-integrin (O), and the merged image (P). Bar represents 10 μm. In (A), (B), (E), and (F), the muscle fiber is shown in dark gray for context.

functionally, and that these cells can proliferate as myoblasts and participate in normal regenerative processes in response to two temporally distinct injuries.

Results

Characterization of Muscle Stem Cells, the Satellite Cells

Muscle stem cells, or satellite cells, are particularly well suited to address the question of whether bone marrow-derived stem cells (BMDC) can give rise to tissue-specific stem cells en route to participating in mature tissue. Unlike most tissue-specific stem cells that are difficult to identify, muscle stem cells, or satellite cells, can be reliably identified based on their highly specific location and morphology. Satellite cells can be visualized by microscopy as mononucleate cells located between the plasma membrane and the basal lamina that ensheathes each myofiber (Mauro, 1961). To avoid analyzing hundreds of transverse sections of muscle tissue, we isolated intact single muscle fibers on which the closely juxtaposed satellite cells can be readily visualized in tissue culture. To isolate individual fibers, the tibialis anterior muscle (TA) from the legs of mice was dissociated and the single fibers isolated with a Pasteur pipette following trituration. Isolated fibers were then cultured overnight, a time period that allowed activation of the transcription factor Myf-5, yet did not induce proliferation of satellite cells or their migration from the fiber (Rosenblatt et al., 1995). Either individual thin optical sections or three-dimensional (3D) reconstructions of serial optical sections of these fibers were analyzed using a laser scanning confocal microscope and antibodies to characteristic proteins. This rigorous analytic method ensures that the colocalization of markers represents true coexpression of different proteins within

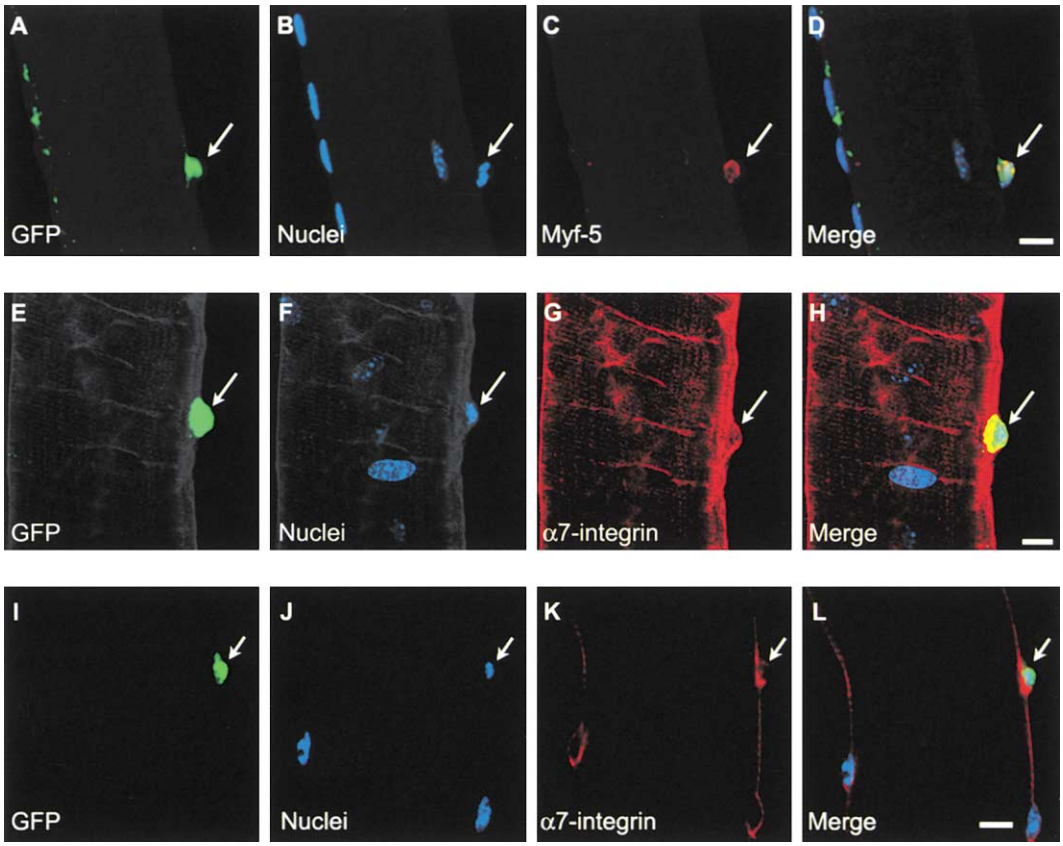

Figure 2. Detection of GFP(+) Bone Marrow Donor-Derived Satellite Cells in Transplant Recipients

Shown here are representative examples from three experiments of single isolated muscle fibers from bone marrow-transplant recipients. A 3D reconstruction of 20 optical sections obtained by laser scanning confocal microscopy shows an isolated muscle fiber juxtaposed to a GFP(+) (A) satellite cell (arrow), with a nucleus stained with ToPro (B), the transcription factor Myf-5 (C), and the coincidence of all three in the merged image (D). In (A) the green labeling on the left is due to nonspecific fluorescence of the sarcolemma, not satellite cells, which are larger in size. As shown here, only cells that strongly stained with all three markers were scored as satellite cells. A similar 3D reconstruction compiled from 20 optical sections shows a single myofiber with an associated GFP(+) satellite cell (arrow) (E), its nucleus (F), α7-integrin (G), and the merged image (H). A single 1.5 μm optical section through an isolated myofiber shows GFP staining, and therefore bone marrow derivation (I), of one of three satellite cells (arrow), their nuclei (J), α7-integrin staining (K), and the merged image (L). Bar represents 10 μm. In (A)–(F), the muscle fiber is shown in dark gray for context.

the same cell (Brazelton et al., 2000; Kornack and Rakic, 2001).

Figure 1 shows four examples of fields of isolated muscle fibers in which satellite cells are present. Two show expression of Myf-5 (Figures 1E–1H and 1M–1P), the earliest expressed of a family of bHLH transcription factors in muscle, a factor critical to initiating the myogenic program in satellite cells (Cossu et al., 1996). The other two show cMet-R (Figures 1A–1D and 1I–1L), a tyrosine kinase receptor that is a well-accepted marker of satellite cells (Cornelison and Wold, 1997). In each case, nuclei were stained and expression of α7β1 integrin (α7-integrin) in the membranes surrounding both the myofiber and satellite cells is shown (Bao et al., 1993). α7-integrin is readily apparent on the surface of satellite cells. In the fields in Figures 1A–1D and 1E–1H, 3D reconstructions of optical sections collected with a laser scanning confocal microscope highlights the satellite cells (arrows) on the upper surface and sides of the myofibers. In the fields shown in Figures 1I–1L and 1M–1P, a single optical section through one satellite cell (arrow) is shown. In all four fields, the satellite cells juxtaposed to the muscle fibers have the characteristic high ratio of nucleus to cytoplasm, and the nucleus appears to occupy most of the space circumscribed by the α7-labeled or cMet-R-labeled satellite cell membrane. As shown here, the membrane protein, α7-integrin, serves as a useful adjunct to the routinely used cMet-R and Myf-5, as it allows visualization of satellite cells in the context of intact individually isolated myofibers.

Progression of Bone Marrow to a Tissue-Specific Stem Cell

We designed experiments to test the hypothesis that BMDC could give rise to satellite cells, mononucleate muscle-specific stem cells. Although several recent reports have shown that BMDC can contribute to mature adult multinucleate skeletal myofibers in bone marrow transplant recipients (Bittner et al., 1999; Ferrari et al., 1998, 2001; Gussoni et al., 1999), given the unexpected nature of these findings and their low frequency (~0.2% of fibers), questions have been raised regarding their biological relevance (Anderson et al., 2001). We designed experiments to determine if BMDC transplanted

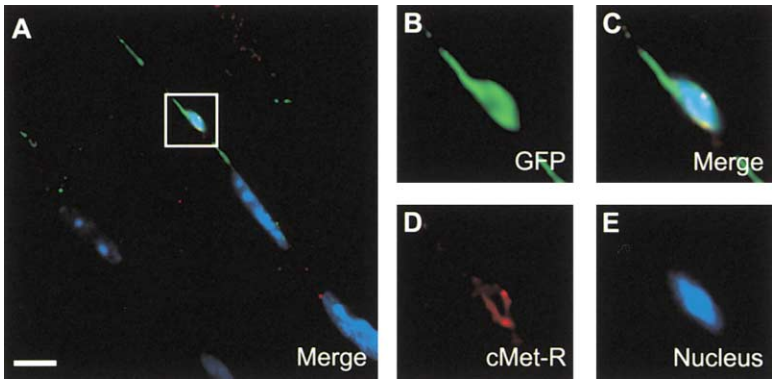

Figure 3. High-Resolution Detection of a GFP(+) Satellite Cell Exhibiting Colocalization of Definitive Markers

