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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

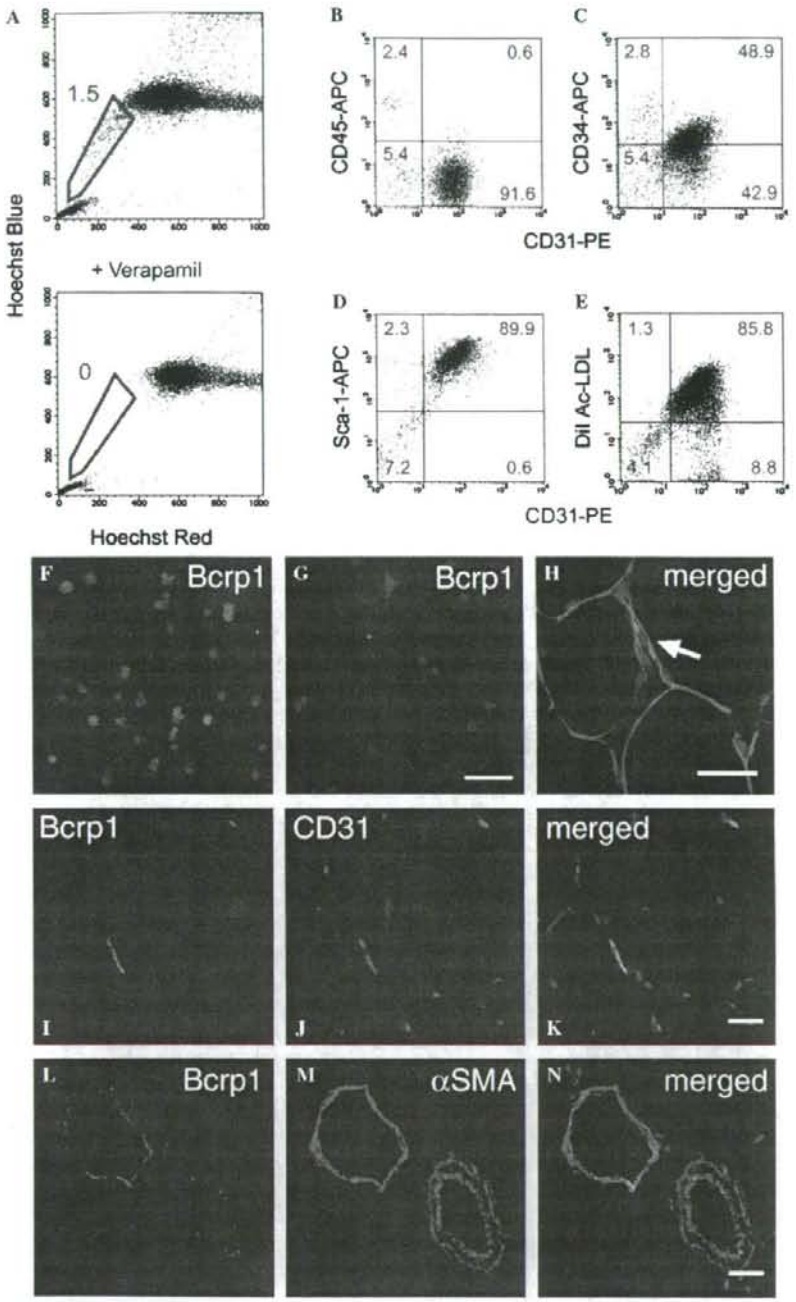

Fig. 1. Characterization of skeletal muscle SP cells. (A) Flow cytometric analysis of muscle-derived mononucleated cells after Hoechst 33342 staining with (lower panel) or without Verapamil (upper panel). The numbers indicate the percentage of SP cells (blue pentagons) in all mononucleated cells. (B–E) 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).

