

# Retrovirally Transduced Muscle-Derived Cells Contribute to Hematopoiesis at Very Low Levels in the Nonhuman Primate Model

Chunji Gao,<sup>1</sup> Elizabeth M. Kang,<sup>1</sup> Ken Kuramoto,<sup>2</sup> Brian A. Agricola,<sup>2</sup> Mark Metzger,<sup>2</sup> Christof von Kalle,<sup>3</sup> Robert E. Donahue,<sup>2</sup> and John F. Tisdale<sup>1,\*</sup>

<sup>1</sup>Molecular and Clinical Hematology Branch, National Institute of Diabetes and Digestive and Kidney Disorders, and

<sup>2</sup>Hematology Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

<sup>3</sup>Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio 45229

\*To whom correspondence and reprint requests should be addressed at Building 10, Room 9N116, Molecular and Clinical Hematology Branch, National Institute of Diabetes and Digestive and Kidney Disorders, National Institutes of Health, Bethesda, MD 20892. Fax: (301) 480-1373.

E-mail: Johntis@intra.niddk.nih.gov.

Recent studies have suggested a remarkable potential of adult stem cells from a variety of organs to give rise to cells of disparate organs, but evidence of such potential at a clonal level is lacking in most if not all studies to date. To assess directly the hematopoietic potential of muscle-derived cells in a relevant large animal, we initiated retroviral-tagging studies in the rhesus macaque to allow tracking at the clonal level by integration site analysis. Four rhesus macaques underwent transplantation with transduced muscle-derived cells after lethal irradiation followed by delayed infusion of an autologous hematopoietic graft. The first animal showed no evidence of hematopoietic recovery and, despite infusion of the backup hematopoietic graft, succumbed due to complications of prolonged cytopenias. In the remaining three animals, the overall contribution of retrovirally tagged muscle-derived cells toward hematopoiesis was exceedingly low. Retroviral integration site analysis among clonally derived muscle cells and bone marrow cells *in vivo* in one animal suggests a common source. These results demonstrate that harvesting disparate organs for cellular therapy is currently highly inefficient at best.

## INTRODUCTION

The concept that renewal of various tissues at steady state or following damage relies upon a small population of locally residing tissue-specific stem cells has remained unchallenged for decades. However, several groups have demonstrated a remarkable capacity of cells derived from one tissue to participate in the repair or repopulation of disparate organs [1–10] thoroughly reviewed in [11]. Two groups have demonstrated not only that muscle-derived cells (MDCs) participate in the hematopoietic reconstitution of irradiated mice but also that their contribution is significant, with a 50% or higher contribution to all hematopoietic lineages, when competed against steady-state bone marrow cells in the second reported study [12,13]. However, subsequent studies have suggested that the hematopoietic potential of MDCs derives from hematopoietic cells residing within muscle, challenging the notion of stem cell plasticity and adding to the controversy heralded by the initial studies [14,15].

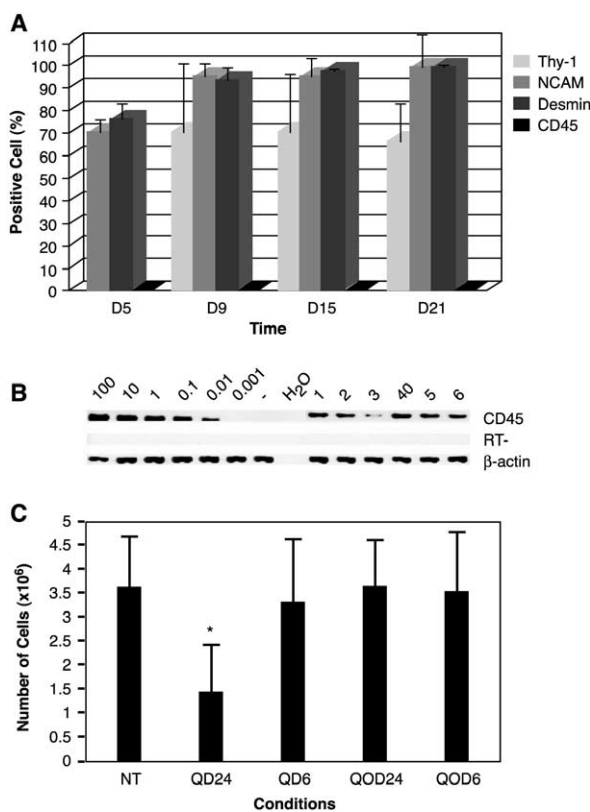
To explore rigorously the hematopoietic potential of MDCs and, further, the potential of adult stem cells, we

