Role of bone marrow cell trafficking in replenishing skeletal muscle SP and MP cell populations

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Summary

The multipotent nature of skeletal muscle-derived side population cells is demonstrated by their myogenic and hematopoietic potential in vivo. However, whether muscle side population cells are derived from the bone marrow is unclear. To study the long-term contribution of the hematopoietic system to muscle side population, whole bone marrow cells from Ly5.1 males or from e-GFP transgenic male mice were transplanted into lethally irradiated Ly5.2 females. Long-term cell trafficking of donor bone marrow cells to muscle side population was monitored 17 times in a 34-week study. Fluorescence-activated cell sorter analyses were used to detect Ly5.1 and GFP$^+$ donor cells, which were confirmed by fluorescence in situ hybridization of the Y-chromosome. Analyses post-transplantation indicated that whereas cells of donor origin could be found in the muscle, donor bone marrow cells had contributed little to the muscle side population. Attempts to increase cell trafficking by induced muscle damage again confirmed that more than 90% of side population cells present in the muscle were derived from the host. These results demonstrate that muscle side population cells are not replenished by the bone marrow and suggest a non-hematopoietic origin for this cell population.

Supplemental figures available online

Key words: Muscle side population, Cell trafficking, Bone marrow transplantation, Fluorescence activated cell sorter (FACS)

Introduction

Adult skeletal muscle is constituted by postmitotic, syncytial multinucleated myofibers which arise from fusion of mononuclear muscle-precursor cells. In addition to myofibers, mononuclear cells are also present within muscle and these include satellite cells and other less-characterized interstitial cells (Seale and Rudnicki, 2000; Zammit and Beauchamp, 2001). Satellite cells are quiescent mononuclear muscle precursors located between the basal lamina and the sarcolemma of myofibers (Mauro, 1961). Upon muscle injury or in the presence of muscle disease, such as muscular dystrophy, satellite cells become activated and participate in muscle repair by fusing into pre-existing myofibers. In addition to satellite cells, bone marrow (BM) cells transplanted via intravenous injection into lethally irradiated wild-type mice can travel to skeletal muscle and fuse with myofibers (Ferrari et al., 1998). This observation was confirmed by studies in *mdx* mice, a mouse model of Duchenne muscular dystrophy (DMD) (Bulfield et al., 1984; Sicinski et al., 1989; Bittner et al., 1999; Gussoni et al., 1999; Ferrari et al., 2001), and in a study of a patient who had received bone marrow transplantation (BMT) at age 1 for severe-combined immune deficiency (SCID) and was diagnosed at age 12 with DMD (Gussoni et al., 2002). BM cells trafficking to skeletal muscle can also contribute to the satellite cell population in wild-type mice following BMT and exercise (LaBarge and Blau, 2002), indicating that bone marrow-derived stem cells (BMSCs) can give rise to other non-BM tissue precursors (LaBarge and Blau, 2002).

Numerous studies have suggested that stem cells exist in tissues other than BM, such as the CNS and skin (Niemann and Watt, 2002; Alonso and Fuchs, 2003; Clarke, 2003). However, because BMSCs can travel to other tissues, it is possible that stem cells in tissues other than the marrow are replenished and supported by BMSC (Anderson et al., 2001). One of these putative stem cell populations within skeletal muscle that remains largely uncharacterized is the muscle side population (muscle SP). Side population cells were first isolated from mouse BM via staining with the vital DNA dye Hoechst 33342, followed by analysis and purification using the fluorescence activated cell sorter (FACS) (Goodell et al., 1996). BM-derived SP cells appear dull (or Hoechst^{low}) due to Hoechst dye efflux mediated by the ABCG2/*brcp1* transporter (Zhou et al., 2001; Zhou et al., 2002). SP cells have been identified in several other tissues (Asakura and Rudnicki, 2002), including skeletal muscle (Gussoni et al., 1999; Asakura et al., 2002). Muscle SP cells exhibit poor ability to differentiate into muscle in vitro (Asakura et al., 2002), whereas muscle main population (MP) cells are enriched for committed myogenic precursors and are able to form myotubes. Despite their limited in vitro myogenic potential, muscle SP cells exhibit both hematopoietic and

myogenic potential in vivo and they can also give rise to satellite cells (Gussoni et al., 1999; Asakura et al., 2002). In *Pax 7* knockout mice, a mutant that lacks satellite cells, muscle SP cells are present at higher percentages than normal, suggesting that muscle SP cells might be satellite cell precursors whose differentiation is blocked by the absence of *Pax7* (Seale et al., 2000).

Studies aimed to address the origin of muscle-derived cells with hematopoietic activity indicated that these cells are $CD45^+$ or of hematopoietic origin (Kawada and Ogawa, 2001; McKinney-Freeman et al., 2002; McKinney-Freeman et al., 2003). However, the majority (>95%) of muscle-derived SP cells are $CD45^-$ and their relationship with the BM is still unclear. Recent studies documented that BM cells trafficking to skeletal muscle may lose expression of CD45 (Issarachai et al., 2002b; Issarachai et al., 2002a), leaving open the possibility that muscle SP cells could be supported by the BM, despite the lack of CD45 expression. A recent study using clones of muscle-derived stem cells (MDSC) isolated via the pre-plating technique demonstrated that MDSCs have long-term hematopoietic and myogenic potential (Cao et al., 2003). Although MDSC and muscle SP cells share similarities ($CD45^-$, c-kit^-, Sca-1^+), it is unclear whether they are the same or different cell populations. Thus, whether $CD45^-$c-kit^-Sca-1^+ MDSC (Cao et al., 2003) and muscle SP cells are related or derive from the hematopoietic system remains to be addressed.

In the present study, long-term BM cellular trafficking to skeletal muscle MP and SP cells was monitored in uninjured and in cardiotoxin-injured muscle following BMT. The study demonstrates that both $CD45^+$ BM-derived cells and BM-derived cells that have silenced the expression of CD45 upon trafficking to skeletal muscle contribute to muscle MP cells. However, little or no contribution of BM-derived cells to muscle SP cells was observed, suggesting that cellular replenishment of muscle-derived SP cells is not provided by the BM.

Materials and Methods

Animals and BMT

Six to eight week old C57BL/6-CD45.2 (Ly5.1) male mice were purchased from Charles River Laboratories (Wilmington, MA) and C57BL/6-CD45.1 (Ly 5.2) female mice were obtained from National Cancer Institute (Bethesda, MD). Animal protocols were approved by the Animal Research Committees at Harvard-MIT and at Children's Hospital. C57BL/6-CD45.2 donor male mice express the Leukocyte Common Antigen (CD45) allele CD45.2, whereas the C57BL/6-CD45.1 mice express the CD45.1 allele. Forty C57BL/6-CD45.1 female mice were lethally irradiated (950-cGy) and transplanted via intravenous (tail vein) injection with 1.5×10^7 whole BM cells from C57BL/6-CD45.2 male mice.

Alternatively, whole BM was harvested from transgenic male mice constitutively expressing enhanced-green fluorescent protein (e-GFP) under the control of the β-actin promoter (Jackson Laboratory, Bar Harbor, ME) (Okabe et al., 1997). Fourteen female C57/Bl6 mice were lethally irradiated with 1100 rads in a single dose and then injected with 1.2×10^7 nucleated e-GFP male whole BM cells. Mice were maintained in autoclaved cages under pathogen free conditions.

Muscle injury

Eight CD45.1 female mice received 15 μg of cardiotoxin from *Naja mossambica mossambica* (Sigma Aldrich) in the left hind limb by intramuscular injections in multiple muscles. The right hind limbs were used as non-cardiotoxin controls. Of the eight mice, three received cardiotoxin at 5 (n=2) and 16 (n=1) weeks after BMT. The skeletal muscles and BM of these mice were harvested 2 weeks after cardiotoxin injection (7 and 18 weeks). The remaining five mice were injected with 15 μg cardiotoxin 1 day before BMT. Skeletal muscles and BM of these four mice were harvested for analysis at 2 (2 mice), 5, 6 and 7 weeks after BMT.

Preparation of muscle and BM cell suspensions for FACS analysis

Starting at 3 and ending at 34 weeks after BMT, one or two recipient female mice were euthanized weekly. The BM and skeletal muscles were harvested and separately processed to obtain mononuclear cell suspensions. BM was flushed out of the femurs and tibias using a $25^{5/8}$ G needle into Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal bovine serum (FBS) (Hyclone). A single cell suspension was obtained by passages through an 18 G needle followed by filtration through a 40 μm cell strainer.