A representative longitudinal 10 μm section of a fixed TA muscle from a GFP(+) bone marrow transplant recipient analyzed with a laser scanning confocal microscope. (A) Shows a low-magnification merged image of bone marrow-derived satellite cell adjacent to a muscle fiber (box). Insets show high-magnification images of the satellite cell stained for GFP (B), cMet-R (D), nuclear DNA (E), and colocalization of the three markers in the merged image (C). Bar represents 10 μm.

from mice transgenic for GFP could replenish some of the satellite cells depleted following irradiation. 10-week-old syngeneic mice received 9.6 Gy whole-body irradiation followed by transplantation via tail vein injection of 10^6 GFP-labeled (GFP(+)) bone marrow cells from age-matched donors. Mice were sacrificed 2–6 months posttransplantation, the TA muscles were dissected, and single isolated myofibers were analyzed by confocal scanning microscopy in conjunction with immunohistochemistry, as described for Figure 1. Figures 2A–2D and 2E–2H show 3D reconstructions derived from a composite of 20 optical sections obtained by laser scanning confocal microscopy of single isolated fibers of the TA. Figures 2A–2D show a GFP(+) satellite cell nucleus expressing Myf-5. A GFP(+) satellite cell is also shown in Figures 2E–2H separated by α7-integrin from its adjacent myofiber. In Figures 2I–2L, a field is shown with a single optical section in which three satellite cells are present, only one of which is GFP(+) and therefore bone marrow derived. All satellite cells have nuclei stained with ToPro and are circumscribed by α7-labeled membranes. In Figure 3 a longitudinal tissue section of TA muscle tissue is shown in which a single GFP(+) satellite cell is juxtaposed to a myofiber (Figure 3A). The higher magnification of the satellite cell in the inset clearly shows the colocalization of GFP, nuclear ToPro, and the satellite cell marker cMet-R on intact myofibers in vivo in muscle tissue (Figures 3B–3E). These data demonstrate that following a bone marrow transplant, GFP(+) cells from the bone marrow can gain access to and occupy the satellite cell niche, as shown in fibers either isolated in culture or present in intact muscles.

BMDC Muscle Stem Cells Exhibit a Heritable Change in Cell Phenotype

We tested whether bone marrow-derived GFP(+) satellite cells had undergone a heritable change, were stably myogenic, and were capable of self-renewal and differentiation as myotubes in culture. Myoblasts, derived from satellite cells (Zammit and Beauchamp, 2001), were isolated by dissociating the muscle tissues from four different GFP(+) bone marrow transplant recipients, as previously described (Rando and Blau, 1994). These cells were sorted twice by FACS and gated so that >99% of the cells collected were GFP(+) and therefore derived from bone marrow and expressed the muscle protein α7-integrin (Figure 4A; Blanco-Bose et al., 2001).

To examine heritability of the myogenic phenotype of bone marrow-derived muscle stem cells, FACS-sorted cells were plated at limiting dilution (0.1 cells/well) to ensure clonality and grown in 96-well plates. The changes in gene expression shown for individual satellite cells in Figures 2 and 3 persisted in their progeny. Two representative clones derived from single satellite cells show coincident expression of the bone marrow marker GFP and the muscle-specific intermediate filament protein desmin (Figures 4D–4I). Clones also expressed cMet-R, Myf-5, and α7-integrin (data not shown). These data show that the reprogramming of BMDC to a muscle-specific stem cell entails a heritable change that is passed on to myogenic progeny upon cell division.

An issue of major interest is whether changes in cell fate arise due to cell fusion or to activation of previously silent genes. To address this question, we analyzed the karyotype of the cells isolated by FACS. To this end, muscle tissue was dissociated and plated for only 3.5 or 5.5 days to allow cells to adhere to tissue culture plates. Minimal cell division occurs during this period. Cells from bone marrow-transplanted mice and from wild-type controls were then FACS-sorted as in Figure 4A and exposed to the microtubule inhibitor, nocodozole, overnight. Following fixation, the metaphase chromosomes of cells were counted to reveal their karyotype. Virtually all were diploid (2N), as shown in Figures 4B and 4C.

To determine whether the clones derived from single cells could differentiate, they were exposed to low-mitogen media. When pools of myoblasts were exposed to differentiation medium, multinucleate myotubes that expressed desmin and the bone marrow marker GFP were evident (Figures 4J–4O). Moreover, 13 clones derived from single cells had myotubes ranging in size from 3 to 10 nuclei.

To determine whether BMDC myoblasts could participate in myogenesis in vivo in mice, approximately 10^5 FACS-sorted bone marrow-derived myoblasts that were both GFP(+) and α7-integrin+ were injected into the TA muscles of six SCID mice. Seven days later, the muscles were assayed histologically in tissue sections. GFP(+) fibers were detected in transverse sections (10 μm thick) from each of the mice. Moreover, the same GFP fiber could be detected in sections separated by 200 μm,

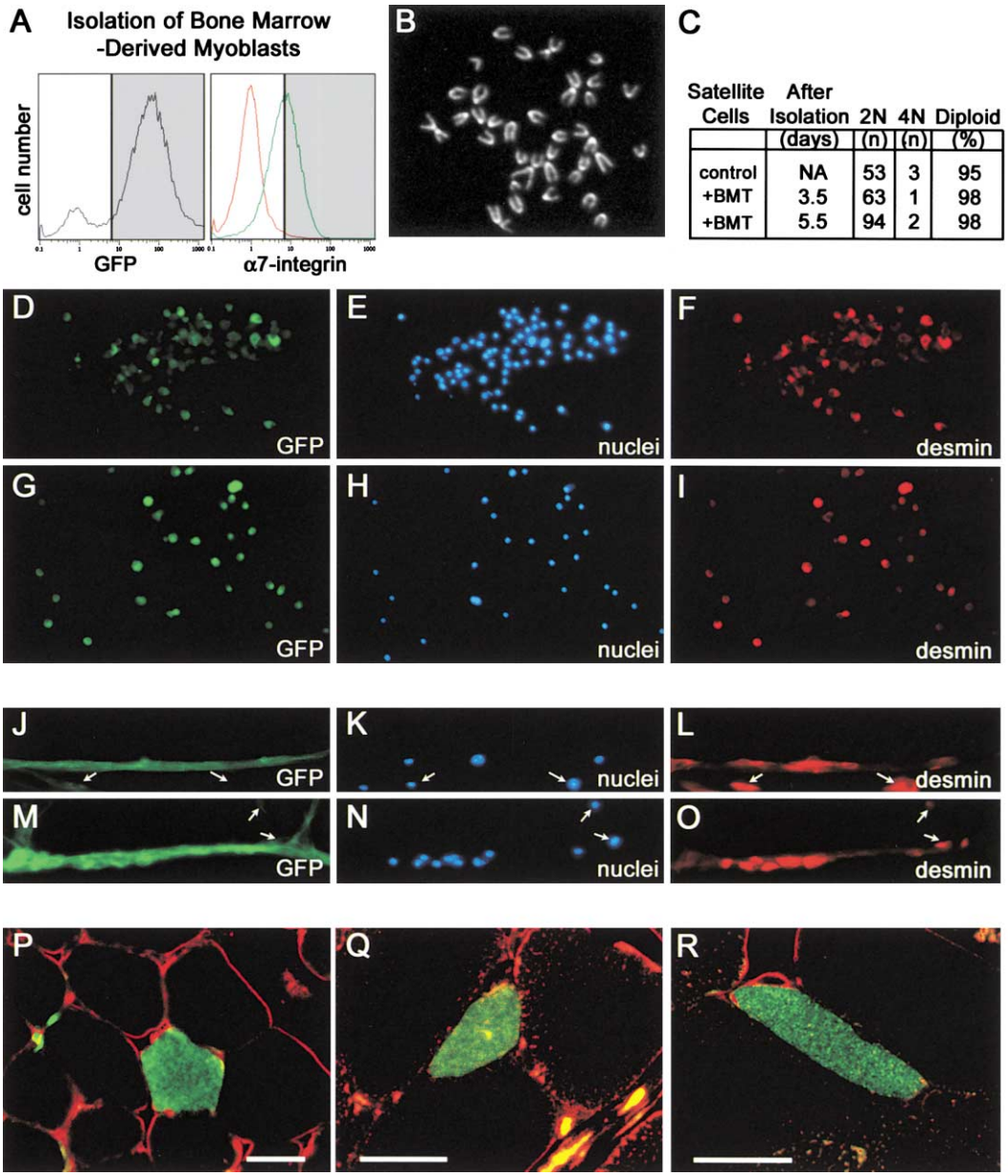

Figure 4. A Heritable Myogenic Phenotype Is Characteristic of Bone Marrow-Derived Satellite Cells

The descendants of satellite cells, myoblasts, were isolated from the skeletal muscle of recipients of transplanted green fluorescent protein (GFP(+)) bone marrow in three independent experiments.

