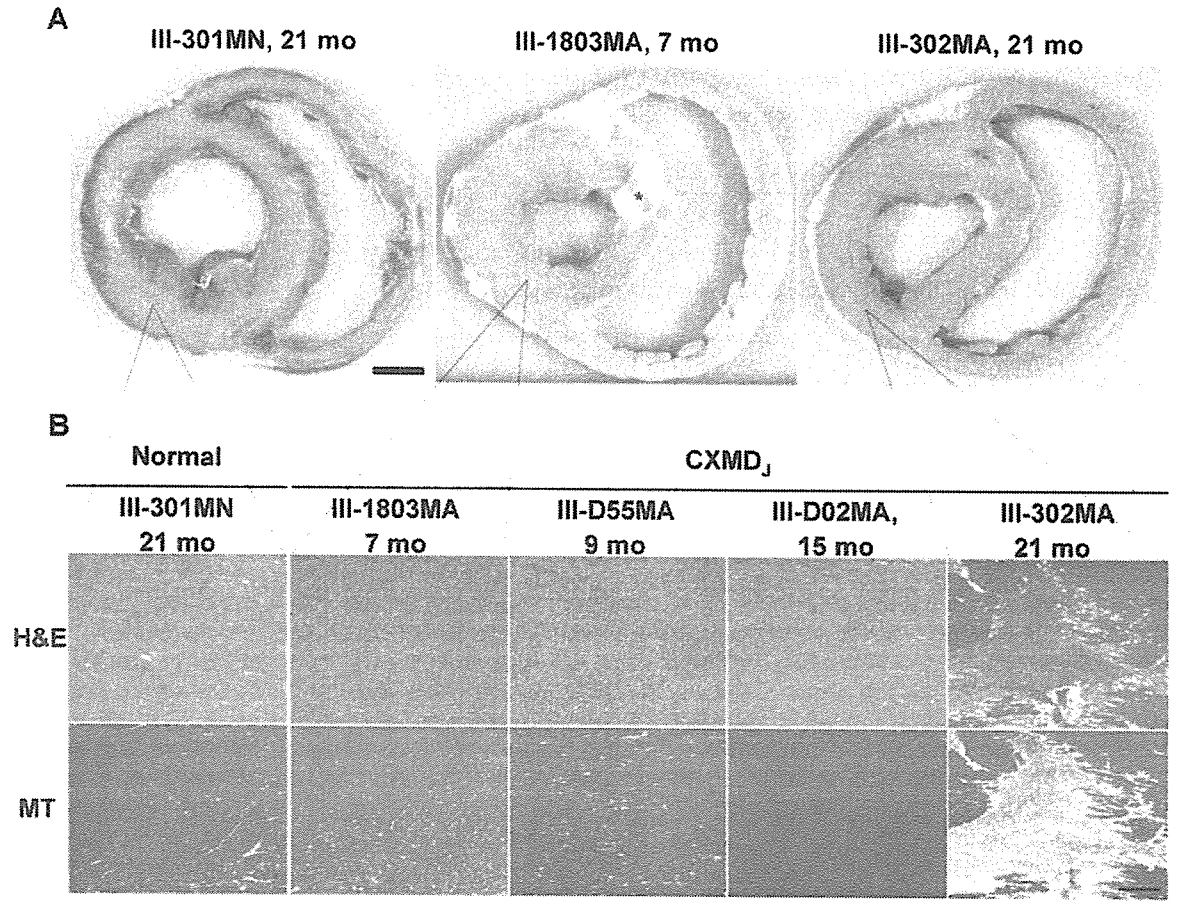

Figure 5
Macroscopic and histopathological findings in CXMD$_J$ hearts A. Macroscopic examinations of the base of the formalin-fixed hearts of a normal littermate III-301MN at 21 months and CXMD$_J$ dogs, III-1803MA at 7 months and III-302MA at 21 months of age. *Aortic valve. Bar shows 1 cm. **B.** Hematoxylin and eosin (H&E) and Masson's trichrome (MT) staining for histopathological evaluation of the left ventricular posterior wall in a normal littermate, III-301MN at 21 months and the CXMD$_J$ dogs, III-1803MA at 7 months, III-D55MA at 9 months, III-D02MA at 15 months, and III-302MA at 21 months of age. Posterior walls of left ventricles of both III-D55MA and III-D02MA were macroscopically normal (data not shown). Bar shows 200 μm.

anisms causing the abnormal Q-waves but also more information on the pathogenesis in the dystrophin-deficient heart.

Competing interests
The author(s) declare that they have no competing interests.

Authors' contributions
NY and NU carried out the electrocardiographic, echocardiographic, and pathological examination and drafted the manuscript. YF performed the electrocardiographic study. MY, KY and MRW participated in the necropsy and pathological examination. MN, YS, MT and AT participated in the maintenance of the dog colony and the design of the study. NM performed the pathological examination. YW participated in the design of the study. AN participated in the statistical analysis and drafted manuscript. ST participated in the design, planning and coordination of the study. All authors read and approved the final manuscript.

Acknowledgements

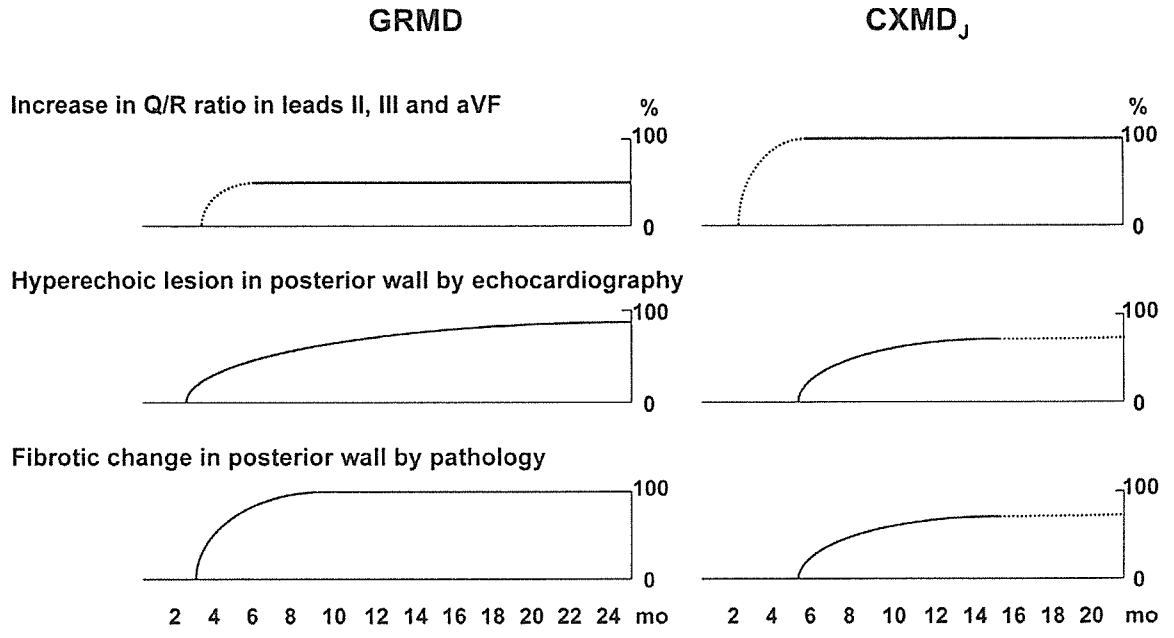

Figure 6

Comparison of cardiac involvement between GRMD and CXMD$_J$ with advancing age. Subjects were compared as follows: increase in Q/R ratio in leads II, III, and aVF in ECG, hyperechoic lesion in posterior wall by echocardiography, and fibrotic change in left ventricular posterior wall by pathology. The data on GRMD was based on the previous literature [22–24]. It is difficult to evaluate Q/R ratio in early stage of GRMD and CXMD$_J$. It is also difficult to evaluate hyperechoic lesion in echocardiogram and fibrotic change in pathology at late stage of CXMD$_J$ due to small numbers of examination ($n < 3$).

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Functional heterogeneity of side population cells in skeletal muscle

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Abstract

Skeletal muscle regeneration has been exclusively attributed to myogenic precursors, satellite cells. A stem cell-rich fraction referred to as side population (SP) cells also resides in skeletal muscle, but its roles in muscle regeneration remain unclear. We found that muscle SP cells could be subdivided into three sub-fractions using CD31 and CD45 markers. The majority of SP cells in normal non-regenerating muscle expressed CD31 and had endothelial characteristics. However, $CD31^-CD45^-$ SP cells, which are a minor subpopulation in normal muscle, actively proliferated upon muscle injury and expressed not only several regulatory genes for muscle regeneration but also some mesenchymal lineage markers. $CD31^-CD45^-$ SP cells showed the greatest myogenic potential among three SP sub-fractions, but indeed revealed mesenchymal potentials in vitro. These SP cells preferentially differentiated into myofibers after intramuscular transplantation in vivo. Our results revealed the heterogeneity of muscle SP cells and suggest that $CD31^-CD45^-$ SP cells participate in muscle regeneration.

Keywords: Side population cells; Muscle regeneration; Mesenchymal differentiation; Transplantation

Adult skeletal muscles have a remarkable ability to regenerate following muscle damage. This regeneration has been attributed to satellite cells that reside between the sarcolemma and the basal lamina. Satellite cells are quiescent mononucleated cells in normal conditions, however, in response to muscle damage, they become activated, proliferate, and then exit the cell cycle either to renew the quiescent satellite cell pool or to differentiate into mature myofibers. Thus, they have been considered to be the myogenic precursor cells that give rise to myoblasts and the sole source of adult myogenic cells [1].

In 1998, Ferrari et al. [2] have demonstrated for the first time that bone marrow (BM)-derived cells contribute to the skeletal muscle after BM transplantation. Side population (SP) cells were first identified in bone marrow based on the ability to exclude Hoechst 33342 dye as an enriched fraction of hematopoietic stem cells (HSCs) [3], later, it has been reported that they also participate in muscle regeneration [4]. Studies using whole BM cells showed that BM-derived mononucleated cells display several characteristics of satellite cells, suggesting that donor-derived BM cells contribute to muscle fibers in a stepwise biological progression [5,6]. However, using single HSC transplantation experiment, Camargo et al. [7] suggested that cells committed to the myeloid lineage contribute to muscle through fusion event. Therefore, multiple mechanisms underlay contribution of BM-derived cells to skeletal muscle regeneration.

SP cells have been also identified in skeletal muscle [4]. Muscle SP cells cannot only reconstitute the hematopoietic system of lethally irradiated mice [4,8], but also differentiate into skeletal muscle cells [4,9]. Furthermore, they have been reported to participate in vascular regeneration [10]. Several lines of evidence suggest that muscle SP cells are a cell population distinct from satellite cells [9,11–13]. While muscle SP cells possess these attractive

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features, they have been reported to be heterogeneous population. In fact, muscle SP cells contain both $CD45^+$ and $CD45^-$ cells, and hematopoietic potential has been exclusively found in $CD45^+$ fraction [8,9]. As regards the myogenic potential, both $CD45^+$ and $CD45^-$ fractions have been shown to differentiate into skeletal muscle cells [9,14], but there is no comparative study dealing with subpopulation of muscle SP cells during muscle regeneration.

In the present study, we have further divided muscle SP cells into three sub-fractions using CD31 and CD45, examined the properties of each sub-fraction, and identified a novel subpopulation ($CD31^-CD45^-$ SP cells) that showed the greatest myogenic potential both in vitro and in vivo. These results provide a new insight for stem cell-based therapy of muscular dystrophy.