Muscles were finely minced using sterile scalpels and digested at 37°C in 1U ml^{-1} dispase (Roche) and 2 mg ml^{-1} collagenase-type 4 (Worthington) for 45 minutes using an Enviro Genie (USA Sci) set at a 20/40 cycle. The enzymatic digestion was inactivated by adding 30 ml of Ham's F10 medium supplemented with 20% FBS (Hyclone). Muscle cell digestion was filtered through 100 μm followed by 40 μm cell strainers (Becton Dickinson). Approximately $2\text{-}3\times10^7$ muscle and $4\text{-}5\times10^7$ BM mononuclear cells were obtained from each transplanted mouse.

Hoechst 33342 staining and flow cytometric analyses

Muscle and BM-derived cell suspensions were centrifuged at 365 ***g*** or 235 ***g***, respectively, for 10 minutes, then resuspended at a concentration of 10^6 cells ml^{-1} in pre-warmed (37°C) PBS with 0.5% BSA (PBS-0.5% BSA). Hoechst 33342 dye (Sigma-Aldrich) was added at a final concentration of 5 μg ml^{-1} for staining of BM cells (Goodell et al., 1996; Gussoni et al., 1999) and at a final concentration of 12.5 μg ml^{-1} for muscle cells (Gussoni et al., 1999). In parallel, as a negative control for SP cell gating, $1\text{-}2\times10^6$ BM- and muscle-derived cells were stained with Hoechst 33342 as described, in the presence of 50 μM verapamil (Sigma-Aldrich). Cells were incubated in PBS-0.5% BSA for 60 minutes at 37°C protected from the light in a water-bath and then washed by adding 3.5-5 volumes of ice-cold PBS-0.5% BSA. For detection of donor-derived BM cells within the BM and skeletal muscle samples of recipient mice, aliquots of 2×10^6 cells stained with Hoechst in the absence of verapamil were incubated with 2 μg of biotin anti-mouse CD45.2 (clone 104) or with 2 μg biotin anti-mouse CD45.1 (clone A20) antibody (BD Pharmingen). Incubation with primary antibodies was performed for 10 minutes on ice. Samples were washed in cold PBS-0.5% BSA and incubated for 10 minutes on ice with streptavidin-phycoerythrin (PE) (Molecular Probes), diluted 1:50 in PBS-0.5% BSA. As a negative control, 2×10^6 cells that were not stained with the primary antibody were incubated with streptavidin-PE diluted 1:50 in PBS-0.5% BSA. Prior to flow cytometric analyses, cells were resuspended in PBS-0.5% BSA containing 2 μg ml^{-1} propidium iodide (PI). Flow cytometric analysis and cell sorting were performed on a dual-laser FACSVantage SE flow cytometer (Becton Dickinson) equipped with two lasers: one with 200 MW power (488 nm) and one at 150 MW power (UV). Both the Hoechst and PI dyes were excited at 350 nm and their fluorescence was measured at 400 long pass (LP) and 600 LP, respectively, with a 550 short pass (SP) dichroic mirror. Fluorescein (FITC) and GFP signals were excited at 488 nm and their fluorescence was detected using a FL1 (530/30) filter. Phycoerythrin was excited at 488 nm and its fluorescence was detected using a FL2 (575/26) filter. For each sample analyzed, 10,000 or 20,000 total cell counts were acquired using CellQuest software, version 3.3 (Becton Dickinson). Further analyses of acquired events were performed using FlowJo software, version 4.1 (Tree Star).

Immunohistochemistry/FISH analysis

Immunohistochemistry

For each time point analyzed, part of the recipient quadriceps muscles were rapidly frozen in cold isopentane and stored at –80°C. Co-detection of CD45.2$^+$ Y$^+$ donor-derived cells within muscle tissue sections was performed as previously described (Gussoni et al., 1996; Gussoni et al., 1999). Briefly, 10 μm cryostat sections were fixed in methanol for 3 minutes, washed in PBS containing 0.01% Tween-20 (PBST) and blocked for 1 hour at room temperature in PBST supplemented with 10% fetal bovine serum (FBS). Sections were incubated overnight at 4°C with biotin anti-mouse CD45.2 (BDPharmingen) diluted 1:50 in PBST-10% FBS, or with rabbit polyclonal anti-desmin (Sigma Aldrich) diluted 1:150 in PBST-10% FBS. After washing three times for 15 minutes in PBST, tissue sections were incubated for 1 hour at room temperature with streptavidin-Alexa 488 (Molecular Probes) diluted 1:1000 in PBST or with Texas Red-conjugated anti-rabbit IgG (Jackson Immunolabs) diluted 1:100 in PBST. Slides were washed again three times as above and mounted in Vectashield (Vector Laboratories) containing 4′-6′ diamidino-2-phenylindole (DAPI) at a final concentration of 100 ng ml^{-1} for nuclear counterstain. Prior to FISH hybridization, slides were examined using a Zeiss Axioplan 2 microscope equipped with a Hamamatsu Orca ER digital camera. Images were acquired using Zeiss F Fluor 40×, Zeiss Plan Apochromat 63× or Zeiss Plan Apochromat 100× oil objectives and Openlab software, version 3.1.5 (Improvision).

FISH hybridization

Following immunofluorescence, tissue sections were fixed in Histochoice (Amresco) for 45 minutes (Gussoni et al., 1996; Gussoni et al., 1999), denatured for 12 minutes in 70% formamide (American Bioanalytical), 2× sodium chloride/sodium citrate (SSC) prewarmed to 70°C in a water-bath and dehydrated in series of cold ethanol as described (Gussoni et al., 1996; Gussoni et al., 1999). The Y-chromosome probe was labeled with digoxigenin-11-dUTP (Roche Applied Science) via nick translation and denatured at 70°C for 25 minutes (Gussoni et al., 1996; Gussoni et al., 1999). 100 ng of denatured probe were added to each slide and sections were hybridized overnight at 37°C in a humidified chamber. Sections were washed three times for 5 minutes in 50% formamide (Sigma-Aldrich, wash grade) in 2×SSC prewarmed to 45°C, followed by three times for 5 minutes in 0.1% SSC prewarmed to 60°C. Slides were incubated in a humidified chamber at 37°C for 30 minutes with digoxigenin blocking solution (Roche Applied Science), then for an additional 30 minutes with anti-DIG antibody conjugated with rhodamine (Roche Applied Science) diluted 1:100 in PBST. Sections were washed three times for 10 minutes in PBST prewarmed to 45°C before being mounted in Vectashield (Vector Laboratories) containing (DAPI) and examined using a Axioplan 2 microscope equipped with a Hamamatsu Orca ER digital camera. Images were acquired using a Zeiss Plan Apochromat 63× or Zeiss Plan Apochromat 100× oil objectives and Openlab software version 3.1.5 (Improvision).

For preparation of cytospins, CD45.2$^+$ BM and CD45.2$^-$ muscle-derived cells from recipient mice were collected using the FACS and fixed on glass slides by cytospin (Thermo-Shandon). Slides were fixed in Histochoice at room temperature for 30 minutes, denatured for 2 minutes in 70% formamide 2×SSC preheated at 70°C. Hybridization, washes and image acquisition were performed as described for tissue sections.

Results

BM cells contribute to muscle MP, but not SP, cell populations

Recipient CD45.1$^+$ (Ly 5.2) females that had been lethally irradiated and transplanted with whole BM cells from CD45.2$^+$ (Ly5.1) donor males were euthanized starting at 3 weeks and ending at 34 weeks after BMT. BM and skeletal muscles were harvested and stained in parallel with Hoechst 33342 to visualize the SP and MP cell populations, followed by staining with the monoclonal antibody CD45.2 for detection of donor-derived cells.

At 3 weeks after transplantation, analysis of SP and MP cells in the BM revealed that 99% of the cells were CD45.2$^+$ and therefore of donor origin (Fig. 1A-D). Parallel analyses by FACS in skeletal muscle samples revealed that 18% of the MP cells were CD45.2$^+$ or of donor origin (Fig. 1G), whereas all muscle SP cells were negative for CD45.2 (Fig. 1H), thus appearing to be host-derived.