(A) A representative flow cytometry analysis (FACS) of a muscle preparation is shown with the sorting gates from which cells were collected indicated by gray boxes. GFP expression (left) and α7-integrin expression on the GFP(+) population (green, right) with control secondary antibody only (red, right).

(B and C) Metaphase chromosome spreads were prepared and chromosome number per cell was counted from FACS-sorted bone marrow-derived GFP(+)/α7-integrin+ myoblasts 3.5 and 5.5 days after isolation and growth in culture and from wild-type C3H primary myoblasts during which adhesion and minimal proliferation occurs.

(D–I) Two representative clones that originate from single donor-derived myoblasts express GFP (D and G), exhibit nuclei stained with Hoechst 3342 (E and H), and express the intermediate filament protein desmin (F and I) (magnification ×200).

(H–O) When induced to differentiate, clones formed multinucleate myotubes in culture that express GFP (J and M), exhibit nuclei stained with Hoechst 3342 (K and N), and express desmin (L and O) (magnification ×200). Arrows show nuclei of myoblasts outside the myotubes.

(P–R) Bulk FACS-sorted donor-derived myoblasts were injected into the TA muscles of SCID mice where they fused with existing skeletal muscle fibers. Transverse sections of TA are shown with antibody staining to GFP (green) and laminin (red) that are representative of three sections taken at 200 μm intervals showing that GFP(+) fibers span up to 200 μm. Scale bar represents 20 μm.

showing that the cells had contributed to intact fibers similar to their unlabeled neighbors (Figures 4P–4R).

In summary, these results show that BMDC can adopt functions characteristic of muscle stem cells. They are diploid and assume an anatomical position either in isolated fibers or in fibers in intact muscle tissues consis-

tent with satellite cells. They grow as clones expressing myogenic markers, showing that their change in gene expression is heritable. When exposed to low-mitogen medium, they fuse like cloned primary myoblasts in tissue culture and when injected into muscle in mice, they are incorporated into myofibers. Thus, by all of these criteria they can be considered muscle-specific stem cells, or satellite cells.

Effect of Irradiation on GFP(+) Satellite Cell Number

It has been well established that irradiation dramatically depletes the muscle stem cell number in the TA (Heslop et al., 2000). To determine whether BMDC could replace some of these lost damaged cells, the following experiments were performed.

First, we sought conditions that would replicate the reported loss in endogenous satellite cells after irradiation. The effects of the 9.6 Gy used routinely for lethal irradiation prior to bone marrow transplantation in all of the experiments described above was compared with the 18 Gy reported previously to deplete satellite cells in muscle. The mice were shielded in a lead-jig such that only their right-hind limb was exposed to the irradiation source. Three weeks postirradiation, satellite cells were counted from a total of 93 muscle fibers of similar length (1600 ± 60 μm, $p > 0.5$) isolated from the dissociated TA muscles of six mice. Isolated fibers from both right (irradiated) and left (nonirradiated control) legs of each mouse were analyzed. These single fibers were cultured in individual wells for 48–60 hr in conditions that permit migration of satellite cells away from the fiber yet minimize proliferation (Rosenblatt et al., 1995). The results of these studies showed that by comparison with nonirradiated control legs (0 Gy), a marked decline in the number of satellite cells per fiber, from 33 ± 5 to 11 ± 1 and 6 ± 1, was observed after exposure to 0 Gy, 9.6 Gy, and 18 Gy, respectively (Figure 5A, Table 1A). With 9.6 Gy the reduction in endogenous satellite cells per fiber approximated 80% when determined 2–6 months posttransplant (Figure 5D, Table 1A), and this value remained constant over time ($p > 0.5$) at 6.9 ± 0.3 satellite cells per fiber.

We then determined whether the marked depletion by irradiation of the endogenous satellite cells was sufficient to open a niche that BMDC could enter. Whole-body irradiated and nonirradiated (control) GFP(+) bone marrow transplant recipients were sacrificed and compared 2 months posttransplant. Single fibers were isolated and cultured, and their associated satellite cells were counted as described above. In the absence of irradiation, no GFP(+) satellite cells were detected, whereas there were on average 0.37 ± 0.1 GFP(+) satellite cells per fiber; thus, 5% of the remaining satellite cells postirradiation were GFP(+) satellite cells (Figure 5B and Table 1A), a number that remained constant 2–6 months after transplantation ($p > 0.5$) (Figure 5E, Table 1A). The GFP(+) and GFP(−) satellite cells that migrated from single isolated fibers were also characterized in culture with respect to their expression of myogenic markers. Fibers from five bone marrow-transplanted and four wild-type control mice were assayed by immunocytochemistry for the muscle proteins cMet-R, Myf-5, and α7-integrin. Frequency of expression of these three markers were similar to wild-type satellite cells and greater than 88% of the GFP(+) cells expressed one or more markers (Table 2). These data show that the majority of the GFP(+) cells that migrate from isolated intact fibers are myogenic and that their frequency of myogenic marker expression is on a par with nontransplanted controls.

Effect of Irradiation on GFP(+) Muscle Fibers

To examine the GFP(+) satellite cell contribution to muscle fibers after irradiation, serial 10 μm thick transverse sections of fixed TA muscles were analyzed by laser scanning confocal microscopy in which on average 200 fibers could be visualized and scored per field. Unlike satellite cells, muscle fibers can be readily identified in transverse sections of adult muscle tissue. By contrast with satellite cells, only one mature GFP(+) muscle fiber was observed 2 months posttransplant among the 1589 fibers analyzed in irradiated transplant recipient mice. These results indicate that although the GFP(+) satellite cells can contribute to muscle fibers postirradiation, they do so at an extremely low frequency, less than 1% (Ferrari et al., 2001; Gussoni et al., 1999). Not surprisingly, the TA muscles of the nonirradiated transplant recipients had no detectable GFP(+) muscle fibers (Figure 5C and Table 1B). The number of fields and total muscle fibers scored 2, 4, and 6 months posttransplant are shown in Table 1B and no significant difference was observed over time (Figure 5F). Taken together, these data suggest that a certain proportion of the satellite cell pool is regenerated within a short time period following irradiation treatment, and that the GFP(+) marrow-derived satellite cells, like endogenous satellite cells, occupy this niche and persist over time in a quiescent state with minimal contribution to muscle fibers.

Effect of Exercise-Induced Damage on GFP(+) Satellite Cells

Continuous exercise is thought to cause damage to intracellular and membrane components of the muscle fibers due to the intense shearing forces. In response to cues resulting from muscle damage, wild-type mononucleate satellite cells become mitotic, fuse into, and actively participate in the regeneration of the injured muscle tissue, resulting in hypertrophic multinucleate fibers. To determine whether marrow-derived GFP(+) cells not only appeared to be satellite cells based on morphological criteria and their behavior in tissue culture, we tested whether they could also function as satellite cells in response to exercise-induced damage.

