Identification of myogenic-endothelial progenitor cells in the interstitial spaces of skeletal muscle

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Putative myogenic and endothelial (myo-endothelial) cell progenitors were identified in the interstitial spaces of murine skeletal muscle by immunohistochemistry and immunoelectron microscopy using CD34 antigen. Enzymatically isolated cells were characterized by fluorescence-activated cell sorting on the basis of cell surface antigen expression, and were sorted as a CD34$^+$ and CD45$^-$ fraction. Cells in this fraction were ~94% positive for Sca-1, and mostly negative (<3% positive) for CD14, 31, 49, 144, c-kit, and FLK-1. The CD34$^+$/45$^-$ cells formed colonies in clonal cell cultures and colony-forming units displayed the potential to differentiate into adipocytes, endothelial, and myogenic cells. The CD34$^+$/45$^-$ cells fully differentiated into vascular endothelial cells and skeletal muscle fibers in vivo after transplantation. Immediately after sorting, CD34$^+$/45$^-$ cells expressed only c-met mRNA, and did not express any other myogenic cell-related markers such as MyoD, myf-5, myf-6, myogenin, M-cadherin, Pax-3, and Pax-7. However, after 3 d of culture, these cells expressed mRNA for all myogenic markers. CD34$^+$/45$^-$ cells were distinct from satellite cells, as they expressed Bcrp1/ABCG2 gene mRNA (Zhou et al., 2001). These findings suggest that myo-endothelial progenitors reside in the interstitial spaces of mammalian skeletal muscles, and that they can potentially contribute to postnatal skeletal muscle growth.

Introduction

Recent identification of tissue specific stem cells in adult brain (Reynolds and Weiss, 1992; Johansson et al., 1999), bone marrow (Prockop, 1997: Ferrari et al., 1998), and skeletal muscle (Gussoni et al., 1999; Jackson et al., 1999) opens new pathways for tissue reconstitution therapy via cell transplantation. For skeletal muscle, the satellite cells, residing between the basal lamina and the plasma membrane of muscle fibers, have been considered the only myogenic source for postnatal growth, repair, and maintenance of skeletal muscle (Grounds, 1991; Schultz, 1996). Recently, a hematopoietic and myogenic stem cell population in the muscle called side population (SP)* cells has been purified based on the efflux of the fluorescent dye Hoechst 33342 (Gussoni et al., 1999; Jackson et al., 1999), which was clearly distinct from satellite cells (Seale et al., 2000).

In a recent study, we have found de novo formation of muscle fibers in the interstitial spaces of skeletal muscles in postnatal growing rats (Tamaki et al., 2002). In the interstitial spaces of muscles of 3-d-old rats, there were many cells expressing myogenic transcription genes such as MyoD and myogenin, with several small fibers also being positive for anti-neonatal myosin heavy chain, indicating that these were new fibers. The total numbers of fibers in the plantaris muscle increased by 28% from 3 to 10 wk of age. We also identified several CD34 (a hematopoietic stem cell marker) (Brown et al., 1991)-positive cells in the interstitial spaces, although they did not coexpress MyoD. A primary question that remains is whether these interstitial CD34$^+$ cells are derived from satellite cells, muscle SP cells, or a distinct myogenic cell population.