At 10 weeks after transplantation, 17% of muscle MP cells were CD45.2$^+$ (donor-derived, Fig. 2C), whereas only 1% CD45.2$^+$ cells were detected in the muscle SP fraction (Fig. 2D). At 30 weeks after BMT, 11% CD45.2$^+$ cells were detected in the MP population (Fig. 2G), and no CD45.2$^+$ cells were detected in the muscle SP cell fraction (Fig. 2H). In 17 independent analyses performed between 3 and 34 weeks after BMT, 80-99% of cells in the BM MP or SP fraction expressed the CD45.2 marker of donor origin (Table 1). By contrast, the percentage of CD45.2$^+$ bone-marrow-derived muscle MP cells ranged from 7-32%, whereas the percentage of CD45.2$^+$ muscle SP cells ranged from 0-5% (Table 1).

These results suggest that after a full reconstitution of the host BM with CD45.2$^+$ donor-derived cells, trafficking and contribution of CD45.2$^+$ cells is predominantly observed within the muscle MP but not SP cell population.

Muscle SP cells do not express the cell surface antigen CD45.

Because recent studies have demonstrated that ~15-20% of muscle SP cells are hematopoietic-derived and express the cell surface antigen CD45 (Asakura et al., 2002; McKinney-Freeman et al., 2003), the recipient BM and muscles were also analyzed for expression of host-derived (CD45.1$^+$) cells (Fig. 3). At 30 weeks after BMT, 0.5% of BM MP cells (Fig. 3B) and 3% of BM SP cells (Fig. 3C) were CD45.1$^+$- (or host-) derived. These results confirmed that after lethal irradiation and BMT, the recipient BM MP and SP cell populations were reconstituted with donor-derived CD45.2$^+$ cells and only few CD45.1$^+$ host-derived cells had survived. Parallel analyses of skeletal muscle indicated that 4.7% of the MP cells expressed the host-derived antigen CD45.1 (Fig. 3E), whereas no CD45.1-positive cells were detected in the muscle SP fraction (Fig. 3F).

These results demonstrate that muscle dissociation and digestion procedures did not damage the CD45 antigen, which is expressed in a total of ~15% of skeletal muscle MP cells (11.2% CD45.2$^+$ and 4.7% CD45.1$^+$) and in only 1% of muscle SP cells. Because the percentage of CD45$^+$ muscle–derived SP cells detected in the current analysis is significantly lower than what previously observed (Asakura et al., 2002; McKinney-Freeman et al., 2002; McKinney-Freeman et al., 2003), we studied whether differences in Hoechst dye concentration might account for this discrepancy. Muscles of C57BL/6 mice were dissociated and stained with either 5 μg ml^{-1}, 10 μg ml^{-1} or 12.5 μg ml^{-1} of Hoechst 33342 dye and parallel analyses of verapamil-treated samples was performed for proper gating of SP cells (see supplemental data Fig. S1,

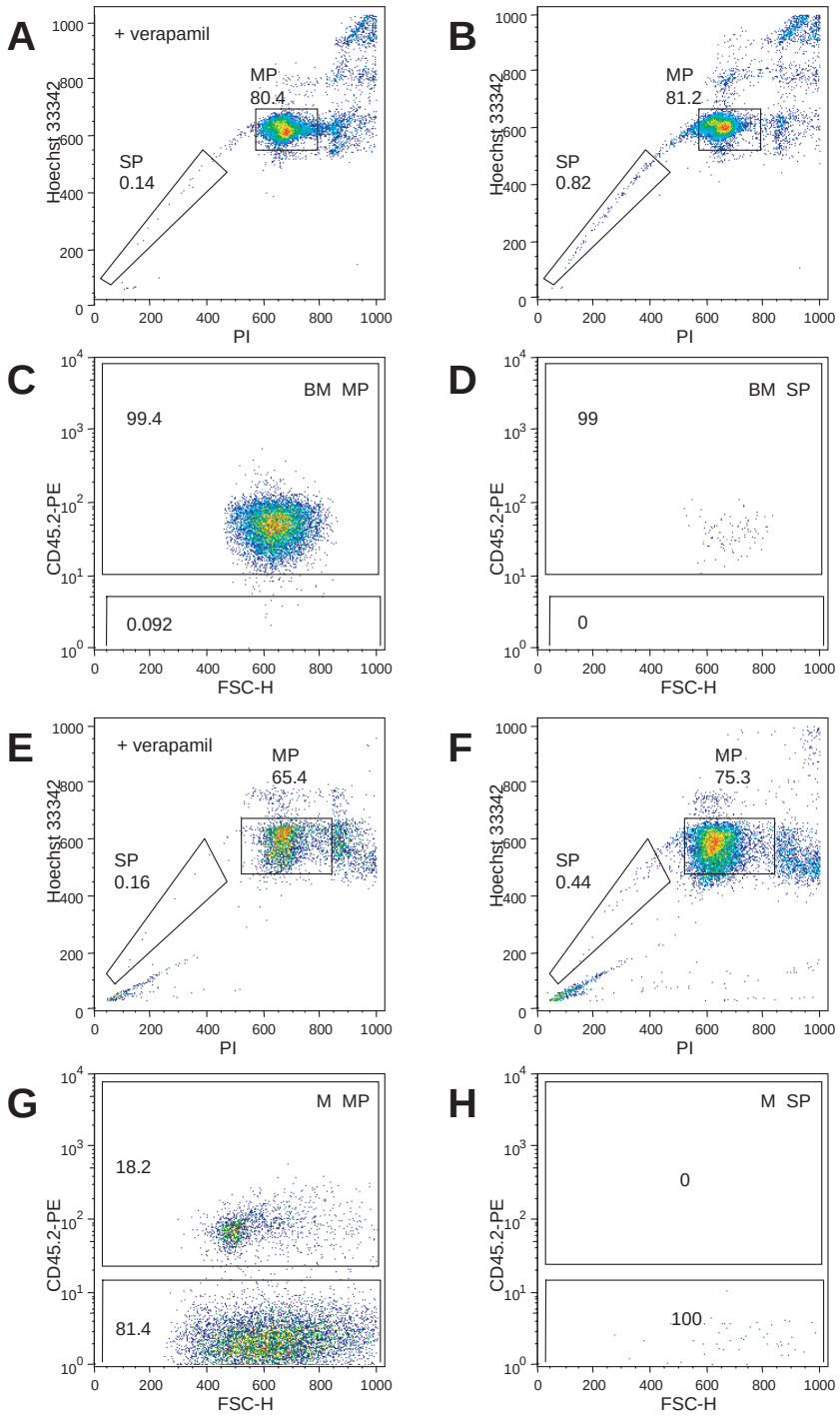

Fig. 1. Cell trafficking from the BM to the skeletal muscle 3 weeks after lethal irradiation and BMT. Detection via FACS analysis of donor-derived CD45.2$^+$ cells within the MP and SP cell populations in the BM (A-D) and the skeletal muscle (E-H) of recipient females. BM cells stained with 5 μg ml^{-1} Hoechst in the presence (A) or absence (B) of verapamil. Over 99% of BM MP cells (C) and SP cells (D) are CD45.2$^+$ or of donor origin. Skeletal muscle mononuclear cells stained with 12.5 μg ml^{-1} Hoechst dye in the presence (E) or absence (F) of verapamil. Approximately 18% of muscle MP cells (G) are CD45.2$^+$ donor-derived, whereas all muscle SP cells are CD45.2$^-$ (H).

http://jcs.biologists.org/supplemental/). Verapamil-treated samples contained 0.57% of drug-insensitive cells within the SP gate when samples were stained with 5 μg ml^{-1} of Hoechst 33342 dye, which decreased to 0.05% and 0.02% when samples were stained with 10 μg ml^{-1} or 12.5 μg ml^{-1} of Hoechst dye, respectively (Fig. S1). In the non-verapamil treated samples, the percentage of muscle SP cells was the highest (3.4%) at the lowest Hoechst concentration (5 μg ml^{-1}) and decreased to 0.48% and 0.32% in the samples stained with 10 μg ml^{-1} and 12.5 μg ml^{-1} of Hoechst dye, respectively (Fig. S1, Fig. 4). These results indicated that cells that do not show verapamil-sensitivity, unlike SP cells, are contained in the SP gate at low Hoechst 33342 dye concentrations. To test this hypothesis, following staining with 5 μg or 12.5 μg ml^{-1} of Hoechst dye, samples were incubated with anti CD45-PE (clone 30F11) (BD Pharmingen) (Fig. 4). As expected, the percentage of CD45$^+$ muscle cells within the total mononuclear cells was the same in both samples because they were derived from the same animal (Fig. 4B,E). CD45$^+$ cells were then analyzed for Hoechst dye exclusion to visualize their distribution within the MP-SP populations. When cells were stained with 5 μg ml^{-1} of Hoechst dye, 23% of CD45$^+$ cells were within the muscle SP cell gate (Fig. 4C). By contrast, no CD45$^+$ cells were within the SP cell gate when the sample was stained with 12.5 μg ml^{-1} of Hoechst dye (Fig. 4F). Thus, in agreement with previously published results (Asakura et al., 2002; McKinney-Freeman et al., 2003), the percentage of CD45$^+$ cells within the muscle SP population is higher when samples are stained with low Hoechst dye concentrations and decreases at high Hoechst dye concentration (Fig. 4C,F). These results demonstrate phenotypical differences within the muscle SP-MP cell populations depending on the staining conditions and might have important implications on the potential of these cells observed in vitro and in vivo.

