

# Intraarterial Injection of Muscle-derived CD34<sup>+</sup>Sca-1<sup>+</sup> Stem Cells Restores Dystrophin in *mdx* Mice

Yuan Torrente,<sup>\*§</sup> Jacques-P Tremblay,<sup>¶</sup> Federica Pisati,<sup>\*</sup> Marzia Belicchi,<sup>\*</sup> Barbara Rossi,<sup>||</sup> Manuela Sironi,<sup>‡</sup> Franco Fortunato,<sup>\*</sup> Mostafa El Fahime,<sup>¶</sup> Maria Grazia D'Angelo,<sup>‡</sup> Nicolas J. Caron,<sup>¶</sup> Gabriela Constantin,<sup>||</sup> Denise Paulin,<sup>\*\*</sup> Guglielmo Scarlato,<sup>\*‡</sup> and Nereo Bresolin<sup>\*‡</sup>

\*Istituto de Ricovero e Cura a Carattere Scientifico (IRCCS) Ospedale Maggiore Policlinico, 20122 Milan, Italy; <sup>‡</sup>IRCCS Eugenio Medea, 20038 Bosisio Parini, Italy; <sup>§</sup>Associazione Amici Centro Dino Ferrari, Institute of Clinical Neurology, University of Milan, 20122 Milan, Italy; <sup>¶</sup>University of Verona, Anatomia Patologica, 37129 Verona, Italy; <sup>||</sup>Laval University, Unité de Génétique Humaine, Centre Hospitalier of Laval University, Ste-Foy, G1K 7P4 Quebec, Canada; and <sup>\*\*</sup>University of Paris 7, Case 7136, 75251 Paris, France

**Abstract.** Duchenne muscular dystrophy is a lethal recessive disease characterized by widespread muscle damage throughout the body. This increases the difficulty of cell or gene therapy based on direct injections into muscles. One way to circumvent this obstacle would be to use circulating cells capable of homing to the sites of lesions. Here, we showed that stem cell antigen 1 (Sca-1), CD34 double-positive cells purified from the muscle tissues of newborn mice are multipotent in vitro and can undergo both myogenic and multimyeloid differentiation. These muscle-derived stem cells were isolated from newborn mice expressing the LacZ gene under the control of the muscle-specific desmin or troponin I promoter and injected into arterial circulation of the hindlimb of *mdx* mice. The ability of these cells to interact and firmly adhere to endothelium in *mdx* muscles microcirculation was demonstrated by intravital microscopy after an intraarterial injection. Donor Sca-1, CD34 muscle-derived stem cells were able to

migrate from the circulation into host muscle tissues. Histochemical analysis showed colocalization of LacZ and dystrophin expression in all muscles of the injected hindlimb in all of five out of five 8-wk-old treated *mdx* mice. Their participation in the formation of muscle fibers was significantly increased by muscle damage done 48 h after their intraarterial injection, as indicated by the presence of 12%  $\beta$ -galactosidase-positive fibers in muscle cross sections. Normal dystrophin transcripts detected enzymes in the muscles of the hind limb injected intraarterially by the *mdx* reverse transcription polymerase chain reaction method, which differentiates between normal and *mdx* message. Our results showed that the muscle-derived stem cells first attach to the capillaries of the muscles and then participate in regeneration after muscle damage.

**Key words:** hemopoietic • dystrophin • gene therapy • muscle-derived cell • transplantation

## Introduction

Duchenne muscular dystrophy (DMD)<sup>1</sup> is an inherited muscle disease characterized by the absence of dystrophin in the membrane-associated cytoskeleton of muscle fibers (Hoffman et al., 1987; Emery, 1989) resulting in a lethal muscle wasting disease. No cure is currently available for DMD. An experimental model of DMD is the *mdx/mdx* mouse where the absence of the membrane-associated dystrophin is caused by a point mutation (Sicinski et al.,

1989). Using this model, different experimental therapies have been investigated, including transplantation of myoblasts from normal donor mice (Partridge et al., 1989). Transplanted mononuclear muscle precursor cells were shown to fuse with mature muscle myofibers of the dystrophic host to produce a hybrid fiber expressing dystrophin. Despite the good efficacy of dystrophin gene transfer obtained in the *mdx* mouse model, myoblast transplantation in DMD patients has been disappointing (Tremblay et al., 1993; Mendell et al., 1995). Very few myoblasts survived in the host muscles after transplantation and only a small proportion of the surviving cells could express dystrophin or its mRNA (Gussoni et al., 1997). This might have been due to inadequate immunosuppression, or to the advanced state of the disease, which results in extensive muscle fibrosis and adipose substitution. The newly introduced myoblasts did not migrate to damaged areas, but remained

Address correspondence to Nereo Bresolin, Institute of Clinical Neurology, University of Milan, Padiglione Ponti, Ospedale Policlinico, via Francesco Sforza 35, 20122 Milan, Italy. Tel.: 39-02-55033817. Fax: 39-02-55190392. E-mail: gpcomi@mailserver.unimi.it

<sup>1</sup>Abbreviations used in this paper: BCECF, 29,79-bis-(carboxyethyl)-5-(and-6) carboxy-fluorescein; DMD, Duchenne muscular dystrophy; gal, galactosidase; IL, interleukin; MHC, myosin heavy chain; PECAM, platelet endothelial cell adhesion molecule; pp, preplate; RT, reverse transcription; Sca-1, stem cell antigen 1; SCF, stem cell factor; TA, tibialis anterior.

relatively close to the site of injection. The large number of myoblasts required to treat all of the affected muscles as well as the inability of the transplanted cells to migrate from the injection site increase the difficulty of using this method to treat DMD (Tremblay et al., 1993; Mendell et al., 1995).

Considerable interest currently surrounds a population of multipotential muscle stem cells that could be transplanted into dystrophic *mdx* and human skeletal muscle and repopulate the diseased tissue. Interestingly, bone marrow transplantation in myeloablated mice resulted not only in myeloid reconstitution, but also in some transplanted cells participating in myogenesis, suggesting that a population of transplanted bone marrow-derived stem cells had myogenic potential (Ferrari et al., 1998). As such, bone marrow transplantation has been performed to correct dystrophin deficiency in the *mdx* mouse, though only a few fibers turned dystrophin positive after transplantation (Gussoni et al., 1999). After bone marrow transplantation, donor-derived cells have also been found in multiple nonhemopoietic tissues, including liver (Petersen et al., 1999), vascular endothelial cells (Shi et al., 1998), astroglia in the brain (Eglitis and Mezey, 1997), and bone (Horwitz et al., 1999). Although bone marrow contains many cell types that could account for this migration in many tissues, it is possible that mesenchymal stem cells are directly or indirectly involved.

In early ontogeny, hemopoiesis is tightly associated with the endothelial network; this is particularly apparent in two spatially and chronologically distinct sites: the extraembryonic blood islands and the dorsal aorta (Pardanaud et al., 1996; Dzierzak et al., 1997). These aspects suggest developmental relationships between these two lineages, i.e., they might derive from a common bipotent endothelial-hematopoietic precursor (hemangioblastic) (Sabin, 1917; Murray, 1932; Wagner, 1981).

CD34 is a sialylated transmembrane glycoprotein that is present in vascular endothelium and myeloid progenitors cells (Berenson et al., 1988; Fina et al., 1990). In mice, stem cell antigen-1 (Sca-1), which belongs to the Ly-6 gene family, is a marker of hematopoietic stem cells (van de Rijn et al., 1989). Both of these cell surface markers have been shown to be present on a population of multipotent stem cells in a murine skeletal muscle (Jackson et al., 1996; Pittenger et al., 1999).

In this study, we used a muscle culture system for facilitating the enrichment and purification of Sca-1<sup>+</sup> and CD34<sup>+</sup> cells. This population was characterized by FACS<sup>®</sup>. These cultured muscle-derived stem cells possess a great proliferative potential resulting in a large expansion of colony-forming cells with myogenic potential.

We used Sca-1<sup>+</sup>CD34<sup>+</sup> enriched muscle-derived cells isolated from normal mouse newborns to evaluate whether these cells are able to migrate from the bloodstream to the muscles after intraarterial injection and proliferate and participate in myogenesis when injected in *mdx* mice. Using the intravital microscopy, we showed that these cells adhere firmly to the lining of *mdx* muscle microvessels. Transgenic newborn mice carrying a reporter LacZ gene were also used to follow the long term fate of injected cells. The LacZ gene was under the control of regulatory elements of desmin or troponin I gene which

are expressed in skeletal muscle and heart (Hallauer et al., 1993; Li et al., 1997). Mice were analyzed for the presence of  $\beta$ -galactosidase ( $\beta$ -gal)-positive cells and dystrophin-positive fibers to trace the distribution of cells in the tissues.

## Materials and Methods

This work was authorized and supervised by the Canadian University Animal Care Committee and was conducted according to the guidelines set by the Canadian Council of Animal Care.

### Transgenic Mice

Muscle-derived cells were established from two strains of transgenic mice. One strain was the TnILacZ 1/29 bred with a CD1 background (gift from Dr. K. Hasting, McGill University, Montreal, Canada). These transgenic mice express the  $\beta$ -gal gene under the control of the promoter of the quail fast skeletal muscle troponin I gene (Hallauer et al., 1993).

The second strain was the Des-LacZ bred onto the C57BL/10J background (gift from Dr. D. Paulin, Paris University, France). These transgenic mice specifically express the reporter gene LacZ in skeletal muscle (des-nlsLacZ) as described previously (Li et al., 1997). In these mice, the *Escherichia coli* LacZ gene carrying a nuclear localizing signal was inserted in-frame into the first exon of the desmin gene. Founder animals were identified by PCR analysis of tail DNA using primers specific for the desmin-lacZ transgene. Primer sequences were as follows: Des1 and LacZ430R (sense), 5'-TTGGGGTTCGTCGCGGTCTAGCC-3'; Des1R (antisense), 5'-GGTCGTCTATCAGGTTGTCACG-3'; LacZ430R (antisense), 5'-GATCGATCTCGCCATACAGCG-3'. These primers yield a product of ~450 bp for the LacZ transgene and a supplementary 350-bp band for the desmin gene. Homozygous male transgenic mice were bred by backcrossing with C57BL/10J females. Thus, all experiments were performed with hemizygous embryos which expressed the LacZ transgene.

### Isolation of Muscle-derived Cells by Preplatings

Muscle-derived cells were isolated using a protocol described previously (Rando and Blau, 1994). The forelimbs and hindlimbs were removed from neonatal desLacZ and TnILacZ mice and the bone was dissected. The remaining muscle mass was minced into a coarse slurry using razor blades. Cells were enzymatically dissociated by adding 0.2% collagenase type XI and 0.1% trypsin for 1 h at 37°C. The muscle cell extract was preplated on the culture flasks using a slight modification of the technique described by Qu et al. (1998), i.e., the flasks were not gelatin coated and a different culture medium was used (see below). Different populations of muscle-derived cells were isolated based on the number of preplates performed on the culture flasks. Preplate 1 (pp1) represented a population of muscle-derived cells that adhered in the 1 h after isolation, pp2 in the next 2 h, and the subsequent preplates were obtained at 24-h intervals (pps 3–6). The proliferation medium was F10-Ham supplemented with 20  $\mu$ g/ml pancreatic insulin, 50 ng/ml stem cell factor (SCF), 15% FBS, and 1% penicillin/streptomycin. All the culture medium supplies were purchased from GIBCO BRL.

### Characterization of Muscle-derived Cells for FACS<sup>®</sup> Analysis

FITC-conjugated RAM34 (anti-CD34; rat IgG2a) and phycoerythrin-conjugated D7 (anti-Ly-6A/E [anti-Sca-1]; rat IgG2a) were purchased from BD Pharmingen. Some antibodies were also received as hybridomas. Hybridomas M1/70.15.11.5 (anti-Mac-1; rat IgG2b), GK1.5 (anti-CD4, rat IgG2b), and 53-6.72 (anti-CD8; rat IgG2a) were purchased from American Type Culture Collection. For antibody staining, cells were suspended in HBSS containing 2% FCS and 1 mM Hepes at 10<sup>6</sup> cells per ml. The antibodies were added at 1:50 to 1:100 dilutions. The lineage cocktail used was comprised of the following: CD4, CD8, and Mac-1. The mixture was incubated on ice for 10 min and resuspended in media containing goat anti-rat antibody conjugated to phycoerythrin and incubated for 10 min on ice. After washing, the cells were resuspended in 1:3 volume rat serum (Cappel Laboratories) and 2:3 HBSS. The method for the reaction of cell suspensions with Sca-1 and CD34 antibodies for flow cytometry has been described previously (Forni, 1979; Lange et al., 1999; Nakamura et al., 1999). Stained cells were then passed through a 70- $\mu$ m nylon filter (Falcon

Plastics) and subjected to a two-color flow cytometric analysis. Gating on the lymphoid region was used to exclude dead cells by size and granularity. Flow cytometric analysis was performed on a FACScan™ flow cytometer using CELLQuest™ software (Becton Dickinson) with 10,000 events recorded for each sample.