initiated retroviral-tagging studies in the nonhuman primate in which transduced MDCs were infused and monitored hematopoietic reconstitution after lethal irradiation. The contribution of MDCs toward hematopoiesis was monitored by conventional PCR and tracking of individual clones by linear amplification-mediated PCR (LAM-PCR) of the retroviral integration sites. The contribution of MDCs toward hematopoiesis was extremely low in our model; however, a common integration site among clonally derived muscle cells and bone marrow cells *in vivo* suggests that adult stem cells may be capable of multiple fates.

## RESULTS

### MDC Culture and *ex Vivo* Transduction

Prior to initiating *in vivo* studies, we performed sartorius muscle resection in nine rhesus macaques. Enrichment for satellite cells occurred as a function of time in culture (Fig. 1A). Expression of muscle markers such as neural cell adhesion molecule (NCAM), a surface glycoprotein expressed on developing and regenerating muscle as well as



**FIG. 1.** Optimization of MDC transduction. (A) Sartorius muscle was harvested from nine animals and cultured for up to 21 days. The expression of the Thy-1, NCAM, desmin, and CD45 is shown from day 5 (D5) through day 21 (D21). (B) Nested RT-PCR for CD45 performed on samples obtained at day 9 of culture/transduction. Controls consist of dilutions of DNA from the producer cell clone at known copy number ranging from 100 to 0.001%;  $\beta$ -actin served as a control. RT-, no reverse transcriptase. (C) MDCs were exposed after 48 h to vector daily for 24 h (QD24), daily for 6 h (QD6), every other day for 24 h (QOD24), or every other day for 6 h (QOD6) for a total 9-day culture period, and the number of cells was compared to that of a nontransduced (NT) control.

muscle satellite cells [23], and desmin, a muscle-specific intermediate filament protein [24], was greater than 90% and expression of CD45 was not observed after day 9 of culture. Further, CD45 mRNA was not detectable by conventional RT-PCR (data not shown), but was present at the limit of detection (0.01%) by nested PCR (Fig. 1B).

We next compared the influence of different transduction conditions on satellite cell growth (Fig. 1C). While cell growth was negatively impacted by daily 24-h vector supernatant exposure compared to the nontransduced fraction ( $P = 0.048$ ), daily 6-h exposure had no significant impact and was associated with transduction rates of >60%.

### In Vivo Transplantation of MDCs

We performed unilateral sartorius muscle harvesting, satellite cell preparation, and transduction in all four ani-

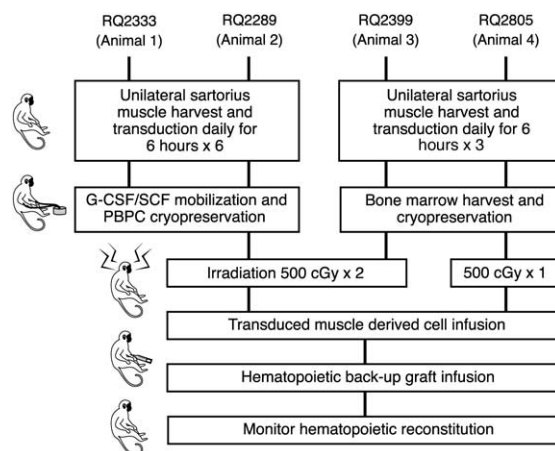
mals, followed by collection of a backup hematopoietic stem cell source to ensure animal survival (Fig. 2). Animals 1, 2, and 3 received myeloablative conditioning with 500 cGy of total body irradiation daily for 2 consecutive days. Animal 4 received a nonmyeloablative dose of 500 cGy delivered only once. We infused transduced MDCs fresh on day 0 and infused backup grafts either on day 14 (animal 1) or earlier on day 7 (animals 2, 3, and 4).