Materials and methods

Animals. All procedures using experimental animals were approved by the Experimental Animal Care and Use Committee at the National Institute of Neuroscience. Eight- to ten-week-old C57BL/6 mice were purchased from Nihon CLEA (Japan). GFP Tg mice were provided by Dr. M. Okabe (Osaka University) and used in cell transplantation experiments. NOD/*scid* mice provided by the Institute for Experimental Animals, Japan, were used as recipients.

To induce muscle regeneration, 100 μl of CTX (10 μM in saline, Wako Chemicals) was injected into the tibialis anterior (TA) muscle with a 29-gauge needle. In FACS analysis experiments, CTX was injected into TA (50 μl), gastrocnemius (50 μl), and quadriceps femoris muscles (25 μl).

BM transplantation was performed as previously described [14]. Mice were subjected to analysis 12 weeks after transplantation.

Antibodies. Mouse Bcrp-1 cDNA was provided by Dr. A.H. Schinkel [15]. A DNA fragment corresponding to cytoplasmic domain of Bcrp1, amino acids 300–337, was fused to GST in a pGEX-4T-2 vector (Amersham Biosciences), and the fusion protein was used to immunize rabbits. The serum obtained was affinity-purified. Other antibodies used in these studies are listed in Table S1.

Cell preparation and FACS analysis. Muscle-derived mononucleated cells were prepared from C57BL/6 mice, GFP Tg mice, or GFP-BM transplanted mice as previously described [14]. Hoechst staining was performed as described by Goodell et al. (http//www.bcm.tmc.edu/genetherapy/goodell/new_site/protocols.html). Cells were re-suspended at 10^6 cells per ml in DMEM (Invitrogen) containing 2% FBS (Trace Biosciences), 10 mM Hepes, and 5 μg/ml Hoechst 33342 (Sigma), and incubated for 90 min at 37 °C in the presence or the absence of 50 μM verapamil (Sigma). During incubation, cells were mixed 3–4 times. For analysis of Ac-LDL uptake, 10 μg/ml DiI-labeled Ac-LDL (Biomedical Technologies) was added. After antibody staining, cells were re-suspended in PBS containing 2.5% FBS and 2 μg/ml propidium iodide (PI) (BD PharMingen). Cell sorting was performed on a FACS VantageSE flow cytometer (BD Biosciences). Debris and dead cells were excluded by forward scatter, side scatter, and PI gating. Cell viability after staining and sorting was comparable to that previously reported [14].

RNA extraction and RT-PCR. Total RNA was extracted from 1×10^4 FACS sorted cells by using a RNeasy Micro Kit (Qiagen) and then reverse transcribed into cDNA by using TaqMan Reverse Transcription Reagents (Roche). The PCRs were performed with 1 μl cDNA product under the following cycling conditions: 94 °C for 3 min followed by 40 cycles of amplification (94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s) with a final incubation at 72 °C for 5 min. Specific primer sequences used for PCR are available on request.

Cell culture. SP cells were cultured alone with growth medium (GM); DMEM containing 20% FBS and 2.5 ng/ml bFGF (Invitrogen) in chamber slides (Nalge Nunc) coated with Matrigel (BD Biosciences) for 3–5 days. For osteogenic differentiation, the medium was changed to a differentiation medium (DM), 5% horse serum in DMEM supplemented with or without 500 ng/ml recombinant human BMP2 (R&D Systems), and cultured for 4–6 days. For adipogenic differentiation, cells were exposed to 3 cycles of 3 days of adipogenic induction medium (Cambrex Bioscience) followed by 1 day of adipogenic maintenance medium (Cambrex Bioscience) and then cells were maintained for five more days in the adipogenic maintenance medium. Alkaline phosphatase (AP) was stained using Sigma kit #85 according to the manufacturer's instructions. To stain lipids, cells were fixed in 10% formalin, rinsed in water and then 60% isopropanol, stained with Oil red O in 60% isopropanol, and rinsed in water. For myogenic differentiation, muSP-31, muSP-45, or muSP-DN purified from GFP Tg mice were co-cultured with myoblasts prepared from C57BL/6 mice as previously described [16,17] in GM. DM was supplied 3–5 days after starting co-culture.

Osteogenic activity and myotube-forming activity were determined by the following formulas: osteogenic activity = [the number of AP^+ cells in seven randomly selected fields (corresponding to one-tenth of the whole area of the well)]/(the number of seeded cells) and myotube-forming activity = (the number of GFP^+ myotubes in seven randomly selected fields)/(the number of seeded cells). In order to measure the extent of adipogenic differentiation, stained oil droplets were extracted for 5 min with 100 μl of 4% Nonidet P-40 in isopropanol, and the absorbance of the dye-triglyceride complex was measured at 520 nm [18]; then, adipogenic activity was determined by the following formula: (the absorbance at 520 nm)/(the number of seeded cells).

Intramuscular transplantation experiments. muSP-DN or muSP-31 cells were purified from GFP Tg mice and were injected directly into the TA muscles of NOD/*scid* mice. One day before transplantation, host TA muscles were treated with CTX. The number of transplanted cells is indicated in Table 1. Three weeks after transplantation, TA muscles were excised and fixed in 4% PFA for 30 min, immersed sequentially in 10% sucrose/PBS and 20% sucrose/PBS, and frozen in isopentane cooled with liquid nitrogen.

Immunohistochemistry. FACS sorted cells were collected by Cytospin3 (ThermoShandon). Cells were fixed with 4% PFA for 5 min. Frozen muscle tissues were sectioned using a cryostat. Specimens were blocked with 5% goat serum (Cedarlane) in PBS for 15 min and incubated with primary antibodies at 4 °C overnight, followed by secondary staining. Stained cells were mounted in Vectashield with DAPI (Vector) and photographed using a fluorescence microscope IX70 (OLYMPUS) equipped with a QuantixTM air-cooled CCD camera (Photometrics) and IP Lab software (Scanalytics Inc.). Stained muscle sections were counterstained with TOTO-3 (1:5000; Molecular Probes), then mounted in Vectashield (Vector), and observed under the confocal laser scanning microscope system TCSSP (Leica).

Statistics. Values were expressed as means ± SD or ± SEM. Statistical significance was assessed by Student's *t* test. In comparison of more than two groups, one-way analysis of variance (ANOVA) followed by the Fisher's PLSD was used. A probability of less than 5% ($P < 0.05$) or 1% ($P < 0.01$) was considered statistically significant.

Table 1
Appearance of GFP^+ myofibers after intramuscular transplantation

Cell type	Experiment No.	Number of injected cells/TA muscle	Number of GFP^+ myofibers/TA muscle
muSP-DN cells	Ex. 1	1.7×10^3	14
	Ex. 2	2.5×10^3	9
	Ex. 3	2.5×10^3	0
muSP-31 cells	Ex. 1	1.6×10^4	3
	Ex. 2	1.6×10^4	0
	Ex. 3	1.6×10^4	0

Results

Most muscle SP cells are found in a subset of capillary or vein endothelial cells in non-regenerating skeletal muscle

We identified verapamil-sensitive SP cells in skeletal muscle after Hoechst staining (Fig. 1A) and analyzed the expression of several markers on them. The majority of muscle SP cells were $CD31^+$, usually recognized as a marker of endothelial cells (Figs. 1B–E), and negative for a pan-hematopoietic marker, CD45 (Fig. 1B). More than half of muscle SP cells were $CD34^+$, and $Sca\text{-}1^+$ cells comprised 90% of muscle SP cells (Figs. 1C and D). Compared to FACS profiles of whole-muscle-derived cells, SP cells were enriched in $Sca\text{-}1^+$ cells (Fig. S1). More than 85% of muscle SP cells were $CD31^+$ and took up acetylated low-density lipoprotein (Ac-LDL), a functional marker for endothelial cells and macrophages (Fig. 1E). These results indicate that most muscle SP cells have endothelial characteristics. Only cells in the main population (MP) were found to be $Pax7^+$, indicating that SP cells do not include muscle satellite cells (data not shown).

To examine the localization of muscle SP cells, we generated a rabbit polyclonal anti-mouse Bcrp1 antibody, because it has been reported that Bcrp1 is the major determinant of the SP phenotype [19]. Our antibody clearly recognized Bcrp1 expression in liver, small intestine, and kidney, as previously reported (Fig. S2) [20,21]. We confirmed that Bcrp1 antibody recognizes more than 80% of SP cells and less than 3% of MP cells collected by cytospin (Figs. 1F and G). In skeletal muscle, $Bcrp1^+$ cells were found outside the muscle basal lamina (Fig. 1H), which clearly distinguished $Bcrp1^+$ cells from satellite cells. Next, Bcrp1 expression in the vascular system was investigated. CD31 staining identified all endothelia from larger vessels to capillaries in muscle sections. Intriguingly, Bcrp1 was expressed by CD31-expressing endothelial cells, and its expression was preferentially observed on a subpopulation of capillary endothelium (Figs. 1I–K) and venous endothelium surrounded by thin vessel walls, as revealed by α-smooth muscle actin (αSMA) expression (Figs. 1L–N). These results, together with the results of FACS analysis, strongly suggest that the majority of muscle SP cells are a subset of endothelial cells present in capillaries or veins in non-regenerating skeletal muscle.

Behavior of muscle SP cells during muscle regeneration

We next examined the kinetics of SP cells during muscle regeneration induced by injection of cardiotoxin (CTX). After CTX injection, the total number of mononuclear cells per muscle weight gradually increased, with a peak at day 3. The number of SP cells also increased and reached its peak at day 3 (Fig. 2A). Muscle SP cells could be divided into three subpopulations based on CD31 and CD45 expression: $CD31^+CD45^-$ SP cells (designated muSP-31 cells), $CD31^-CD45^+$ SP cells (muSP-45 cells), and $CD31^-CD45^-$ SP cells (muSP-DN cells). muSP-31 cells and muSP-DN cells distributed throughout the SP tail, but muSP-45 cells were located close to the shoulder (data not shown). The majority of muscle SP cells in untreated muscle were muSP-31 cells (Fig. 1B). During regeneration, however, muSP-45 cells and muSP-DN cells increased in both their ratios and their numbers (Figs. 2B and C). Although $CD45^+$ cells were abundant in whole muscle-derived cells during regeneration and most of them were F4/80 antigen-positive mature macrophages, SP cells did not contain any mature inflammatory cells, as previously reported (data not shown) [14].

To clarify the origin of each subpopulation of SP cells, BM transplantation experiments were performed. We confirmed that muSP-45 cells were mobilized from bone marrow as previously reported (Figs. 3A and B) [14]. In contrast, both $CD45^-$ SP fractions are residents of skeletal muscle (Figs. 3A and B), consistent with the results reported by Rivier et al. [22].

Next, to determine whether each subpopulation of SP cells proliferates in damaged muscle, cells were stained with Ki67 antibody. Most muSP-45 cells (Figs. 3C and D) and muSP-31 cells (Figs. 3G and H) prepared from regenerating muscle were negative for Ki67, suggesting that the proliferation activities of these two fractions were low. On the other hand, about 60% of muSP-DN cells were positive for Ki67 (Figs. 3E and F), indicating that muSP-DN cells actively proliferated during muscle regeneration.

We next examined Bcrp1 expression on three sub-fractionated SP cells and found that only muSP-31 cells were Bcrp1-positive (Fig. 3K). These results suggest that some ABC transporters other than Bcrp1 are responsible for the phenotype of $CD31^-$ SP cells.