### ***In Vitro Self-Renewal and Pluripotentiality of Muscle-derived Cells***

The ability of muscle-derived cells to undergo differentiation into hematopoietic lineages was tested with methylcellulose culture using a method described previously (Delassus and Cumano, 1996). In our experiments, 1 ml of culture medium contained an appropriate number of fresh preplating cells ( $\sim 10^5$ ), 1.2% methylcellulose (Fisher Scientific), IMDM containing 15% FBS, 1% deionized BSA (Sigma-Aldrich), 0.1 mmol/liter 2-mercaptoethanol (Eastman Kodak Co.), 150 ng/ml recombinant mouse SCF (Sigma-Aldrich), and 10% (vol/vol) X63 interleukin (IL)-3-conditioned medium. The mixture was incubated in 35-mm nontissue culture dishes (Falcon Labware) under humidified 5% CO<sub>2</sub> atmosphere at 37°C. The number of colonies was scored after 8 d of culture using an inverted microscope. Colony types were confirmed by lifting them from the semisolid medium on day 8 of culture, and cytospin preparations were stained with May-Grunwald-Giemsa. To determine the myogenic ability, muscle-derived cells were maintained in proliferation medium as they reached 60–70% confluence. At this time, cells were exposed to differentiation medium consisting of Ham's F10 supplemented with 5% FBS and antibiotics as described above. After 14 d of cultures, enumeration of differentiated myoblasts containing two or more nuclei (i.e., fusion index) was assessed in all preplates under mitotic or differentiation medium. Differentiated myoblasts were detected by immunostaining with antibody reactive to slow myosin heavy chains (MHCs), desmin, and dystrophin. To determine whether pp6 cells exhibit stem cell characteristics of self-renewal, individual clones were analyzed. To establish clones, pp6 were plated at  $\sim 10$  cells/cm<sup>2</sup>, grown to 50–150 cells per colony, isolated with cloning cylinders, and transferred to separate wells and finally to individual flasks.

### ***In Vitro Immunohistochemistry of Muscle-derived Cells***

For immunocytochemistry, cells were plated in a Lab-tek chamber slide (Life Technologies) until confluence, fixed in ethanol 70% in PBS for 1 min, and permeabilized for 5 min with 0.5% Triton X-100 in PBS. Cells were then incubated with primary antibodies: M-cadherin (1:50), platelet endothelial cell adhesion molecule (PECAM; 1:100; Santa Cruz Biotechnology, Inc.), Mac-1 (1:50; Santa Cruz Biotechnology, Inc.); and anti-desmin monoclonal antibodies (1:100; Sigma-Aldrich) overnight at 4°C. After washing with PBS, cells were incubated with FITC-conjugated goat anti-mouse IgG for 1 h at room temperature and examined by epifluorescence microscopy. A slow MHC antibody (Ylem) was visualized using an HRP-coupled secondary antibody (Bio-Rad Laboratories) in PBS containing 0.6 mg/ml diaminobenzidine (Sigma-Aldrich).

### ***Dystrophin and Myosin Western Blotting***

To determine the in vitro myogenic ability of the muscle-derived cells, proteins were extracted as described previously (Anderson and Davison, 1999) from cells obtained from all preplatings (pp0–pp6) after culture in a differentiation medium for 14 d. The concentration of proteins was determined using the Lowry technique. 100 µg of extracted proteins was separated on 6% polyacrylamide gels and electrotransferred onto nitrocellulose membranes (Bio-Rad Laboratories). To ensure that equivalent amounts of proteins were loaded for each sample, the membrane was stained with Ponceau S. (Sigma-Aldrich). Membranes were subsequently incubated with a monoclonal antibody directed against dystrophin (NCL-DYS 2; Novocastra) and MHCs and revealed using a commercially available chemiluminescence kit (Ultra ECL; Pierce Chemical Co.). Membranes were then exposed to BioMax autoradiographic films (Eastman Kodak Co.), which were developed and scanned with a densitometer.

### ***Intravital Microscopy***

Animals were anesthetized with an intraperitoneal injection of physiologic saline containing ketamine (5 mg/ml) and xylazine (1 mg/ml). The right common carotid artery was cannulated for the injection of fluorescent cells toward the aortic arch, then the skin covering the pectoral muscle was reflected and a coverslip (24 × 24 mm) was applied and fixed with silicon grease. A round camera with 11-mm internal diameter was at-

tached on the coverslip and filled with water. An intravital microscope (BX50WI; Olympus) equipped with water immersion objectives (Achromplan, focal distance 3.3 mm, NA 0.5 $\times$ ; Olympus) was used for these experiments. 148 kD of FITC-dextran (Sigma-Aldrich) was injected to evaluate the microvascular dimensions. The muscle-derived cells were fluorescently labeled with 2',7'-bis-(carboxyethyl)-5-(and-6) carboxy-fluorescein (BCECF; Molecular Probes). After staining for 30 min at 37°C (2 µg BCECF/10<sup>7</sup> cells), the cells were washed and resuspended at 10<sup>7</sup> cells/ml in RPMI 1640 (BioWhittaker) containing 10% FBS.  $7 \times 10^5$  of BCECF-labeled cells were injected in the right carotid artery by a digital pump (SP100; World Precision Instruments) at a flow rate of 1 µl/s. The recipient was maintained at 37°C by a stage-mounted strip heater Linkam CO102 (Olympus). All scenes were recorded on videotape using a silicon-intensified-target videocamera (VE 1000-SIT; Dage MTI), a time and frame counter (ELCA), and a high picture quality SVHS VCR (Panasonic). The recordings were digitalized on Casablanca digital system (MS MacroSystem Computer GmbH).

### ***Intraarterial Transplantation of Sca-1, CD34 Muscle-derived Cells into mdx***

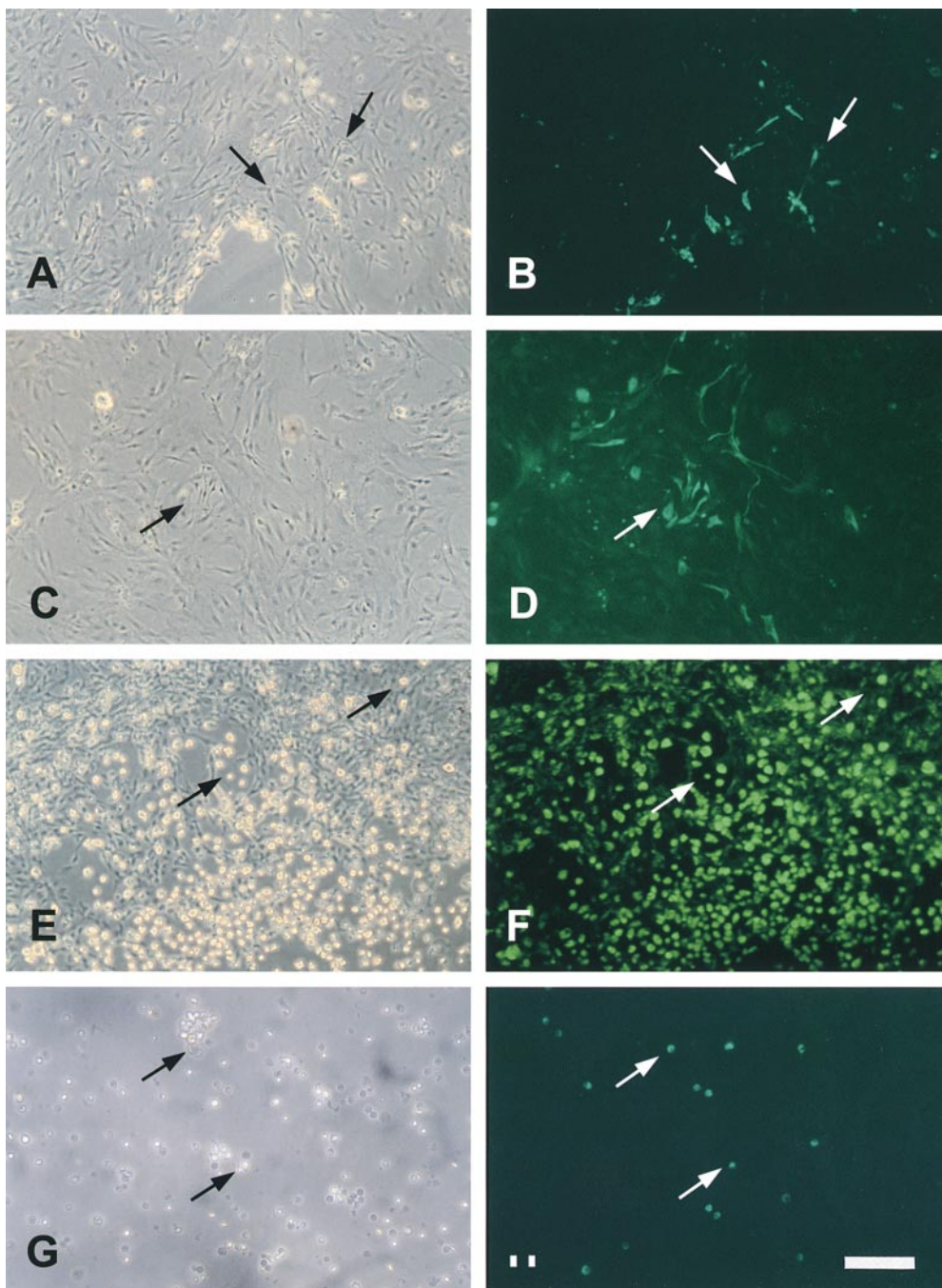
Approximately  $5 \times 10^5$  cells prepared from pp6 of newborn Des-LacZ mice were injected into five 3-mo-old C57BL/10ScSn *mdx/mdx*. Mice were anesthetized with an intraperitoneal injection of physiologic saline (10 ml/kg) containing ketamine (5 mg/ml) and xylazine (1 mg/ml) and a limited low midline laparotomy was performed. The intestines were exposed and cells were injected via a 0.20-mm-diameter needle inserted into the iliac artery. The needle was connected to a peristaltic pump by a heparinized Tygon tube (Ika Labortechnik). This Tygon tube was connected to a sterile Eppendorf tube containing  $5 \times 10^5$  cells/50 µl. Cells were delivered by laminar flow (5 µl/s) over a period of 10 s. The blood flow was not stopped before or during this procedure. There was no visible damage to the vessel wall during or after operation. The body wall muscle was closed with sutures and the skin with surgical staples. Intraarterial delivery of  $5 \times 10^5$  cells of preplating six prepared from the newborn TnILacZ mice was also performed in six CD1 mice. In these mice, mechanical muscle damage was induced with three needle insertions in the tibialis anterior (TA) of the injected hindlimb 48 h after the intraarterial injection. The *mdx* and CD1 mice were killed 21 d after injection. To evaluate the effect of muscle degeneration–regeneration on the intraarterial injection of pp6, we tested the effects of an intense swim on six mice. A group of three *mdx* mice was also used to evaluate the fate of pp6 cells injected intraarterially 48 h after an intense swim. For all the transplantations, the immunosuppressor FK506 (Fujisawa Pharmaceutical Co., Ltd.) was administered intraperitoneally at 2.5 mg/kg beginning on the day of the injection and maintained until the animals were killed.