We utilized the optimized 9-day culture with daily 6-h vector exposure in the first two animals (animals 1 and 2). We utilized a shorter, 5-day *ex vivo* culture/transduction in the second two animals (animals 3 and 4). The longer culture period resulted in both a higher cell number and a higher purity (Table 1). Additionally, higher transduction efficiency was achieved with the longer culture duration (67–69% versus 15–36%, Table 1). CD45-positive cells were not detectable by flow cytometric analysis in cultured MDCs from any of the four transplanted animals (Table 1).

We did not observe hematopoietic reconstitution in animal 1, who succumbed due to complications of prolonged pancytopenia (Fig. 3). In animals 2, 3, and 4, for whom the backup hematopoietic grafts were infused 1 week earlier, we did observe hematopoietic reconstitution, but it was delayed compared to historical control macaque recipients of retrovirally transduced mobilized peripheral blood CD34-positive cells in whom the median time to recovery was 8 days (mean 8.8 days, range 5–14 days,  $n = 17$ ).

### Posttransplantation Analysis for *in Vivo* Gene Marking

We analyzed samples of blood, bone marrow, muscle, liver, spleen, kidney, and lymph nodes obtained postmortem from animal 1 for marking by nested, semiquantitative PCR, and they were negative except for marking at 0.001% in an axillary lymph node (data not shown). From



**FIG. 2.** MDC transplantation protocol.

**TABLE 1:** Characteristics of the MDC and hematopoietic grafts

Animal	Age (y)	Muscle <sup>a</sup> (g)	MDCs <sup>b</sup> (10 <sup>6</sup> /kg)	MDC markers (% positive) <sup>c</sup>				Txn eff (%) <sup>d</sup>	MNC/CFU <sup>e</sup>
				NCAM	Thy-1	CD45	Desmin		
1	4	6	3.3	90.5	54.5	Neg	98	69	5.6/8.3
2	4.5	12	8.2	97.5	16.7	Neg	98	67	3.6/13.2
3	6	7.7	0.4	70.9	49.9	Neg	90	36	0.3/6.3
4	3.5	7.5	0.1	63.2	4.2	Neg	88	15	0.3/5.1

<sup>a</sup>Harvested muscle weight in grams prior to digestion.

<sup>b</sup>MDC dose  $\times 10^6$  obtained at the end of culture/transduction.

<sup>c</sup>Expression of NCAM, Thy-1, CD45, and desmin at the end of culture/transduction.

<sup>d</sup>Transduction efficiency assessed by PCR performed on clonally derived MDCs from the end of culture/transduction.

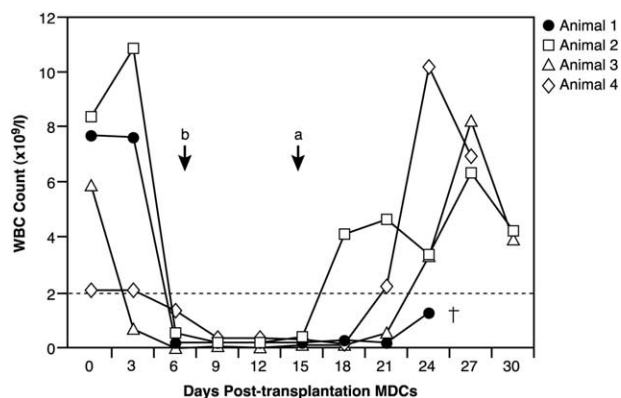
<sup>e</sup>Total mononuclear cell ( $\times 10^9$ ) and CFU ( $\times 10^5$ ) content of the backup peripheral blood (animals 1 and 2) and bone marrow (animals 3 and 4) grafts.