Recently, CD34 antigen has been shown to be a myogenic cell marker (Beauchamp et al., 2000; Lee et al., 2000; Torrente et al., 2001), and the muscle SP cells have also been reported to be a mixture of CD34-positive and
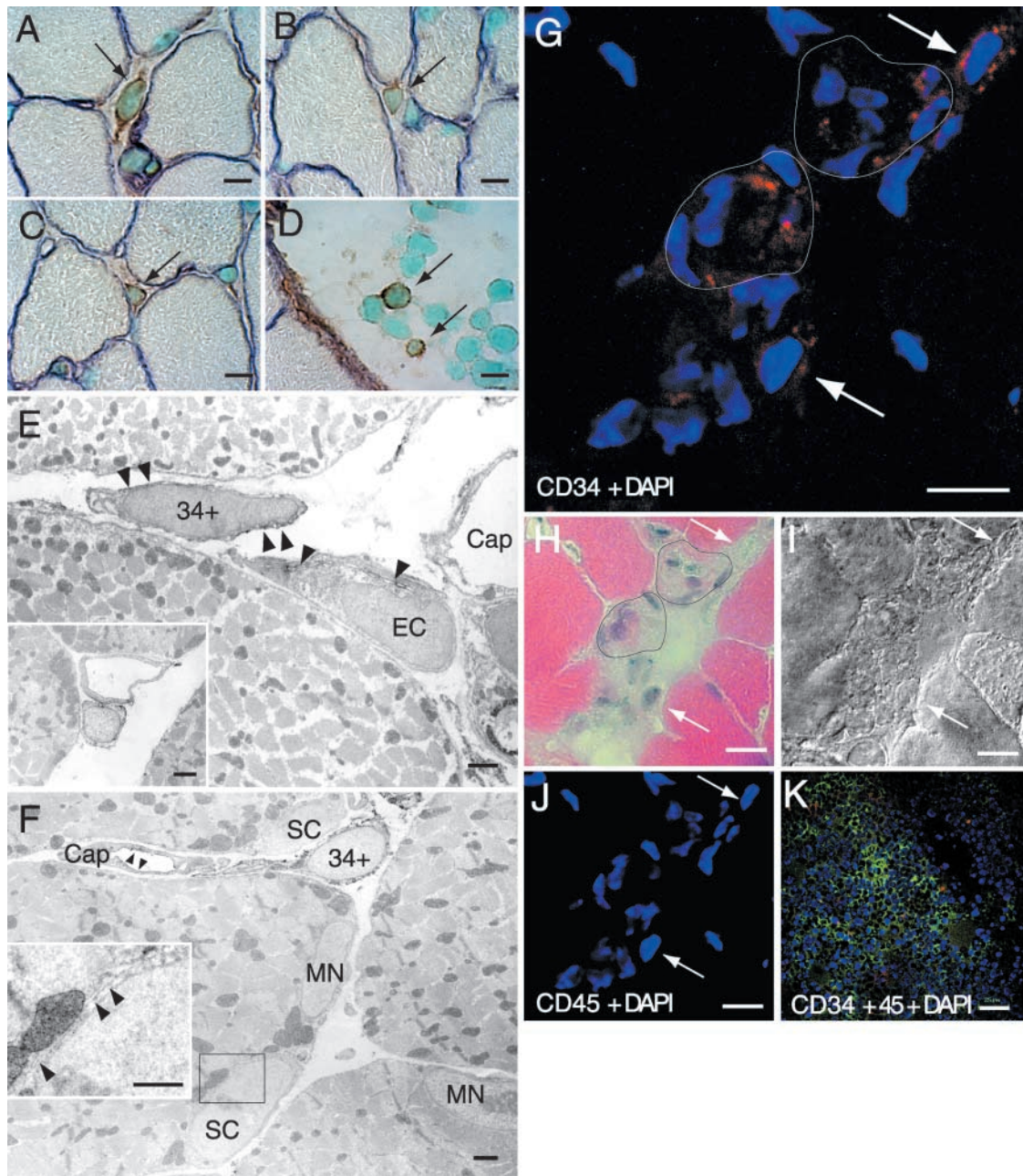
Figure 1. **Localization of CD34$^+$ cells in the interstitial spaces of skeletal muscles of 3-wk-old mice.** Small mononucleated CD34$^+$ cells were observed in the interstitial spaces (surrounding brown reaction, arrows in A–D). The CD34$^+$ cells are located outside of the basal lamina (laminin staining, dark purple). The location, outside of the basal lamina was also confirmed by immunoelectron micrograph (E and inset). The CD34$^+$ cell shows lower nuclear/cytoplasmic ratio representing immature cell. Reaction products of DAB can be seen in surface of CD34$^+$ cell as well as in the lumina of endothelial cell (arrowheads in E). However, note that the satellite cells shows CD34$^-$, whereas CD34$^+$ cell can be seen within the interstitial spaces (F). Inset in F shows higher magnifications of the corresponding square areas on the photograph. Cell membrane of both satellite cell and muscle cell is evident (arrowheads). Immunofluorescent costaining with CD34 (G, Rhodamine-red) and CD45 (J, FITC-green) shows that CD34$^+$ cells are CD45$^-$ (G and J, arrows), and they are located in the interstitial spaces (H and I, arrows). Vasculature related CD34$^+$ reactions are also observed in enclosed portions in G and H. Staining in the marrow of the tibia on the same sections with A–C and with G–J are shown in D (CD34, brown) and K (CD34, red and CD45, green) as a positive control. The mean frequencies of CD34$^+$ and CD34$^-$ mononuclear cells appearing in a unit area of 3-wk-old mice muscles were 1.9 ($\pm$0.3, SE) and 2.9 ($\pm$0.4), respectively. The data were obtained from four to six unit areas (175 $\times$ 130 μm per unit area) in 20 sections taken from three mice, i.e., corresponding to $\sim$40% of the total interstitial cells in the unit area. Note that there were no CD34$^+$ cells inside the basal lamina. Green reactions in A–D, nuclear stain with methyl green. Blue staining in G and J, nuclear stain with DAPI. HE, hematoxylin-eosin staining; TV, transmission view of confocal laser scanning microscope; 34+, CD34 positive cell; EC, endothelial cell; Cap, capillary; SC, satellite cell; MN, myonuclear. Bars, (A–D and G–J) 10 μm; (E and F) 1 μm; (K) 25 μm.