### ***Endothelial Cell Line***

We used an immortalized endothelial cell line isolated from a transgenic animal carrying a thermolabile SV40 T antigen and LacZ gene reporter under the control of the human vimentin promoter (Vicart et al., 1994). To produce this cell line, enriched fractions of microvessels were obtained by gradient centrifugation on a 15% BSA cushion of brain homogenates and preincubated with 0.2% collagenase for 10 min at 37°C. Thereafter, the microvessel-enriched fraction was treated with trypsin for 10 min at 37°C, plated in gelatin-coated petri dishes, and grown in DME supplemented with 20% FBS. The endothelial cell line proliferated indefinitely under the permissive condition (i.e., 33°C) and underwent normal differentiation at 37°C.

### ***Muscle-derived Cells and Endothelial Cell Transplantation Directly in the TA Muscle***

To determine whether the origin of the muscle-derived cells influenced their myogenic ability, intramuscular injections of pp6 and of endothelial cell line were performed. In these experiments, 12 CD1 mice (The Jackson Laboratory),  $\sim 3$  mo old, were injected with the pp6 ( $n = 6$ ) derived from the TnILacZ mice or with the endothelial cell line ( $n = 6$ ). A cardiotoxin (5 µg/µl) pretreatment was performed in the right TA muscle 48 h before cell transplantation. The mice were anesthetized as described above. The skin was excised to expose the right TA muscle and 15 µl containing  $2 \times 10^6$  cells was slowly injected with a glass micropipette with a 50-µm tip (Drummond Scientific Co.). The skin was then closed with fine sutures and immunosuppression with FK506 was administered until the animals were killed 30 d after the transplantation.



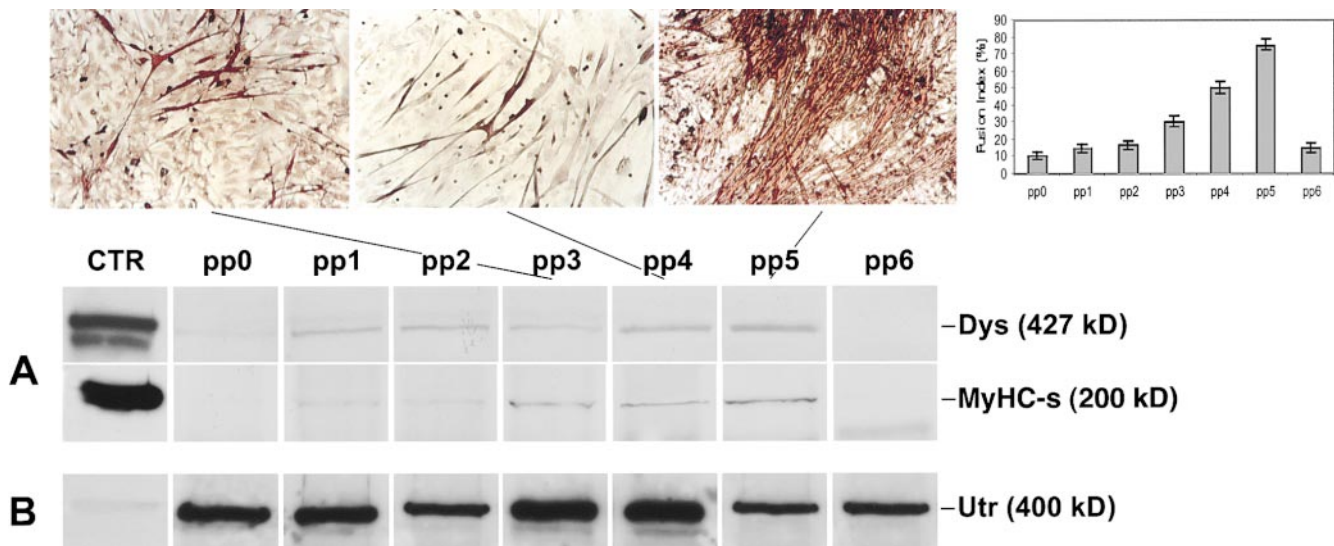
**Figure 1.** Characterization of muscle-derived cells from TnILacZ and Des-LacZ transgenic mice. The desmin immunofluorescence is shown in B, D, F, and H, and the corresponding phase-contrast field is shown in A, C, E, and G. The adherent cells of the earlier preplates display a low desmin immunoreactivity ranging from 5 to 15% (A and B), whereas the sequential preplates were enriched in their content of desmin-positive cells: pp3 = 37% (C and D); pp5 = 85% (E and F). Muscle-derived cells isolated at pp6 contained only 10% desmin-positive cells (G and H). Bar, 120  $\mu$ m.

### Detection of Dystrophin, $\beta$ -Actin, and desLacZ Genes

The brain, lungs, diaphragm, liver, kidneys, heart, bones with bone marrow, and muscles were taken from the mice injected intraarterially with Sca-1, CD34 positive cells (i.e., pp6) and divided in two identical halves. One half was used to isolate genomic DNA and total RNA, the other half was cryostat-sectioned and used for X-gal histochemistry and immunodetection of dystrophin. Distribution of muscle-derived cells (i.e., pp6) in the tissues of mdx mice was assessed by PCR for dystrophin and desLacZ genes. Genomic DNA was isolated as described previously (Shrager et al., 1992). To detect the normal (injected pp6) and mutant (mdx tissues) dystrophin genes, primers were located on exon 23 of the mouse dystrophin gene which contains a point mutation in the *mdx* mouse (Sicinski et al., 1989). The primer dystrophin forward was designed to introduce a new site for the restriction enzyme MaeIII in the sequence of normal dystrophin cDNA, but not in the mutated cDNA of *mdx* mice (Asselin et al., 1995; Torrente et al., 2000). An initial denaturation at 94°C for 5 min was followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C

for 45 s, extension at 72°C for 30 s, and a final extension at 72°C for 10 min. The 200-bp PCR product was extracted with phenol/chloroform, precipitated with ethanol resuspended in 15 ml of H<sub>2</sub>O, and incubated with MaeIII at 55°C for at least 5 h.

For reverse transcription (RT)-PCR, total RNA was extracted using the RNeasy<sup>TM</sup> B isolation kit (Qiagen, Crawley, UK) and was reverse-transcribed for 1 h at 42°C using random hexamers and the GeneAmp RNA PCR kit (PerkinElmer). Each amplification mix contained equal amounts of cDNA, 1.5 mM MgCl<sub>2</sub>, 30 pmol of each primer, 5% dimethylsulfoxide, 200 mM of each dNTP (Boehringer), and 2 U of Taq DNA polymerase (Amplitaq; PerkinElmer) in 1 $\times$  PCR buffer (PerkinElmer). Control reactions were performed in the absence of reverse transcriptase. The sequences and amplification parameters of the desLacZ, dystrophin, and  $\beta$ -actin have been described previously (Russo et al., 1998; Torrente et al., 2000). PCR products were separated by electrophoresis on a 1% agarose, 2.5% NuSieve gel, stained with ethidium bromide, and photographed under ultraviolet light. The 257-bp dystrophin PCR products were cleaned in a Microspin S-400 column (Amersham Pharmacia Bio-



**Figure 2.** Immunoblotting analysis of dystrophin and slow MHCs of different populations of muscle-derived cells when cultivated in fusion medium. The first lane of all immunoblotting corresponds to a homogenate of normal muscle as control (CTR). The populations (pp0–pp6) had a variable ability to differentiate into myotubes when cultivated in a fusion medium for 14 d, resulting in an increase in dystrophin and MHC synthesis in pp3–pp5 (A). In contrast, a significant reduction in the amount of this myogenic marker expression was observed in pp6. Utrophin (Utr) immunoblotting (B) indicated a presence in all preplatings including pp6. These data were also confirmed by sMHC immunostaining (A, top, corresponding to pp3–pp5). The enumeration of differentiated myoblasts containing two or more nuclei (i.e., fusion index) is shown graphically beside Western blotting condition. The error bars represent the SEM from three independently derived preplating cultures.

tech), digested with MaeIII at 55°C for >5 h, and separated on a 17.5% nondenaturing polyacrylamide gel.

### Histochemistry and Immunocytochemistry on Muscle Tissue

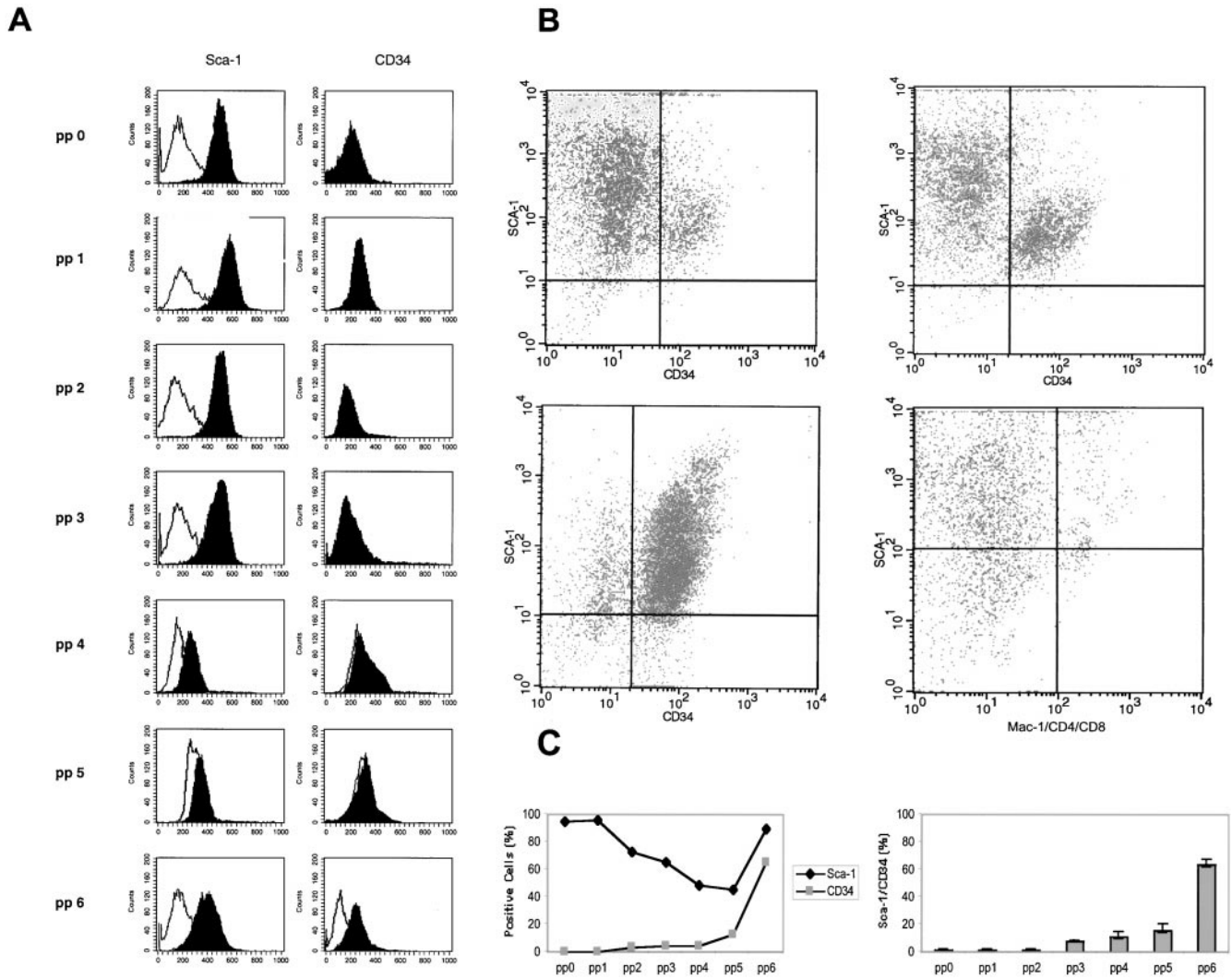
For histochemistry on tissue sections, samples were frozen in liquid nitrogen, cooled in isopentane, and cryostat-sectioned. Serial sections of different thickness were examined (12 μm for histochemical detection of β-gal activity, 8 μm for immunohistochemical analysis, and 8 μm for hematoxylin and eosin). Tissue sections were transferred to gelatin-coated glass slides and fixed by dipping the slides in a cold solution of 0.25% glutaraldehyde in PBS, pH 7.4, for 15 min, rinsed twice for 5 min in PBS, and stained overnight at 32°C with 1 mg/ml X-gal solution. Slides were examined by light microscopy for β-gal-positive myofibers (Li et al., 1993). Immunohistochemical detection of dystrophin was performed with a polyclonal antibody against the COOH 60-kD Dys fragment as described previously (Prelle et al., 1992). A polyclonal antibody anti-PECAM was obtained from Santa Cruz Biotechnology, Inc. and used at a dilution of 1:100. Monoclonal anti-mouse vimentin was obtained from Sigma-Aldrich and used at a dilution of 1:200 as described previously (Vicart et al., 1994). A polyclonal antiserum against SV40 large T protein was obtained from BD PharMingen and used at a dilution of 1:200 (Vicart et al., 1994). LacZ and dystrophin positive myofibers were counted from adjacent hematoxylin and eosin-stained sections, and expressed as a percentage of the total cryosectioned fibers. Thus, β-gal and dystrophin percentage reliably reflected the β-gal activity and dystrophin expression of the segment of muscle from which the sections were taken.