The following experimental protocol was used. One week following a GFP(+) bone marrow transplant, three mice were given a running wheel to allow voluntary exercise and compared with three controls that did not exercise in this manner for a 6-month period. At the time of sacrifice, both TA muscles of each of the six mice were dissected. From each mouse, one TA muscle was used to isolate single fibers, and the numbers of GFP(−) endogenous satellite cells and GFP(+) donor-derived satellite cells were quantified as described above. The other TA muscle was fixed and crosssectioned to quantify fiber numbers in the muscle. GFP expression in a fiber served to indicate that bone marrow-derived GFP(+) satellite cells had responded to signals released in re-

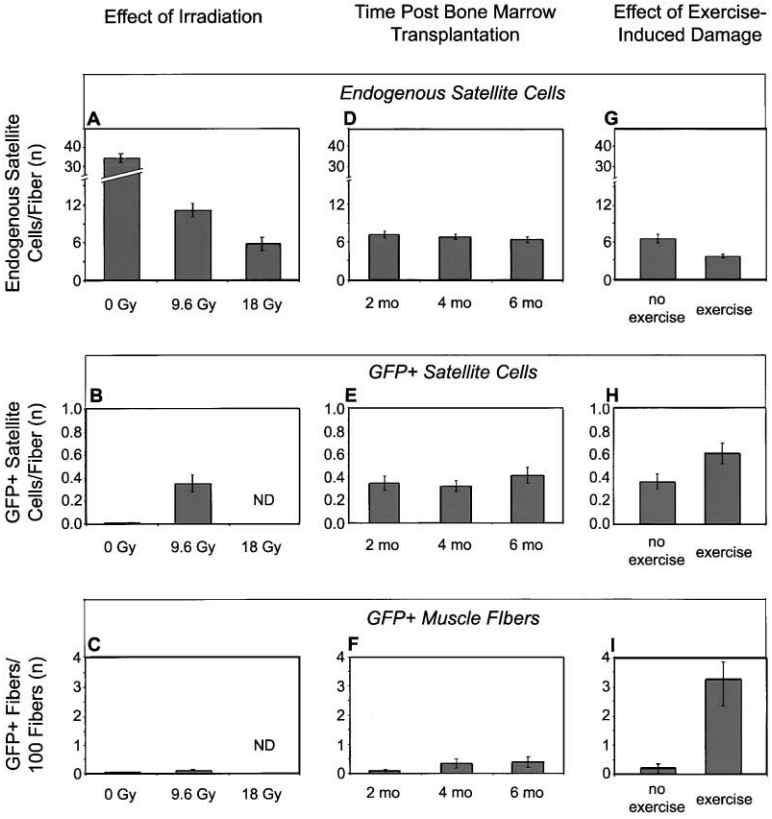

Figure 5. Radiation- and Exercise-Induced Damage Enhances the Contribution of GFP(+) Bone Marrow-Derived Cells to the Satellite Cell Niche

(A) Satellite cells from single muscle fibers isolated 3 weeks after irradiation of TA muscles and from contralateral nonirradiated controls were analyzed to determine the effect of irradiation on the endogenous satellite cells in the tissue-specific stem cell niche. 9.6 Gy elicits a 3-fold decrease and 18 Gy a 5-fold decrease in satellite cell number relative to nonirradiated controls ($p < 0.001$). Three animals were analyzed for each irradiation condition. Differences in average fiber lengths among groups were not significantly different and did not contribute to differences in satellite cell number (1600 ± 60 μm, $p > 0.5$).

(B) GFP-expressing satellite cells derived from GFP(+) bone marrow were quantified on single isolated myofibers 2 months posttransplant. Transplant recipients that received no irradiation prior to transplant were compared with those that received 9.6 Gy. Lethal irradiation, 9.6 Gy, enhances GFP(+) satellite cell contribution ($p < 0.01$).

(C) Fixed tissue transverse sections of the transplant recipient and control TA muscles were analyzed for GFP(+) fibers, an indication of regeneration by GFP(+) bone marrow-derived cells. After 9.6 Gy, one GFP(+) muscle fiber was detected among a total of 1589 fibers scored, none were detected in the control.

(D) GFP(−) endogenous satellite cell number remains relatively constant with slight decreasing trend over time ($p < 0.01$). Endogenous GFP(−) satellite cells analyzed from single muscle fibers of GFP(+) bone marrow transplant recipients. Satellite cell numbers were assayed 2, 4, and 6 months posttransplant. Average numbers of satellite cells per muscle fiber following radiation were somewhat lesser, but in the same range as those in (A) after 9.6 Gy.

(E) GFP(+) bone marrow-derived satellite cells per fiber remained constant over time (approximately 0.37 ± 0.01 GFP(+) cells/fiber or ~5% on average, $p > 0.5$). Differences in average fiber length among groups were not significant and did not contribute to differences in satellite cell number (1698 ± 40 μm, $p > 0.5$). GFP(+) satellite cells per fiber were in good agreement with those in (B).

(F) The numbers of GFP(+) muscle fibers per 100 fibers scored remained essentially constant; the apparent increase over time from 2 to 6 months is not significant, but may reflect a trend ($p > 0.5$). Muscle fibers in fixed tissue were analyzed for GFP(+) muscle fibers in 26 transverse sections to determine the contribution of bone marrow-derived cells to the regeneration of the adult fibers over time. Between 1000 and 2000 muscle fibers were analyzed at each time point.

(G) Endogenous GFP(−) satellite cells were quantified from single myofibers isolated from exercised and nonexercised-control GFP(+) bone marrow transplant recipients and a 40% decrease in endogenous cell number was evident in the exercised group versus nonexercised ($p < 0.01$). Graphs represent satellite cells counted following 48–60 hr in culture from single myofibers isolated from control and exercised groups, respectively (three mice per group).

(H) The number of GFP(+) satellite cells per fiber remained essentially the same with or without exercise. However, a nonsignificant 1.7-fold increase in the exercise group relative to the control group may represent a trend (0.61 ± 0.09 relative to 0.36 ± 0.09 GFP(+) cells/fiber, $p > 0.5$). Differences in average fiber lengths among groups were not significant and did not contribute to differences in satellite cell number (1750 ± 60 μm, $p > 0.5$).

(I) GFP(+) muscle fibers increase 20-fold analyzed in fixed TA muscle transverse sections from exercised relative to nonexercised mice ($p < 0.01$). GFP(+) myofibers are indicative of regeneration from GFP(+) satellite cells.

The numerical data is represented in Table 1, bars represent standard error of the mean, and p values were determined with a Student's t test.

sponse to exercise-induced injury and regenerated damaged muscle fibers.

Overall satellite cell numbers per fiber (GFP(+) or GFP(−)) did not change markedly in response to exercise. Endogenous GFP(−) cells were reduced somewhat (Figure 5G and Table 1A), whereas a slight increase in the number of GFP(+) satellite cells per muscle fiber was observed following exercise (Figure 5H and Table 1A).

Effect of Exercise-Induced Damage on GFP(+) Muscle Fibers

By contrast with satellite cells, exercise-induced damage resulted in a marked increase of 20-fold GFP(+) myofibers after 6 months exposure to a running wheel (from 0.16 to 3.52 GFP(+) myofibers/100 myofibers). This contribution to muscle fibers of GFP(+) satellite cells was determined by scoring the number of GFP(+) myofibers in transverse sections of the fixed TA muscle (Figure 5I and Table 1B). The 20-fold difference reflects the proportion of GFP(+) myofibers in the group that did not exercise relative to the group that did, 0.17% relative to 3.52% of total fibers analyzed (1165 and 1905 muscle fibers, respectively) (Table 1B). Moreover, although the GFP(+) muscle fibers were sometimes dispersed (Figure 6A), they were often observed in clusters (Figures 6B and 6C), suggesting that bone marrow-

Table 1. Quantitation of Satellite Cells and Muscle Fibers in Bone Marrow Transplant Recipient and Wild-Type Mice

(A) Satellite Cells Quantified from Single Muscle Fibers

Time Postirradation & Transplant	γ Irradiation (Gy)	Months of Exercise	Animals (n)	Muscle Fibers Evaluated (n)	Endogenous Satellite Cells (n)	GFP(+) Satellite Cells (n)	Endogenous Satellite Cells/Fiber	GFP(+) Satellite Cells/Fiber	GFP(+)/ Total (%)
3 weeks[a]	0	NA	3	28	948	NA	33.86	NA	NA
3 weeks[a]	9.6	NA	3	36	415	NA	11.53	NA	NA
3 weeks[a]	18	NA	3	29	192	NA	6.62	NA	NA
2 months[b]	0	NA	3	49	1563	0	31.90	0.00	0.00
2 months[b]	9.6	NA	4	47	345	16	7.34	0.34	4.43
4 months[b]	9.6	NA	5	108	735	33	6.93	0.31	4.30
6 months[b]	9.6	NA	6	80	516	36	6.45	0.45	6.52
6 months[c]	9.6	0	3	45	301	16	6.69	0.36	5.05
6 months[c]	9.6	6	3	61	250	37	4.10	0.61	12.89