the subsequent three animals, we obtained peripheral blood and bone marrow mononuclear and granulocyte fractions at various time points posttransplantation. The majority of samples were negative (Fig. 4A) with the exception of intermittent positivity at the 0.001% or 1 cell per 100,000 level beyond 6 weeks posttransplantation. Individual bone marrow-derived colony-forming units (CFU) plated at regular time points posttransplantation were also negative for the transferred gene. However, CFU derived from bone marrow mononuclear cells plated at high concentration ( $10^6$ – $10^8$ /ml) in methylcellulose medium supplemented with G418 revealed CFU-GM colonies positive for the *neo* gene by PCR in 8 of 16 colonies from two separate time points in animal 2 and in 10 of 29 from three separate time points in animal 4, indicating very low level hematopoiesis originating from the MDC graft (Fig. 4A).

### Retroviral Integration Site Analysis by LAM-PCR

To allow the simultaneous detection of multiple retroviral integration sites among both clonally derived muscle cells

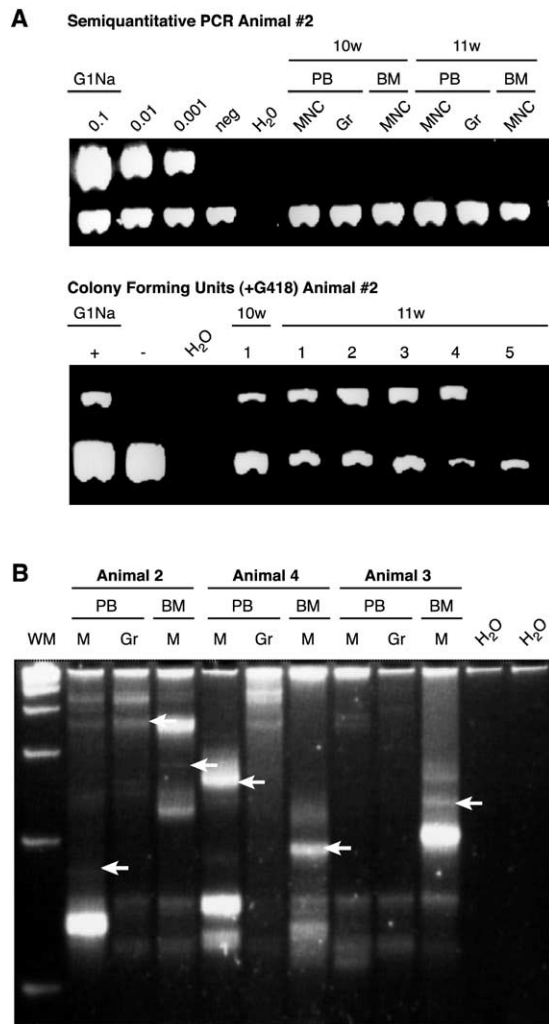
*in vitro* and bone marrow-derived cells *in vivo*, we employed LAM-PCR. Several unique integration sites were confirmed among peripheral blood and bone marrow mononuclear cells for all three animals (Fig. 4B). Because of the high degree of nonspecific products present when LAM-PCR is performed in the context of very low level marking, we verified all amplified products by the presence of the ligation cassette, the restriction site, genomic DNA, and the vector LTR sequence. To determine whether a single cell contributed to both muscle cells *in vitro* and hematopoietic cells *in vivo*, we devised a strategy to compare retroviral integration sites among the two tissues (Fig. 5A). We subjected cells from the end of transduction or clonally derived MDCs prepared by single-cell sorting (Coulter EPICS Elite ESP, Coulter, FL) along with peripheral blood or bone marrow samples to analysis by LAM-PCR. The use of GeneScan software allowed sequencing of only those products of identical size from the disparate tissues to determine identity. Alignment of a product amplified from clonally derived muscle cell culture and bone marrow mononuclear cells from animal 2 obtained 12 months posttransplantation revealed a common integration site and thus a common progenitor (Fig. 5B).