-negative (Gussoni et al., 1999) cells. Therefore, it is possible that more primitive cells, distinct from satellite cells, reside in the interstitial spaces, and that these cells differ-

entiate into myogenic cells and contribute to new fiber formation. To further investigate the role of interstitial CD34$^+$ cells in the muscle, we switched our experimental
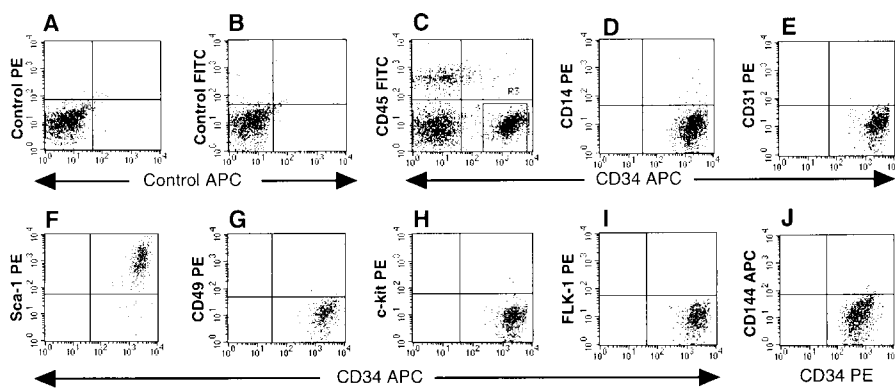
Figure 2. **Isolation and characterization by flow cytometry of EECs and Sk-34 cells from skeletal muscles of the hind limbs of 3-wk-old mice.** The EEC was divided into four subpopulations: $CD34^-/45^+$, $34^+/45^+$, $34^+/45^-$ (Sk-34) and $34^-/45^-$ (C). The mean frequencies of each population were $19.8 \pm 2.8\%$, $11.6 \pm 1.7\%$, $49.7 \pm 4.1\%$, and $17.7 \pm 4.0\%$ ($n = 20$ mice), respectively. The frequencies of coexpression of other markers on the Sk-34 cells ($n = 5$) were: $2.6 \pm 1.3\%$ for CD14 (D); $1.7 \pm 1.2\%$ for CD31 (E); $93.7 \pm 1.3\%$ for Sca-1 (F); $1.0 \pm 0.4\%$ for CD49 (G); $2.4 \pm 2.0\%$ for c-kit (H); $0.2 \pm 0.3\%$ for FLK-1 (I); and $0.9 \pm 0.8\%$ for CD144 (J). (A and B) Isotype controls. Values, mean $\pm$ S.E.

Results and discussion

Immunohistochemical staining with anti-CD34 and laminin (a marker for the basal lamina) on the entire lower hind limb of 3-wk-old mice revealed numerous $CD34^+$ cells outside the basal lamina, i.e., a location distinct from that of satellite cells (Fig. 1, A–D). Positive staining in the marrow cells of the tibia (Fig. 1 D, used as a positive control) was evident; however, there were no CD34-positive reactions within the basal lamina where satellite cells would be located normally. A location of $CD34^+$ cell that outside the basal lamina was also confirmed by immunoelectron microscopy, representing immature shape with a less cytoplasm and cell–organelle (Fig. 1 E and inset).

To examine the $CD34^+$ cells in the interstitial spaces of skeletal muscles, we obtained the interstitial cell enzymatically and characterized them by flow cytometry using several cell surface markers. We used an extremely mild enzymatic digestion based on the living single fiber isolation method associated with satellite cells as described by Bischoff (1986), and used only the extracted fractions, i.e., all remaining muscle fiber elements were discarded. However, skeletal muscles are highly vascular and contain a variety of mononuclear cells, including those in blood, endothelial cells, fibroblasts, and myogenic satellite cells. In addition, MyoD and/or myogenin expressing myoblasts that exist in the interstitial spaces in growing animal (Tamaki et al., 2002), also would be extracted during this procedure. To avoid contamination of these cell types, we first sorted the enzymatically extracted cells (EECs) by their reaction to CD34 and CD45 (a hematopoietic cell marker) antigens (Fig. 2). The EEC was divided into four subpopulations, $CD34^-/45^+$, $CD34^+/45^+$, $CD34^+/45^-$, and $CD34^-/45^-$ (Fig. 2 C). The CD45-positive fractions ($CD34^-/45^+$ and $CD34^+/45^+$) were considered to be hematopoietic cells mainly consisting of monocytes and/or macrophages (Thomas, 1989), and were not used in this study. However, the possibility still remains that CD34-positive cells are endothelial cells (Asahara et al., 1997). Because the skeletal muscle–derived $CD34^+/45^-$ fraction (Sk-34) may include endothelial cells, we char-