Gene expression of muscle SP cells during muscle regeneration

Our analysis revealed that each subpopulation of SP cells showed distinct kinetics during muscle regeneration. To better understand the traits of muscle SP cells, we analyzed gene expression during muscle regeneration. Three subpopulations of SP cells (in following experiments, muSP-45 cells from untreated muscle were omitted because of their low yield) or MP cells were collected from each time point during muscle regeneration, and RT-PCR was performed. We chose several myogenic (*Pax3*, *Pax7*, and *myf5*), endothelial (*Tie2*, *Flk1*, and *vWF*), and mesodermal-mesenchymal-associated (*αSMA*, *PPARγ*, *Runx2*, *PDGFRα*, and *PDGFRβ*) genes to clarify lineage characteristics of the target cells. We also examined expression of genes of developmental regulators (*msx1*, *Frizzled4* (*Fzd4*), *Patched1* (*Ptc1*), and *BMPR1A*), angiogenic factors (*angiopoietin-1* (*ang1*) and *VEGF*), and TGF-β superfamily antagonists (*follistatin* and *DAN*). muSP-DN cells from untreated muscles expressed only *PDGFRβ*, *Ptc1*, *ang1*, *follistatin*, and *DAN* (Fig. 4, cont, lane 1). Neither myogenic nor other lineage-specific markers could be detected in

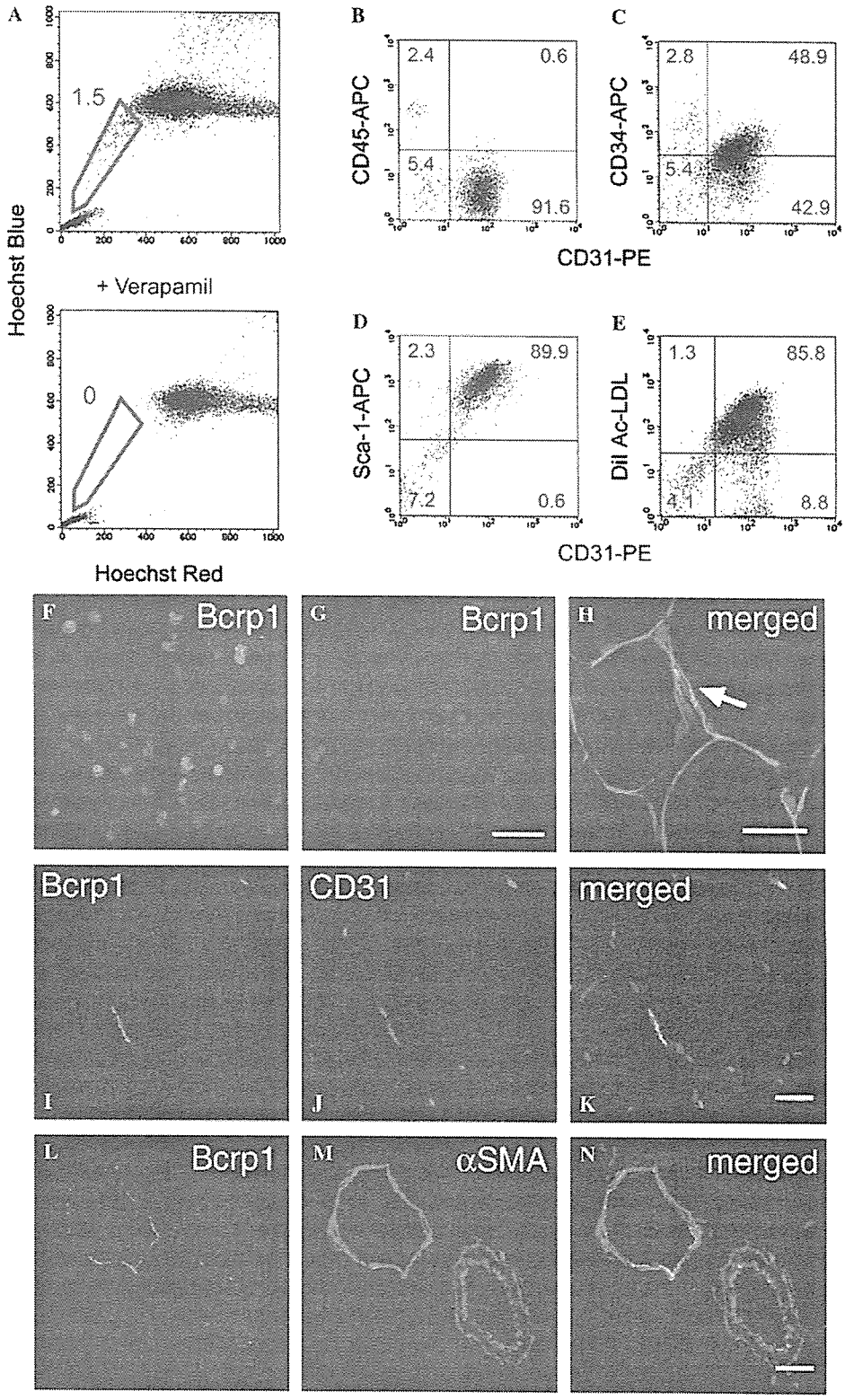

Fig. 1. Characterization of skeletal muscle SP cells. (A) Flow cytometric analysis of muscle-derived mononucleated cells after Hoechst 33342 staining with (lower panel) or without Verapamil (upper panel). The numbers indicate the percentage of SP cells (blue pentagons) in all mononucleated cells. (B–E) The expression of CD45 (B), CD34 (C), Sca-1 (D), and DiI-Ac-LDL uptake (E), and CD31 (B–E) on muscle SP cells. The percentage of cells in each quadrant is shown in the panel. (F,G) Immunofluorescent staining for Bcrp1 (green) and DAPI counterstaining (blue) of freshly sorted SP (F) and MP (G) cells. Immunofluorescent staining for Bcrp1 (green) and laminin α2 chain (red) (H), Bcrp1 (green) and CD31 (red) (I–K), and Bcrp1 (green) and α-smooth muscle actin (red) (L–N). TOTO-3 nuclear staining is shown in merged images (blue in H, K, and N). Bcrp1-positive cells are located outside the basal lamina (arrow), and they are partially overlapped with endothelial cells of capillary (I–K) and vein (L–N). Bars: 50 μm in (F,G), 20 μm in (H–N).

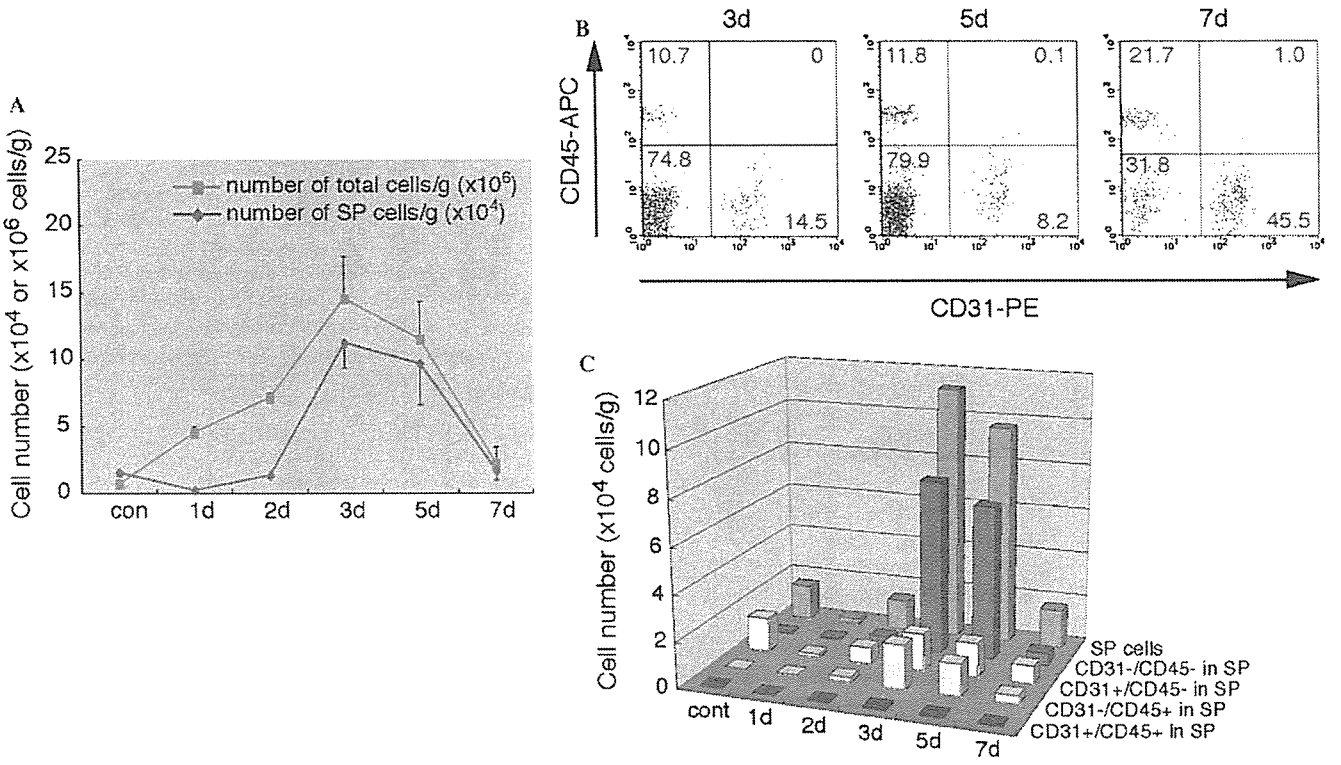

Fig. 2. Behavior of subpopulations of SP cells during muscle regeneration. (A) At 1 day (1d), 2 days (2d), 3 days (3d), 5 days (5d), and 7 days (7d) after CTX injection, the number of total cells (pink line) and SP cells (blue line) per gram of muscle weight was quantified. (B) At 3 days (3d), 5 days (5d), and 7 days (7d) after CTX injection, muscle SP cells prepared from regenerating muscle were analyzed for CD31 and CD45 expression. (C) Cell numbers in subpopulations of SP cells. muSP-45 cells (light blue bar) and muSP-DN cells (dark red bar) were significantly increased in number during muscle regeneration. Values (A,C) are the average of three independent experiments. Error bars represent SD.

this population indicating that muSP-DN cells do not contain cells committed to the lineages tested. At day 3 after CTX injection, muSP-DN cells began to express developmental regulator genes (Fig. 4, 3d, lane 1), and then at day 5, they also began to express several other lineage-specific genes (*Tie2*, *αSMA*, *PPARγ*, and *Runx2*). Angiogenic factors and TGF-β superfamily antagonists were also strongly expressed at this time point (Fig. 4, 5d, lane 1). In contrast, muSP-31 cells continuously expressed all three endothelial genes analyzed throughout the regeneration process (Fig. 4, lane 2). Expression of mature endothelial marker, such as *vWF*, suggests that muSP-31 cells represent committed endothelial cells. muSP-45 cells expressed only low levels of *αSMA*, *PDGFRβ*, and *follistatin* at day 5 after CTX injection (Fig. 4, lane 3). Myogenic markers, *Pax7* and *myf5*, were detected only in the MP fraction (Fig. 4, MP) indicating that myogenic cells are completely sorted into the MP fraction even during the process of muscle regeneration.

Differentiation potential of muscle SP cells for mesenchymal lineages

muSP-DN cells showed a unique gene expression pattern during muscle regeneration process: they began to express several mesenchymal genes at a late phase of muscle regeneration. Therefore, we examined the mesenchymal potentials of muscle SP subpopulations. muSP-DN cells from untreated muscle readily gave rise to alkaline phosphatase (AP)-positive cells when cultured in the presence of bone morphogenetic protein 2 (BMP2) (Figs. 5A and C). With adipogenic induction, they also differentiated into adipocytes containing numerous lipid droplets in the cytoplasm (Figs. 5A and D). Reflecting the results of gene expression analysis, muSP-DN cells from regenerating muscle more efficiently differentiated into osteogenic cells and adipocytes than those from untreated muscle did (Figs. 5B–D). Unexpectedly, muSP-DN cells from regenerating muscle also differentiated into adipocytes without adipogenic induction (Figs. 5B and D), suggesting that they are susceptible to adipogenesis under our culture condition. In contrast, muSP-31 cells did not possess these differentiation potentials (Figs. 5A–D). Nor did muSP-45 cells, which were dramatically mobilized from BM into regenerating muscle (Figs. 5B–D). The attribute of differentiation potential is therefore a feature of muSP-DN.