CD45.2$^-$ muscle cells are not BM-derived

The current analyses indicated that BM cell trafficking to skeletal muscle contributes predominantly to muscle MP, but very little to muscle SP cells. Previous reports have demonstrated that a proportion of BM cells trafficking to the skeletal muscle might lose expression of CD45 (Issarachai et al., 2002b; Issarachai et al., 2002a). In addition, CD45$^-$ mesenchymal BM cells could also contribute to muscle (Deschaseaux et al., 2003). These observations urged us to use markers other than CD45 to monitor cellular contribution from the BM to skeletal muscle SP cells. Two experimental procedures were performed. First, CD45$^-$ muscle cells were purified by FACS from the transplanted mice and hybridized by FISH using a Y-chromosome probe, a genetic

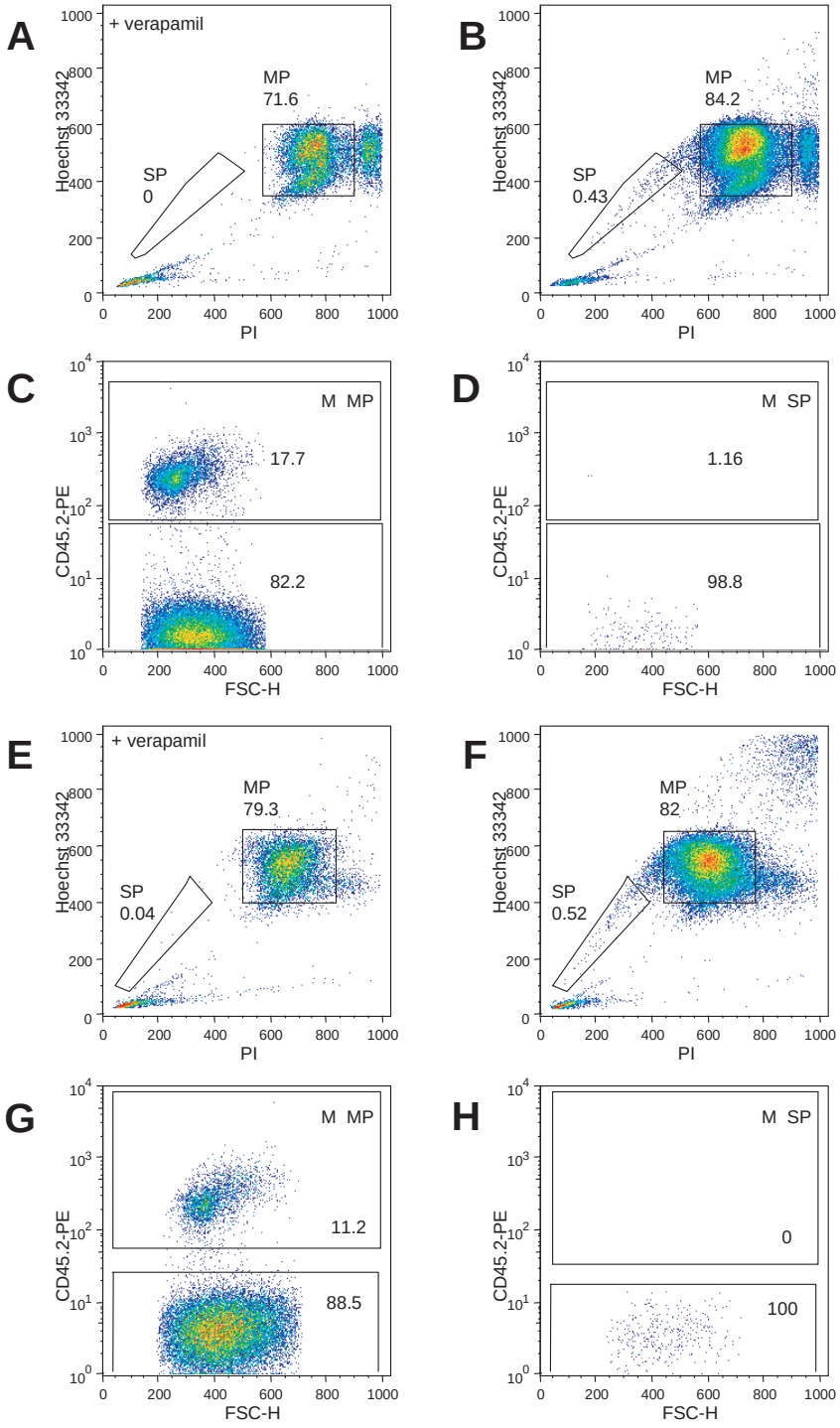

Fig. 2. Cell trafficking and detection of donor-derived CD45.2$^+$ cells within the muscle MP and SP cell populations of recipient females 10 weeks (A-D) and 30 weeks (E-H) after lethal irradiation and BMT. Muscle cells were stained with 12.5 μg ml^{-1} Hoechst dye in the presence (A,E) or absence (B,F) of verapamil. Approximately 17% (C) and 11% (G) of muscle MP cells at 10 and 30 weeks after BMT, respectively, are CD45.2$^+$ donor-derived. By contrast, 1% (D) and 0% (H) of muscle SP cells are CD45.2$^+$ at 10 and 30 weeks after BMT.

marker specific to the donor BM cells. Cytospin preparations of BM-derived CD45.2$^+$ cells were hybridized in parallel as a positive control. In two separate experiments, 80% and 95% of CD45.2$^+$ BM cells were also Y$^+$, as expected (Fig. 5A). By contrast, none of the CD45.2$^-$ cells isolated from muscle hybridized with the Y-chromosome probe, demonstrating that these cells were host-derived (Fig. 5B). This finding confirms that CD45$^-$ muscle SP cells did not receive contribution from the BM and that they were not 'false negative' cells of BM origin.

To further corroborate the results obtained by FISH hybridization, fourteen C57/BL6 recipient female mice were lethally irradiated and injected with 1.2×10^7 whole BM cells obtained from male e-GFP transgenic mice. Two of these mice were analyzed at 8 weeks after BMT. BM nuclei from the transplanted mice hybridized in two independent experiments using FISH demonstrated that 111 of 121 (91%) and 136 of 150 nuclei (90%) were Y-positive or of donor origin, as expected after lethal irradiation and BMT. FACS analyses of skeletal muscle MP and SP cells of these mice (Fig. 5C) indicated that 20% of MP cells (Fig. 5D) and 2.6% of SP cells (Fig. 5E) were GFP$^+$, confirming our results. Parallel FACS analysis of muscle SP cells prepared from e-GFP transgenic mice demonstrated that >98% of muscle SP express e-GFP (Fig. 5F), demonstrating that the low percentage of GFP$^+$ muscle SP cells in mice transplanted with e-GFP$^+$ BM cells is probably because of reduced BM cellular trafficking to the muscle SP cell-niche rather than because of muted expression of the GFP marker in muscle SP cells.

Muscle injury by cardiotoxin increases BM cell trafficking to the muscle.