acterized them further by endothelial cell related markers such as CD14, 31, 49, and 144, c-kit, FLK-1, as well as with a Sca-1, known as stem cell antigen (Fig. 2, D–J). The Sk-34 cells were $94 \pm 1.3\%$ positive for Sca-1 (Fig. 2 F) and mostly negative for all other markers (>3% positive cells) (Fig. 2, D–E and G–J). Endothelial progenitor cells are positive for Sca-1 as are the Sk-34 cells, but also are positive for CD31, FLK-1, and CD144 (VE-cadherin) (Asahara et al., 1997, 1999; Yamashita et al., 2000). Therefore, the Sk-34 fraction contained very few hematopoietic and endothelial cells. The muscle SP cells, a hematopoietic and myogenic stem cell population in skeletal muscle (Gussoni et al., 1999; Jackson et al., 1999), also are positive for Sca-1 and negative for c-Kit and CD45. However, the muscle SP cells are a mixture of CD34-positive and -negative (Gussoni et al., 1999) cells, thus they might be sorted both in the $CD34^-/45^-$ and $CD34^+/45^-$ fraction.

To determine the growth characteristics and multilineage differentiation of purified Sk-34 cells in vitro, we performed clonal cell culture in a semisolid medium. The Sk-34 cells began to form colonies within 4–5 d when cultured in medium containing methylcellulose (Fig. 3 A). As the number of cells gradually increased, two populations of cells were found in a colony at day 6: a floating and/or weakly attaching cell population and an adhesive and spreading cell population (Fig. 3 B, arrows). Both cell populations continuously increased their numbers (Fig. 3 C), and small tubes were observed after 10 d of culture (Fig. 3 C, arrowheads). At this time, the colonies consisted of large, round cells and small tubes showing spontaneous and intermittent contractions (Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200112106). After 14 d of culture, many myotubes were formed in the colony (Fig. 3 D) and these also contracted actively and spontaneously (Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200112106).

To characterize the cells comprising these colonies after 6 d of culture, floating and/or weakly attaching cells in individual colonies were picked up by micropipette and made cytospins and stained for MyoD. The majority of the cells were positive for MyoD (Fig. 2 G). For the remaining adhesive and spreading cell populations, the medium containing methylcellulose was washed out by DME containing 5% FCS, and the cells prepared for analyzing the uptake of DiI-
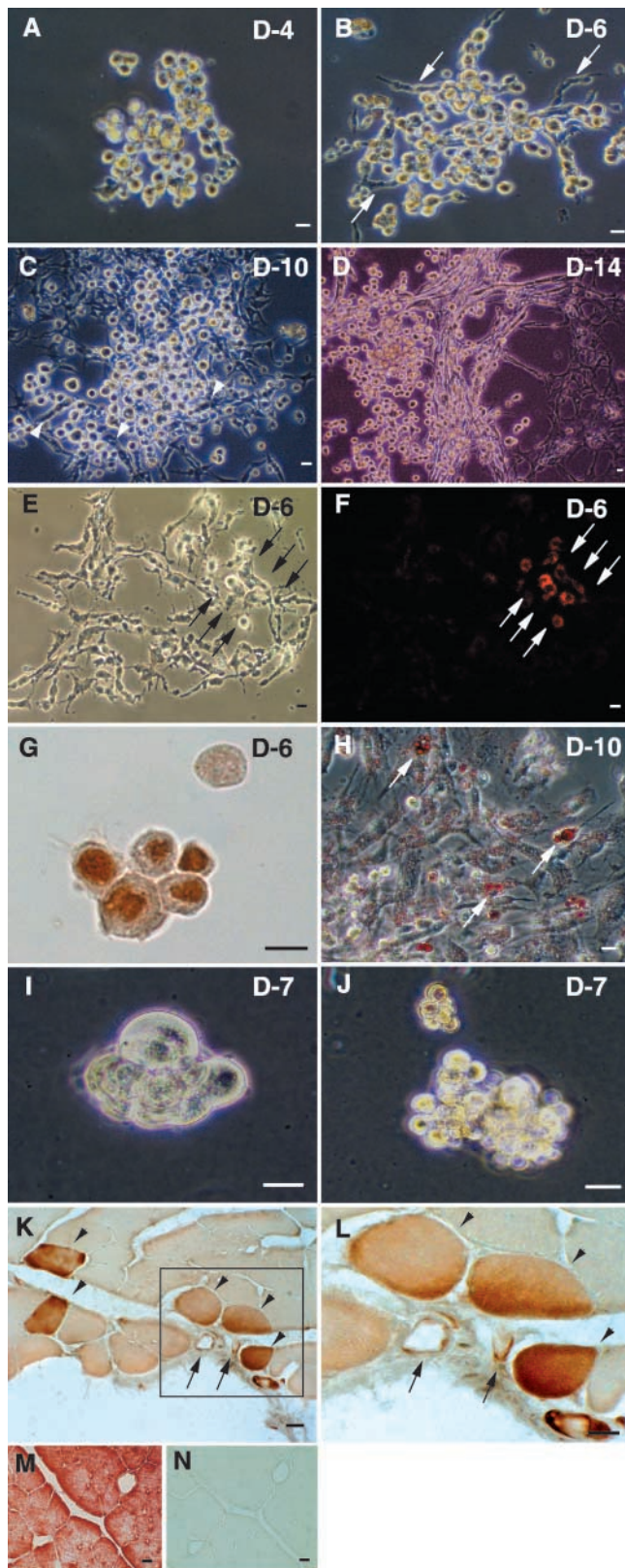
Ac-LDL (a functional marker for endothelial cells and macrophages). Several cells demonstrated uptake of DiI-Ac–LDL, indicating a differentiation to endothelial cells (Fig. 3, E and F, arrows). The same procedures were performed for the samples after 10 d of culture, and the remaining adhesive and spreading cells were stained with oil-red O, a marker of lipid deposition. Several cells showed oil droplet-like staining typical of fat cells (Fig. 3 H, arrows). Combined, these observations confirmed the multilineage potential (myogenic, endothelial, and adipogenic) of a colony-forming unit (CFU) in the Sk-34 cells in vitro. The colonies containing three lineage was formed 75 ± 58 out of 108 ± 61 total colonies when 1×10^4 cells were plated. Remaining 30 colonies were composed by endothelial and adipogenic cell populations, and there were no colonies composed by exclusively single population such as myogenic, endothelial, or adipogenic cell. Interestingly, our preliminarily data using Hoechest 33342 suggested that Sk-34 cells included about 0.6% of SP cells (unpublished data) as similar to a percentage of the CFU in Sk-34 cells (0.75 to 1.1%). To further examine the potential of Sk-34 cells, we performed clonal cell culture on soft agar–coated dishes to avoid cell attachment to the plates. After 7 d of culture, a large number of floating colonies appeared (Fig. 3, I and J). Several cells in these colonies fused to each other and formed sphere-like shapes (Fig. 3 I), and others remained as individual in a colony (Fig. 3J). Interestingly, the double negative fraction (CD34$^-$/45$^-$ cells) did not grow well in the same clonal methylcellulose culture in contrast to the Sk-34 cells, and the rate of myotube formation in the Sk-34 cells apparently was reduced in a liquid culture system (unpublished data).