Myogenic potential of muscle SP cells in vitro

We next evaluated the myogenic potential of muscle SP cells in vitro. When SP cells were cultured alone, they never differentiated into skeletal muscle cells (data not shown). Each subpopulation of SP cells was prepared from GFP Tg mice and co-cultured with wild type (WT) primary

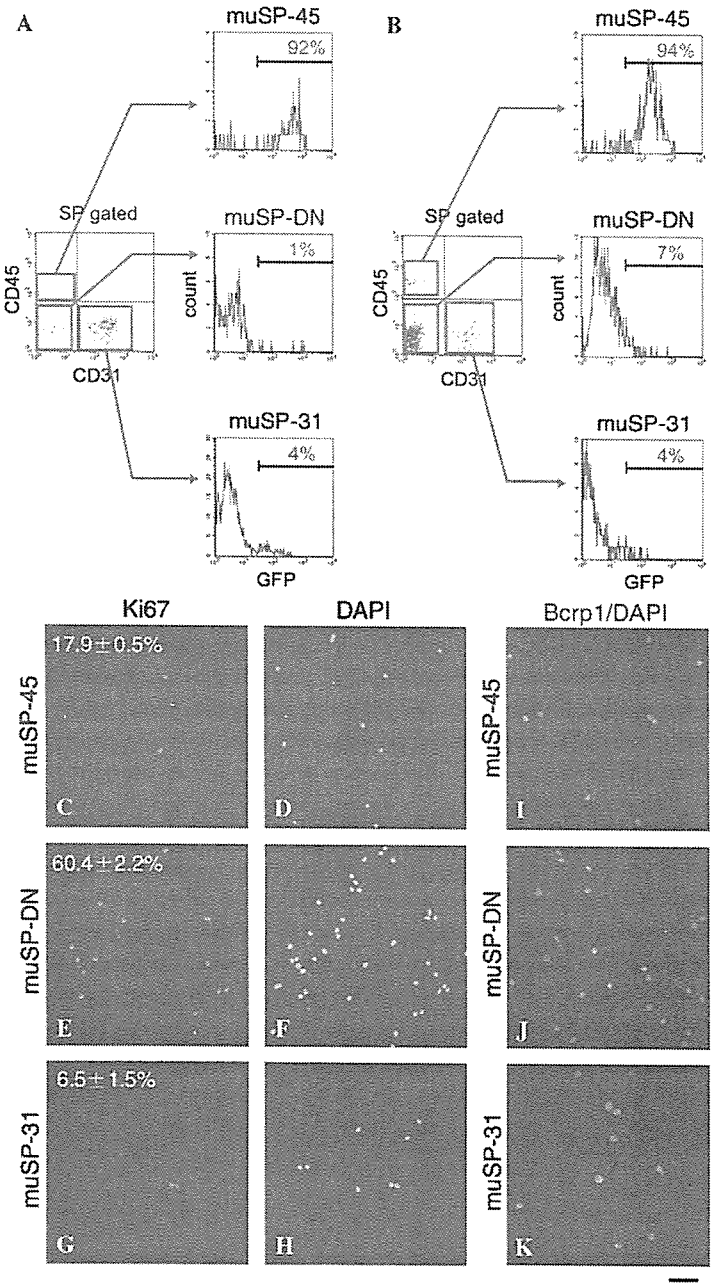

Fig. 3. Origin, proliferative activity, and Bcrp1 expression of subpopulations of muscle SP cells. (A,B) C57BL/6 mice were transplanted with whole BM from GFP Tg mice, and 3 months later, SP cells from untreated muscle (A) or regenerating muscle (3 days after CTX injection) (B) were further analyzed for CD31, CD45, and GFP expression. Note that $CD45^-$ SP cells (middle and lower panels) are almost all negative for GFP, indicating that they do not originate from BM. In contrast, more than 90% of muSP-45 cells were GFP^+ (upper panels). (C–H) Ki67 expression (green) and nuclei stained with DAPI (blue) on muSP-45 (C,D), muSP-DN (E,F), and muSP-31 (G,H) cells. The percentages of Ki67-positive cells were expressed as means ± SD of three independent experiments. muSP-45 (I), muSP-DN (J), and muSP-31 (K) were sorted from regenerating muscle and stained for Bcrp1 (green) and nuclei (blue). Only muSP-31 cells were stained positive for Bcrp1 (K). Bar: 50 μm.

myoblasts derived from satellite cells. muSP-DN cells from untreated muscle rapidly proliferated in vitro as observed in regenerating muscle (Fig. 2C). On the contrary, muSP-31 cells hardly expanded. After 2–3 weeks co-culture, both muSP-DN cells and muSP-31 cells differentiated not only into multinucleated myotubes co-expressing GFP and sarcomeric-α-actinin (Figs. 5E–G, only muSP-DN culture is shown) but also mononucleated myocytes (shown in insets). The frequency of mononucleated myocytes was too low to quantify, but existence of these cells suggests that myogenic differentiation of SP cells could occur without fusion. Strikingly, the myotube-forming activity (the frequency of GFP^+ myotubes, see Materials and methods for details) of muSP-DN cells was approximately 10-fold that of muSP-31 cells (Fig. 5H, lane for cont, 0.026 ± 0.007 vs 0.002 ± 0.001). In the experiments using SP cells from regenerating muscle at 3 days after CTX injection, muSP-DN cells showed the highest

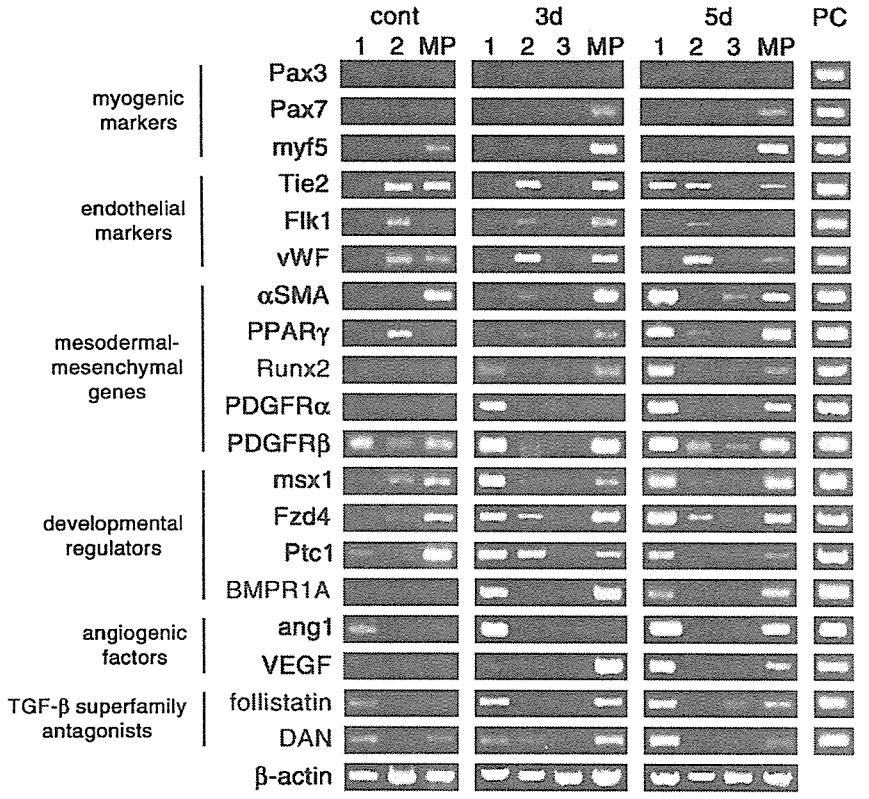

Fig. 4. Gene expression profiles of muscle SP cells during muscle regeneration. muSP-DN (lane 1), muSP-31 (lane 2), muSP-45 (lane 3), or MP cells were collected from untreated (cont) and regenerating muscle at 3 days (3d) or 5 days (5d) after CTX injection, and RT-PCR was performed against the indicated genes. Total embryo extract (E13) was used as a positive control (PC). β-actin was amplified to confirm that the quantities of mRNA were equal.

myotube-forming activity, although each SP subpopulation did form myotubes co-expressing GFP and sarcomeric-α-actinin (Fig. 5H, lane for CTX3d). This clearly demonstrates that muSP-DN cells have the highest myogenic potential among SP sub-fractions in vitro. For comparison, we quantified the myotube-forming activity of satellite cell-derived myoblasts. The value was 0.09 ± 0.01, indicating that myogenic activity of myoblasts is much higher than that of muSP-DN cells.

Myogenic potential of muscle SP cells in vivo

To evaluate the myogenic potential of muscle SP cells in vivo, we performed transplantation experiments. muSP-DN or muSP-31 cells from untreated muscle of GFP Tg mice were directly transplanted into CTX-treated TA muscles of immunodeficient NOD/*scid* mice. Three weeks after transplantation, muSP-DN cells had generated myofibers more efficiently than muSP-31 cells (Figs. 6A and B, and Table 1), indicating that muSP-DN cells had relatively higher myogenic potential in vivo as well as in vitro. Contrary to our expectation, muSP-DN cells formed no GFP-positive adipocytes after transplantation.

Discussion

Muscle SP cells have been suggested to be multipotent and can contribute to skeletal muscle regeneration [4,9,10,23]. However, most of these studies dealt with whole muscle SP cells as one functional unit. We subdivided, for the first time, muscle SP cells using CD31 and CD45 markers and revealed functional heterogeneity of muscle SP cells. $CD31^+CD45^-$ SP cells (muSP-31 cells) are a main subpopulation in non-regenerating muscle, but $CD31^-CD45^-$ SP cells (muSP-DN cells) which represent a minor subpopulation in non-regenerating muscle have the greatest differentiation potentials and become predominant subpopulation of SP cells upon muscle injury.

Differentiation potential of muscle SP cells

Phenotypic and immunohistochemical analysis suggested that muSP-31 cells are a subset of endothelial cells of capillaries and veins. They poorly proliferate after injury or in in vitro culture, and their differentiation potentials are limited both in vitro and in vivo.