## Results

### Expression of Muscle Markers in Cells Obtained after Various Numbers of Preplatings

Different populations of muscle-derived cells isolated from hindlimb muscles by different numbers of preplatings were observed. The adherent cells of the earlier preplates display a low desmin immunoreactivity ranging from 5 to 15%, whereas the sequential preplates were en-

riched in their content of desmin-positive cells (pp3 = 37%; pp4 = 78%; pp5 = 85%) (Fig. 1). These data suggested that the attached cells of pp0–2 are mostly fibroblasts, as confirmed by their typical morphology and the many desmin-positive cells remaining as floating cells in the first 48 h of preplating. Surprisingly, the muscle-derived cells isolated at pp6 contained only 10% desmin-positive cells (Fig. 1, G and H). Since these cells were mostly desmin negative, we believe that our pp6 are different than the muscle-derived cells described by Qu et al. (1998). The morphology of these pp6 cells was predominantly similar to medium-sized blast cells. Endothelial-like elongated cells were also present at this preplate. A number of the pp6 cells (10%) detached from the plastic surface and appeared similar to small lymphocytes. Few M-cadherin-positive cells were found, especially in pp5. These cell populations (pp0–pp6) consequently had a variable ability to differentiate into myotubes when cultivated into a fusion medium for 14 d. Differentiated myoblasts were detected by immunostaining with antibody reactive with MHCs. The number of myotubes obtained in earlier preplates was much lower than in pp4 and pp5. In contrast, the population of pp6 displayed a poor ability to differentiate into myotubes (data not shown). The fusion index of differentiated preplate cultures showed an increased kinetics of differentiation as follows: pp0 = 10%, pp1 = 15%, pp2 = 17%, pp3 = 30%, pp4 = 50%, pp5 = 75%, and pp6 = 12% (graphical in Fig. 2). Few myotubes were also found in grown conditions (pp3 = 7% and pp4–pp5 = 8%). These data were confirmed by immunoblotting experiments after maintaining the cells in a fusion medium for 14 d (Fig. 2). Dystrophin and slow MHCs were threefold higher in pp4 and pp5 than in the other preplates (Fig. 2).



**Figure 3.** Immunophenotyping of the muscle-derived cells using FACScan™ analysis. Muscle-derived cells were tested for CD34 and Sca-1 single (A) and double (B) expression. A distinct population of Sca-1<sup>+</sup> cells was found in all seven preplates, but CD34<sup>+</sup> cells were observed only in pp6 (A). The morphology distribution of muscle-derived cells after flow cytometry showed different subpopulations within the Sca-1<sup>+</sup> cell population (B). The profile of these cells varied from cells with large diameters (pp0–4) to small diameters (pp5–6). The majority of cells derived from pp3 (B, top left quadrant) and pp5 (B, top right quadrant) highly express Sca-1, but were also poorly CD34<sup>+</sup>. Muscle-derived cells from pp6 showed 69% double-positive cells for CD34 and Sca-1 markers (B, bottom left quadrant). However, all Sca-1<sup>+</sup> cells were not CD34<sup>+</sup> and a fraction of the population of pp6 was single-positive for CD34 (7%). The Sca-1, CD34 double-positive muscle-derived cell population was gated in a region of small cell diameter, a phenotype characteristic of hematopoietic progenitors. Labeling of cells with antibodies against Sca-1 and the lymphoid CD4 and CD8 markers was also performed (B, bottom right quadrant). Dead cells were excluded from analysis. Calculation of Sca-1 and CD34 single (C, left) and double (C, right) positive cells as the percentage of cells contained within all preplates is shown graphically.

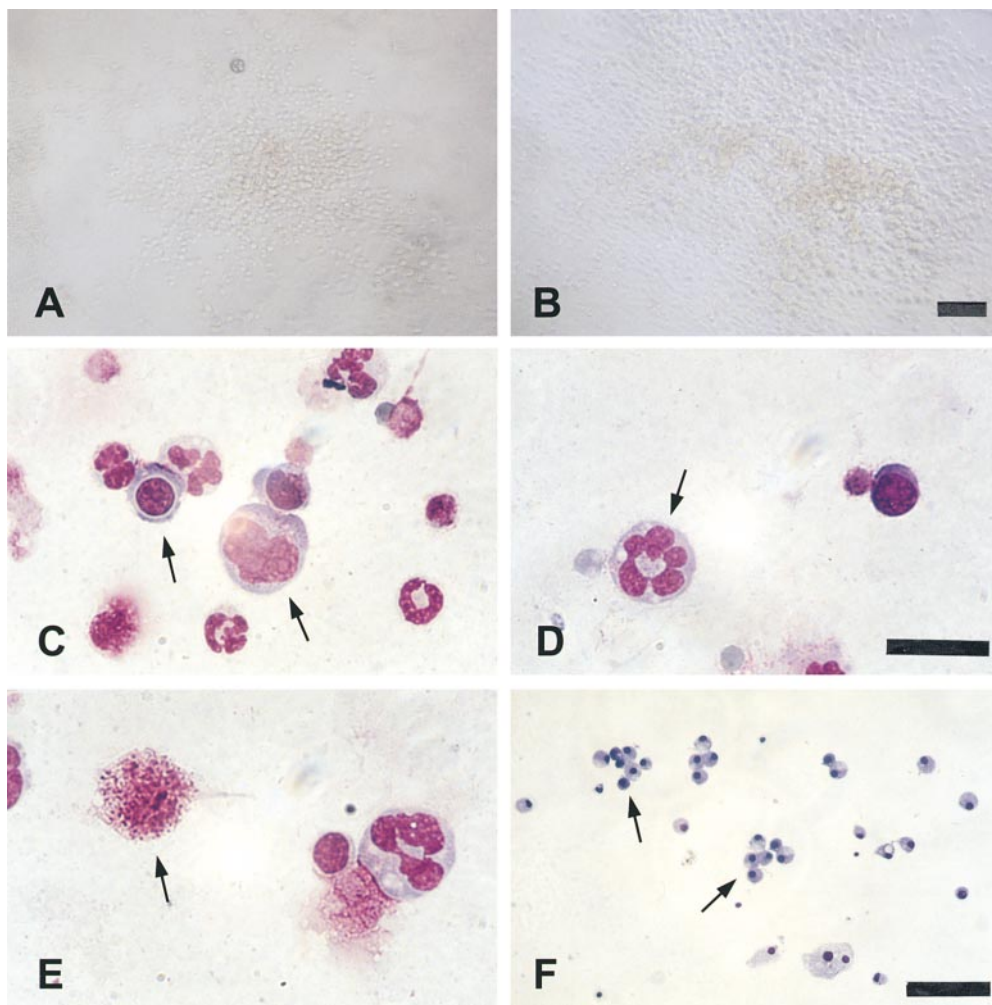
### Specific Sca-1, CD34 Expression in Muscle-derived Cells

Flow cytometric analysis of muscle-derived cell preplates from hindlimb muscles was performed. As shown in Fig. 3, a distinct population of Sca-1<sup>+</sup> cells was found in all seven preplates, but CD34<sup>+</sup> cells were observed only in pp6 (Fig. 3, A and C). The morphological distribution of muscle-derived cells after flow cytometry showed different subpopulations within the Sca-1<sup>+</sup> cell population. The profile of these cells varied from cells with large diameters (pp0–4) to cells with small diameters (pp5–6). Double staining of muscle-derived cells with Sca-1 and CD34 (Fig. 3, B and C) showed that 69% of cells at pp6 coexpressed both markers. However, all Sca-1<sup>+</sup> cells were not CD34<sup>+</sup> and a fraction of the population of pp6 was only positive for CD34 (7%).

The Sca-1, CD34 double-positive muscle-derived cell population was gated in a region of small cell diameter, a phenotype characteristic of hematopoietic progenitors. Since it had been demonstrated previously that Sca-1 is expressed on a high percentage of activated T lymphoid cells, a double staining with antibodies against Sca-1 and the lymphoid CD4 and CD8 markers was performed. As expected, no lymphoid markers were detected in any preplates (Fig. 3 B).

### Clonogenic Ability of Sca-1, CD34 Double-positive Cells and Self-Renewal

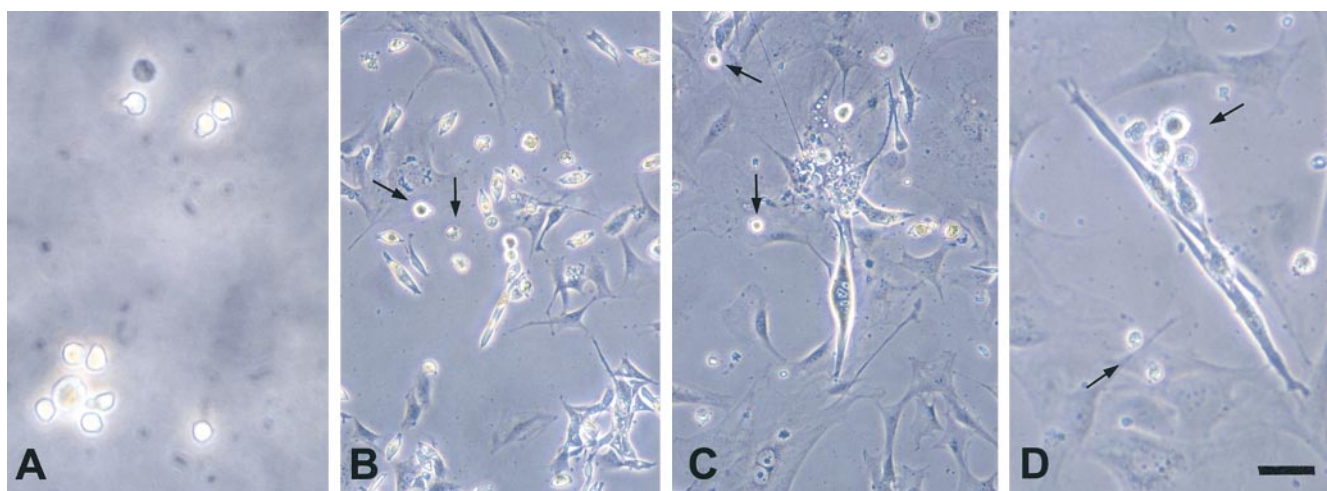
To verify whether the Sca-1 and CD34 double-positive cells (i.e., pp6 cells) or muscle-derived cells contained hematopoietic progenitor cells, a colony-forming unit in culture assay in methylcellulose was performed (Delassus