To study whether BM cell trafficking to skeletal muscle MP-SP cells increases after induced muscle injury, intramuscular injections of 15 μg cardiotoxin were performed in three CD45.1 female mice at 5 weeks (n=2) and 16 weeks (n=1) after BMT with CD45.2 donor BM male cells. In each animal, cardiotoxin was injected in one leg and the contralateral leg was used as control. Mice were analyzed 2 weeks after cardiotoxin injury (7 and 18 weeks after BMT) (Fig. 6). Cardiotoxin injury increased the proportion of donor CD45.2$^+$ cells in the muscle MP cell population four- to tenfold (Fig. 6E; Table 2, mice A-C) and caused an up to twofold increase of CD45.2$^+$ cells in the SP cell population (Fig. 6F; Table 2). In separate experiments, intramuscular injection of 15 μg of cardiotoxin was administered in one leg of five host female CD45.1 mice 24 hours prior to lethal irradiation and BMT using 2.2×10^7 CD45.2$^+$ donor male BM cells (Table 2, mice D-H). Recipients were euthanized and skeletal muscle and BM analyzed for the presence of CD45.2$^+$ donor cells at 2 (n=2), 5, 6 and 7 weeks

after BMT (Table 2, mice D-H). Analysis of the BM revealed that, as expected, >90% of the cells were of donor origin (data not shown). Analyses of skeletal muscles demonstrated that up to 55% of muscle MP cells were CD45.2$^+$ donor-derived in the cardiotoxin-injured limb, whereas, by contrast, the highest percentage of donor-derived muscle SP cells after cardiotoxin injury was 7.8% (Table 2, mouse E). Statistical analysis using the analysis of variance (ANOVA) test demonstrated that the percentage of donor CD45.2$^+$ in muscle MP cells was significantly higher in the cardiotoxin-injected muscle compared to non-injured muscle (P<0.0001). An increase in the percentage of host-derived CD45.1$^+$ muscle MP cells was also sporadically observed (Table 2), although ANOVA analysis showed no evidence of statistical significance (P=0.2). The increased percentage of CD45.1 host-derived cells after cardiotoxin damage is in agreement with recent observations by Polesskaya et al. (Polesskaya et al., 2003). However, because the mice in the current study had received lethal irradiation, it is possible that the contribution of endogenous CD45.1 cells might have been impaired by it, explaining the lack of statistical significance. Analysis of muscle SP cells demonstrated that neither CD45.2$^+$ donor-derived cells nor CD45.1$^+$ host-derived cells increased significantly in control versus cardiotoxin-injured muscle (P=0.1 and P=1, respectively).

Table 1. Percentage of donor-derived CD45.2$^+$ cells detected in the bone marrow and muscle SP/MP cell fractions from 3-34 weeks after bone marrow transplantation.

Time after BMT	CD45.2$^+$MP cells		CD45.2$^+$SP cells	
	BM	Muscle	BM	Muscle
3w	99.4	18.2	99	0
4w	92.8	11.9	100	3.2
5w	97.2	19.7	98.1	0
6w	94.1	28.8	95.4	5.2
7w	98.8	25.4	97.3	0.9
8w	99.3	21.1	100	0
9w	92.4	27.9	92.1	0
10w	97.1	17.7	90.8	1.1
11w	96.9	32.2	96.4	4.8
12w	98.7	24.9	97.9	4.4
13w	99.0	16.2	97	4.6
18w	99.3	11.1	99.8	4.1
19w	98.8	21.8	97.7	0
22w	91.5	20.7	81.5	0.8
25w	98.4	15.4	84.4	0
30w	99.0	11.2	93.6	0
34w	97.9	7.6	93.4	0.2

Most of the CD45.2$^+$ donor cells are detected in the MP population within the muscle. By contrast, little or no contribution of donor bone marrow-derived cells to the muscle SP cell fraction is observed.

CD45.2$^-$ muscle cells purified from the cardiotoxin-injured muscles using FACS were placed on glass slides via cytospin centrifugation and hybridized by FISH with the Y-chromosome probe. No hybridization signals were observed (0 of 443), confirming that the CD45.2$^-$ cells present in muscle were all of host origin. Parallel hybridization by FISH on BM-derived CD45$^+$ cells revealed a positive hybridization signal in 90 of 113 of the nuclei (80%) (data not shown).

CD45$^+$ cells are Y-chromosome$^+$ and appear as desmin-negative, interstitial cells in skeletal muscle tissue sections.

To localize donor-derived BM cells within the muscles of

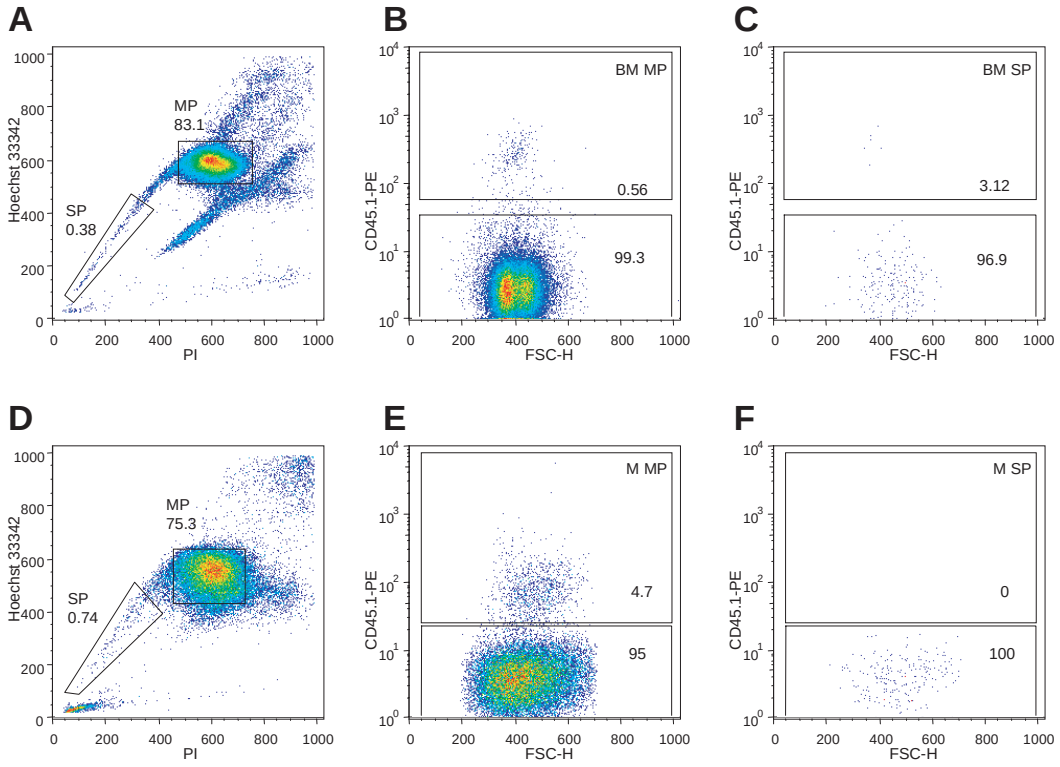

Fig. 3. Analysis of host-derived CD45.1$^+$ cells in the BM (A-C) and skeletal muscle (D-F) of MP and SP cell populations 30 weeks after BMT. As expected, very few CD45.1$^+$ host-derived cells are detected in the MP (B) or SP population (C) of the BM after lethal irradiation and BMT, because the BM was replaced by CD45.2$^+$ donor cells. In the skeletal muscle, host-derived CD45.1$^+$ cells constitute 4% (E) of the MP population, whereas no host-derived CD45.1$^+$ cells are detected within the muscle SP population (F).