$CD45^+$ muscle SP cells (muSP-45 cells) were shown to have both hematopoietic and myogenic potentials, and hematopoietic potential of muscle-derived cells was exclusively found in this fraction [8,9]. We previously reported the contribution of muSP-45 cells to muscle regeneration [14]. In this study, we identified novel subpopulation that possesses much higher myogenic potential than muSP-45, muSP-DN.

muSP-DN cells showed the highest differentiation potential of all the mesenchymal lineages tested among

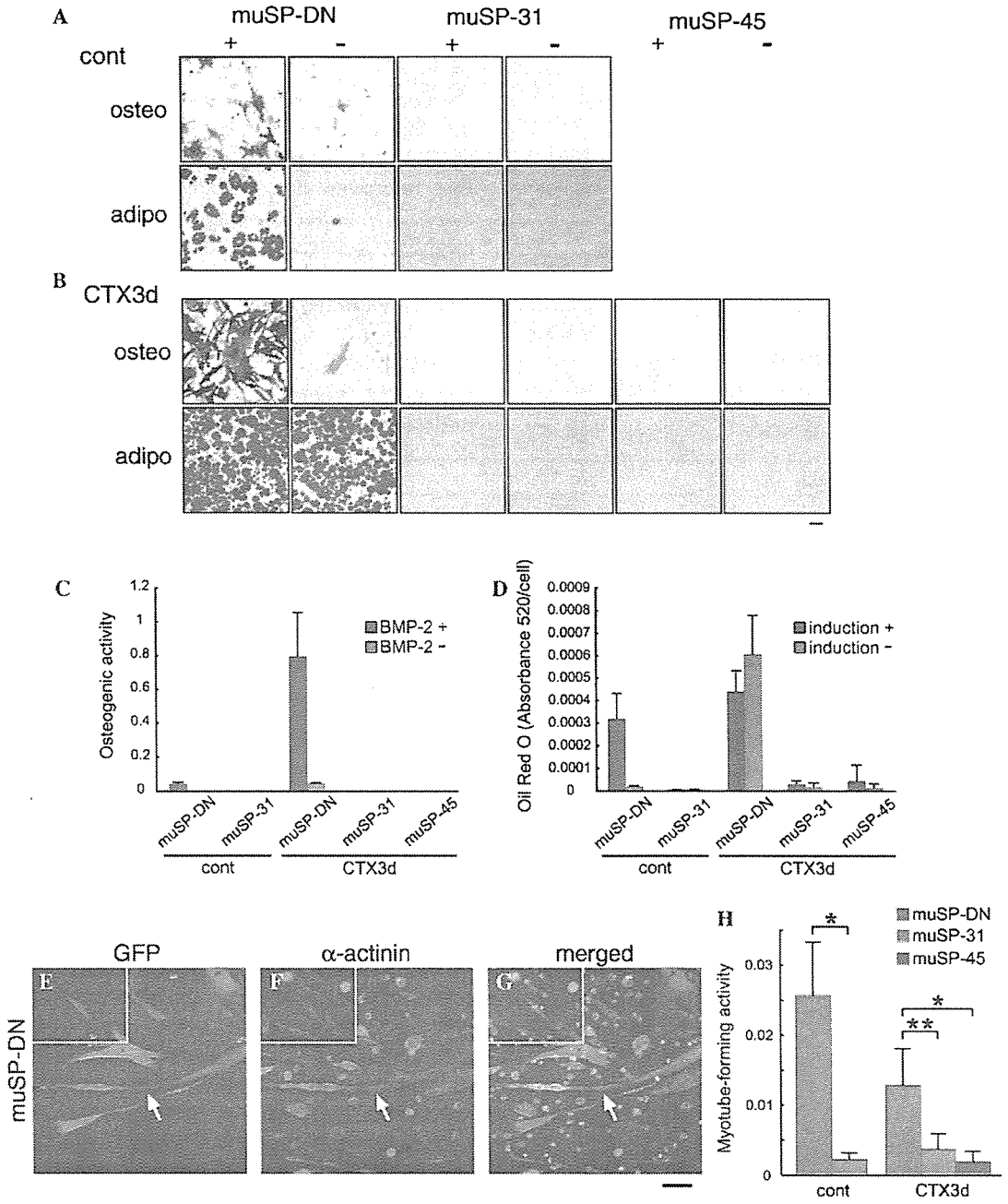

Fig. 5. muSP-DN cells differentiate into osteogenic cells, adipocytes, and skeletal muscle cells. (A,B) Three subpopulations of SP cells prepared from untreated (A) or regenerating (B) muscle were induced to differentiate into osteogenic or adipogenic cells. Uninduced cells (−) and induced cells (+) were then examined for alkaline phosphatase expression (osteo) or oil deposits (adipo). Bar: 50 μm. (C,D) Osteogenic (C) and adipogenic (D) activities of subsets of SP cells prepared from control (cont) or regenerating muscle at 3 days after CTX injection (CTX3d) were quantified. Values are the average of three independent experiments. Error bars represent SD. (E–G) Co-culture of muscle SP cells with myoblasts. muSP-DN cells from GFP Tg mice were sorted and co-cultured with WT primary myoblasts in differentiation medium. Cells were stained with anti-GFP (green) and anti-sarcomeric α-actinin (red) antibodies. Nuclear staining with DAPI (blue) is shown in merged images (G). Insets show GFP^+ mononucleated myocyte. Bar: 50 μm. (H) Myotube-forming activities of muSP-DN cells (red bars), muSP-31 cells (blue bars), and muSP-45 cells (green bar) are shown. Each subpopulation was prepared from untreated (cont) or CTX-treated regenerating muscle (CTX3d). Values are the average of three independent experiments. Error bars represent SD. $^*P < 0.01$, $^{**}P < 0.05$.

SP subpopulations. They were negative for lineage-specific markers under the non-regenerating condition, but after muscle injury or in in vitro expansion, they actively proliferated and were readily induced to express several mesenchymal genes. Their differentiation potential seems to be restricted to mesenchymal lineages because we did not detect hematopoietic colonies derived from muSP-DN cells in vitro and muSP-DN cells failed to rescue the lethally irradiated mice (data not shown). These observations indicate that muSP-DN cells are enriched for primitive mesenchymal cells. This notion is further supported by gene expression pattern of muSP-DN cells. muSP-DN cells

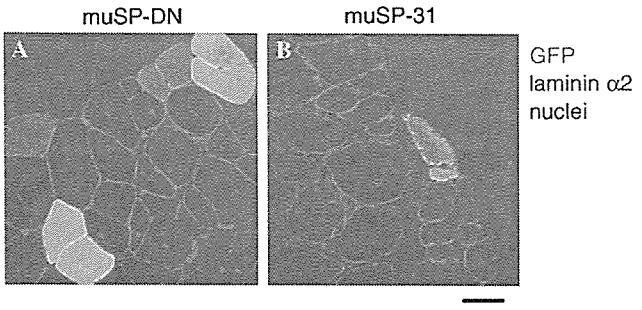

Fig. 6. muSP-DN cells participate in myofiber formation during muscle regeneration. (A,B) muSP-DN (A), muSP-31 (B) were transplanted into CTX-injected NOD/*scid* muscles. Each subpopulation was prepared from untreated muscle of GFP Tg mice. Muscle sections were stained with anti-GFP (green) and anti-laminin α2 (red) antibodies 3 weeks after transplantation. More GFP-positive myofibers were detected in muSP-DN-transplanted muscles (A) than in muscles transplanted with muSP-31 cells (B). Bar: 40 μm.

specifically expressed *angl* under the non-regenerating condition and during the early phase of regeneration (Fig. 4, lane 1, cont or 3d). Perivascular cells, such as pericytes, express angl [24,25], and several groups suggest that multipotent mesenchymal stem cells may be derived from pericytes [26–28]. A recent report demonstrated that vascular mural precursor cells are negative for endothelial markers but positive for Tie2 and smooth muscle cell markers [29]. Likewise, muSP-DN cells were negative for *Flkl* and *vWF* throughout the regeneration process (Fig. 4, lane 1), but began to express *Tie2* and *αSMA* during late phases of regeneration (Fig. 4, lane 1, 5d). Given the similarity between muSP-DN cells and those reported perivascular primitive cells, muSP-DN cells would represent perivascular primitive mesenchymal cells in skeletal muscle.

Roles of muscle SP cells in muscle regeneration

muSP-DN cells actively proliferated and significantly increased in number upon muscle injury. The precise fate of muSP-DN cells has remained to be determined, since the number of muSP-DN cells returned to normal level at late stage of muscle regeneration.

We noted that angiogenic factors and TGF-β superfamily antagonists were strongly expressed in muSP-DN cells during muscle regeneration. Previous reports showed that $Ptcl^{+}$ interstitial mesenchymal cells in muscle produce angiogenic factors, including *angl*, and promote muscle regeneration after ischemia [30,31]. Some members of the TGF-β superfamily, such as myostatin and TGF-β1, are known to act as negative regulators of myogenesis [32,33]. Inversely, one of the TGF-β superfamily antagonists, follistatin, has been reported to promote myoblast recruitment and fusion [34]. Therefore, muSP-DN cells might promote muscle regeneration by producing regeneration-regulating factors.

muSP-DN cells preferentially differentiate into myogenic cells after intramuscular transplantation, implying that normal muscle environment facilitates myogenic differentiation of muSP-DN cells. However, we revealed that muSP-DN cells have a high tendency to differentiate into osteogenic or adipogenic cells in vitro. Therefore, it is possible that muSP-DN cells differentiate into osteogenic or adipogenic cells in some pathological conditions such as Duchenne muscular dystrophy [35,36]. Recent finding that microvascular pericytes can differentiate into adipocytes [37] further supports the notion that muSP-DN cells might be implicated in pathological changes.

In conclusion, we identified novel subpopulation of muscle SP cells, $CD31^{-}CD45^{-}$ SP cells, which possesses capacity of mesenchymal differentiation in vitro and reveals myogenic differentiation potential in vivo. Our findings might provide new insights that may well be useful in understanding adult skeletal muscle regeneration and in designing therapeutic strategies of muscular dystrophy.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.01.037.

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Bone Marrow Stromal Cells Generate Muscle Cells and Repair Muscle Degeneration

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Bone marrow stromal cells (MSCs) have great potential as therapeutic agents. We report a method for inducing skeletal muscle lineage cells from human and rat general adherent MSCs with an efficiency of 89%. Induced cells differentiated into muscle fibers upon transplantation into degenerated muscles of rats and mdx-nude mice. The induced population contained Pax7-positive cells that contributed to subsequent regeneration of muscle upon repetitive damage without additional transplantation of cells. These MSCs represent a more ready supply of myogenic cells than do the rare myogenic stem cells normally found in muscle and bone marrow.

Cell transplantation therapy offers hope for the treatment of intractable muscle degenerative disorders. Embryonic stem (ES) cells and stem cells derived from muscle have been considered as candidates for transplantation therapy (*1–7*). Although they have great potential, they face limitations inherent in procurement from fetal tissue, including problems relating to histocompatibility and ethical concerns. Although muscle stem cells and satellite cells can be isolated from adult and prenatal tissues (*2*, *4–6*), the number of cells that can be harvested may be limited. Bone marrow is another source of myogenic stem cells (*3*, *8*); however, because the stem cell population is very small, the problem of inadequate tissue supply for therapeutic scale again arises.

Because bone marrow stromal cells (MSCs) are easy to isolate and expand rapidly from patients without leading to major ethical and technical problems, they have great potential as therapeutic agents. However, despite their potential for use in cell transplantation therapy, practical application to human muscle degenerative diseases depends on the ability to control their differentiation into functional skeletal muscle cells with high efficiency and purity. Recently we reported that efficient induction of neurons, without glial differentiation, from human and rat MSCs could be achieved by Notch1 intracellular domain (NICD) gene transfer and administration of certain trophic factors (*9*). Further addition of glial cell line–derived neurotrophic factor (GDNF) effectively induced dopamine-producing cells and resulted in functional recovery when those cells were grafted into the brains of Parkinson's disease model rats (*9*). Here we report a method to systematically and efficiently induce skeletal muscle lineage cells

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with high purity from a large population of adherent MSCs, rather than from a rare subpopulation of myogenic stem cells contained in the bone marrow. The induced population effectively differentiated into mature myotubes with some cells persisting as Pax7-positive satellite cells that continued to function in host muscle to restore degenerating muscles in the absence of repeated transplantations. Because our induction system uses a large population of adherent MSCs, which can be easily isolated and expanded, functional skeletal muscle cells including satellite cells can be obtained on a therapeutic scale in a short time period.