To evaluate the ability of Sk-34 cells to reconstitute muscles in vivo, we prepared Sk-34 and CD34$^-$/45$^-$ cells from GFP transgenic mice (Okabe et al., 1997) and transplanted them into the tibialis anterior (TA) muscles of male immune-deficient NOD/scid mice. 6 wk after the injection, engrafted anti–GFP-positive cells were observed in the TA muscles of three of the five Sk-34–injected mice, but in none of the CD34–/45$^-$ injected mice. Injected donor cells differentiated into complete muscle fibers (Fig. 3, K and L, arrowhead) and vascular endothelial cells (Fig. 3, K and L, arrows) in the recipient muscles. The combined results of the culture and transplantation experiments indicate that purified Sk-34 cells can differentiate into myogenic, endothelial and fat cells in vitro and fully differentiate into skeletal muscle and vascular endothelial cells in vivo.

To assess the myogenic profile of the Sk-34 cells, we examined the expression of myogenic cell related gene mRNAs. The

Figure 3. **Growth and differentiation of Sk-34 cells in vitro and in vivo.** Colonies derived from the Sk-34 cells after 4 (A), 6 (B), 10 (C), and 14 (D) days of culture. Typically, colonies were composed of floating and/or weakly attaching large round cells and adherent spread cells, and the frequency of these mixed colonies was 75 ± 58 out of 1×10^4 cells ($n = 10$ culture dishes). (E) An adherent cell colony at the bottom of the plate after washing-out the semisolid medium with the weakly attached cell populations. (F) Uptake of DiI-Ac-LDL in the adherent cells populations shown in E (E and F, arrows). (G) MyoD-positive cells in the cytospin of weakly attaching cell populations as shown in B. (H) Fat cells in a colony after 10 d of culture stained with oil red O (arrows). (I and J) Sphere and floating colony in a semisolid medium culture on a soft agar medium. (K) Engrafted Sk-34 cells prepared from GFP transgenic mice forming vascular endothelial cells (brown reactions, arrows) and myofibers (brown reactions, arrowheads) in the muscle of NOD/SCID mice. (L) High magnification of the inset in K. (M) Positive control using muscle tissue section from a GFP transgenic mouse. (N) Negative control stained with the secondary antibody alone. Bars, 10 μm.
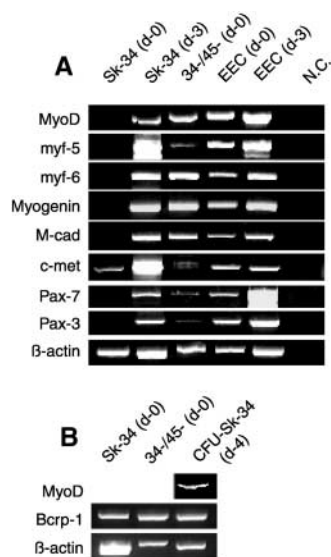
Figure 4. **Expression of the MyoD family gene mRNAs, M-cadherin (M-cad), c-met, Pax-7, and Pax-3 in EEC, Sk-34, and CD34⁻/45⁻ cells (A).** Bcrp-1 in CD34⁻/45⁻, Sk-34, and CFU-Sk-34 cells (B). N.C., negative control without template. d-0: immediately after sorting or enzymatic extraction; d-3 and d-4: after 3 and 4 d of culture. The RT-PCR product for β-actin in the same samples was used as a control.