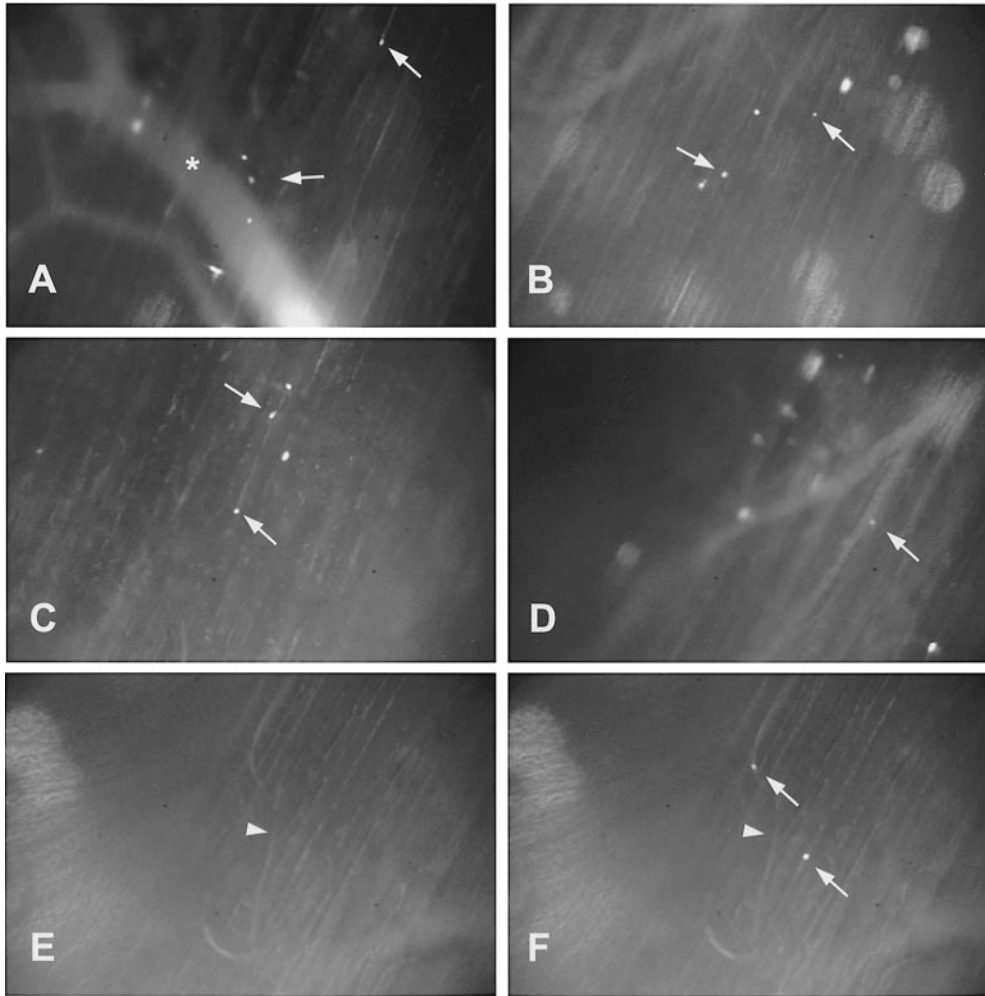
**Figure 4.** In vitro differentiation potential of muscle-derived cells under myeloid conditions. Photomicrographs A and B show the pp6 forming clones in methylcellulose under myeloid conditions. Verification of the multimyeloid nature of the resulting cells was performed by May-Grunwald-Giemsa staining. Two types of clones derived from Sca-1, CD34 positive cells (pp6) were identified: (1) multipotent clones that displayed a multimyeloid potential (mainly macrophages, megakaryocytes, and granular polymorphonuclear cells) (C, D, and E); and (2) clones with a restricted myeloid potential that were able to differentiate into either macrophages or mast cells (F). Bars: (A and B) 120  $\mu$ m; (C and D) 50  $\mu$ m; (E and F) 120  $\mu$ m.

and Cumano, 1996). Cells were cultured under conditions that promote myeloid cell development (i.e., presence of IL-3 and SCF). The cloning efficiency was 22% ( $\sim$ 1 cell in 5; Fig. 4) and two types of clones derived from Sca-1,

CD34 positive cells were identified: (1) clones with a restricted myeloid potential that were able to differentiate into either macrophages or mast cells; (2) multipotent clones that displayed a multimyeloid potential (mainly



**Figure 5.** To determine whether individual muscle-derived cells (i.e., pp6) exhibit stem cell characteristics of self-renewal and pluripotentiality, individual clones were analyzed. To establish clones, pp6 were plated at  $\sim$ 10 cells/cm<sup>2</sup>. Single cells replicated as typical muscle-derived stem cells (A) and differentiated after 15 (B), 30 (C), and 45 (D) d of culture. Undifferentiated, rounded (indicated by arrows) muscle-derived stem cells were evident in each clonal line. Therefore, clones derived from single cells give rise to both muscle-derived stem cells and differentiated cells, indicating stem cell characteristics. Bar, 120  $\mu$ m.



**Figure 6.** Intravital microscopy: muscle-derived cells (pp6) firmly adhere in muscle capillaries. To improve contrast between the intra- and extravascular compartment, the animals were injected intravenously with a low dose of FITC-dextran. Venules (A) and arterioles (E) are indicated by asterisks and arrowheads, respectively. The BCECF-labeled cells (bright dots indicated by arrows) can be seen in the muscle capillaries (A, B, and C) 1 min after intraarterial injection. Arrows in D indicate a migrated BCECF-labeled cell in the perivascular space of a muscle venule 1 h after injection. Images before (E) and during the injection (F) are also presented.

macrophages, megakaryocytes, and granular polymorphonuclear cells). Verification of the multimyeloid nature of the resulting cells was performed by May-Grunwald-Giemsa staining (Fig. 4). In some experiments, cultures of pp0–5 in methylcellulose supplemented with IL-3 and SCF showed no colonies, even after 28 d of culture. These data suggest that there is a strong relationship between the expression of CD34 and the *in vitro* myeloid differentiation. To determine whether muscle-derived cells exhibit stem cell characteristics of self-renewal, pp6 were plated at  $\sim 10$  cells/cm<sup>2</sup>, isolated with cloning cylinders, and transferred to separate wells and finally to individual flasks. Single cells replicated as typical muscle-derived stem cells and underwent morphologic differentiation (Fig. 5). Using immunohistochemistry, these cells were found to be positive for the expression of several markers, such as PECAM (endothelial and hemopoietic cells), desmin (myoblasts and smooth muscle cells), and Mac-1 (macrophages). Undifferentiated muscle-derived stem cells were evident in each clonal line. Therefore, clones derived from single cells give rise to both muscle-derived stem cells and differentiated cells, indicating stem cell characteristics.

#### **Adhesion of Sca-1, CD34 Positive Cells in Muscle Microcirculation**

Since intravenous injection of precursor cells offers the advantages of a total body distribution, we believe that this

approach is also limited by physiological dispersion of injected cells in the blood-stream of several tissues (especially in liver and lungs). In our experiments, we verified the possibility in an elective biodistribution in muscle limbs after intraarterial injection of muscle-derived stem cells. We tested the adhesive behavior of Sca-1, CD34 positive cells to muscle vessels. Boluses of  $7 \times 10^5$  fluorescently labeled cells were injected through a carotid artery catheter and their adhesion to the pectoral muscle vessels was recorded (Fig. 6). Sca-1, CD34 cells had a small diameter and interacted especially with muscle capillaries, but rarely with venules. The Sca-1, CD34 positive cells (in pp6) were distinguishable from the other preplates by their significant adhesive properties to the capillaries (data not shown). Adhesion fractions of injected pp6 were determined by counting the number of interacting cells in each muscle vessel per number of cells that passed through the same vessel during an injection. Injected cells adhered prevalently within muscle capillaries and the adhesion fraction was  $\sim 89\%$ .

#### **Distribution of Donor Des-LacZ Positive Sca-1, CD34 Cells after Intraarterial Injection in mdx Mice**

Cells obtained from pp6 of newborn Des-LacZ mice were intraarterially injected into five C57BL/10ScSn *mdx/mdx* mice. PCR analysis for the LacZ gene and for the normal dystrophin genes in various tissues from these mice indi-



**Table I.  $\beta$ -Gal and Dystrophin Detection by PCR Localization in Tissues of Mice Injected Intraarterially with Muscle-derived  $CD34^+Sca-1^+$  Cells**

Tissues analyzed 30 d after intraarterial injection	n positive mice/total treated mice
Soleus muscle	5/5
Quadriceps femoralis muscle	2/5
TA muscle	2/5
Triceps femori muscle	1/5
Gastrocnemius muscle	5/5
Controlateral limb muscles	0/5
Brain	0/5
Liver	0/5
Heart	0/5
Kidney	0/5
Lung	0/5
Bone and bone marrow	0/5
Diaphragm	0/5
Triceps brachii	0/5

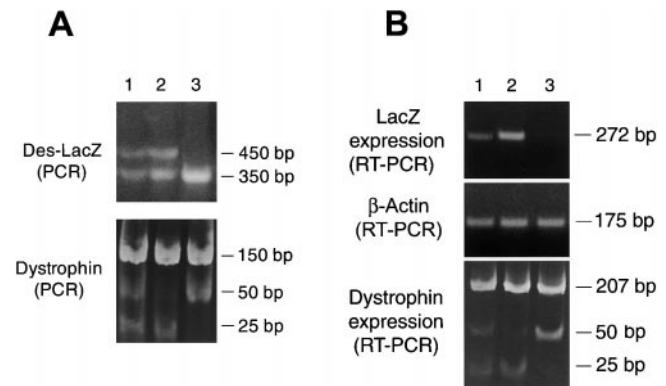
ated that donor cell distribution remains close to the injected hindlimb (Table I). Donor cells were found in the hindlimb soleus and gastrocnemius muscles of all mice injected; two of these mice also had donor cells in the TA and quadriceps muscles. Lungs, liver, brain, kidneys, bones with bone marrow, triceps brachii, diaphragm, and controlateral untreated hindlimb muscles were negative (Table I). Tissues from untreated mice scored negative.

#### Detection of LacZ and Dystrophin Transcripts in mdx Mice Injected Intraarterially with Sca-1, CD34 Cells

To assess the in vivo ability of pp6 cells to differentiate through the myogenic lineage, RT-PCR for dystrophin and LacZ mRNAs analysis was performed on *mdx* mice which were injected intraarterially with Sca-1, CD34 positive cells obtained from the Des-LacZ donor. Tissues which were shown by standard PCR to contain donor cells were evaluated for desmin-LacZ and dystrophin transcripts. Total RNA extracted from untreated and treated *mdx* mice was used as a template for RT-PCR. Digestion of the dystrophin RT-PCR reaction products by MaeIII allowed us to distinguish between normal and mutated dystrophin mRNAs. The normal dystrophin gene gave a 207-bp band plus two 25-bp fragments, whereas the mutated dystrophin gave a 207-bp band plus a single 50-bp fragment. Normal dystrophin and LacZ mRNAs were found in the soleus, TA, gastrocnemius, and quadriceps muscles of treated mice (Table I and Fig. 7), suggesting that transplanted Sca-1, CD34 positive cells participated in myogenesis. Neither LacZ or dystrophin transcriptional activity was detected in the controlateral hindlimb muscles (Table I).