(B) Muscle Fibers Quantified in Crosssections of TA Muscle

Time Posttransplant	γ Irradiation (Gy)	Months of Exercise	Animals (n)	Total Fibers (n)[d]	GFP(+) Fibers (n)	GFP(+)/ Total (%)
2 months[e]	0	NA	2	1291	0	0.00
2 months[e]	9.6	NA	3	1589	1	0.06
4 months[e]	9.6	NA	2	1215	4	0.33[f]
6 months[e]	9.6	NA	4	1940	7	0.36[f]
6 months[g]	9.6	0	2[h]	1165	2	0.17[i]
6 months[g]	9.6	6	3	1905	67	3.52

(A) BMDC (GFP(+)) and endogenous (GFP(−)) satellite cells were counted after their migration off isolated muscle fibers.
(B) GFP(+) muscle fibers were counted in transverse sections of tibialis anterior muscle.
[a] Only the right hind leg was exposed to irradiation (see Figure 5A).
[b] Whole-body irradiation plus bone marrow transplant (see Figures 5D and 5E) and no irradiation plus bone marrow injection (see Figure 5B).
[c] Whole-body irradiation plus bone marrow transplant (see Figures 5G and 5H).
[d] Data were obtained by analysis of 6, 9, 6, 11, 6, and 9 fields, respectively, containing ~180–200 fibers each.
[e] Whole-body irradiation plus bone marrow transplant (see Figure 5F) and no irradiation plus bone marrow injection (see Figure 5C).
[f] Indicates $p < 0.1$ relative to 2 months determined with a Student's t test.
[g] Whole-body irradiation plus bone marrow transplant (see Figure 5I).
[h] Tibialis anterior of one of three animals was lost.
[i] Indicates $p < 0.02$ relative to the exercised group determined with a Student's t test.

derived regeneration may have originated from single satellite cell clones (Hughes and Blau, 1990). A 3D reconstruction of a GFP(+) satellite cell expressing Myf-5 juxtaposed to a GFP(+) muscle fiber isolated from one of the exercised mice is shown in Figures 6D–6G. For comparison, in the same field are three nuclei from a nearby myofiber that is not GFP(+).

In summary, these data show that irradiation has a major impact on the incorporation of GFP(+) marrow derived cells into the satellite cell (stem cell) compartment, whereas exercise has a major impact on the incorporation of these cells into muscle fibers. Moreover, contribution of bone marrow cells to these two steps in the biological progression typical of muscle can be dissociated using two different sequential damage-inducing treatments.

Discussion

Biological Progression in Response to Temporally Distinct Injury-Induced Signals

By separating the transit from blood to muscle into two phases, we were able to provide evidence that the contribution of cells within bone marrow to a specialized nonhematopoietic tissue follows a biological progression in response to biological stimuli. We first showed that genetically marked GFP(+) bone marrow-derived cells (BMDC) occupy the muscle-specific stem cell niche

Table 2. Confirmation of the Myogenic Phenotype of BMDC Satellite Cells That Migrated Off Isolated Muscle Fibers

Bone Marrow Transplant (Y/N)	Animals (n)	GFP Expression (+/−)	Cells Scored (n)	F4/80+	Myf-5	−	+	−	−	+	−	Total Cells Expressing ≥1 Myogenic Markers (%)
					cMet-R	+	−	−	+	−	−	
					α7-integrin	+	+	+	−	−	−	
						(n)	(n)	(n)	(n)	(n)	(n)	
Yes	5[a]	+	124	0		38	16	20	14	3	12	88
Yes	5[a]	−	139	0		32	21	16	31	1	22	82
No	4	−	211	0		65	61	25	5	0	5	96

After migration off isolated muscle fibers, the frequency of myogenic marker expression on satellite cells from bone marrow transplant recipients and wild-type mice was determined.
[a] GFP(+) and GFP(−) satellite cells were scored in the same single fiber preparations from transplant recipient mouse tibialis anterior muscles.

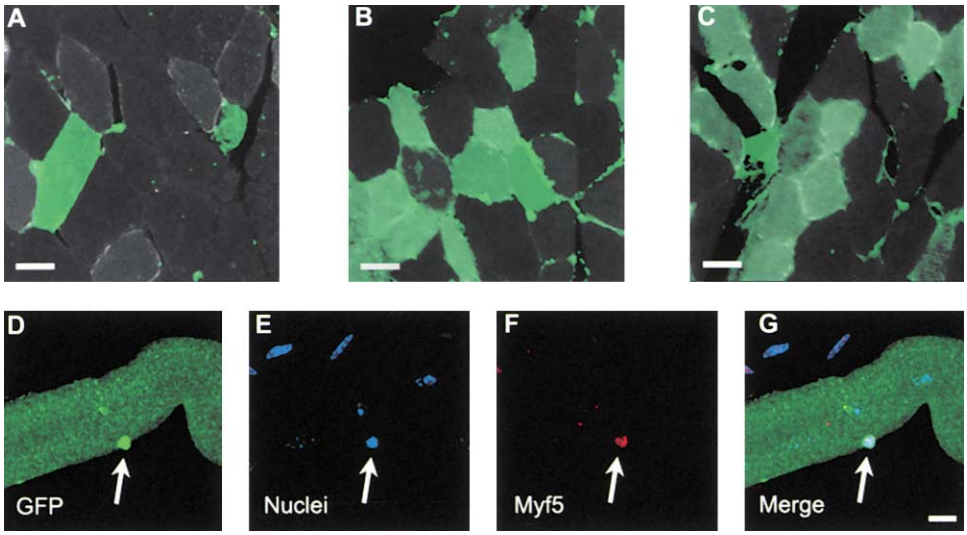

Figure 6. Exercise-Induced Damage Enhances Contribution of GFP(+) Bone Marrow-Derived Satellite Cells to Regenerating Muscle

(A–C) Representative samples of GFP(+) fibers in the exercised group of mice are shown with the background GFP(−) myofibers in dark gray. GFP(+) muscle fibers were dispersed (A) or in clusters (B and C), suggesting that regeneration could result from single GFP(+) satellite cell clones in these regions. A representative 3D reconstruction of a GFP(+) muscle fiber from an exercised mouse juxtaposed to a GFP(+) satellite cell (arrow) expressing GFP (D), nuclear DNA (E), Myf-5 (F), and the merged image (G). Three myonuclei residing within a nearby GFP(−) myofiber can be seen in the same field adjacent to the GFP(+) myofiber. In (A), (B), and (C), bars represent 30 μm, and in (G) bar represents 10 μm.

following depletion by radiation of the endogenous satellite cells. These cells were heritably altered, were capable of self-renewal as myogenic clones, generated multinucleate muscle cells in tissue culture in response to media that promotes differentiation, and contributed to myofibers upon injection into muscle tissue. Using laser scanning confocal microscopy, GFP(+) cells were shown in thin optical sections and 3D reconstructions to coexpress characteristic proteins and to be morphologically indistinguishable from endogenous satellite cells. GFP(+) cells in isolated single myofibers were mononucleate and circumscribed by a membrane, in which α7-integrin, cMet-R, and Myf-5 proteins were expressed. Thus, they were distinct from, yet juxtaposed to, myofibers.

However, GFP(+) cells in the satellite cell niche remained constant in number over a 6 month time period and rarely contributed to the multinucleate muscle fibers with which they were associated. In order to increase their contribution to muscle fibers, a second injury or metabolic stress was required. After voluntary exercise on a running wheel, the number of GFP(+) satellite cells per fiber increased less than 2-fold, whereas the number of GFP(+) muscle fibers increased 20-fold over a 6 month period. These results suggest that the GFP(+) cells, which morphologically and biochemically appear to be satellite cells, also appear to function as satellite cells, presumably undergoing sequential asymmetric divisions as they participate in the regeneration of damaged muscle fibers over time. Moreover, these findings provide evidence that the contribution of bone marrow cells to muscle is not a random low-frequency event, as it reaches 3.5% under selective pressure of exercise-induced damage over a period of only 6 months. The increase observed is more than one order of magnitude greater than in any previous reports of bone marrow to muscle (Ferrari et al., 1998, 2001; Gussoni et al., 1999). These data demonstrate clearly that a biological stepwise progression from adult bone marrow to muscle-specific stem cell to differentiated muscle fiber occurs in muscle. Such a progression may well be typical of cell type conversions in other tissues.