General adherent MSCs were established as described [(*10*), Note1]. After three passages, induction was initiated. The induction procedure and corresponding phase contrast images taken at each step are shown (Fig. 1, A and B). Human and rat MSCs plated at a set cell density ([(*10*), Note1] were treated with basic fibroblast growth factor (bFGF), forskolin (FSK; known to up-regulate intracellular cyclic adenosine 3′,5′-monophosphate), platelet-derived growth factor-AA (PDGF), and neuregulin for 3 days (cells at this stage are referred to as C-MSCs). The C-MSCs were then transfected with an NICD expression plasmid by lipofection followed by G418 selection and allowed to recover to 100% confluency (referred to as CN-MSCs). Although MyoD expression was detected in CN-MSCs (Fig. 2J), the frequency of spontaneous cell fusion (the fusion index) was very low ["percentage nuclei incorporated in myotubes (*11*)" was <0.1%] in both rat and human CN-MSCs 5 days after cells reached 100% confluency. To confirm the potential of CN-MSCs to differentiate into multinucleated myotubes, we supplied cells with either 2% horse serum or ITS (insulin-transferrin-selenite) serum-free medium, both of which promote differentiation of myoblasts to myotubes (*11*, *12*). The fusion index was ~24% at 5 days after administration of 2% horse serum or 12% by ITS serum-free medium (Fig. 1A). A much higher production of differentiated myotubes was observed based on the appearance of a muscle phenotype that mainly arose from the spontaneous differentiation of original MSCs (*13*). Because horse serum is not appropriate for clinical usage, and cell survival and myotube formation were unsatisfactory in ITS serum-free medium, we searched for alternative conditions. We found that the supernatant of the original MSCs was also an effective inducer, with a fusion index of about 20% at 5 days after administration and plateauing at ~40% 14 days after induction (Fig. 1C). In the following experiments, we used MSC supernatants for the fusion induction and refer to CN-MSCs treated with supernatant of MSCs as M-MSCs (muscle-MSCs). Rat CN-MSCs and M-MSCs displayed the same features as human MSC-derived cells. Some multinucleated cells in both rat and human M-MSCs exhibited spontaneous contraction in vitro. Furthermore, these multinucleated cells expressed MyoD, myogenin (Fig. 2, A and B), skeletal myosin (Fig. 2F), myosin heavy chain (MHC) (Fig. 2, A, B, and D), and troponin (Fig. 2E), exhibiting skeletal myotube characteristics (*11*). The multinucleated cells appeared postmitotic as determined by p21 immunostaining (Fig. 2C, arrows) and 5-bromo-2′-deoxyuridine (BrdU) incorporation (Fig. 2D) (*12*). In addition to multinucleated cells and MyoD-positive mononucleated cells, cells immunopositive for Pax7 (Fig. 2F, arrows) and c-MetR (Fig. 2E, arrows), both markers for muscle satellite cells (*14*, *15*), were detected. These data suggest that M-MSCs consist of skeletal muscle lineage cells.

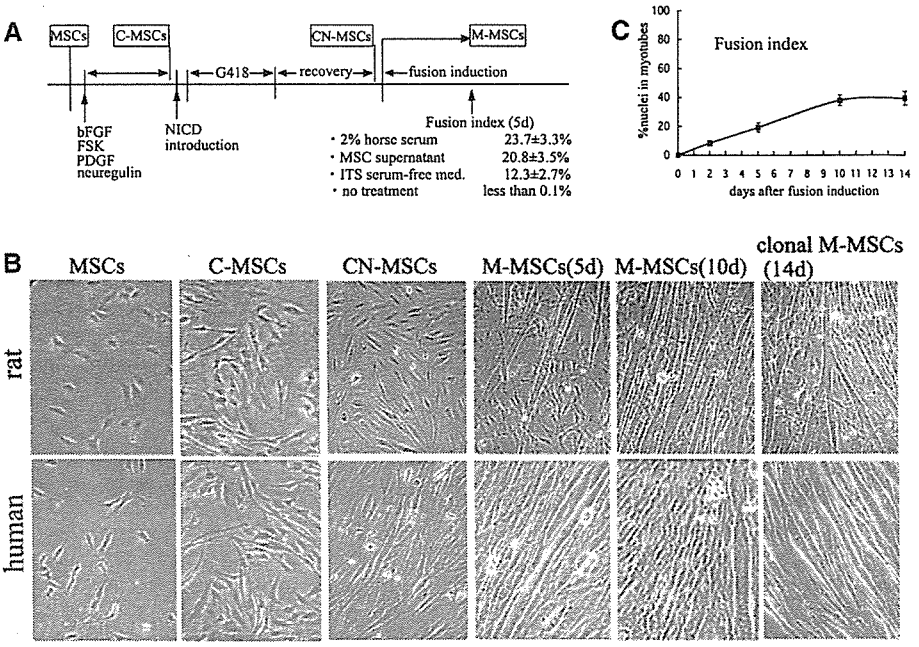
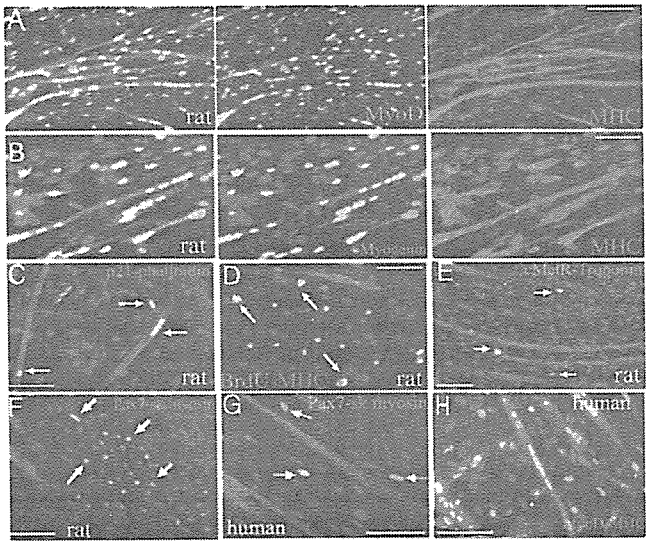


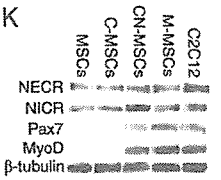
Fig. 1. Induction of skeletal muscle lineage cells. (A) Schematic diagram of the induction process. When human CN-MSCs reached 100% confluency, fusion induction was initiated. Fusion indexes were estimated after 5 days in human M-MSCs. For the cytokine treatment, omission of bFGF resulted in a major reduction of the fusion index in human M-MSCs (5 days; 0.5 ± 0.1%). Singular omission of Neuregulin, PDGF, or FSK singly resulted in fusion indexes of 1.8 ± 0.6%, 2.1 ± 0.4%, and 2.5 ± 0.7%, respectively. (B) Phase contrast microscopy of rat and human cells at each step and of clonal-M-MSCs (14 days). (C) Fusion indexes of human M-MSCs upon administration of human MSC supernatant.

Although most M-MSCs seemed to consist of skeletal muscle lineage cells, the possible existence of nonmuscle elements could not be neglected. We therefore subjected human and rat M-MSCs to single-cell clonal culturing (clonal-M-MSCs) and showed that ~89% of viable clones formed multinucleated cells at 14 days in vitro (Fig. 1B). Our results indicated that a large majority of proliferation-competent cells in M-MSCs possess myogenic potential. Clonal-M-MSCs were also shown to develop into MHC, skeletal myosin and MyoD-expressing multinucleated cells, MyoD-positive mononucleated cells, and Pax7-positive mononucleated cells as observed in their parental M-MSC population (Fig. 2, G and H). The ratios of MyoD-, myogenin-, and Pax7-positive cells to the total clonal-M-MSC cell number are shown in Fig. 2I.

To understand the induction events leading from MSCs to M-MSCs, we investigated the expression of genes related to myogenesis in these cells by means of reverse transcription–polymerase chain reaction (RT-PCR) (Fig. 2J). In MSCs, Pax3, Six1, and Six4 were detected, whereas Pax7, MyoD, and myogenin were not. In C-MSCs, Pax3 was down-regulated, whereas Pax7 expression was detected [(*10*), Note 2], which persisted in CN-MSCs and M-MSCs. Expression of MyoD and myogenin was found in CN-MSCs and M-MSCs. These results were confirmed by Western blot analyses (Fig. 2K). Myf6/MRF4, a marker for mature skeletal muscle (*16*), was detectable only in M-MSCs (Fig. 2J). Whereas expression of Six1 and Six4 persisted in M-MSCs, another myogenic factor, myf5, was not detected in any MSC-derived cells (Fig. 2J). This induction process mimicked some aspects of conventional skeletal muscle development in that Pax3, Pax7, MyoD, Myogenin, and Myf6/MRF4, all of which are related to muscle development (*11*, *12*, *14*, *16*), could be detected in a sequential manner. However, because the characteristics of MSCs used in this induction system are different from those of the conventional myogenic progenitor cells, it is possible that some of the mechanisms might differ, especially in the initial step in which MSCs are converted to MyoD-positive CN-MSCs. For this initial step, cytokine pre-



I	2 day	5 day
Pax7	17.9±1.7	3.3±0.8
MyoD	65.3±2.8	67.2±1.2
Myogenin	11.0±1.1	19.1±1.5



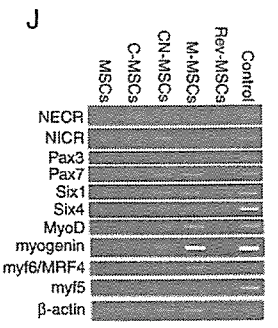


Fig. 2. Characterization of induced cells. (A to H) Immunocytochemical analysis of rat M-MSCs (10 days) (A to F) and human clonal-M-MSCs (14 days) (G and H). In (D), arrows indicate BrdU-incorporated mononucleated cells after 2 hours' incubation. Bars, 50 μm. (I) Ratios of Pax7-, MyoD-, and Myogenin-positive cells in rat clonal-M-MSCs. (J) RT-PCR and (K) Western blot of rat MSCs, C-MSCs, CN-MSCs and M-MSCs (5 days), and Rev-MSCs (5 days after fusion induction). In RT-PCR, the positive control (Control) is C2C12 cells, except for Pax3, which used ES cells. Notch extracellular region (NECR; corresponding to endogenous Notch) and intracellular region (NICR; corresponding to endogenous plus exogenous Notch) were detected in MSCs, suggesting that MSCs are endogenously expressing a small amount of Notch. After transfection with an NICD expression plasmid (CN-MSCs), NICR was up-regulated. The down-regulation of NECR in Rev-MSCs corresponds to the neuronal induction data in our previous report; when MSCs are first transfected with NICD, endogenous expression of Notch is down-regulated (9). β-tubulin was used as a loading control.

treatment and the subsequent NICD transfection are critical for MSC-derived cells to acquire competence for myogenic induction. Indeed, when we reversed the order of cytokine treatment and NICD transfection, muscle-lineage markers were not detected (Fig. 2J; Rev-MSCs), nor were multinucleated cells observed (*17*). The expression profiles of Notch and Hes genes during myogenic induction processes and effects of Notch/Hes signaling in the muscle induction system are described in (*10*), Note 3. Furthermore, we induced re-expression of NICD in CN-MSCs and estimated its effects on myogenic differentiation by analyzing the expression of MyoD and the fusion induction [(*10*), Note 3].

Bone marrow contains a small population of myogenic stem cells known to express c-Kit, CD45 and CD34 (*2*–*7*). However, the major population of MSCs is negative to these markers [(*10*), Note 1]. To exclude the possibility that the production of muscle-lineage cells was due to the vast proliferation of myogenic stem cells contained in MSCs, we isolated human MSCs negative for c-Kit, CD45, and CD34 by fluorescence-activated cell sorting (FACS) and subjected them to the induction process (Fig. 3A). We confirmed that isolated cells could also be driven to become muscle-lineage cells as efficiently as the unsorted MSCs. The data from rat MSCs were essentially identical to those from human MSCs. Thus, in our system, it appears that the major population of MSCs, rather than a small fraction of bone marrow–derived myogenic stem cells, contributes to the production of muscle lineage cells.