#### Colocalization of Dystrophin and LacZ Expression

To identify the ability of intraarterially injected tissues to transcribe normal dystrophin and LacZ mRNAs, histochemical staining on cryostat sections of muscles, brain, liver, lungs, kidneys, and bones were performed.  $\beta$ -Gal-positive nuclei were detected only in striated muscles of the injected hindlimb. Positive LacZ myofibers were found in restricted areas. Muscle fibers scoring positive had peripheral nuclei and fiber diameters varied from 20 to 60  $\mu$ m. 10 different fibers having  $\beta$ -gal-positive nuclei

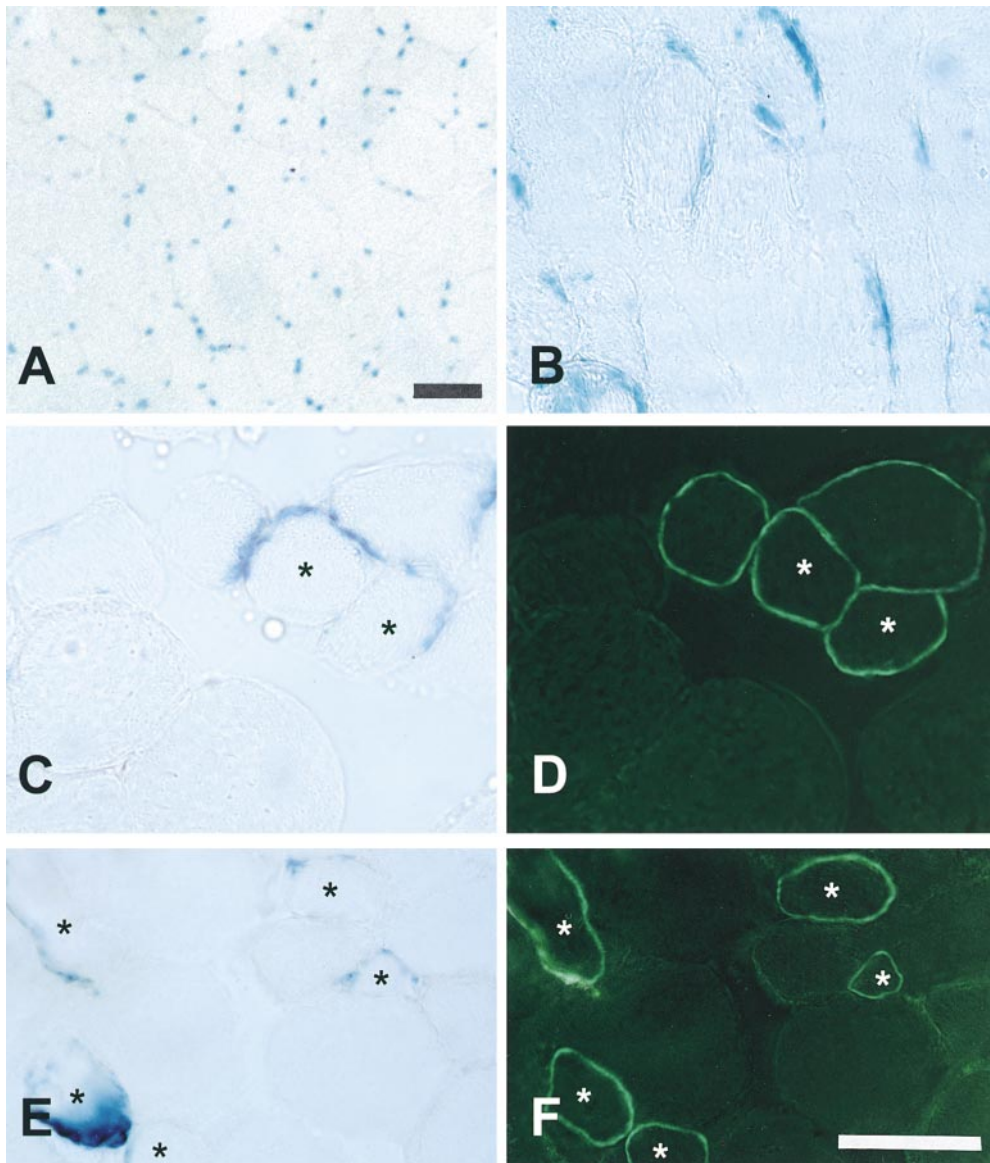


**Figure 7.** Detection by PCR of donor cells in the tissues of *mdx* mice. (A) Muscle-derived cells from Des-LacZ transgenic mice were injected intraarterially into *mdx/mdx* mice and the animals were examined 21 d later. Genomic DNA was extracted and analyzed by PCR for the des-LacZ transgene and the normal (25 bp) and *mdx* (50 bp) dystrophin gene. (B) Actin, the normal (25 bp) and *mdx* (50 bp) dystrophin, and des-LacZ expression was detected by RT-PCR. The 450-bp band of the des-LacZ transgene, indicating injected cells in *mdx* mice, was found in the analyzed muscle tissues. LacZ expression, indicated by the 272-bp band in RT-PCR, was also found in the same tissues showing the myogenic program. Actin expression was detected in all tissues. Normal dystrophin expression was indicated by the 25-bp band for wild-type dystrophin. Lane 1, treated muscle tissues; lane 2, control des-LacZ; lane 3, control *mdx/mdx*.

were counted in the soleus, corresponding to  $\sim 1\%$  of total fibers per cross sectional area, and 15 positive fibers, corresponding to 0.5% of total fibers in a given cross section, were counted in the quadriceps. The pattern of LacZ gene expression was compared with that of dystrophin, and typical immunofluorescence for dystrophin was found in the same fibers that contained  $\beta$ -gal-positive nuclei (Fig. 8). The dystrophin antibody did not cross-react with autosomal dystrophin-related proteins such as utrophin. Positive fiber always seemed to exist as isolated small groups (one to five positive fibers), suggesting that there might have been clonal proliferation of some donor cells, which contributed to the formation of the fibers in that region of the muscle. Control *mdx* muscles contained rare revertant fibers that were positive for dystrophin (Hoffman et al., 1990), but also  $\beta$ -gal negative.

#### $\beta$ -Gal Histochemistry in TA of CD1 Mice Injected Intraarterially and Damaged 48 h Later

21 d after intraarterial injection of Sca-1, CD34 positive muscle-derived cells derived from the pp6 of TnILacZ, clusters of  $\beta$ -gal-positive myofibers ( $12\% \pm 0.8$  per cross section) of 20–60- $\mu$ m diameter were observed (Fig. 9, B–D). These fibers had peripherally located nuclei and were along the needle tracks made in the TA 2 d after the intraarterial injection of the pp6 cells. Fibers of the same section exhibited different levels of LacZ activity, some fibers being more intensely stained than others. Most of the labeling was restricted to regions near the three needle tracks done in the muscles where most of the muscle regeneration was present. TA controlateral muscle did not show any positive cells. In a second similar experiment, the damage to the muscle fibers 48 h after the intraarterial injection of the same type of cells was achieved by a pro-



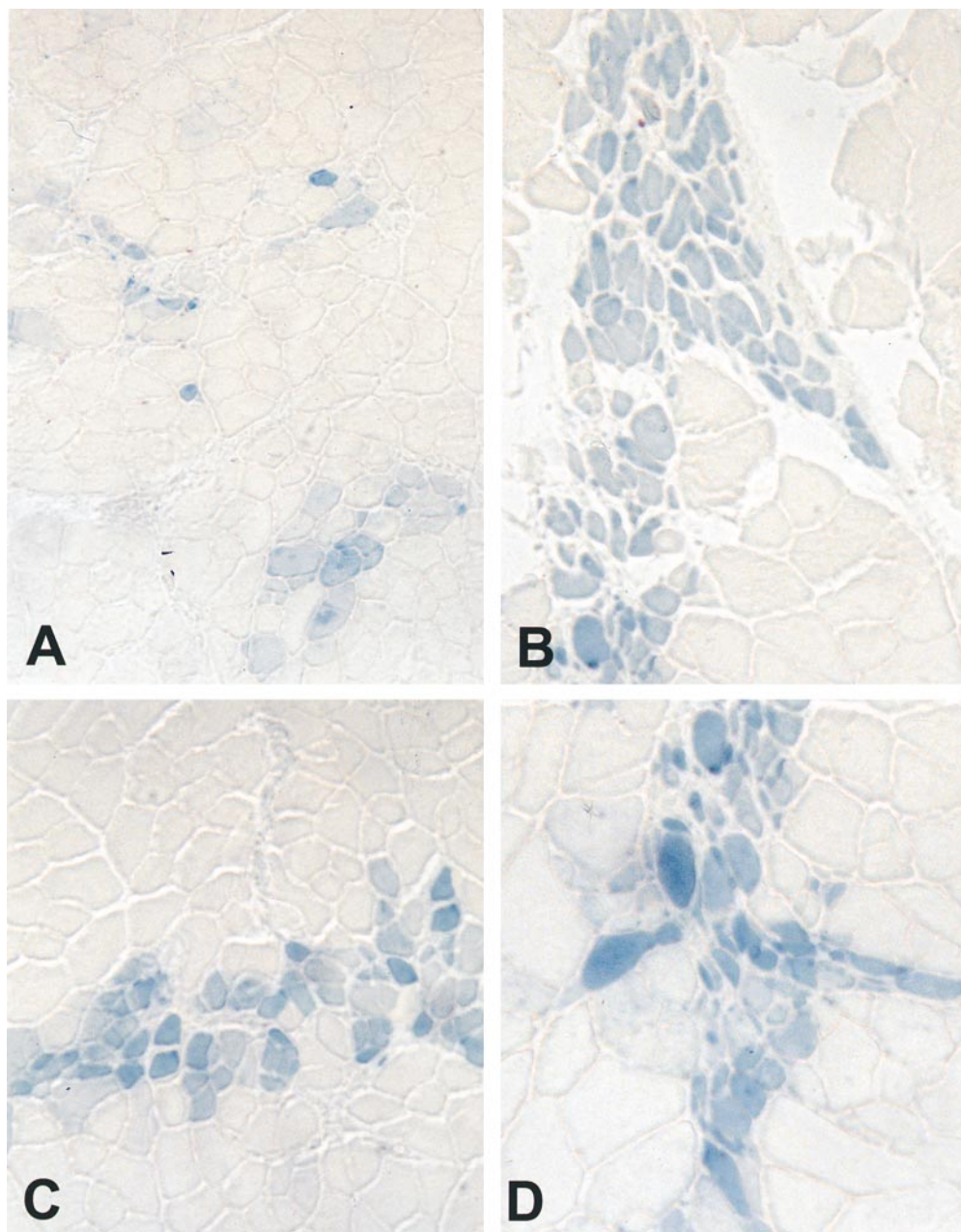
*Figure 8.* Sections of the muscles of *mdx/mdx* mice injected intraarterially with muscle-derived pp6 cells showing dystrophin and des-LacZ gene expression. LacZ staining of muscle sections from donor des-LacZ mice was performed as a positive control (A and B). Some LacZ-positive myofibers (C and E) were found in the intraarterially injected *mdx* muscles that colocalized with dystrophin-positive myofibers of the adjacent sections (D and F). Bars, 50  $\mu$ m.

longed (60-min) swimming exercise. A preliminary experiment has demonstrated that this type of activity damaged 25% of the muscle fibers in the TA muscle, as indicated by the detection of intracellular calcium with alizarin red staining (data not shown). The histology of muscles of these mice presented foci of degenerating and regenerating fibers 24 h after the swimming exercise. In fact, a significant number of basophilic myotubes with vesicular myonuclei and numerous myotubes of small diameters with central nuclei were evident (data not shown). An inflammatory cellular infiltrate was also present. The cell infiltration decreased rapidly and disappeared starting 3 d after exercise. As in the first experiment, the animals were killed 21 d after the intraarterial injection. Quite surprisingly, this type of exercise-induced damage did not increase the percentage of  $\beta$ -gal-positive muscle fibers, as only 1% of them were positive.

#### ***LacZ Expression in Muscle Injected with Muscle-derived and Endothelial Cells***

In an attempt to investigate whether the Sca-1, CD34 positive cells have characteristics of bilineage-committed pre-

cursors (hemopoietic and endothelial), the intramuscular injection of pp6 was compared with the injection of an endothelial cell line. The ability of injected pp6, labeled with a LacZ transgene, to fuse with host muscle fibers was investigated 30 d after intramuscular transplantation. A small number of  $\beta$ -gal myofibers surrounding the injected area were observed in these muscles ( $\sim$ 3% per cross section; Fig. 9 A). The endothelial cell line was isolated as described in Materials and Methods. These cells expressed the vimentin and the  $\beta$ -gal placed under a vimentin promoter. It has been shown that myoblasts and regenerating myofibers express the vimentin protein (Tokuyasu et al., 1984; Sarnat, 1992). The fibers which had been colonized by the endothelial cells expressed the LacZ. They also contained the large T antigen with a thermosensitive mutation to achieve inactivation of large T antigen by temperature shift when necessary. Thus, these cells were able to differentiate when injected in the TA muscles of animals having a body temperature of 37°C. 30 d after transplantation, intensely  $\beta$ -gal- and vimentin-positive mononuclear cells were observed in different areas of the cross section of the injected TA muscle (Fig. 10). The positive



**Figure 9.** Intramuscular transplantation of muscle-derived stem cells. (A) A small number of  $\beta$ -gal myofibers surrounding the injected area were observed in muscles injected with pp6, labeled with a LacZ transgene, and investigated 30 d after intramuscular transplantation. We also verified whether Sca-1, CD34<sup>+</sup> stem cells remain in the muscle after intraarterial injection without differentiation and participate actively to muscle formation only after muscle damage. Thus, muscle damage with needle insertions was induced 48 h after intraarterial injection of muscle-derived pp6 cells in the TA muscle of injected hindlimb. The myogenic potentialities of the injected cells was determined by LacZ staining. TA muscle damaged with needle insertions showed a higher increase of LacZ expression (B, C, and D) compared with intramuscular injections (A). In these experiments, fibers of the same section exhibited different levels of LacZ activity, some fibers being more intensely stained than others. Most of the labeling was restricted to regions near the three needle tracks made in the muscles where most of the muscle regeneration was present.