The lack of evidence that the BMDC could occupy a tissue-specific stem cell niche could have been due to difficulties in identifying tissue-specific stem cells in heart, epithelium, liver, skeletal muscle, and brain (Bittner et al., 1999; Brazelton et al., 2000; Ferrari et al., 1998, 2001; Fukada et al., 2002; Gussoni et al., 1999; Jackson et al., 2001; Krause et al., 2001; Lagasse et al., 2000; Mezey et al., 2000; Orlic et al., 2001). Unlike the satellite cells of muscle, the tissue-specific stem cells in many tissues are often difficult to identify. In the studies reported here, we capitalized on an advantageous feature specific to muscle: muscle stem cells, or satellite cells, are anatomically and biochemically distinct (Cornelison and Wold, 1997; Mauro, 1961). Moreover, muscle stem cells can be readily analyzed on freshly isolated single muscle fibers, as these preparations include the fiber-associated satellite cells (Bischoff, 1986; Blaveri et al., 1999; Rosenblatt et al., 1995). Although such studies are not easy, due to these properties of muscle, we were able to monitor the sequential effects of two distinct damage-inducing procedures and temporally dissociate BMDC conversion to mononucleate satellite cells from their subsequent contribution to multinucleate myofibers. Taken together, these results suggest that BMDC may constitute a previously unrecognized reservoir of cells that is capable of contributing to a tissue-specific stem cell pool, thereby serving as an alternative or back-up source of cells for repairing damaged adult tissues.

Bone Marrow Cell Conversion to Muscle Stem Cells Is Heritable

If BMDC give rise to muscle-specific stem cells, or satellite cells, they should be heritably altered. Clones derived from single BMDC that are GFP(+) would be expected to express muscle-specific proteins in vivo and in vitro and be capable of self-renewal and differentiation in tissue culture as well as following injection into muscle tissues of mice. As shown in this report, these characteristics are true of the BMDC satellite cells analyzed here. Depletion of the endogenous satellite cells by irradiation leads to an unoccupied niche. The microenvironment of that niche that normally supports and maintains the endogenous muscle stem cells (satellite cells) can exert similar effects on BMDC that enter that niche. These cells remain quiescent until induced to proliferate, self-renew, or differentiate. Only a subset of the total BMDC satellite cells contribute to myofibers in the absence of further damage, a proportion similar to that observed for clones of primary myoblasts isolated by others (Baroffio et al., 1996; Beauchamp et al., 1999).

Bone Marrow-Derived Satellite Cells Function in Repair of Muscle Damage

Exercise-induced damage to skeletal muscle is associated with satellite cell activation, increased satellite cell number, and an increase in the number of satellite cell-derived nuclei in muscle fibers (Grounds, 1998; Kadi and Thornell, 2000). Based on the observed 20-fold increase in GFP(+) muscle fibers detected in the exercised group of mice, an increase in the number of muscle fiber nuclei that originated from GFP(+) satellite cells is clear (Figure 5I). It has long been known that irradiation does not detectably damage mature muscle fibers (Goyer and Yin, 1967; Warren, 1943), but does prevent satellite cells with proliferative potential from participating in regeneration (Gulati, 1987; Rosenblatt et al., 1994). Although the satellite cell population is largely ablated (20% remain) due to irradiation, some remaining satellite cells are presumably the radiation-resistant cells described by others (Heslop et al., 2000) which, together with the proportion of BMDC GFP(+) satellite cells (5%), suffice to allow for muscle regeneration in response to damage such as exercise. The survival advantage of nonirradiated BMDC satellite cells (GFP(+)) in vivo is further evidenced in culture when the cells are exposed to mitogen-rich media that favor proliferation (Figure 4A). Similarly, in studies of other tissues, irradiation was necessary for BMDC conversion, for example to liver (Theise et al., 2000; Wang et al., 2002), but usually in conjunction with other strong damage-inducing selective pressures, either genetic or chemical (Ferrari et al., 1998; Gussoni et al., 1999; Lagasse et al., 2000).

Is Cellular Plasticity due to Fusion or Changes in the Extracellular Microenvironment?

Two recent studies suggest, based on karyotype analysis, that the reported instances of bone marrow-derived tissues could be the result of fusion between two cells instead of de novo conversion (Terada et al., 2002; Ying et al., 2002). Fusion does not appear to explain our findings regarding BMDC GFP(+) myoblasts. Within 3.5 days or 5.5 days following isolation, a period during which the cells were primarily attaching to the dishes following tissue dissociation, karyotypes were analyzed. A metaphase chromosome complement of 40, or 2N, was observed in 98% of cells, on a par with wild-type controls (Figures 4B and 4C). These data suggest that instead of fusion, the change in gene expression observed in BMDC satellite cells is due to the microenvironment of the niche they occupy, which reveals their inherent plasticity.

Summary

The data presented here suggest that the frequency of conversion of BMDC to a tissue-specific cell type is low unless damage to the tissue occurs. In the absence of damage, the contribution of bone marrow to tissue is a rare, but detectable, event (Castro et al., 2002; Wagers et al., 2002). However, reports that have used damage-induced stress (genetic, chemical, or metabolic) in addition to the irradiation necessary to reconstitute the bone marrow following a transplant provide evidence that damage-induced stimuli increase the conversion of BMDC to tissues other than blood. Thus, we propose that the following precepts may be generalizable to studies of BMDC conversions: (1) ablation of the endogenous bone marrow milieu to allow engraftment of donor bone marrow cells, (2) reduction in the number of tissue-specific stem cells to decrease the regenerative potential within a tissue and increase the demand for new cells in that tissue, and (3) exacerbation of the needs of repair and regeneration of a tissue by injury to that tissue in order to increase BMDC contribution.

Further studies are required to define the factors that recruit cells from bone marrow to diverse tissue-specific stem cell niches. Once in the niche, these tissue-specific stem cells can be maintained in a quiescent state. In the case of muscle, this period can be at least 6 months, as shown here for GFP(+) satellite cells. In addition, the disparate injury-induced signals resulting from irradiation and exercise that cause the BMDC to become quiescent satellite cells or to proliferate and fuse into multinucleate muscle fibers of the host also remain to be elucidated. Other studies suggest that chemical or genetic damage may release factors key to cell type conversion. Knowledge of the relevant factors may override the need for bone marrow transplantation, which currently serves to mark the cells in order to track them. In addition, identification of the responsible cell type in bone marrow has yet to be resolved and may require retroviral marking or single cell transplantation experiments. An understanding of both the signaling cascades and the origin of the cells responsible will contribute to our overall understanding of stem cell plasticity and its role in development and regeneration.

Experimental Procedures

Bone Marrow Transplantation

Marrow was sterilely isolated from 8- to 10-week-old male C57BL/6 transgenic mice that ubiquitously expressed enhanced green fluorescent protein (GFP) (Okabe et al., 1997) and non-GFP, C57BL/6 control mice (Stanford). Donor mice were killed by cervical dislocation, were briefly immersed in 70% ethanol, and had their skin peeled back from a midline, circumferential incision. After the femurs, tibias, and humeri were removed, all muscle was scraped away with a

razor blade and the bones were placed in 10 ml of calcium- and magnesium-free Hank's balanced salt solution (HBSS, Irvine Scientific) with 2% fetal bovine serum (FCS) on ice for up to 90 min. The tips of the bones were removed and a 25-gauge needle containing 1 ml of ice-cold HBSS with 2% FCS was inserted into the marrow cavity and used to wash the marrow out into a sterile culture dish. Marrow fragments were dissociated by triturating through the 25-gauge needle and the resulting suspension was filtered through sterile 70 μm nitex mesh (Falcon). The filtrate was cooled on ice and spun for 5 min at 250 × g, and the pellet was resuspended in ice-cold HBSS with 2% FCS to 8×10^6 nucleated cells per ml. Simultaneously, 8- to 10-week-old C57BL/6 mice (Stanford) were lethally irradiated with two doses of 4.8 Gy 3 hr apart. Each irradiated recipient received 125 μl of the unfractionated marrow cell suspension by tail vein injection within 2 hr of the second irradiation dose.

Muscle Fiber Isolation and Satellite Cell Quantification

Single muscle fibers were isolated from the tibialis anterior according to Rosenblatt et al. (1995). Briefly, the tibialis anterior was carefully dissected with a razor blade and fine forceps, handling the muscle only by the tendons at the ankle to minimize damage to the fibers. The muscle was then incubated in DMEM/0.2% type I collagenase (Sigma-Aldrich) while constantly rolling at 37°C for 2 hr. Muscles were triturated using fire-polished pipettes to gently disaggregate the muscle fibers. Using a dissecting microscope, single fibers were extracted and transferred serially into fresh dishes containing 8 ml of DMEM/10% horse serum (HS) (GIBCO)/0.5% chick embryo extract (GIBCO) so that no debris surrounded the fibers and their attached satellite cells.