family of muscle specific basic helix-loop-helix transcription factors (the MyoD family) (Weintraub, 1993; Ontell et al., 1995; Cornelison and Wold, 1997) and several quiescent satellite cell markers such as M-cadherin (Irintchev et al., 1994), c-met (Cornelison and Wold, 1997; Birchmeier and Gherardi, 1998), Pax7 (Seale et al., 2000), and Pax3 (Tajbakhsh et al., 1997) were examined by RT-PCR analysis (Fig. 4 A). Immediately after enzymatic digestion (d-0), the EEC showed expression of all markers, and the expression levels became stronger after 3 d of culture (d-3). Therefore, the EEC contained myogenically committed precursors. This was expected since the muscles of growing animals include large numbers of MyoD and/or myogenin positive cells in the interstitial spaces as well as activated satellite cells (Tamaki et al., 2002). In contrast, immediately after sorting the Sk-34 fraction (d-0) expressed only c-met, whereas the CD34⁻/45⁻ fraction expressed all myogenic cell marker mRNAs. This finding indicates that myogenically committed cells in the EEC were negative for CD34, and they were sorted in the CD34⁻/45⁻ fraction, and that the purified Sk-34 fraction did not contain myogenically committed cells. After 3 d of culture (d-3), the purified Sk-34 cells strongly expressed mRNAs for all markers, indicating a commitment to the myogenic precursor cells. This result is consistent with the myogenic differentiation of Sk-34 cells in vitro (Fig. 3, A–D) and in vivo (Fig. 3, K–L).

The presence of CD34⁺ cells in skeletal muscles has been reported previously (Beauchamp et al., 2000; Lee et al., 2000; Torrente et al., 2001). These reports consistently suggest that the CD34⁺ cells are satellite cells based on their location inside the basal lamina sheath of the muscle fibers in vivo (Lee et al., 2000), and isolated single fiber with satellite cells (Beauchamp et al., 2000). However, our immunohistochemical and electronmicroscopic analyses revealed that

CD34⁺ cells clearly located outside of the basal lamina of the muscle fibers, whereas no CD34⁺ cells were detected in the satellite cell position beneath the basal lamina (Fig. 1, A–F). The reasons for this discrepancy are unknown. However, it is likely that the CD34⁺ cells identified in the present study are a different population of cells than those reported elsewhere, as the expression patterns for specific markers are different. For example, Beauchamp et al. (2000) reported that ∼80% of the satellite cells associated with isolated single muscle fibers were CD34⁺, myf-5⁺, and M-cadherin–positive, and a yet undefined minority population of satellite cells were negative for all three markers. These characteristics do not correspond to the properties of the Sk-34 cells described in the present paper.

We also found that Sk-34 cells are Pax3 and Pax7 negative on day 0, but positive after 3 d of culture. Seale et al. (2000), demonstrating that there was an absence of satellite cells in the skeletal muscles of Pax7 knockout mice, whereas muscle SP cells were present. Their finding suggested that the induction of Pax7 in muscle-derived stem cells induced satellite cell specification by restricting alternate developmental programs and clearly demonstrated that satellite cells were a distinct population from muscle SP cells. Furthermore, these data suggested that muscle SP cells may form a reservoir of satellite cells during the latter stages of embryonic muscle development and may persist in adult skeletal muscle to maintain a steady-state number of satellite cells (Seale et al., 2000). The recent work of Zhou et al. (2001) demonstrated that Bcrp1/ABCG2 gene is an important determinant of the SP phenotype, and the non-SP fraction (including satellite cells) did not express Bcrp1 mRNA in mouse muscle. We also examined the expression of Bcrp1 mRNA in the Sk-34 (d-0 and CFU of d-4) and CD34⁻/45⁻ (d-0) fraction using RT-PCR analysis (Fig. 4 B). Both the Sk-34 (d-0) and CD34⁻/45⁻ (d-0) fractions expressed Bcrp1 mRNA. In addition, the CFU of Sk-34 cells expressed both Bcrp1 and MyoD, indicating that Bcrp1-positive cells differentiated into myogenic cells. In this regard, it is likely that the Sk-34 cell populations included one of the phenotypes of muscle SP cell populations, and that CD34⁻/45⁻ fractions also included SP cell fractions. Combined, these results suggest that Sk-34 cells are distinct from satellite cells, as they express Bcrp1 mRNA. However, it is likely that Sk-34 cells have the potential to become satellite cells in newly formed fibers since they have the ability to express Pax7 after 3 d of culture.