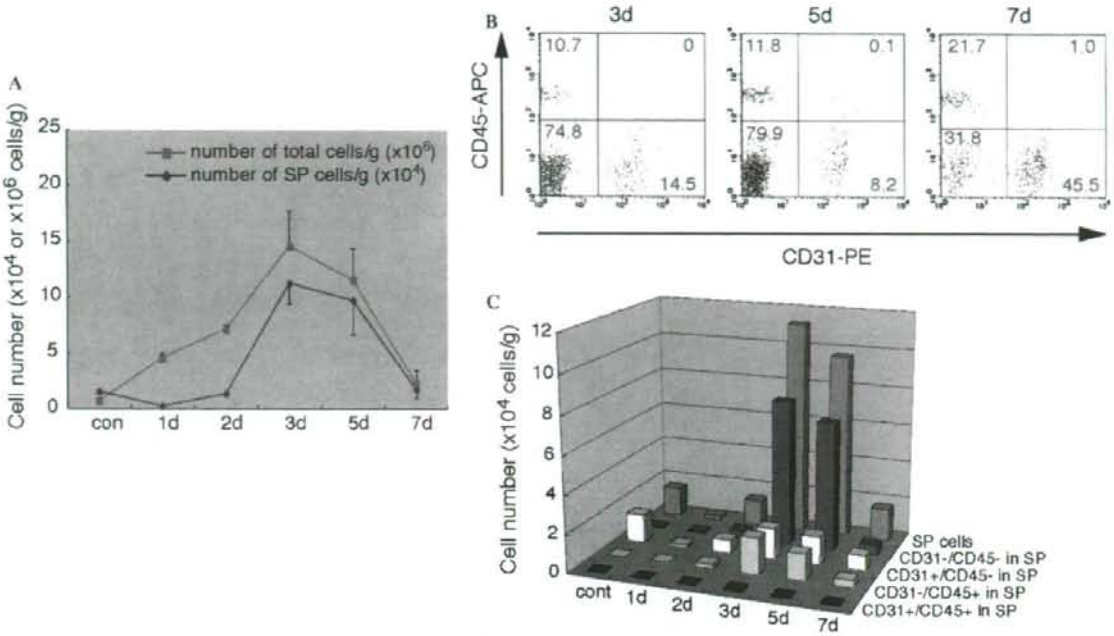

Fig. 2. Behavior of subpopulations of SP cells during muscle regeneration. (A) At 1 day (1d), 2 days (2d), 3 days (3d), 5 days (5d), and 7 days (7d) after CTX injection, the number of total cells (pink line) and SP cells (blue line) per gram of muscle weight was quantified. (B) At 3 days (3d), 5 days (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

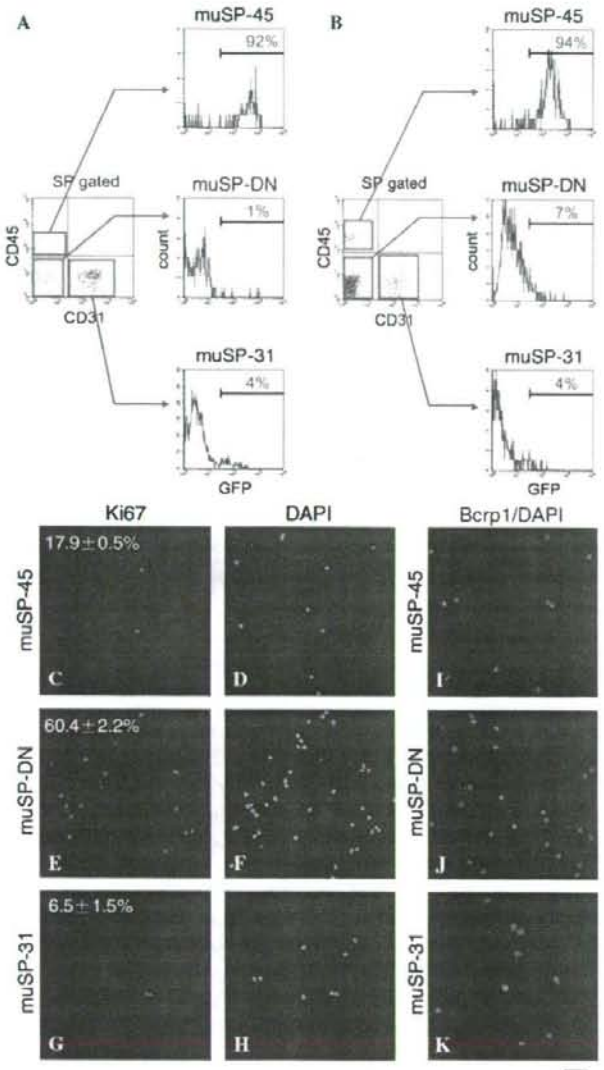

Fig. 3. Origin, proliferative activity, and Bcrp1 expression of subpopulations of muscle SP cells. (A,B) C57BL/6 mice were transplanted with whole BM from GFP Tg mice, and 3 months later, SP cells from untreated muscle (A) or regenerating muscle (3 days after CTX injection) (B) were further analyzed 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