**FIG. 3.** Hematopoietic reconstitution. The lower limit of normal for white blood cell count is indicated by the dashed line. Infusion of a backup hematopoietic graft on day 14 in animal 1 and on day 7 in animals 2, 3, and 4 is indicated by an (a) and (b), respectively. †, animal deceased.

### DISCUSSION

The notion that adult-derived stem cells might be capable of forced differentiation toward a variety of specific tissue fates has shifted much of the framework in the field of stem cell biology, yet a great deal of controversy has been raised by the reported observations to date. Prompted by the report by Jackson *et al.* [13], we sought to explore the notion of stem cell plasticity in a large animal by using retroviral-tagging studies to follow the contribution of MDCs toward hematopoiesis in a rigorous *in vivo* model. Despite the infusion of an equivalent number of cultured MDCs ( $0.1$ – $8.2 \times 10^6$ /kg) using conditions nearly identical to those used in the Jackson study ( $\sim 1 \times 10^6$ /kg assuming a 20-g mouse), hematopoietic reconstitution was not observed in the first animal and was delayed in



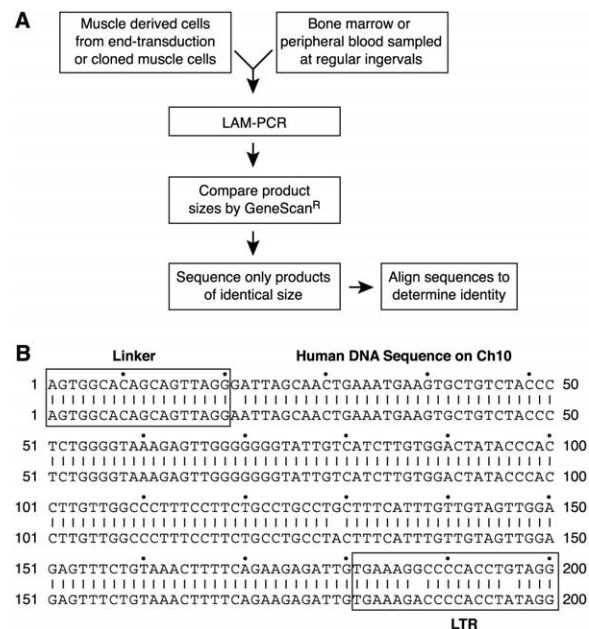
**FIG. 4.** *In vivo* gene marking. (A) Top: Peripheral blood (PB) mononuclear cells (MNC) and granulocytes (Gr) and bone marrow (BM) mononuclear cells from the 10- (10W) and 11-week (11W) time points from animal 2 were assessed for a contribution by MDCs using semiquantitative PCR for the *neo* gene as shown. Bottom: Individual bone marrow-derived colony-forming units (CFUs) plated in methylcellulose supplemented with 500 ng/ml active concentration G418 plucked and analyzed by PCR for the *neo* gene from the same time points from animal 2 are shown. (B) PB mononuclear cells (M) and Gr and BM M collected from animals 2, 4, and 3 at 11 months, 19 weeks, and 17 weeks posttransplantation, respectively, analyzed by LAM-PCR are shown. Arrows indicate bands that were confirmed to have the correct orientation of ligation sequence, genomic DNA, and vector LTR by sequencing.

the subsequent animals compared to historical control recipients of genetically modified hematopoietic grafts, suggesting the backup graft as the hematopoietic source for the initial recovery.

The introduction of a clonal tag through the use of an integrating retroviral vector allows assessment of the contribution of transduced MDCs toward hematopoiesis for the life of the animal. Using semiquantitative PCR to

examine bone marrow and peripheral blood for the presence of the marker gene, only intermittent marking was observed at the level of 0.001%, suggesting an upper limit of marking of 1 cell per 100,000. Though it is conceivable that nontransduced MDCs contributed to hematopoiesis, the death of the first animal and the delayed recovery in the three surviving animals suggest that their contribution is not significant, at least early postinfusion. To derive clonal hematopoietic cells for confirmation of marking within the hematopoietic compartment, bone marrow mononuclear cells were plated at the highest concentrations possible in methylcellulose supplemented with G418, the antibiotic to which the neomycin phosphotransferase confers resistance, at moderate stringency. While only a limited number of resistant hematopoietic colonies that contained the marker gene were obtained from two of the animals despite plating on multiple occasions, their derivation from an MDC graft grown in muscle-specific medium, which was devoid of hematopoietic markers, suggests that such marking originated from muscle cells.