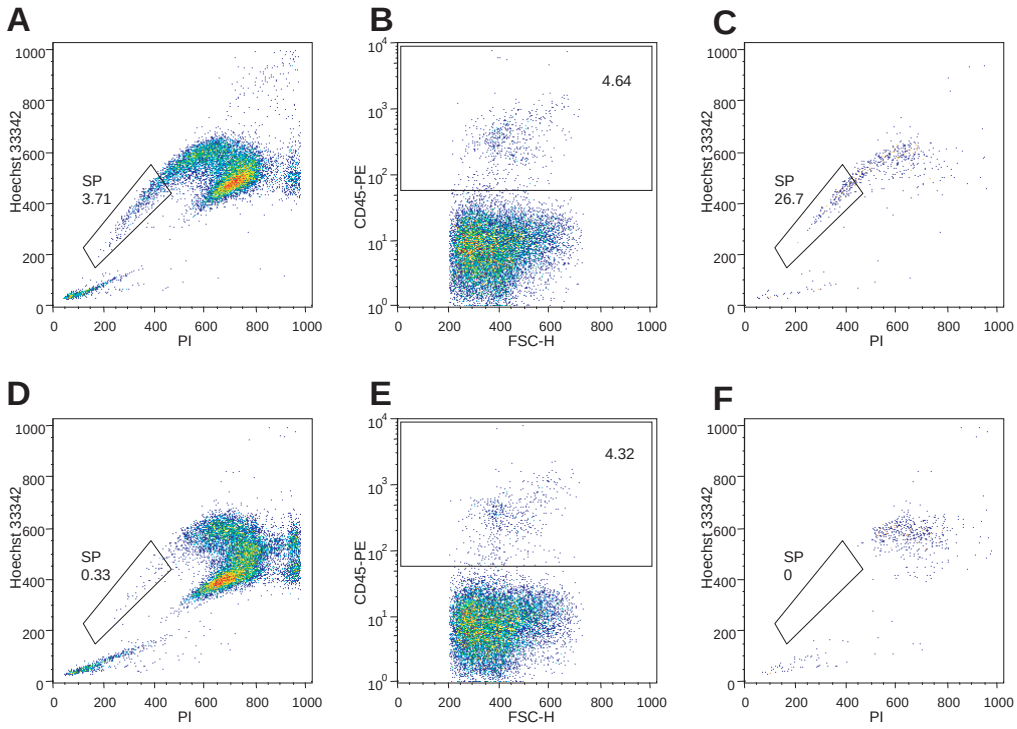

Fig. 4. Phenotypical differences of muscle SP cells detected using varying Hoechst dye concentrations. Parallel samples stained with 5 μg ml^{-1} (A) or with 12.5 μg ml^{-1} (D) of Hoechst dye show different percentages of SP cells. As expected, the total percentage of CD45$^+$ cells within the samples does not vary (B,E). However, when CD45$^+$ cells are analyzed for Hoechst exclusion properties, 26% of CD45$^+$ cells fall within the SP cell gate when the sample is stained with 5 μg ml^{-1} of Hoechst dye (C), whereas no CD45$^+$ cells are present in the SP gate when the sample is stained with 12.5 μg ml^{-1} Hoechst dye (F).

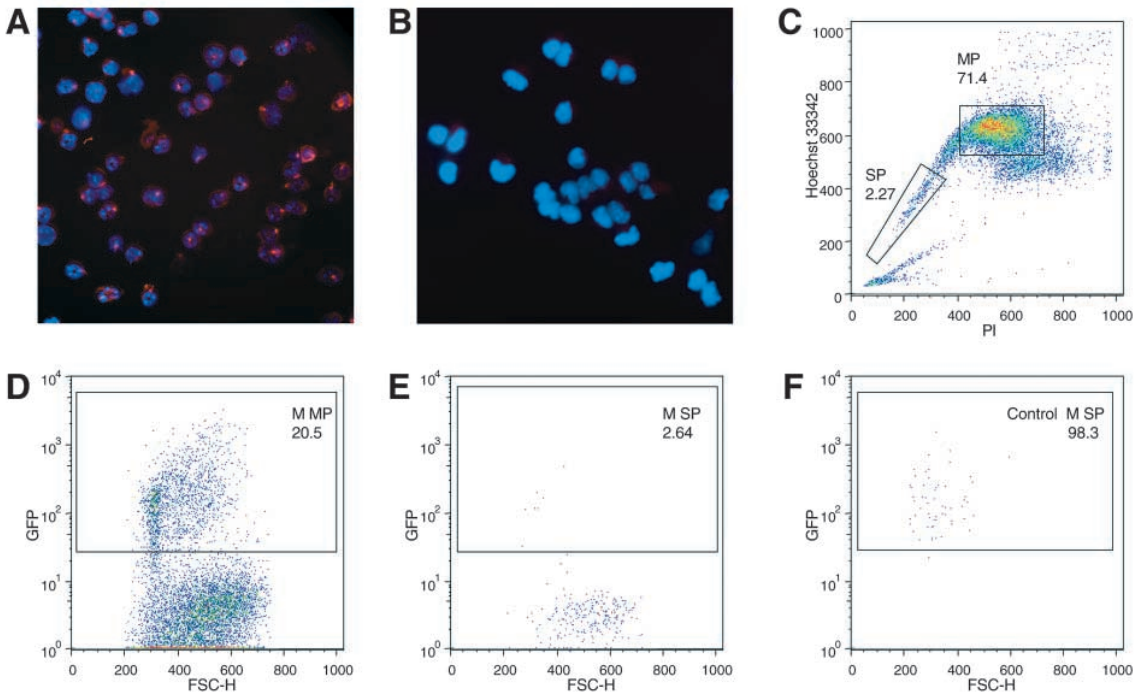

Fig. 5. (A) CD45.2-PE$^+$ BM cells purified by FACS and hybridized with the Y-chromosome probe by FISH (red). A red-orange outline at the periphery of the nuclei indicates the CD45.2-PE signal that persisted after FISH. Nuclei are counter-stained with DAPI (blue). (B) CD45.2-PE-negative muscle cells sorted by FACS and hybridized with the Y-probe by FISH. No hybridization signals were observed. (C) FACS analysis of a muscle sample, which was taken 8 weeks after BMT from a mouse injected with e-GFP plus BM. SP (2.2%) and MP (71.4%) cell gates are shown. (D) Percentage of GFP$^+$ BM-derived cells in muscle MP cells and in muscle SP cells (E). (F) More than 98% of muscle SP cells from an e-GFP transgenic mouse are positive for e-GFP.

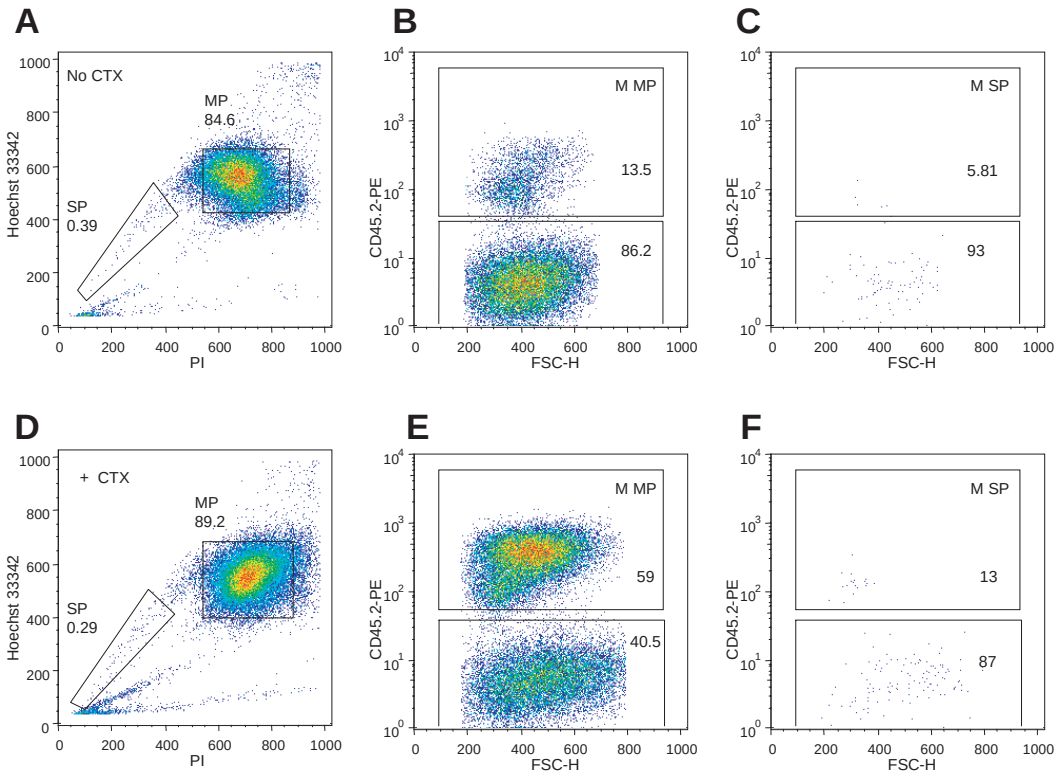

Fig. 6. Analysis of BM-cell trafficking to skeletal muscle MP and SP cells in the absence (A-C) or in the presence (D-F) of cardiotoxin injury (Mouse B in Table 2). FACS profiles and percentages of SP and MP cells in control (A) or cardiotoxin-injected muscles (D). Percentage of CD45.2$^+$ donor-derived cells in muscle MP cells in the absence (B) or presence (E) of cardiotoxin injury. A near 5-fold increase of donor-derived cells is observed upon cardiotoxin injury in the MP population. Percentage of CD45.2$^+$ donor-derived cells in muscle SP cell population in control (C) or in cardiotoxin-injured (F) muscles.