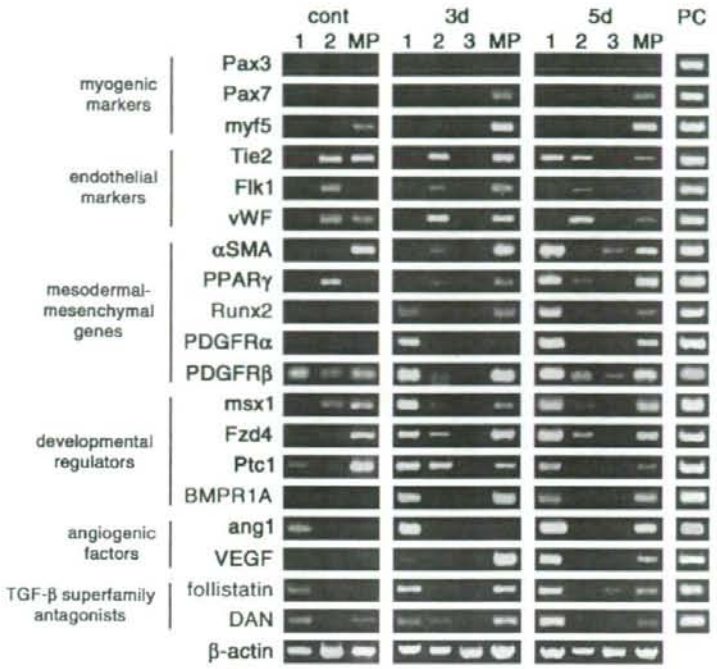

Fig. 4. Gene expression profiles of muscle SP cells during muscle regeneration. muSP-DN (lane 1), muSP-31 (lane 2), muSP-45 (lane 3), or MP cells were collected from untreated (cont) and regenerating muscle at 3 days (3d) or 5 days (5d) after CTX injection, and RT-PCR was performed against the indicated 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