Recent work suggests that satellite cells can be derived from endothelial precursors associated with the embryonic vasculature (De Angelis et al., 1999). Seale et al. (2000) also suggest that progenitor cells associated with the embryonic vasculature, either directly or indirectly, give rise to satellite cells during the embryonic stage of development. These authors further suggest that putative vasculature-associated precursors could give rise to pluripotent stem cells in the adult. Young et al. (1995, 1999) also proposed that there were mesenchymal stem cells within the connective tissues of fetal and adult human skeletal muscle and several tissues and organs on the chick embryo. There are consistent with the possibility that embryonic myo-endothelial progenitor cells could remain in the interstitial spaces of skeletal muscles and express CD34 as observed in the Sk-34 cells.

Based on these observations, we conclude that Sk-34 cells are putative myo-endothelial progenitor cells located in the interstitial spaces of skeletal muscle and are distinct from satellite cells. It is possible that Sk-34 cells are a reservoir of satellite cells and one of the phenotypes of muscle SP cell populations remaining in the interstitial spaces of postnatal and/or adult skeletal muscle. After all, in the present study, we first demonstrated in vivo location of newly myogenic cell populations distinct from satellite cells, and possibly they are one of phenotypes of muscle SP cells. Our finding of myo-endothelial progenitors in skeletal muscle may provide important new insight into muscle cell biology, and possible therapeutic strategies for cell transplantation studies. Tissue specific stem cells, including Sk-34 cells, may share a common embryonic origin and possess the capacity to activate diverse genetic programs in response to peculiar environmental stimulation.

Materials and methods

Mouse strain
C57BL/6 mice were used for the cell cultures, immunohistochemistry, immunoelectron microscopy, and flow cytometric characterizations. Green fluorescent protein transgenic mice (C57BL/6 TgN[act EGFP]Osb Y01) were provided by Dr. M. Okabe (Osaka University, Osaka, Japan) and used in cell transplantation studies as donor mice, and NOD/shi-scid mice as recipients.

Cell purification and characterization
Interstitial cells were extracted from the thigh and lower leg muscles of 3-wk-old mice based on an isolation method for intact, living single muscle fibers associated with satellite cells described previously (Bischoff, 1986). Muscles were treated with 0.06% collagenase type IA (Sigma-Aldrich) in DME containing 10% FCS with gentle agitation for 2 h at 37°C. Extracted cells were filtered through a 40 and then a 20 μm nylon mesh, washed, and resuspended in DME containing 20% FCS. The cells were stained with a combination of FITC-, phycoerythrin-, and biotin-conjugated anti–mouse CD34 (RAM34), CD45 (30-F11), CD14 (rmC5-3), CD31 (MEC13.3), CD49d (R1-2), CD117 (c-kit, 2B8), FLK-1 (Ly-73), Sca-1 (Ly6A/E), and CD144 (11D4.1). All antibodies were purchased from PharMingen. Cells were fractionated using a FACS Vantage flow cytometer (Becton Dickinson) equipped with an argon laser tuned to 488 nm.

Clonal cell culture
Purified CD34$^+$/45$^-$ cells (1 × 10^4 cells/ml) were plated in Methocult GFH4434V (StemCell Tech.) complete methylcellulose medium and incubated at 37°C in 5% CO_2 and 95% O_2 with humidity. All cultures were performed in quadruplicate, and scored at 14 d of culture using an inverted microscope. The growing ability of purified CD34$^+$/45$^-$ cells was also determined by clonal cell culture on soft agar–coated (0.7%) dishes in methylcellulose medium.