recipient mice, immunohistochemistry was performed on frozen tissue sections using the CD45.2 antibody on samples harvested at different time points after transplantation. At 11w, 18 w, 25 w and 34 w after BMT, CD45.2-positive cells were detected as interstitial cells between muscle fibers (Fig. 7A-C). Hybridization by FISH using the Y-chromosome probe revealed that nearly all CD45.2$^+$ cells were also Y$^+$, confirming their donor origin (Fig. 7A-C). Parallel analyses performed on muscle tissue section from recipient females that had received muscle injury by cardiotoxin, again revealed the presence of donor-derived CD45.2$^+$,Y$^+$ cells clustered within the interstitial spaces, demonstrating increased trafficking of BM-derived cells following muscle damage (Fig. 7D,E). To investigate the presence of donor-derived CD45.2$^+$-desmin$^+$ satellite cells, immunofluorescence analysis was performed on muscle tissue sections using the anti-CD45.2 and anti-desmin antibodies simultaneously. Double-positive cells were not detected, because all the CD45.2 positive cells appeared as desmin

Table 2. Percentage of CD45.2$^+$ (donor) and CD45.1$^+$ (host) muscle MP and SP cells detected in recipient mice in the presence or in the absence of cardiotoxin (CT) injury

			MP-CD45.2		SP-CD45.2		MP-CD45.1		SP-CD45.1	
Mouse	CT	Analysis	Control	CT	Control	CT	Control	CT	Control	CT
A	5w	7w	6.8	77.7	5.0	11.1	2.8	9.6	0	0
B	5w	7w	13.6	59.1	6.9	13	4.0	47.2	0	0
C	16w	18w	11.1	42.1	4.1	1.9	ND	ND	ND	ND
D	–1d	2w	4.6	39.7	0	6.6	1.5	10.3	0	0.6
E	–1d	2w	5.8	55.4	1.5	7.8	2.1	38.6	0.7	2.3
F	–1d	5w	3.0	5.5	0	4.7	1.6	2.5	0	0
G	–1d	6w	14.5	33.9	4.2	0	3.2	6.9	0	0
H	–1d	7w	16.9	7.3	6.6	5.6	2.4	3.8	0	0

Mice A-C received cardiotoxin injection in one hindlimb at 5 and 16 weeks (w) after bone marrow transplantation (BMT). The cardiotoxin-injected and control contralateral hindlimbs were analyzed 2 weeks after cardiotoxin injury. Mice D-H received cardiotoxin injury 1 day prior to BMT and were analyzed 2 weeks (n=2), 5, 6 and 7 weeks after BMT.

CT, time at which cardiotoxin was injected (day 0=bone marrow transplantation). Analysis, time at which muscles were harvested for analysis. ND, not determined.

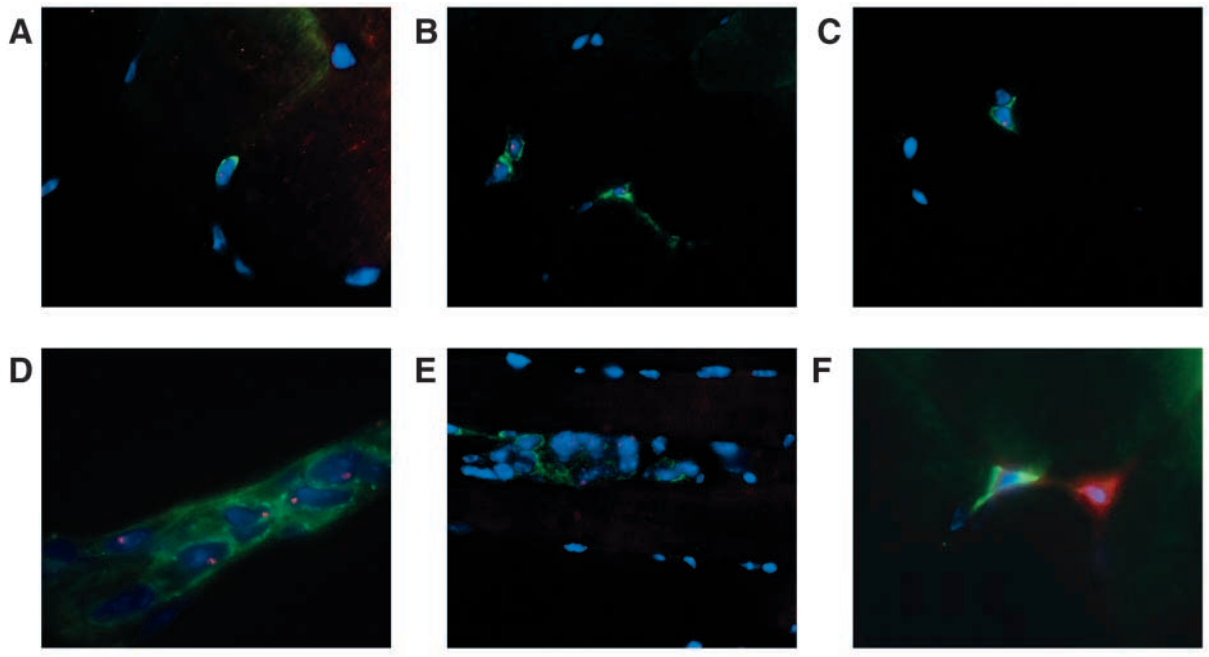

Fig. 7. Combined immunohistochemistry/FISH analysis on muscle-tissue sections of recipient female mice injected with donor male BM cells. Tissue sections were collected from samples harvested at 11 w (A), 19 w (B) and 34 w (C) after BMT. Samples (D) and (E) are muscles analyzed 18 w after transplantation and 2 w after cardiotoxin injury. (A-E) The green signal represents the expression of CD45.2 (donor-derived cells antigen). Nuclei are stained in blue by DAPI. Red dots within nuclei represent FISH hybridization using the Y-chromosome probe to confirm the donor-cell origin. (F) Immunohistochemistry using anti-CD45.2 (green) and anti-desmin (red) antibodies. A host-derived satellite cells (red) and a donor-derived CD45.2$^+$ BM cell (green) that has migrated to the muscle are shown.

negative and vice versa (Fig. 7F), suggesting that loss of CD45 expression by BM-derived cells might be necessary for their transition to the skeletal muscle satellite cell compartment (LaBarge and Blau, 2002).

Discussion

Studies have demonstrated that BM cells delivered via BMT can traffick to the skeletal muscle of mice and humans with muscular dystrophy and fuse with pre-existing myofibers (Ferrari et al., 1998; Bittner et al., 1999; Gussoni et al., 1999; Ferrari et al., 2001; Gussoni et al., 2002; LaBarge and Blau, 2002). These studies opened the possibility to use BM cells for optimizing cell-based therapies of muscle disorders (Pennisi, 1998).

SP cells were first isolated from the BM based on their ability to exclude the vital DNA dye Hoechst 33342 (Goodell et al., 1996). This property is attributed to the activity of the ABCG2/*brcp1* transporter (Zhou et al., 2001; Zhou et al., 2002). Studies in mice demonstrated that BM SP cells are enriched of highly primitive cells (Goodell et al., 1996). SP cells were found in many other tissues (Asakura and Rudnicki, 2002), but their role, origin, contribution to local tissues and relationship with other SP cells remain largely unknown. One attractive hypothesis is that tissue-specific SP cells derive from a common, circulating stem cell that harbors in other tissues and has the potential to integrate and become a committed progenitor of that tissue (Howell et al., 2003). Because the existence of stem cells within the BM has been well demonstrated and these cells are known to circulate, it seems reasonable to hypothesize that stem cells found in other tissues might also derive from and maintain a relationship with the BM. Although early analyses of muscle SP cells suggested that these cells might be a muscle-derived cell population (Gussoni et al., 1999), this concept remains largely unproven. The current study investigated cell trafficking and relationship between the BM and muscle-derived SP cells.

Cell trafficking from the BM to muscle SP and MP cell populations was monitored up to 34 weeks after BMT in the presence or absence of induced muscle injury, by using multiple donor cell markers. Our results consistently demonstrate that cell trafficking from the BM to skeletal muscle does occur both in the presence and absence of muscle injury. Most if not nearly all BM-derived cells that maintain the expression of CD45 in the muscle become part of the MP, not the SP, cell compartment. Previous studies using GFP$^+$ donor-derived BM cells demonstrated that upon transition and trafficking to the skeletal muscle, a proportion of BM-derived cells loose expression of CD45 (Issarachai et al., 2002b; Issarachai et al., 2002a). In this study, we extended these observations by determining that GFP$^+$CD45$^-$ BM-derived cells contribute mainly to muscle MP, not SP, cells. Thus, CD45$^-$ muscle SP cells appear to be a population independent from the BM.