Since the original publication by Jackson *et al.* [13], there have been a number of studies that support the notion that muscle contains cells that possess hematopoietic potential [12,14,15,25–28]; however, the cell of origin has remained controversial and the inability to track cells



**FIG. 5.** Comparison of vector integration sites among muscle and blood. (A) Strategy used to screen for common integrants among both muscle cells *in vitro* and blood cells *in vivo*. (B) Alignment of an integration site from clonally derived MDCs grown from the end of transduction (upper sequence) with that obtained from bone marrow at 12 months posttransplantation (lower sequence) from animal 2. The flanking genomic sequence was homologous to that of human DNA on chromosome 10.

at the clonal level in most studies has left open the possibility that the blood-forming activity results from resident hematopoietic stem cells. A primitive population of cells defined by DNA dye efflux, termed side population or SP cells [29], was suggested as a candidate by early studies in which these cells were either injected intravenously after sorting [12] or identified among cultured muscle satellite cell preparations [13]. The lack of expression of the hematopoietic marker CD45 among muscle SP cells capable of hematopoietic regeneration argued against blood cells as the source [12]. However, experiments by Kawada and Ogawa suggested that hematopoietic activity within fresh muscle derives entirely from the hematopoietic compartment [14]. Furthermore, Goodell and colleagues subsequently fractionated fresh muscle cells on the basis of Sca-1 and CD45 expression and were able to detect minimal hematopoietic activity only from the Sca-1/CD45-positive fraction, although no such analysis was performed on the cultured satellites they had previously found to contain such remarkable hematopoietic potential [15]. Additionally, two groups have recently demonstrated hematopoietic engraftment by cultured MDCs that lacked CD45 expression by both flow and RT-PCR [27,28]. Furthermore, hematopoietic engraftment by clonal MDCs that expressed myogenic markers but were devoid of CD45 expression in both primary and secondary recipients has been described [25].

Our cultured satellite cell preparations were negative for CD45 by both flow cytometry and conventional RT-PCR for CD45 mRNA, although nested RT-PCR performed on six products of the optimization protocol revealed the presence of CD45 message at the limit of detection at 0.01%. Indeed, one could argue that such low level contamination could be responsible for the marking we and others have observed, but our MDC doses ranged from 419,000 to 36,000,000 total such that blood cell contamination rates of 42 to 3600 cells are estimated. It is possible that blood stem cells were present among these contaminating cells; however, using retroviral tagging and insertion site analysis in the rhesus autologous transplantation model, we have previously estimated a frequency of hematopoietic stem cells within the marrow at around  $5 \text{ per } 10^7$  mononuclear cells [20]. These calculations make it unlikely that hematopoietic stem cells were among the relatively small number of contaminating blood cells estimated. Moreover, the finding of a common integration site among clonally derived muscle cells *in vitro* and bone marrow mononuclear cells *in vivo* suggests that adult stem cells may contribute to disparate organs at very low levels.