We next tested the differentiation of clonal-M-MSCs in vivo by transplantation into animals. Human clonal-M-MSCs were labeled by means of a green fluorescent protein (GFP)–encoding retrovirus and then transplanted by local injection (L.I.) into muscles or by intravenous injection (I.V.) into immunosuppressed rats whose gastrocnemius muscles were damaged with cardiotoxin pretreatment (*18*). Two weeks after transplantation, GFP-labeled clonal-M-MSCs incorporated into newly formed immature myofibers, and most of the GFP-positive myofibers exhibited centrally located nuclei in both L.I.- (*17*) and I.V.- (Fig. 3, B and D) treated animals. The incorporation ratios of human and rat GFP-positive cells at 2 weeks are indicated in (*10*), Note 4. Four weeks after transplantation, 60 to 70% of the GFP-positive myofibers exhibited mature characteristics with peripheral nuclei just beneath the plasma membrane (Fig. 3, E to G). Functional differentiation of grafted human clonal-M-MSCs was also confirmed by the detection of human dystrophin in GFP-labeled myofibers (Fig. 4A). In both L.I.- and I.V.-treated animals (4 weeks after injection), GFP-labeled human-derived cells were not detected in the host brain, heart, liver, kidney, and nondamaged muscles (*17*), suggesting that transplanted cells incorporate only into the damaged tissues. However, in the lung, a small number of rat and human GFP-positive cells were detected in the I.V.-treated animals (4 weeks), but not in the L.I.-treated animals. These findings indicate that clonal-M-MSCs are able to incorporate into damaged muscles and contribute to regenerating myofiber formation, regardless of the transplantation method.

In addition, some of the transplanted cells were observed between the plasma membrane and laminin-positive basal lamina that surround distinct myofibers (Fig. 3I). Because these cells expressed the satellite cell marker Pax7 (*14*) (Fig. 3H), they might be retained as satellite cells and/or developed into satellite cells in the host muscle. The ratios of transplanted Pax7/GFP-positive cells within total Pax7-positive satellite cells (transplanted and host satellite cells) are described in (*10*), Note 4. It is believed that muscle satellite cells contribute to regenerating myofiber formation upon muscle damage (*19*). We examined whether the transplanted satellite-like cells were able to function as satellite cells in vivo. Four weeks after transplantation of human clonal-M-MSCs (I.V.), cardiotoxin was readministered into the same muscles without additional transplantation just after the muscles were biopsied. The biopsies confirmed that 60 to 70% of GFP-positive myotubes displayed peripheral nuclei (Fig. 4A). Two weeks after the second cardiotoxin treatment (6 weeks after initial transplantation), we observed many regenerating GFP-positive myofibers with centrally located nuclei (Fig. 4B), and 16.5 ± 4.7% (mean ± SD; n = 4) of myofibers in the damaged area were GFP-positive. These results suggest that the Pax7-positive cells retained in the host muscle function as satellite cells, contributing to muscle repair. This implies that, upon transplantation of clonal-M-MSCs to muscles of patients, cells retained as satellite cells in clonal M-MSCs should be able to continue to contribute to future muscle regeneration. Similar characteristics were observed with rat clonal-M-MSCs (*17*).

Transplantation of muscle-lineage cells offers a potential therapeutic approach for the treatment of muscle degenerative disorders such as Duchenne muscular dystrophy. We therefore locally injected GFP-labeled human clonal-M-MSCs into cardiotoxin-pretreated

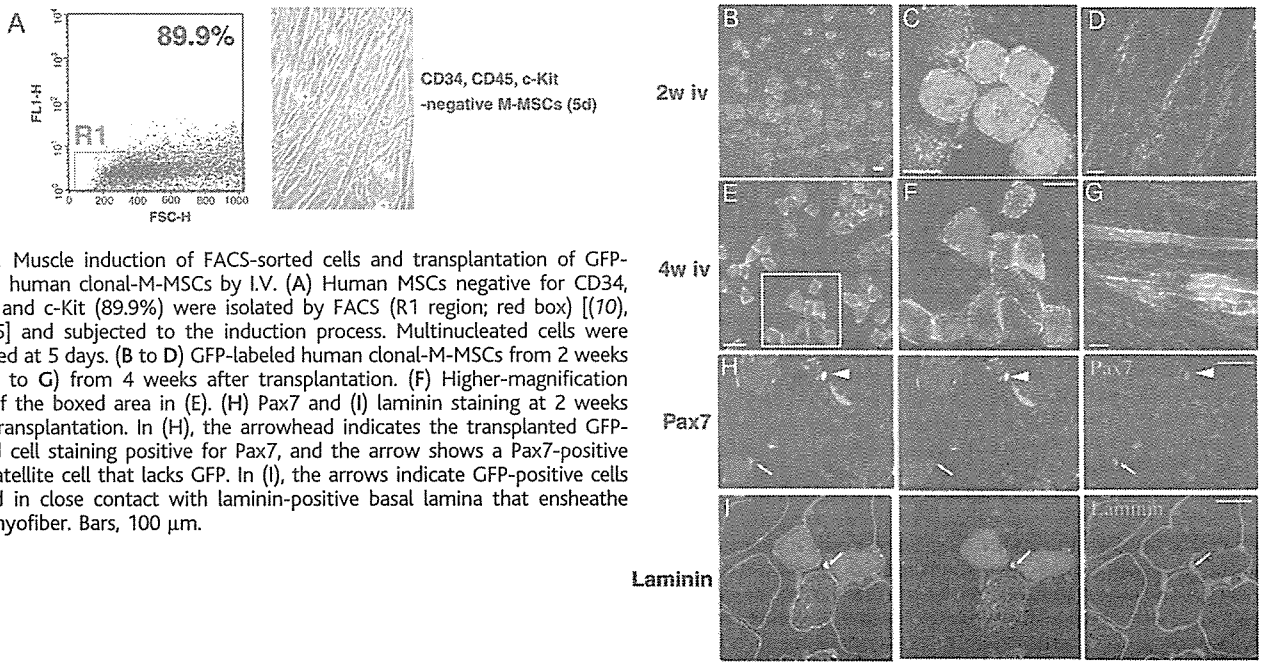


Fig. 3. Muscle induction of FACS-sorted cells and transplantation of GFP-labeled human clonal-M-MSCs by I.V. (A) Human MSCs negative for CD34, CD45, and c-Kit (89.9%) were isolated by FACS (R1 region; red box) [(*10*), Note 5] and subjected to the induction process. Multinucleated cells were observed at 5 days. (B to D) GFP-labeled human clonal-M-MSCs from 2 weeks and (E to G) from 4 weeks after transplantation. (F) Higher-magnification view of the boxed area in (E). (H) Pax7 and (I) laminin staining at 2 weeks after transplantation. In (H), the arrowhead indicates the transplanted GFP-labeled cell staining positive for Pax7, and the arrow shows a Pax7-positive host satellite cell that lacks GFP. In (I), the arrows indicate GFP-positive cells located in close contact with laminin-positive basal lamina that ensheathe each myofiber. Bars, 100 μm.

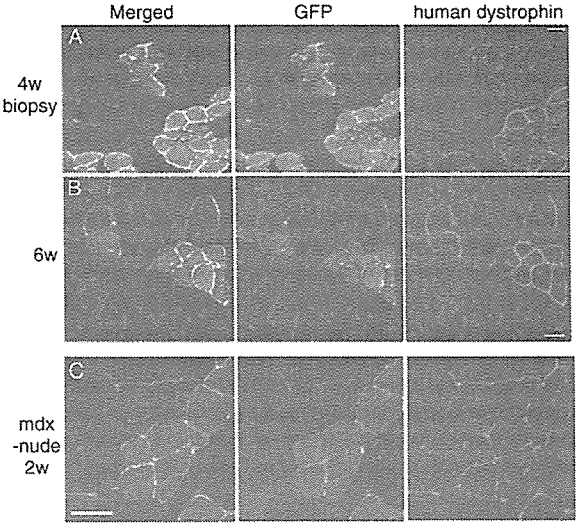


Fig. 4. Regeneration of human clonal-M-MSC transplanted rat (A and B) and mdx-nude mouse (C) gastrocnemius muscles after cardiotoxin treatment. (A) Specimen obtained from biopsy at 4 weeks by I.V. transplantation. Human dystrophin (red) is expressed by GFP-labeled transplanted cells. (B) After biopsy, cardiotoxin was administered and 2 weeks later (6 weeks after human clonal-M-MSC transplantation), gastrocnemius muscles were examined. Human dystrophin could be detected in GFP-labeled regenerating muscle fibers with centrally located nuclei. (C) Expression of human dystrophin in GFP-labeled cells in mdx-nude mouse gastrocnemius muscle after 2 weeks. Bars, 100 μm.

muscles of mdx-nude mice, which genetically lack dystrophin expression. Immunohistochemistry revealed the incorporation of transplanted cells into newly formed myofibers, which expressed human dystrophin 2 weeks after transplantation (Fig. 4C).

Compared to the various stem cell systems that have been reported (*1*, *20*–*22*), our MSCs offer several important advantages. First, MSCs can easily be obtained from patients or bone marrow banks and can be expanded efficiently in vitro. In the case of MSCs derived from inherited muscle dystrophy patients, genetic manipulation is possible after the isolation and expansion of MSCs. Second, transplantation of MSC-derived cells should encounter fewer ethical problems, because the use of these cells avoids the embryonic stem cell controversy and is in theory similar to bone marrow transplantation, which is currently in wide use for patients with leukemia, refractory anemia, etc. Third, autologous transplantation of MSC-derived muscle cells or transplantation of these cells with the same HLA (human leukocyte antigen) subtype from a healthy donor should minimize the risks of rejection. Because our induction system does not depend on a rare stem cell population, but can use the general population of adherent MSCs, which can be easily isolated and expanded, functional skeletal muscle cells can be obtained within a reasonable time on a therapeutic scale. At present, there are no effective therapeutic approaches for muscle dystrophy. Although the mechanism of muscle induction by NICD introduction remains to be clarified, we believe that our MSC differentiation system may contribute substantially to a major advance toward eventual cell-based therapies for muscle disease.

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Materials and Methods
Figs. S1 to S7
Tables S1 and S2
References

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Cellular and Molecular Life Sciences

Review

Insights into autotransplantation: the unexpected discovery of specific induction systems in bone marrow stromal cells

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Abstract. Many kinds of cells, including embryonic stem cells and tissue stem cells, have been considered candidates for transplantation therapy for neuro- and muscle-degenerative diseases. Bone marrow stromal cells (MSCs) also have great potential as therapeutic agents since they are easily isolated and can be expanded from patients without serious ethical or technical problems. Recently, new methods for the highly efficient and specific induction of functional neurons and skeletal muscle cells have been developed for MSCs. These induced cells were transplanted into animal models of stroke, Parkinson's disease and muscle degeneration, resulting in the successful integration of transplanted cells and improvement in the behavior of the transplanted animals. Here I describe the discovery of these induction systems and focus on the potential use of MSC-derived cells for 'autocell transplantation therapy' in neuro- and muscle-degenerative diseases.

Keywords. Mesenchymal cell, transdifferentiation, regenerative medicine, cell therapy, transplantation, Schwann cell, neuronal differentiation, myogenic differentiation.

Introduction

Neurodegenerative diseases, such as Parkinson's disease and brain ischemia, and muscle-degenerative diseases, such as muscular dystrophy, are responsible for a decline in neuronal and muscular function which often limits the life span. While transplantation of liver, kidney, and bone marrow has already been performed on thousands of patients, transplantation of the nervous system and general muscle tissue has faced many limitations. Effective therapeutic strategies still need to be developed. In the central nervous system (CNS), where neurons become post-mitotic after birth, neural cell transplantation is one potential treatment of such neurologic disorders. As for muscles, satellite cells are considered stem cells in adult muscle tissue, although the difficulty isolating a sufficient number of pure satellite cells has precluded their use in cell-based tissue repair [1, 2] Furthermore, there is a need to establish cell therapies using healthy donors since muscle dystrophies are inheritable diseases.