Immunostaining and immunoelectron microscopy
Localization of CD34$^+$ cells in the interstitial spaces of skeletal muscles was detected by rat anti–mouse CD34 monoclonal antibody (RAM34; PharMingen) and rat anti-laminin monoclonal antibody (Chemicon). The entire lower hindlimb from 3-wk-old mice were quick frozen in isopentane, and 7-μm thick serial cryostat sections were obtained, and then fixed in cold (–20°C) acetone and stained by anti-CD34 (1:200 for over night at 4°C) and laminin (1:2,000 for 2 h at room temperature). Reactions were visualized by streptavidin–biotin complex and 3,3-diaminobenzidine (DAB) and 4-chloro-1-naphthol (both from Wako Pure Chemical). Coexpression of CD45 in the interstitial CD34$^+$ cells were determined by an immunofluorescence analysis using FITC-conjugated rat anti–mouse CD45 monoclonal antibody (PharMingen). Reactions of CD34 was visualized by rhodamine-conjugated goat anti–rat secondary antibody (Chemicon) and observed by confocal laser scanning microscope (LSM 418; Zeiss). The specific cellular composition of each colony was determined with monoclonal anti-MyoD (5.8 A; Dako), uptake of acetylated low density lipoprotein labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine perchlorate (DiI-Ac-LDL; Biomedical Technologies Inc.), and staining with oil-red O (Sigma-Aldrich). When staining for putative myogenic colonies, each colony was manually picked up by fine-tip grass pipette and fixed with 4% paraformaldehyde for 10 min, and prepared for cytospin (1,000 rpm) and then stained with monoclonal anti-MyoD. Adhesive and spreading cells were cultured in DME containing 10 μg/ml DiI-Ac-LDL with 5% FCS for overnight. For an immunoelectron microscopy, 3-wk-old mouse were perfused with warm ringer and 4% para-formaldehyde/0.1 M phosphate buffer (4% PFA/PB). The lower hindlimb muscles were removed and immersed in 4% PFA/PB for overnight, and then quick frozen in isopentane. Several 7-μm thick cross-sections were cut and immunostaining of CD34 was performed. Reactions were visualized by DAB after fixation of 1% glutaraldehyde-0.1 M phosphate buffer, and prepared for electron microscopic analysis.

Cell transplantation
The Sk-34 and CD34$^-$/45$^-$ cells were purified from hindlimb muscles of GFP transgenic mice, and 2 × 10^4 cells were injected directly into the TA muscle of NOD/scid mice (n = 5 for each cell). For detection of engrafted Sk-34 cells from GFP transgenic mice, recipient mice were perfused with warm ringer and 4% PFA/PB. The TA muscles were removed and immersed in 4% PFA/PB overnight. Samples were treated by graded sucrose (5–25%)/PBS and then quick frozen in isopentane. Serial 7-μm thick cross-sections (20–25 sections) were cut. Immunostaining was performed using rat anti-GFP monoclonal antibody (JFP-K2, produced by S.C. Fujita and colleagues at the Mitsubishi Institute of Life Sciences (Tokyo, Japan), 1:10 for 2 h at room temperature). Reactions were visualized by visualized by streptavidin–biotin complex and DAB.

RT-PCR
Total RNA was extracted from EEC, Sk-34 and CD34$^-$/45$^-$ fractions using a total RNA isolation kit (Wako Pure Chemical). Equal amounts of RNA were reverse-transcribed using the RNA-PCR kit version 2.1 (Takara). The paired primers used were as follows: mouse MyoD: ACA TAG ACT TGA CAG GCC CCG A/AGA CCT TCG ATG TAG CGG ATG G (451 base pair); myf-5: GTC AAC CAA GCT TTC GAG ACG/CGG AGC TTT TAT CTG CAG CAC (305 base pair); myf-6: ATT CTG CGG AGT GCC ATC A/TGT TCC AAA TGC TGG CTG AG (356 base pair); Myogenin: TAC GTC CAT CGT GGA CAG CAT/TCA GCT AAA TTC CCT CGC TGG (263 base pair); M-cadherin: TGG AGC GTC AGC CAG ATT AAC/TTG TCC CGA AGG TCC TCT TGT (359 base pair); c-met: CCA AGC CGC GTA TGT CAG TAA/AAT AAG TCG ACG CGC TGC A (304 base pair); Pax-7: GAA AGC CAA ACA CAG CAT CGA/ACC CTG ATG CAT GGT TGA TGG (466 base pair); and Pax-3: CCT GGA ACC CAC GAC CAC GGT GTC/AAC GTC CAA GGC TTA CTT TG (183 base pair) (Goulding et al., 1991). β-Actin: AAC ACC CCA GCC ATG TAC GTA/AAG GAA GGC TGG AAA AGA GCC (409 base pair) was used as the control. The samples were denatured at 94°C for 5 min, followed by amplification rounds consisting of 94°C for 30 s (denaturing), 65°C for 30 s (annealing), and 72°C for 30 s (extension) for 30 cycles, and 72°C for 10 min. For Fig. 4 A, cells were cultured in DME containing 20% FCS without methylcelluose for 3 d, and for Fig. 4 B, Sk-34 cells was cultured in methylcelluose medium for 4 d.

Online supplemental material
Videos 1 and 2, available online at http://www.jcb.org/cgi/content/full/jcb.200112106, show spontaneous, intermittent, and active contractions of large, round cells in Fig. 3 C and many myotubes in Fig. 3 D.

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