Overall, our results indicate that MDCs contribute poorly to hematopoiesis in a relevant large animal model, although marking within CFU demonstrates a contribution to bona fide hematopoietic cells *in vivo* and integration site analysis suggests that a common cell can contribute to muscle cells *in vitro* and hematopoietic cells *in vivo*. There are, however, several limitations to the inter-

pretation of our clonality data. First, although the clonally derived muscle cell preparations were sorted after approximately 2 weeks of culture, a time point when muscle purity is high, and the single-cell preparations possessed typical muscle morphology, including myotube formation, these single-cell cultures were originally initiated to determine the efficiency of transduction, and no formal analysis for muscle-specific markers or transcription factors was performed before DNA extraction. Second, although a number of G418-resistant clonogenic progenitors obtained from two of the animals were confirmed to contain the transferred gene by both conventional PCR and LAM-PCR, the matching integration in animal 2 was obtained from bone marrow mononuclear cells prepared by density gradient centrifugation. Attempts to identify the matching integration site among CD34-selected bone marrow cells or G418-resistant CFU from the animal by LAM-PCR or conventional nested PCR using integration-site-specific primers failed to document a contribution by this clone at later time points (data not shown). Finally, fusion [30–32] of transduced MDCs to hematopoietic cells surviving the myeloablative conditioning or infused as a backup cannot be ruled out given the low level of contribution to hematopoiesis by the MDCs. Taken together, our results suggest that the harnessing of disparate organs for cellular therapy is currently at best a highly inefficient process.

## METHODS

### *Preparation of muscle-derived cells, culture, and ex vivo transduction.*

Young rhesus macaques were housed and handled in accordance with the guidelines set forth by the Committee on Care and Use of Laboratory Animals (DHHS Publication No. NIH 85-23) and the protocol was approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute. We harvested unilateral sartorius muscle under general anesthesia and prepared muscle by minor modifications of the protocols described by Jackson *et al.* [13] and Rando *et al.* [16]. Vector supernatant was prepared by adding F-10 medium (Life Technologies, GIBCO BRL) with 5% chick embryo extract (Life Technologies, GIBCO BRL) and 20% FCS (Hyclone, Logan, UT) to confluent G1Na producers for 12 h, and supernatant was collected and passed through a 0.22- $\mu\text{m}$  filter before transduction of target MDCs. Transduction was performed with fresh vector supplemented with 8  $\mu\text{g/ml}$  protamine sulfate (Sigma, St. Louis, MO) 6 h daily beginning 48 h after transfer to gelatin-coated plates. Following transduction, MDCs were removed from plates using 0.05% trypsin-EDTA solution (Life Technologies, GIBCO BRL) and harvested in cold PBS solution with 0.5% bovine serum albumin (Sigma) and then infused immediately for transplantation.

### *Immunofluorescence staining and flow cytometric analysis.*

Desmin staining: Aliquots of cultured MDCs were plated immediately after transduction in Lab-Tek chamber slides (Nalge Nunc, Naperville, IL). Twenty-four hours later, the cells were analyzed for desmin expression after fixation for 5 min in methanol at  $-20^\circ\text{C}$  and incubation with primary monoclonal mouse anti-human desmin (DAKO, Denmark) for 30 min at room temperature. After washing with PBS, a secondary antibody, FITC-conjugated goat anti-mouse IgG (Sigma) was applied and the cells were incubated for another 30 min at room temperature. After three rinses in PBS, immunolabeled cells were observed using a Leitz fluorescence microscope.

**Flow cytometry:** Three additional monoclonal antibodies, mouse anti-human Thy-1-FITC (5E10), NCAM-PE (NCAM16.2), and CD45-PE (Tü116) (all from Becton–Dickinson Pharmingen, San Diego, CA) were used for analysis of muscle satellite cell purity by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA) using CellQuest software. Appropriate isotypic controls were used in all studies.

**Analysis of transduction.** An aliquot of transduced MDCs was placed back into culture on the day of harvesting for an additional 7 days and clonal cultures were then prepared by single-cell sorting (Coulter EPICS Elite ESP, Coulter) and growth in 96-well plates for an additional 14–20 days. Individual colonies were then transferred to larger flasks. Confluent muscle cultures were harvested and DNA extracted. Simultaneous PCR for *neo* and  $\beta$ -actin sequences were performed on each collected colony to determine the percentage of transduced colonies.

**Rhesus PB progenitor cells and bone marrow harvesting.** Peripheral blood or bone marrow progenitor cells were harvested and cryopreserved as previously described [17].