Single fibers were transferred to individual wells of a 24-well plate that were coated with DMEM/10% Matrigel (Beckton-Dickinson). When each well contained one fiber, the plates were placed in a humidified 37°C incubator for 10 min to allow adhesion to the substratum, then 0.5 ml of DMEM/10% HS/0.5% chick embryo extract was added very slowly. Fibers were cultured in a humidified, 37°C, 5% CO_2 chamber for 48–60 hr, and satellite cells crawled off the fiber and attached to the matrix. Typically, using this procedure we isolated 12–24 surviving fibers 1–3 mm in length per tibialis anterior. GFP(+) and GFP(−) satellite cells were counted on an inverted stage fluorescent microscope (Zeiss LSM510). Samples were also obtained in DMEM/5% Matrigel-coated 4-well chamber slides (Beckton-Dickinson) that were subsequently stained with antibodies against c-MetR (Santa Cruz), Myf-5 (Santa Cruz), F4/80 (Caltag), and α7-integrin (Sierra Biosource) to confirm the identity of the migrating satellite cells as described in the following section.

To determine the effects of 9.6 Gy and 18 Gy of γ irradiation on the endogenous satellite cell niche, mice were anesthetized with IP Nembutal (50 mg/kg) and irradiated inside a lead jig that exposed the right leg and protected the rest of the body. Three weeks postirradiation, the animals were sacrificed (n = 3 per radiation level) and about 12–20 muscle fibers of similar lengths were isolated from the tibialis anterior of the right and left legs to facilitate satellite cell counting (left leg served as the nonirradiated control).

Immunofluorescence of Isolated Myofibers

Muscle fibers were isolated from bone marrow transplant recipients and littermate controls, as above, and added to poly-L-lysine-treated chamber slides (Beckton-Dickinson) that were also coated with DMEM/10% Matrigel, and incubated in a humidified 37°C incubator for 2 hr to allow adhesion to the substratum. Each well was then carefully filled to maximum capacity (about 2 ml) with 4% EM grade paraformaldehyde (Polysciences) for 5 min at 37°C. Samples were blocked for 2 hr at room temperature in PBS/20% normal goat serum (NGS) (GIBCO)/0.3% triton-100. Primary antibodies were incubated at 4°C for 16–40 hr in 0.35% lambda-carraggeennan (Sigma) in the following concentrations: anti-α7 integrin-A594 (1:200, rat IgG clone CA5.5, Sierra Biosource), anti-cMet receptor (1:200, Santa Cruz Biotechnology), anti-Myf5 (1:400, Santa Cruz Biotechnology), anti-GFP (1:1000, Molecular Probes). Secondary antibodies (1:400) and nuclear stain, ToPro 3 (1:2000, Molecular Probes), were added in PBS/5% NGS for 2 hr at room temperature. Clone CA5.5 has been shown to specifically stain membranes of primary cultured myoblasts and not NIH3T3 fibroblasts (Blanco-Bose et al., 2001). Three 15 min washes in PBS were performed between each incubation and after fixation. Cover slips were mounted with Fluoromount-G (Southern Biotechnology Associates) (Beauchamp et al., 2000).

Each fiber was analyzed for antibody staining by laser scanning confocal microscopy (Zeiss LSM510). Data were collected by sequential excitation with different lasers to eliminate any possibility of bleed-through. 1.5 μm optical sections were obtained every 1.0 to 1.5 μm either to visualize individual optical sections or to reconstruct a three-dimensional representation of each cell.

Immunohistochemistry

To examine sections of whole TA muscle, bone marrow transplant recipient mice were overdosed with IP Nembutal (150 mg/kg) and then perfused with potassium phosphate buffer (0.1 M [pH 7.4]) for 3 min immediately followed by perfusion with freshly prepared 4% EM grade paraformaldehyde for 15 min. Perfusion fixing was necessary in order to retain GFP within cells, as freezing alone led to rapid loss of the GFP signal. The TA was then dissected and frozen in embedding medium (Tissue-Tek, Sakura) and sectioned as 10 μm thick transverse or longitudinal sections.

Samples were blocked for 2 hr at room temperature in PBS/20% NGS/0.3% triton-100. Primary antibodies were incubated with the sections at room temperature for up to 5 hr in a solution of PBS/5% NGS with antibodies at the following concentration: anti-cMet receptor (1:200), anti-GFP (1:1000). Secondary antibodies and ToPro 3 (1:2000) were incubated with the sections at room temperature for 2 hr in a solution of PBS/5% NGS. Three 15 min washes in PBS were performed between each incubation and after fixation. Cover slips were mounted with Fluoromount-G. Each section was analyzed as above using sequential laser excitation to eliminate bleed-through.

Exercise Regimen

To determine the effect of exercise-induced damage on donor-derived and endogenous satellite cells, GFP(+) bone marrow transplant recipients were placed in cages with running wheels 1 week after transplantation. They remained in their "enriched environments" for a period of 6 months until they were sacrificed for analysis. Littermate controls were maintained as usual in a healthy but nonstimulating environment.

Myoblast Isolation and Cell Culture

Primary cultures were prepared from muscle slurry according to Rando and Blau (1994). After 9 days of expansion in F-10/20% FBS/bFGF (20 ng/ml) (Promega), cells were released from the collagen-coated plate with PBS/0.1 mM EDTA and passed through a 70 μm mesh strainer. After a centrifugation step, cells were stained with an antibody to α7-integrin and then double sorted for GFP and α7-integrin expression. Cells were sorted twice using these two markers to reduce the frequency of error to 0.0001 (Moflo, Cytomations). Cells were then replated at clonal density by limiting dilution into DMEM/5% Matrigel (Beckton-Dickinson)-rinsed 96-well plates, grown into individual colonies and then switched into DMEM/2% HS for more than 7 days to induce differentiation. Differentiated myotubes, pooled populations, or myoblast colonies were fixed with 4% paraformaldehyde for 5 min at room temperature, blocked, and stained with anti-GFP (1:1000, Molecular Probes), anti-Desmin (1:400, Chemicon), and Alexa 488 or Alexa 594 (Molecular Probes), respectively, -conjugated secondary antibodies (Molecular Probes) and Hoechst 3342 DNA stain (1:1000, Sigma).

Cytology

Cells were harvested from bone marrow transplant crude preparations, and GFP(+)/α7-integrin+ myoblasts were double sorted 3.5 and 5.5 days postinitiation of culture then, side-by-side with control primary C3H myoblasts, were cultured overnight in F10/20% FCS/bFGF (20 ng/ml) with 500 μg/ml nocodozole (Sigma). Cells received a hypotonic shock in 75 mM KCl, followed by four rounds of fixation in methanol:acetic acid (3:1), then cells were dropped and dried onto methanol-washed slides where their metaphase chromosomes were stained with Hoechst 3342 and counted. More than 50 spreads were evaluated from each sample.

Myoblast Implantation

SCID mice (Stanford) were anesthetized with IP Nembutal (50 mg/kg), followed by a 10 μl injection of double sorted GFP(+)/α7-integrin+ bone marrow-derived myoblasts 10^7 cells/ml in PBS/2% FCS using insulin syringes (Beckton-Dickinson). Seven days following the injection, the animals were sacrificed and their TA muscles were fixed in 4% pfa, sectioned by cryostat (10 μm), blocked, and stained with anti-GFP (1:1000, Molecular Probes), anti-laminin (1:200, Chemicon), and Alexa-488 or Alexa-594 secondaries (1:400, Molecular Probes).

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References

Anderson, D.J., Gage, F.H., and Weissman, I.L. (2001). Can stem cells cross lineage boundaries? Nat. Med. *7*, 393–395.

Bao, Z.Z., Lakonishok, M., Kaufman, S., and Horwitz, A.F. (1993). Alpha 7 beta 1 integrin is a component of the myotendinous junction on skeletal muscle. J. Cell Sci. *106*, 579–589.

Baroffio, A., Hamann, M., Bernheim, L., Bochaton-Piallat, M.L., Gabbiani, G., and Bader, C.R. (1996). Identification of self-renewing myoblasts in the progeny of single human muscle satellite cells. Differentiation *60*, 47–57.

Beauchamp, J.R., Morgan, J.E., Pagel, C.N., and Partridge, T.A. (1999). Dynamics of myoblast transplantation reveal a discrete minority of precursors with stem cell-like properties as the myogenic source. J. Cell Biol. *144*, 1113–1122.

Beauchamp, J.R., Heslop, L., Yu, D.S., Tajbakhsh, S., Kelly, R.G., Wernig, A., Buckingham, M.E., Partridge, T.A., and Zammit, P.S. (2000). Expression of CD34 and Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. J. Cell Biol. *151*, 1221–1234.