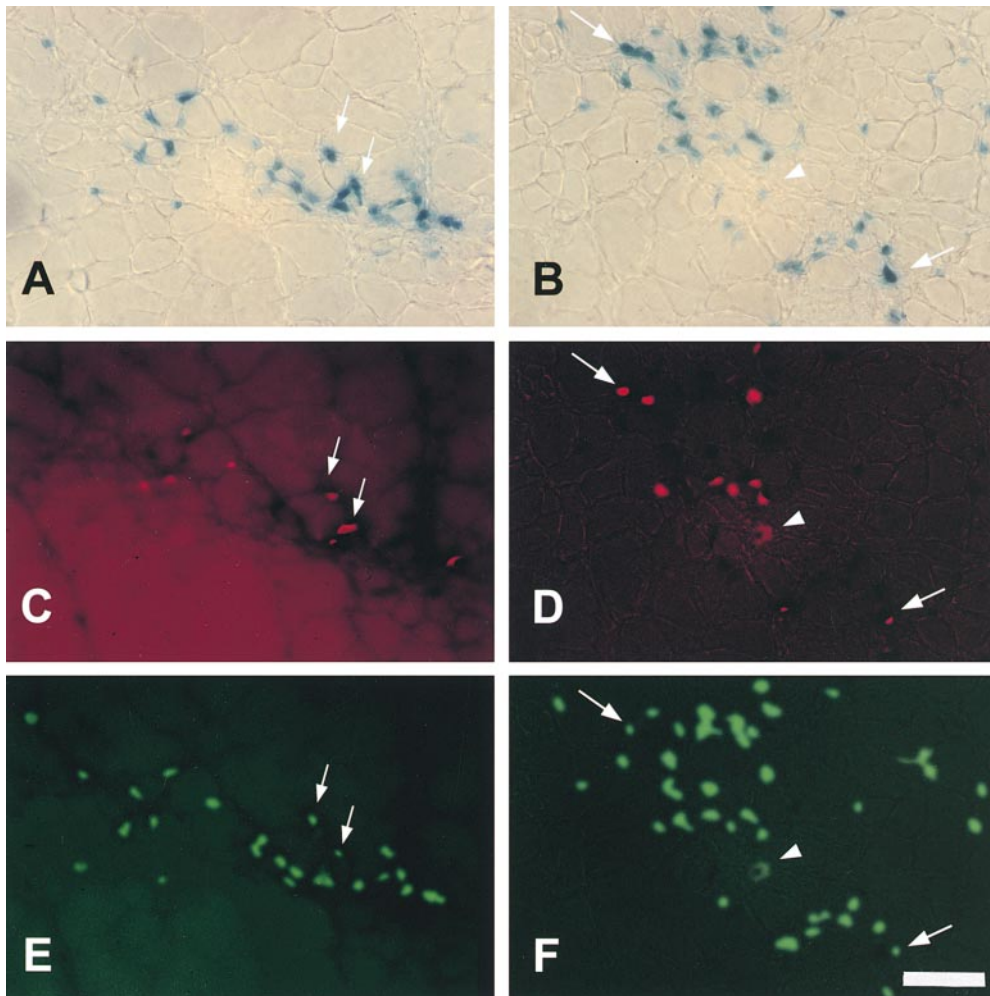
cells were spread throughout the muscle, but fused poorly with the host's fibers. The PECAM staining confirmed that injected cells were still endothelial cells (data not shown). Interestingly, endothelial injected cells remained as mononuclear cells close to the muscle fibers. Very few cells expressed the large T antigen in their nuclei. Few of the perithelial layer of muscle vessels in the injected area were positive to LacZ and vimentin staining, indicating an incorporation of the endothelial injected cells in these sides (Fig. 10, B, D, and F).

### Discussion

We have further investigated a new strategy to restore dystrophin expression in mdx mouse muscles by an intraarterial injection of normal purified Sca-1, CD34 muscle-derived stem cells. Marker transgenic DNA was found in muscles of mdx/mdx mice demonstrating the migration

of these cells from the blood vessels. These muscle-derived cells crossed the vessel barriers thus leading to a mosaic expression of the marker gene in muscles of the injected hindlimb of adult mice. The precise pathway used by the transplanted cells to home to host muscle tissues is not known. However, the adhesion-initiating vascular diapedesis of these cells is high in capillaries. Based on our findings, the cells might have actively migrated in response to chemoattractants, as described previously, after bone marrow transplantation (Mazo et al., 1998; Deog-Yeon et al., 2000).

The fate of injected Sca-1, CD34 positive cells was evaluated by examining tissues of five mdx/mdx mice. We found transcription and expression of LacZ and normal dystrophin genes in muscles of the injected hindlimbs. Unexpectedly, PCR and RT-PCR analysis revealed the absence of injected cells in other muscles and tissues of the treated mice. Histochemical analysis of muscles showed



**Figure 10.** Intramuscular injection of an endothelial cell line. These cells expressed vimentin and the  $\beta$ -gal placed under a vimentin promoter. 30 d after transplantation, intensely  $\beta$ -gal (A and B) and vimentin (E and F) positive mononuclear cells were observed in different areas of the cross sections of the injected TA muscle. Few injected cells still remained large T antigen positive (C and D). Few perithelial layers of muscle vessels in the injected area were positive to LacZ and vimentin staining, indicating an incorporation of the endothelial injected cells in these sides (arrow-heads in B, D, and F). Bar, 50  $\mu$ m.

colocalization of dystrophin and  $\beta$ -gal in muscle fibers. Peripherally nucleated double-positive fibers were generally grouped in some segments of the muscles and always seemed to exist in small groups, suggesting that there might have been clonal proliferation of some donor cells, later contributing to the formation of several muscle fibers. Thus, muscle-derived cells injected into arterial circulation of a congenital dystrophinopathy partly restored normal dystrophin production in striated muscles.

In this study, we used a muscle culture system for facilitating the enrichment and purification of Sca-1<sup>+</sup> and CD34<sup>+</sup> cells. We found a lower content of desmin-positive cells in a population of purified muscle-derived cells that display both CD34 and Sca-1 as markers of progenitor cells. This population was obtained with a purification method described in Qu et al. (1998), but with a different medium and growth conditions. However, these cells showed a poor ability of myogenic differentiation, indicating that they are distinct from the pp6 cells described in Qu et al. (1998). These cells could represent a pool of stem cells capable of commitment to more than one lineage (hemopoietic, endothelial, and muscular) given the right environmental cues. However, the major problem encountered in this study was the poor ability of the pp6 cells to undergo muscle differentiation when injected directly in the muscle. It is conceivable that engraftment efficiency may depend on the inflammatory consequences of muscle

directly injected compared with the arterial injection of the hindlimb where histologic features of muscles are invaried. These data do not preclude the possibility of the muscle-derived Sca-1, CD34 double-positive cells representing the immediate progenitors to satellite cells. The descendants of the activated satellite cells, myogenic precursor cells, undergo multiple rounds of division before fusing to existing or new fibers. Quiescent satellite cells express c-met and M-cadherin proteins, but do not express markers of committed myoblasts such as Myf-5, MyoD, and desmin (Cornelison and Wold, 1997; Sabourin et al., 1999). The total number of satellite cells in muscle remains relatively constant, suggesting that a capacity for self-renewal in the satellite cell compartment maintains the population of quiescent cells (Bischoff, 1994). However, the mechanism by which satellite cells undergo self-renewal in skeletal muscle is poorly understood. Muscle stem cells may undergo asymmetric cell division to generate two daughter cells: a committed myogenic precursor and a pluripotent "self." Moreover, it remains possible that muscle-derived stem cells represent an independent stem cell population separate from the satellite cells. Additionally, circulating pluripotent cells derived from the bone marrow might be the common putative stem cell population present in many different tissues.

To analyze the potential role of the endothelial CD34<sup>+</sup> progenitor cells of our purified muscle-derived cells, we

performed in vivo studies focusing on the muscle injections of endothelial cells. Interestingly, endothelial cells injected intramuscularly remained as mononuclear cells under the basal lamina of the host muscle fiber in the same location as muscle satellite cells and fused poorly with host fibers. Few LacZ- and vimentin-positive cells were also located at the perithelial sides of muscle vessels.

It is extremely interesting to note that the efficiency of Sca-1, CD34 muscle engraftment increased significantly to 12% when TA muscle of the injected hindlimb was damaged 48 h after intraarterial injection. This observation suggests that these cells first attached to the capillaries as Sca-1, CD34 positive stem cell remaining in the muscle without differentiation and participate actively in muscle formation only after muscle damage. Thus, the myogenic potentialities of the injected cells are determined by the location of the precursors at the beginning of muscle and blood vessel damage. In another experiment, the damage to the leg muscles of mdx mice 48 h after the intraarterial injection of TnILacZ Sca-1<sup>+</sup>CD34<sup>+</sup> cells (pp6) was done by a swimming exercise which was shown to damage the muscle fibers of mdx mice. 1 mo later, we observed only 1% of  $\beta$ -gal-positive fibers in the leg injected arterially. There are two possible hypotheses to interpret the results of these two experiments with muscle damage produced with a needle and with exercise. The first is that the Sca-1<sup>+</sup>CD34<sup>+</sup> cells are attached to the capillaries and participate in muscle regeneration when not only the muscle fibers, but also the blood vessels, are damaged. The second hypothesis is that the Sca-1<sup>+</sup>CD34<sup>+</sup> cells are still present in the blood circulation and are attracted to the damaged muscle when the blood vessels are damaged. The first hypothesis means that there are two types of muscle regeneration. The first type (low regeneration), which would occur after damage only to the muscle fibers, would involve the participation of only the satellite cells attached to the muscle fibers. The second type of muscle regeneration (intense regeneration) would occur after a damage to the muscle involving not only the muscle fibers, but also the blood vessels. With this more intensive damage, the regeneration would involve not only the participation of satellite cells, but also of stem cells already attached to the blood vessels or present in the blood circulation. The low regeneration would occur after intense muscle activity. The intense regeneration would occur after a major injury to the muscle (an accident, a bite, or a crush). The presence of precursor cells attached to the blood vessels and participating in muscle regeneration only after damage to these vessels would explain why Grounds and McGeachie (1992) observed that within a few hours after a muscle crush there were more myogenic cells than satellite cells. This intense regeneration hypothesis also explains why there is muscle regeneration after muscle irradiation when notexin is injected intramuscularly (Heslop et al., 2000), since these injections also damaged the blood vessels. More importantly, this hypothesis would account for the absence of muscle regeneration in DMD patients despite the presence of stem cells originating from the bone marrow or other sources already attached to the blood vessels and capable of forming myogenic cells.

During embryonic vasculogenesis, primordial perithelial cells, which later would generate smooth muscle and connective tissue of the vessel wall, develop in the outermost

portion of vasculogenic clusters (DeRuiter et al., 1997). It is possible that these multipotent mesodermal progenitors remain associated to the tissue vasculature as perivascular cells and acquire a commitment depending on the tissue (Bianco and Cossu, 1999). In this model, the mesodermal progenitors would represent the source of cells to develop and regenerate tissue. Thus, when muscle stem cells are injected by intraarterial circulation, they would become associated with the vasculature and would be able to differentiate into the myogenic lineage after muscle and blood vessel damage. In contrast, the direct muscle injection of these cells did not give the correct lineage relationship between endothelial and perithelial progenitor cells.

This raised the possibility that the precursor Sca-1, CD34 muscle-derived cells with characteristics of bilineage-committed precursors (hemopoietic and muscular) may prevent irreversible pathological damage and allow engraftment in many distant areas affected by the disease. It would also be important to know the muscle-derived signals governing cell migration. Thus, further accurate studies of the Sca-1, CD34 muscle-derived cells will be essential to improve transplantation efficiency.

We thank S. Salani, B. Roy, and M. Goulet for technical assistance.

This work has been supported by the Association Francaise contre Les Myopathies, the Muscular Dystrophy Association, and by the Associazione Amici Dino Ferrari.