**Autologous transplantation.** Animals underwent transplantation conditioning with 500 cGy of total body  $\gamma$ -irradiation daily for 1 or 2 days. On the following day (transplant day 0), the transduced MDCs were infused via a central venous catheter. One or two weeks later, the cryopreserved cells (PB or BM) were thawed at 37°C and then infused. Recombinant human G-CSF (Amgen) 5  $\mu$ g/kg/day was administered by daily subcutaneous injection until the white blood cell count reached 6000/ $\mu$ l. Standard supportive care was administered posttransplantation. CFU assays of thawed cells were performed using methylcellulose medium as previously described [18].

**Analysis posttransplantation.** PB and BM samples were obtained at regular time points following recovery. Mononuclear cells of PB or BM were purified by density gradient centrifugation as previously described [18]. DNA was extracted from mononuclear cells and granulocytes as directed using the Wizard genomic DNA purification kit (Promega, Madison, WI).

To confirm marking within the hematopoietic compartment, BM mononuclear cells were plated at high concentration ( $10^6$  to  $10^8$  cells/ml) in methylcellulose medium (StemCell Technologies, Vancouver, BC, Canada) containing recombinant human granulocyte–macrophage colony stimulating factor (10 ng/ml; Sandoz), IL-3 (10 ng/ml; Sandoz), stem cell factor (100 ng/ml; Amgen), recombinant human erythropoietin (5 u/ml; Amgen), supplemented with the antibiotic G418 (500 ng/ml; Gemini Bio-Products, Woodland, CA). Additionally, CD34-positive cells were isolated from the bone marrow of animal 2 as previously described [19] following a simple 3- to 5-ml aspirate or after a full bone marrow harvest and were similarly plated. Individual colonies were plucked after 10–14 days and DNA was extracted for PCR analysis. DNA from individual colonies was obtained as previously described [20].

**PCR assay.** Purified DNA (0.5  $\mu$ g) from PB, BM, or other tissues, including lymph nodes, lung, liver, spleen, and muscle, or 20  $\mu$ l of colony DNA was added to a master mix containing buffer, dNTPs, and *Tag* polymerase (Roche, Applied Biosystems, Branchburg, NJ) and then divided equally between two tubes for amplification using vector-specific primers or control primers for  $\beta$ -actin as previously described [21]. For RT-PCR, total cellular RNA was collected from transduced muscle-derived cells using RNA STAT-60 (Tel-Test, Friendswood, TX), purified with DNase, and reverse transcribed using an RNA PCR Core kit (Roche Molecular Systems). Nested PCR for CD45 was performed with outer primers for 26 cycles and with inner primers for 20 cycles: denaturation at 94°C (1 min), primer annealing at 55°C (1 min), and primer extension at 72°C (1 min). The outer primers were 5'GGAATTGTTCTCGTCTG and 5'GCTTTGCCCT-GTCACAAAT. The inner primers were 5'AACAGTGGAGAAAGGACGCA and 5'TGTGTCCAGAAAGGCAAAGC. Serial dilutions of RNA from rhesus peripheral blood mononuclear cells into G1Na cell line RNA were used for semiquantitative RT-PCR.

**Insertion site analysis.** Linear amplification-mediated PCR was performed as previously described [22]. The method combines an initial linear amplification with an LTR-specific biotinylated primer followed by magnetic

capture and second-strand synthesis using random hexanucleotide primers. Digestion and ligation of an oligonucleotide ligation cassette then allowed for nested PCR using primers specific for the vector LTR and the ligation cassette, resulting in amplification of the flanking genomic sequence. To detect amplified sequences of identical size, the LTR III primers were replaced by fluorescent primers of the same sequence labeled at their 5' end with 5-carboxyfluorescein (FAM or HEX) and the products separated and analyzed by automatic fluorescence qualification and size determination using the computer program GeneScan 672 (ABI 373A, Applied Biosystems, Weiterstadt, Germany).

**Statistical evaluation.** Student's two-tailed *t* test was performed for comparison using Microsoft Excel 2000 (Microsoft, Seattle, WA).

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