Recently, embryonic stem (ES) cells and tissue stem cells have aroused a great deal of interest because of their potential for treating degenerative diseases. ES cells are known to differentiate into various kinds of cells including neurons and skeletal muscle cells, either by spontaneous differentiation or following certain induction methods [3–5].

Tissue-specific stem cells have been identified in various tissues at more advanced developmental stages. Neural precursors and/or progenitors have been identified in developing and adult CNS tissues [6–10]. These cells have the ability to self-renew and the potential to differentiate into neurons, astrocytes and oligodendrocytes. For neuronal cell replacement, transplantation of neural stem cells (NSCs) has been attempted in a wide range of animal models of diseases and injuries such as Parkinson's disease, Huntington's disease, stroke, and spinal cord injury,

and functional improvement has often been reported [11–15]. Stem cells and satellite cells isolated from adult and prenatal muscle tissue [16–19] and myogenic stem cells from bone marrow [20, 21] are considered to be sources of cell replacement, and several attempts have been made to ameliorate muscle degeneration by transplantation of these muscle stem cells [20]. Although tissue stem cells have great potential, they face limitations inherent in procurement from fetal tissue, including problems of histocompatibility and ethical concerns.

Bone marrow contains a category of nonhematopoietic cells that can be cultivated *in vitro* as plastic adherent cells, namely bone marrow stromal cells (MSCs) [22]. MSCs are mesenchymal elements that normally provide structural and functional support for hematopoiesis and express mesenchymal markers but lack hematopoietic surface markers [23, 24]. The great benefit of MSCs is that they are easily accessible through aspiration of the bone marrow from patients. This strategy avoids ethical issues, enabling us to use them for 'auto-cell transplantation therapy'. They are also easily expanded on a large scale; for example, 20–100 ml of bone marrow aspirate provides 10^7 cells within 2–3 weeks, a plentiful number for transplantation.

At the present time, the benefits of MSCs for transplantation therapy are twofold. First, the transient trophic effect of MSCs can delay cell death and restore the tissues [25–29] and, second, the multipotency of MSCs gives rise to 'cells with a purpose' for cell-based transplantation therapy.

According to a hierarchical paradigm, MSCs differentiate into mesenchymal lineage cells such as osteocytes, chondrocytes and adipocytes [22, 30, 31]. Recently, however, the unorthodox plasticity of MSCs has been described, as they have the ability to cross oligolineage boundaries which were previously thought to be impenetrable. In fact, it has been suggested that various kinds of cells are inducible from MSCs both *in vivo* and *in vitro*. The possibility of MSC plasticity and transdifferentiation was initially described in *in vivo* experiments, where transplanted donor bone marrow-derived cells differentiated into glial cells in the recipient brain [32]. In the case of muscle, infused bone marrow cells integrated into host muscles and supported regeneration [20]. While these studies suggested the plasticity of MSCs because of the expression of donor markers and cell-specific markers, the clonality and functions of these transdifferentiated cells were not clearly evaluated in some cases. Moreover, there has been the suspicion that these phenomena are based on cell fusion or spontaneous transdifferentiation at a very low frequency [33, 34].

Apart from these *in vivo* experiments, there have been several *in vitro* attempts to induce MSCs into purposeful cells such as cardiomyocytes with cardiac muscle properties, hepatocytes, insulin-producing cells, and airway epithelial cells. However, some of these reports had a low induction efficiency [35–38]. Nevertheless, the potential of MSCs to transdifferentiate from mesenchymal lineages to other lineages is now of great interest. It is clear that MSCs will represent good candidates for practical cell-based therapy if their differentiation into target cells can be controlled with high efficiency and purity.

Recently, a method was developed which systematically induced neurons, skeletal muscle cells (Fig. 1) and Schwann cells from human and rat MSCs on a therapeutic scale [39–41]. This review describes the discovery of systemic induction, the properties of induced cells, and finally their potential, advantages, and disadvantages for clinical application in neurodegenerative and muscle-degenerative diseases. Schwann cells, peripheral glia known to support axonal regeneration both in the peripheral nervous system (PNS) and CNS, are also inducible from human and rodent MSCs [39, 42, 43]. MSC-derived Schwann cells elicited axonal regeneration and functional recovery in spinal cord injury. The utility of these induced Schwann cells has been reviewed elsewhere [44, 45].

Systems for inducing neurons and skeletal muscle cells from MSCs; the fruit of unexpected discovery

Specific induction of neurons from MSCs

Recently, my research team established a new method to induce neurons systematically from human and rat MSCs. Highly efficient and specific induction of post-mitotic, functional neuronal cells, without glial differentiation, can be achieved by gene transfer of Notch1 intracellular domain (NICD) followed by the administration of certain trophic factors [40] (Fig. 1). However, all these findings are the fruit of an unexpected discovery.

The initial goal of this MSC study was to develop an efficient Schwann cell induction system from MSCs for application to spinal cord injury. A series of experiments demonstrated that transplanted Schwann cells can delay nerve cell death and promote regeneration of nerve fibers and functional recovery when supplied to the damaged spinal cord [46]. However, it is difficult to obtain a sufficient amount of Schwann cells. To cultivate Schwann cells for autologus transplantation in humans, for example, another PNS must be sacrificed. Furthermore, there are other technical difficulties in harvesting and expanding Schwann cells from PNS. Therefore, it would be more desirable to establish cells with Schwann cell characteristics from sources other than the PNS that are easily accessible and capable of rapid expansion. MSCs were thought to be a good candidate.

As described previously, induction of Schwann cells was finally established using a reducing reagent, retinoic acid, and trophic factors related to Schwann cell development [44, 45]. However, I first tried to induce Schwann cells

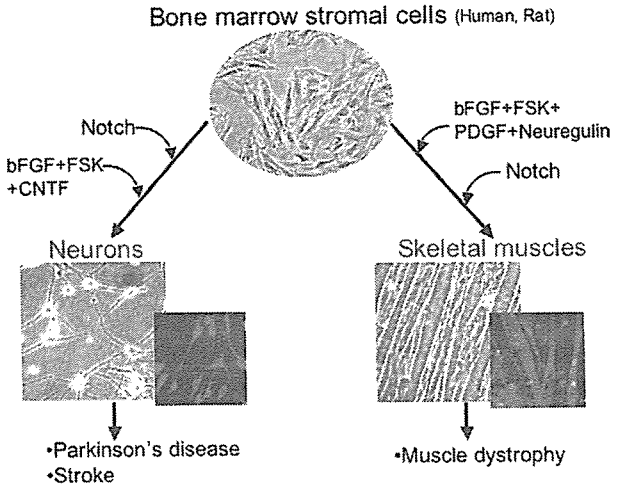

Figure 1. Schematic diagram of the induction system for neurons and skeletal muscle cells. Neurons are induced by Notch intracellular domain gene transfer followed by administration of trophic factors basic fibroblast growth factor (bFGF), forskolin (FSK) and ciliary neurotrophic factor (CNTF). The final population consisted mostly of neurons immunopositive for neuronal markers such as neurofilament. Skeletal muscle cells could be obtained by the reverse treatment, namely trophic factor treatment with bFGF, FSK, Platelet-derived growth factor (PDGF) and neuregulin, followed by Notch gene transfer.

from MSCs by Notch transfection. The Notch gene encodes a 300-kDa single transmembrane cell surface receptor protein that is activated by Delta/Serrate/Lag-1 ligands presented by neighboring cells [47]. Upon ligand binding, the intracellular portion of the Notch receptor is cleaved and enters the nucleus, where it influences the expression of numerous transcription factors related to progenitor pool maintenance, cell fate, and, in the case of the nervous system, terminal specification as glial cells [47–49]. In fact, a series of studies have shown that when Notch signaling is activated, astrocytes and Schwann cells differentiate from NSCs and neural crest stem cells, respectively [48, 49]. Initially, it was expected that MSCs would shift from mesenchymal to Schwann cell characteristics by Notch introduction when combined with administration of trophic factors related to Schwann cell development, such as basic fibroblast growth factor (bFGF), ciliary neurotrophic factor (CNTF) and forskolin (FSK), known to upregulate intracellular cyclic AMP [50]. After such treatment, however, it was very surprising to see a small population of neuron-like cells induced in the final product. The experiment was repeated and the original method was improved to establish the neuronal induction system from MSCs and to examine the properties of induced cells.

The mouse NICD cDNA was subcloned into pCI-neo, a cytomegalovirus (CMV) promoter-containing mammalian expression vector, and transfected into human and rat MSCs by lipofection followed by G418 selection [40]. After transfection with NICD, the MSCs substantially upregulated markers related to NSCs and/or neuronal progenitor cells (NPCs), such as the glutamate transporter GLAST, 3-phosphoglycerate dehydrogenase (3-PGDH) and nestin [51, 52]. This suggested that MSCs may acquire some of the characteristics of NSCs/NPCs when NICD is introduced (Fig. 2).

Next, cells were subcultured once (60–70% confluence) with administration of the trophic factors bFGF, FSK, and CNTF for 5 days, which resulted in a highly efficient and specific induction of cells with neuronal characteristics (Fig. 2) [40]. It was crucial that the cell density of NICD-transfected MSCs be reduced by subculture just before the administration of trophic factors. Some cells already started to extend neurite-like processes 6 h after trophic factor administration. However, if the cell density was too high, neurites attached to the neighboring cells soon after their extension, thereby retracting their neurites and preventing the differentiation of a neuron-like morphology. Therefore, adequate intercellular distance and timing of trophic factor stimulation are crucial for the MSCs to become neurons. Nontransfected as well as control vector-transfected MSCs could not be induced to neurons by trophic factors, indicating that NICD transfection is necessary for MSCs to acquire neuronal potential [40].

These MSC-derived neuronal cells (MSC-Ns) extended neurite-like processes with abundant varicosities and expressed neuronal markers such as MAP-2ab, neurofilament-M, and beta-tubulin isotype3. Approximately 96% of cells were immunopositive for MAP-2ab, although nearly 2% of nestin-positive cells could also be recognized. MSC-Ns did not proliferate when subcultured after trypsin treatment. Indeed, Brd-U incorporation performed 5 days after trophic factor administration showed

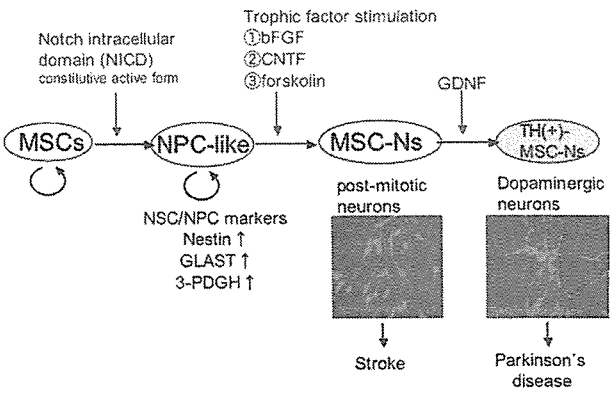

Figure 2. An outline of the neuronal induction system. After NICD transfection, MSCs become similar to NSCs/NPCs (NPC-like), since they express nestin, GLAST and 3-PGDH. After trophic factor stimulation, cells became post-mitotic neurons (MSC-Ns) expressing neuronal markers such as neurofilament. These neurons are effective in the stroke rat model. After administration of GDNF, post-mitotic neurons became dopamine-producing cells [TH(+)-MSC-Ns], useful in the Parkinson's disease model.