Bischoff, R. (1986). Proliferation of muscle satellite cells on intact myofibers in culture. Dev. Biol. *115*, 129–139.

Bittner, R.E., Schofer, C., Weipoltshammer, K., Ivanova, S., Streubel, B., Hauser, E., Freilinger, M., Hoger, H., Elbe-Burger, A., and Wachtler, F. (1999). Recruitment of bone-marrow-derived cells by skeletal and cardiac muscle in adult dystrophic mdx mice. Anat. Embryol. (Berl.) *199*, 391–396.

Blanco-Bose, W.E., Yao, C.C., Kramer, R.H., and Blau, H.M. (2001). Purification of mouse primary myoblasts based on alpha 7 integrin expression. Exp. Cell Res. *265*, 212–220.

Blaveri, K., Heslop, L., Yu, D.S., Rosenblatt, J.D., Gross, J.G., Partridge, T.A., and Morgan, J.E. (1999). Patterns of repair of dystrophic mouse muscle: studies on isolated fibers. Dev. Dyn. *216*, 244–256.

Brazelton, T.R., Rossi, F.M., Keshet, G.I., and Blau, H.M. (2000). From marrow to brain: expression of neuronal phenotypes in adult mice. Science *290*, 1775–1779.

Castro, R.F., Jackson, K.A., Goodell, M.A., Robertson, C.S., Liu, H., and Shine, H.D. (2002). Failure of bone marrow cells to transdifferentiate into neural cells in vivo. Science *297*, 1299.

Cornelison, D.D., and Wold, B.J. (1997). Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. Dev. Biol. *191*, 270–283.

Cossu, G., Tajbakhsh, S., and Buckingham, M. (1996). How is myogenesis initiated in the embryo? Trends Genet. *12*, 218–223.

Ferrari, G., Cusella-De Angelis, M.G., Coletta, M., Paolucci, E., Stornaiuolo, A., Cossu, G., and Mavilio, F. (1998). Muscle regeneration by bone marrow-derived myogenic progenitors. Science *279*, 1528–1530.

Ferrari, G., Stornaiuolo, A., and Mavilio, F. (2001). Failure to correct murine muscular dystrophy. Nature *411*, 1014–1015.

Fukada, S., Miyagoe-Suzuki, Y., Tsukihara, H., Yuasa, K., Higuchi, S., Ono, S., Tsujikawa, K., Takeda, S., and Yamamoto, H. (2002). Muscle regeneration by reconstitution with bone marrow or fetal liver cells from green fluorescent protein-gene transgenic mice. J. Cell Sci. *115*, 1285–1293.

Goyer, R.A., and Yin, M.W. (1967). Taurine and creatine excretion after x-irradiation and plasmocid-induced muscle necrosis in the rat. Radiat. Res. *30*, 301–306.

Grounds, M.D. (1998). Age-associated changes in the response of skeletal muscle cells to exercise and regeneration. Ann. N Y Acad. Sci. *854*, 78–91.

Grounds, M.D. (1999). Muscle regeneration: molecular aspects and therapeutic implications. Curr. Opin. Neurol. *12*, 535–543.

Gulati, A.K. (1987). The effect of X-irradiation on skeletal muscle regeneration in the adult rat. J. Neurol. Sci. *78*, 111–120.

Gussoni, E., Soneoka, Y., Strickland, C.D., Buzney, E.A., Khan, M.K., Flint, A.F., Kunkel, L.M., and Mulligan, R.C. (1999). Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature *401*, 390–394.

Heslop, L., Morgan, J.E., and Partridge, T.A. (2000). Evidence for a myogenic stem cell that is exhausted in dystrophic muscle. J. Cell Sci. *113*, 2299–2308.

Hughes, S.M., and Blau, H.M. (1990). Migration of myoblasts across basal lamina during skeletal muscle development. Nature *345*, 350–353.

Jackson, K.A., Majka, S.M., Wang, H., Pocius, J., Hartley, C.J., Majesky, M.W., Entman, M.L., Michael, L.H., Hirschi, K.K., and Goodell, M.A. (2001). Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J. Clin. Invest. *107*, 1395–1402.

Kadi, F., and Thornell, L.E. (2000). Concomitant increases in myonuclear and satellite cell content in female trapezius muscle following strength training. Histochem. Cell Biol. *113*, 99–103.

Korbling, M., Katz, R.L., Khanna, A., Ruifrok, A.C., Rondon, G., Albitar, M., Champlin, R.E., and Estrov, Z. (2002). Hepatocytes and epithelial cells of donor origin in recipients of peripheral-blood stem cells. N. Engl. J. Med. *346*, 738–746.

Kornack, D.R., and Rakic, P. (2001). Cell proliferation without neurogenesis in adult primate neocortex. Science *294*, 2127–2130.

Krause, D.S., Theise, N.D., Collector, M.I., Henegariu, O., Hwang, S., Gardner, R., Neutzel, S., and Sharkis, S.J. (2001). Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell *105*, 369–377.

Lagasse, E., Connors, H., Al-Dhalimy, M., Reitsma, M., Dohse, M., Osborne, L., Wang, X., Finegold, M., Weissman, I.L., and Grompe, M. (2000). Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nat. Med. *6*, 1229–1234.

Mauro, A. (1961). Satellite cells of muscle skeletal fibers. J. Biophys. Biochem. *9*, 493–495.

Mezey, E., Chandross, K.J., Harta, G., Maki, R.A., and McKercher, S.R. (2000). Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. Science *290*, 1779–1782.

Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T., and Nishimune, Y. (1997). 'Green mice' as a source of ubiquitous green cells. FEBS Lett. *407*, 313–319.

Orlic, D., Kajstura, J., Chimenti, S., Jakoniuk, I., Anderson, S.M., Li, B., Pickel, J., McKay, R., Nadal-Ginard, B., Bodine, D.M., et al. (2001). Bone marrow cells regenerate infarcted myocardium. Nature *410*, 701–705.

Quaini, F., Urbanek, K., Beltrami, A.P., Finato, N., Beltrami, C.A., Nadal-Ginard, B., Kajstura, J., Leri, A., and Anversa, P. (2002). Chimerism of the transplanted heart. N. Engl. J. Med. *346*, 5–15.

Rando, T.A., and Blau, H.M. (1994). Primary mouse myoblast purifi-

cation, characterization, and transplantation for cell-mediated gene therapy. J. Cell Biol. *125*, 1275–1287.

Rosenblatt, J.D., Yong, D., and Parry, D.J. (1994). Satellite cell activity is required for hypertrophy of overloaded adult rat muscle. Muscle Nerve *17*, 608–613.

Rosenblatt, J.D., Lunt, A.I., Parry, D.J., and Partridge, T.A. (1995). Culturing satellite cells from living single muscle fiber explants. In Vitro Cell. Dev. Biol. Anim. *31*, 773–779.

Schofield, R. (1978). The relationship between the spleen colony forming cell ant the haematopoietic stem cell. Blood Cells *4*, 7–25.

Spradling, A., Drummond-Barbosa, D., and Kai, T. (2001). Stem cells find their niche. Nature *414*, 98–104.

Terada, N., Hamazaki, T., Oka, I., Hoki, M., Mastalerz, D.M., Nakano, Y., Meyer, E.M., Morel, L., Petersen, B.E., and Scott, E.W. (2002). Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature *416*, 542–545.

Theise, N.D., Badve, S., Saxena, R., Henegariu, O., Sell, S., Crawford, J.M., and Krause, D.S. (2000). Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. Hepatology *31*, 235–240.

Wagers, A.J., Sherwood, R.I., Christensen, J.L., and Weissman, I.L. (2002). Little evidence for developmental plasticity of adult hematopoietic stem cells. Science *297*, 2256–2259.

Wang, X., Montini, E., Al-Dhalimy, M., Lagasse, E., Finegold, M., and Grompe, M. (2002). Kinetics of liver repopulation after bone marrow transplantation. Am. J. Pathol. *161*, 565–574.

Warren, S. (1943). Effects of radiation on normal tissues. XIV. Effects on striated muscle. Arch. Pathol. *35*, 347.

Watt, F.M., and Hogan, B.L. (2000). Out of Eden: stem cells and their niches. Science *287*, 1427–1430.

Ying, Q., Nichols, J., Evans, E.P., and Smith, A.G. (2002). Changing potency by spontaneous fusion. Nature *416*, 545–548.

Zammit, P., and Beauchamp, J. (2001). The skeletal muscle satellite cell: stem cell or son of stem cell? Differentiation *68*, 193–204.