Submitted: 7 August 2000

Revised: 7 December 2000

Accepted: 11 December 2000

#### References

- Anderson, L., and K. Davison. 1999. Multiplex Western blotting system for the analysis of muscular dystrophy proteins. *Am. J. Pathol.* 154:1017–1022.
- Asselin, I., M. Tremblay, J.T. Vilquin, B. Guerette, R. Roy, and J.P. Tremblay. 1995. Quantification of normal dystrophin mRNA following myoblast transplantation in mdx mice. *Muscle Nerve.* 18:980–986.
- Berenson, R.J., R.G. Andrews, W.I. Bensinger, D.F. Kalamaz, G. Knitter, C.D. Buckner, and I.D. Bernstein. 1988. Antigen CD34+ marrow cells engraft lethally irradiated baboons. *J. Clin. Invest.* 81:951–955.
- Bianco, P., and G. Cossu. 1999. Uno, nessuno e centomila: searching for the identity of mesodermal progenitors. *Exp. Cell Res.* 251:257–263.
- Bischoff, R. 1994. The satellite cell and muscle regeneration. In *Myogenesis*. Vol. 2. A.G. Engel and C. Franzini-Armstrong, editors. McGraw-Hill, Inc., New York. 97–118.
- Cornelison, D.D., and B.J. Wold. 1997. Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. *Dev. Biol.* 191:270–283.
- Delassus, S., and A. Cumano. 1996. Circulation of hematopoietic progenitors in the mouse embryo. *Immunity.* 4:97–106.
- Deog-Yeon, J., S. Rafii, H. Tsuneyoshi, and A.S. Moore. 2000. Chemotaxis of primitive hematopoietic cells in response to stromal cell-derived factor-1. *J. Clin. Invest.* 105:101–111.
- DeRuiter, M.C., R.E. Poelman, J.C. VanMunsteren, V. Mironov, R.R. Markwald, and A.C. Gittenberger-de Groot. 1997. Embryonic endothelial cells transdifferentiate into mesenchymal cells expressing smooth muscle actins in vivo and in vitro. *Circ. Res.* 80:444–451.
- Dzierzak, E., M.J. Sanchez, A. Muller, C. Miles, A. Holmes, H. Tidcombe, and A. Medvinsky. 1997. Hematopoietic stem cells: embryonic beginnings. *J. Cell. Physiol.* 173:216–218.
- Eglitis, M.A., and E. Mezey. 1997. Hematopoietic cells differentiate into both microglia and macroglia in the brains of adult mice. *Proc. Natl. Acad. Sci. USA.* 94:4080–4085.
- Emery, A.E. 1989. Clinical and molecular studies in Duchenne muscular dystrophy. *Prog. Clin. Biol. Res.* 306:15–28.
- Ferrari, G., G. Cusella-De Angelis, M. Coletta, E. Paolucci, A. Stornaiuolo, G. Cossu, and F. Mavilio. 1998. Muscle regeneration by bone marrow-derived myogenic progenitors. *Science.* 279:1528–1530.
- Fina, L., H.V. Molgaard, D. Robertson, N.J. Bradley, and P. Monaghan. 1990. Expression of the CD34 gene in vascular endothelial cells. *Blood.* 75:2417–2426.
- Forni, L. 1979. Reagents for immunofluorescence and their use for studying lymphoid cell products. In *Immunological Methods*. Vol. 151. I. Pernis, editor. Academic Press, New York. 15–33.

- Grounds, M.D., and J.K. McGeachie. 1992. Skeletal muscle regeneration after crush injury in dystrophic mdx mice: an autoradiographic study. *Muscle Nerve*. 15:580–586.
- Gussoni, E., H.M. Blau, and L.M. Kunkel. 1997. The fate of individual myoblasts after transplantation into muscles of DMD patients. *Nat. Med.* 3:970–977.
- Gussoni, E., Y. Soneoka, C.D. Strickland, E.A. Buzney, M.K. Khan, A.F. Flint, L.M. Kunkel, and R.C. Mulligan. 1999. Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature*. 401:300–304.
- Hallauer, P.L., H.L. Bradshaw, and K.E.M. Hasting. 1993. Complex fiber-type-specific expression of fast skeletal muscle troponin I gene constructs in transgenic mice. *Development*. 119:691–701.
- Heslop, L., J.E. Morgan, and T.A. Partridge. 2000. Evidence for a myogenic stem cell that is exhausted in dystrophic muscle. *J. Cell Sci.* 113:2299–2308.
- Hoffman, E.P., R.H. Brown, and L.M. Kunkel. 1987. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell*. 51:919–928.
- Hoffman, E.P., J.E. Morgan, S.C. Watkins, and T.A. Partridge. 1990. Somatic reversion/suppression of the mouse mdx phenotype in vivo. *J. Neurol. Sci.* 99:9–25.
- Horwitz, E.M., D.J. Prockop, L.A. Fitzpatrick, W.W. Koo, P.L. Gordon, M. Neel, M. Sussman, P. Orchard, J.C. Marx, R.E. Pyeritz, and M.K. Brenner. 1999. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. *Nat. Med.* 5:309–313.
- Jackson, K.A., T. Mi, and A. Goodell. 1996. Hematopoietic potential of stem cells isolated from murine skeletal muscle. *Proc. Natl. Acad. Sci. USA*. 96:14482–14486.
- Lange, C., C. Kaltz, K. Thalmeier, H.J. Kolb, and R. Huss. 1999. Hematopoietic reconstitution of syngeneic mice with a peripheral blood-derived, monoclonal CD34<sup>+</sup>, Sca-1<sup>+</sup>, Thy-1<sup>low</sup>, c-kit<sup>+</sup> stem cell line. *J. Hematother. Stem Cell Res.* 8:335–342.
- Li, Z.L., P. Marchand, J. Humbert, C. Babinet, and D. Paulin. 1993. Desmin sequence elements regulating skeletal muscle-specific expression in transgenic mice. *Development*. 117:947–959.
- Li, Z., M. Mericskay, O. Agbulut, G. Butler-Browne, L. Carlsson, L.E. Thornell, C. Babinet, and D. Paulin. 1997. Desmin is essential for the tensile strength and integrity of myofibrils but not for myogenic commitment, differentiation, and fusion of skeletal muscle. *J. Cell Biol.* 139:129–144.
- Mazo, I.B., J.C. Gutierrez-Ramos, P.S. Frenette, R. Hynes, D.D. Wagner, and U.H. Von Andrian. 1998. Hematopoietic progenitor cell rolling in bone marrow microvessels: parallel contributions by endothelial selectins and vascular cell adhesion molecule 1. *J. Exp. Med.* 188:465–474.
- Mendell, J.R., J.T. Kissel, A.A. Amato, W. King, L. Signore, T.W. Prior, Z. Sahenk, S. Benson, P.E. McAndrew, and R. Rice. 1995. Myoblast transfer in the treatment of Duchenne's muscular dystrophy. *N. Engl. J. Med.* 28:832–838.
- Murray, P.D.F. 1932. The development in vitro of blood of the early chick embryo. *Proc. R. Soc. C*. 11:497–521.
- Nakamura, Y., K. Ando, J. Chargui, H. Kawada, T. Sato, T. Tsuji, T. Hotta, and S. Kato. 1999. Ex vivo generation of CD34<sup>+</sup> cells from CD34<sup>−</sup> hematopoietic cells. *Blood*. 94:4053–4059.
- Pardanaud, L., D. Luton, M. Prigent, L.M. Bourcheix, M. Catala, and F. Dieterlen-Lièvre. 1996. Two distinct endothelial lineages in ontogeny, one of them related to hemopoiesis. *Development*. 122:1363–1371.
- Partridge, T.A., J.E. Morgan, G.R. Coulton, E.P. Hoffmann, and L.M. Kunkel. 1989. Conversion of mdx myofibers from dystrophin-negative to positive by injection of normal myoblasts. *Nature*. 337:176–179.
- Petersen, B.E., W.C. Bowen, K.D. Patrene, W.M. Mars, A.K. Sullivan, N. Murase, S.S. Boggs, J.S. Greenberg, and J.P. Goff. 1999. Bone marrow as a potential source of hepatic oval cells. *Science*. 284:1168–1170.
- Pittenger, M.F., A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, and D.R. Marshak. 1999. Multilineage potential of adult human mesenchymal stem cells. *Science*. 284:143–147.
- Prelle, A., M. Moggio, G.P. Comi, A. Gallanti, N. Checcarelli, N. Bresolin, P. Ciscato, F. Fortunato, and G. Scarlato. 1992. Congenital myopathy associated with abnormal accumulation of desmin and dystrophin. *Neuromuscul. Disord.* 2:169–175.
- Qu, Z., L. Balkir, J.C.T. Van Deutekom, P.D. Robbins, R. Pruchnic, and J. Huard. 1998. Development of approaches to improve cell survival in myoblast transfer therapy. *J. Cell Biol.* 142:1257–1267.
- Rando, T.A., and H.M. Blau. 1994. Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. *J. Cell Biol.* 125:1275–1287.
- Russo, S., D. Tomatis, G. Collo, G. Tarone, and F. Tatò. 1998. Myogenic conversion of NIH3T3 cells by exogenous MyoD family members: dissociation of terminal differentiation from myotube formation. *J. Cell Sci.* 111:691–700.
- Sabin, F.R. 1917. Origin and development of the primitive vessels of the chick and of the pig. *Contrib. Embryol. Carnegie Inst.* 226:61–124.
- Sabourin, A.L., A. Girgis-Gabardo, P. Seale, A. Asakura, and M.A. Rudnicki. 1999. Reduced differentiation potential of primary MyoD<sup>−/−</sup> myogenic cells derived from adult skeletal muscle. *J. Cell Biol.* 144:631–643.
- Sarnat, H.B. 1992. Vimentin and desmin in maturing skeletal muscle and developmental myopathies. *Neurology*. 42:1616–1624.
- Shi, Q., S. Rafii, M.H. Wu, E.S. Wijelath, C. Yu, A. Ishida, Y. Fujita, S. Kothari, R. Mohle, and L.R. Sauvage. 1998. Evidence for circulating bone marrow-derived endothelial cells. *Blood*. 92:362–367.
- Shrager, J.B., A. Naji, A.M. Kelly, and H.H. Stedman. 1992. A PCR-based assay for the wild-type dystrophin gene transferred into the mdx mouse. *Muscle Nerve*. 15:1133–1137.
- Sicinski, P., Y. Geng, A.S. Ryder-Cook, E.A. Barnard, M.G. Darlison, and P.J. Barnard. 1989. The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. *Science*. 244:1578–1580.
- Tokuyasu, K.T., P.A. Maher, and S.J. Singer. 1984. Distributions of vimentin and desmin in developing chick myotubes in vivo. Immunofluorescence study. *J. Cell Biol.* 98:1961–1972.
- Torrente, Y., M.G. D'Angelo, Z. Li, R. Del Bo, S. Corti, M. Mericskay, A. DeLiso, A. Fassati, D. Paulin, G.P. Comi, G. Scarlato, and N. Bresolin. 2000. Transplacental injection of somite-derived cells in mdx mouse embryos for the correction of dystrophin deficiency. *Hum. Mol. Genet.* 9:1843–1852.
- Tremblay, J.P., F. Malouin, R. Roy, J. Huard, J.P. Bouchard, A. Satoh, and C.L. Richards. 1993. Results of a triple blind clinical study of myoblast transplantations without immunosuppressive treatment in young boys with Duchenne muscular dystrophy. *Cell Transplant.* 2:99–112.
- van de Rijn, M., S. Heimfeld, G.J. Spangrude, and I.L. Weissman. 1989. Mouse hematopoietic stem cell antigen Sca-1 is a member of the Ly-6 antigen family. *Proc. Natl. Acad. Sci. USA*. 86:4634–4638.
- Vicart, P., B. Schwartz, A. Vandewalle, M. Bens, C. Delouis, J. Panthier, S. Pournin, C. Babinet, and D. Paulin. 1994. Immortalization of multiple cell types from transgenic mice using a transgene containing the vimentin promoter and a conditional oncogene. *Exp. Cell Res.* 214:35–45.
- Wagner, R.C. 1981. Endothelial cell embryology and growth. In *Advances in Microcirculation*. Vol. 9. H. Harders, editor. Karger, Basel/New York. 45–75.