Another conclusion from this study is that muscle-derived SP cells do not express CD45. However, CD45-positive muscle SP cells can be detected when low concentrations of the Hoechst are used to visualize these cells. Thus, although with both low (5 μg ml^{-1}) and high (12.5 μg ml^{-1}) Hoechst dye concentrations, muscle SP cells sensitive to verapamil are detected, the phenotype of these SP cells differ, as demonstrated by variations in the percentage of CD45-positive cells. These findings have important consequences for studies attempting to define the differentiation potential of muscle SP cells into

hematopoietic, muscle (or other cell types) in vitro and in vivo, and strongly suggest that Hoechst dye staining should be accompanied by the characterization of cell surface markers to define the subfractions of muscle SP cells being studied.

In summary, our findings clearly demonstrate that $CD45^+$ BM-derived cells provide very little or no contribution to the $CD45^-$ muscle SP cell niche and suggest the possibility that tissues other than the BM might contribute to the normal replenishment of muscle SP cells. Lack of expression of CD45 on muscle SP cells is consistent with the phenotype of multipotent mesenchymal cells isolated from muscle as well as other tissues (Young et al., 2001; Jiang et al., 2002). One hypothesis is that muscle SP cells are indeed a population of muscle-derived multipotent progenitors cells that are rather radiation-resistant. This hypothesis is supported by the findings that, high doses of radiation do not ablate certain populations of myogenic stem cells resident within the muscle (Heslop et al., 2000), *Pax7*-knockout mice, which lack satellite cells, have a higher content of muscle SP cells compared to normal mice (Seale et al., 2000), and that muscle SP cells have the ability to fuse to myofibers and give rise to satellite cells in vivo (Asakura et al., 2002). All the above findings appear consistent with the hypothesis that muscle SP cells are quiescent primitive progenitors that self-renew upon muscle injury and also give rise to a progeny of satellite cells, which in turn participate more actively in muscle regeneration and repair. Clearly, more studies are necessary to confirm or deny this hypothesis.

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References

Alonso, L. and Fuchs, E. (2003). Stem cells of the skin epithelium. *Proc. Natl. Acad. Sci. USA* **100**, Suppl. 1, 11830-11835.

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

Asakura, A. and Rudnicki, M. A. (2002). Side population cells from diverse adult tissues are capable of in vitro hematopoietic differentiation. *Exp. Hematol.* **30**, 1339-1345.

Asakura, A., Seale, P., Girgis-Gabardo, A. and Rudnicki, M. A. (2002). Myogenic specification of side population cells in skeletal muscle. *J. Cell Biol.* **159**, 123-134.

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.

Bulfield, G., Siller, W. G., Wight, P. A. L. and Moore, K. J. (1984). X chromosome-linked muscular dystrophy (mdx) in the mouse. *Proc. Natl. Acad. Sci. USA* **81**, 1189-1192.

Cao, B., Zheng, B., Jankowski, R. J., Kimura, S., Ikezawa, M., Deasy, B., Cummins, J., Epperly, M., Qu-Petersen, Z. and Huard, J. (2003). Muscle stem cells differentiate into haematopoietic lineages but retain myogenic potential. *Nat. Cell Biol.* **5**, 640-646.

Clarke, D. L. (2003). Neural stem cells. *Bone Marrow Transplant* **32**, Suppl. 1, S13-S17.

Deschaseaux, F., Gindraux, F., Saadi, R., Obert, L., Chalmers, D. and Herve, P. (2003). Direct selection of human bone marrow mesenchymal stem cells using an anti-CD49a antibody reveals their CD45med,low phenotype. *Br. J. Haematol.* **122**, 506-517.

Ferrari, G., Cusella-De Angelis, 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.

Goodell, M. A., Brose, K., Paradis, G., Conner, A. S. and Mulligan, R. C. (1996). Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J. Exp. Med.* **183**, 1797-1806.

Gussoni, E., Bennett, R. R., Muskiewicz, K. R., Meyerrose, T., Nolta, J. A., Gilgoff, I., Stein, J., Chan, Y. M., Lidov, H. G., Bonnemann, C. G. et al. (2002). Long-term persistence of donor nuclei in a Duchenne muscular dystrophy patient receiving bone marrow transplantation. *J. Clin. Invest.* **110**, 807-814.

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

Gussoni, E., Wang, Y., Fraefel, C., Miller, R. G., Blau, H. M., Geller, A. I. and Kunkel, L. M. (1996). A method to codetect introduced genes and their products in gene therapy protocols. *Nat. Biotechnol.* **14**, 1012-1016.

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 (Pt 12)**, 2299-2308.

Howell, J. C., Lee, W. H., Morrison, P., Zhong, J., Yoder, M. C. and Srour, E. F. (2003). Pluripotent stem cells identified in multiple murine tissues. *Ann. NY Acad. Sci.* **996**, 158-173.

Issarachai, S., Priestley, G. V., Nakamoto, B. and Papayannopoulou, T. (2002a). Bone Marrow-Derived $CD45^+$ and $CD45^-$ Cells Reside in Skeletal Muscle. *Blood Cells Mol. Dis.* **29**, 69-72.

Issarachai, S., Priestley, G. V., Nakamoto, B. and Papayannopoulou, T. (2002b). Cells with hemopoietic potential residing in muscle are itinerant bone marrow-derived cells. *Exp. Hematol.* **30**, 366-373.

Jiang, Y., Jahagirdar, B. N., Reinhardt, R. L., Schwartz, R. E., Keene, C. D., Ortiz-Gonzalez, X. R., Reyes, M., Lenvik, T., Lund, T., Blackstad, M. et al. (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* **418**, 41-49.

Kawada, H. and Ogawa, M. (2001). Bone marrow origin of hematopoietic progenitors and stem cells in murine muscle. *Blood* **98**, 2008-2013.

LaBarge, M. A. and Blau, H. M. (2002). Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury. *Cell* **111**, 589-601.

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

McKinney-Freeman, S. L., Jackson, K. A., Camargo, F. D., Ferrari, G., Mavilio, F. and Goodell, M. A. (2002). Muscle-derived hematopoietic stem cells are hematopoietic in origin. *Proc. Natl. Acad. Sci. USA* **99**, 1341-1346.

McKinney-Freeman, S. L., Majka, S. M., Jackson, K. A., Norwood, K., Hirschi, K. K. and Goodell, M. A. (2003). Altered phenotype and reduced function of muscle-derived hematopoietic stem cells. *Exp. Hematol.* **31**, 806-814.

Niemann, C. and Watt, F. M. (2002). Designer skin: lineage commitment in postnatal epidermis. *Trends Cell Biol.* **12**, 185-192.

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.

Pennisi, E. (1998). Bone marrow cells may provide muscle power. *Science* **279**, 1456.

Polesskaya, A., Seale, P. and Rudnicki, M. A. (2003). Wnt signaling induces the myogenic specification of resident CD45+ adult stem cells during muscle regeneration. *Cell* **113**, 841-852.

Seale, P. and Rudnicki, M. A. (2000). A new look at the origin, function, and "stem-cell" status of muscle satellite cells. *Dev. Biol.* **218**, 115-124.

Seale, P., Sabourin, L. A., Girgis-Gabardo, A., Mansouri, A., Gruss, P. and Rudnicki, M. A. (2000). Pax7 is required for the specification of myogenic satellite cells. *Cell* **102**, 777-786.

Sicinski, P., Geng, Y., Ryder-Cook, A. S., Barnard, E. A., Darlison, M. G. and Barnard, P. J. (1989). The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. *Science* **244**, 1578-1580.

Young, H. E., Duplaa, C., Young, T. M., Floyd, J. A., Reeves, M. L., Davis, K. H., Mancini, G. J., Eaton, M. E., Hill, J. D., Thomas, K. et al. (2001). Clonogenic analysis reveals reserve stem cells in postnatal mammals: I. Pluripotent mesenchymal stem cells. *Anat Rec* **263**, 350-360.

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

Zhou, S., Morris, J. J., Barnes, Y., Lan, L., Schuetz, J. D. and Sorrentino, B. P. (2002). *Bcrp1* gene expression is required for normal numbers of side population stem cells in mice, and confers relative protection to mitoxantrone in hematopoietic cells in vivo. *Proc. Natl. Acad. Sci. USA* **19**, 12339-12344.

Zhou, S., Schuetz, J. D., Bunting, K. D., Colapietro, A. M., Sampath, J., Morris, J. J., Lagutina, I., Grosveld, G. C., Osawa, M., Nakauchi, H. et al. (2001). The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat. Med.* **7**, 1028-1034.