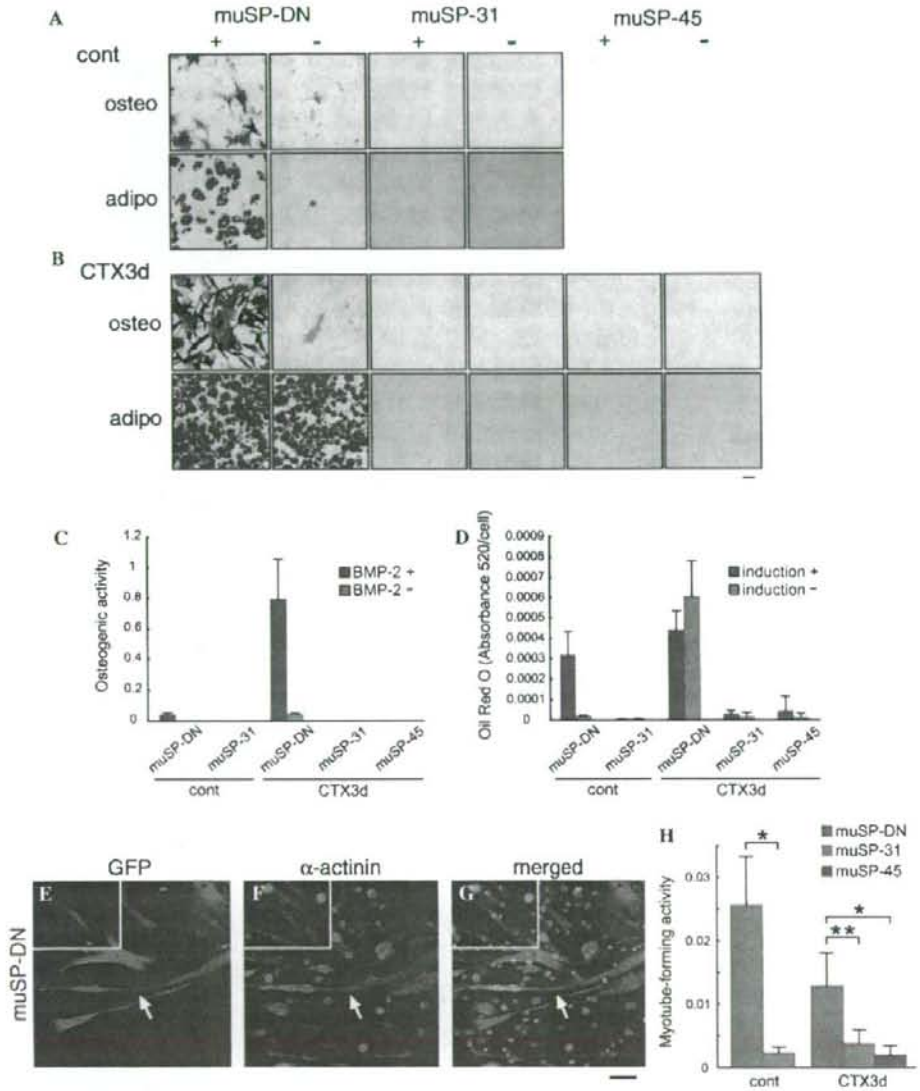

Fig. 5. muSP-DN cells differentiate into osteogenic cells, adipocytes, and skeletal muscle cells. (A,B) Three subpopulations of SP cells prepared from untreated (A) or regenerating (B) muscle were induced to differentiate into osteogenic or adipogenic cells. Uninduced cells (−) and induced cells (+) 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

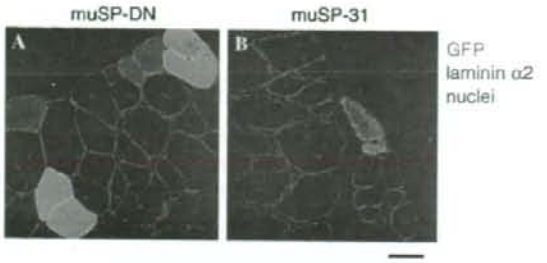

Fig. 6. muSP-DN cells participate in myofiber formation during muscle regeneration. (A,B) muSP-DN (A), muSP-31 (B) were transplanted into CTX-injected NOD/*scid* muscles. Each subpopulation was prepared from untreated muscle of GFP Tg mice. Muscle sections were stained with anti-GFP (green) and anti-laminin 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 *Flk1* 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 *Ptc1*$^{+}$ 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.

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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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筋ジストロフィーと細胞移植治療
――骨髄間葉系細胞からの骨格筋細胞の誘導

Cell-based therapy for muscle degenerative diseases：induction of skeletal muscle cells from bone marrow stromal cells

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◎幹細胞を用いる細胞移植治療は，次世代の医療としてその発展が期待されている．とりわけ，重篤な疾患である筋ジストロフィーについてもその早い確立が切望されているが，移植細胞の候補として筋芽細胞や各種幹細胞，筋肉に含まれている増殖可能な幹細胞，ES 細胞，あるいは胎児の細胞から筋肉細胞を誘導する方法などが検討されている．今回，著者らは骨髄間葉系細胞から効率よく筋細胞を誘導する方法を開発したので紹介する．

Key word：間葉系幹細胞，分化転換，Notch，筋ジストロフィー，筋衛星細胞

筋ジストロフィーを代表とする筋肉変性疾患に対しては遺伝子治療や薬剤投与などをはじめ各種の方法が試みられており，そのひとつとして細胞移植治療の開発も取り組まれてきた．さまざまな種類の細胞が移植治療の対象として検証されてきており，筋芽細胞(myoblast)は，Duchenne muscular dystrophy(DMD)モデルマウスに移植するとdystrophin を発現する筋肉になるということで先駆的な研究として注目を浴びた[1]．しかし，1990年代に臨床試験が行われたが機能改善がみられないという結果に終わっており，それは免疫拒絶によって生着する細胞数が減少するからであろうとの結論であった[2]．筋芽細胞のほかに mesangioblasts(vessel-associated progenitor cell ともいわれている)[3]，血液あるいは筋肉由来の $CD133^+$細胞[4]，side population(SP)細胞[5]なども筋変性モデルへ移植され，その有効性が報告されており，細胞移植治療の候補として考えられている．

とりわけ重要なものに骨格筋幹細胞があげられるであろう．骨格筋組織に存在する幹細胞(muscle-derived stem cells：MDSC)は，筋線維とそれを取り巻く基底膜との間に存在する筋衛星細胞であることが知られており，当然ながらこの細胞は筋肉組織の再生の元となるため，細胞の分離と樹立が研究されている[6]．しかし，生体外では増殖力が低下するため，移植に必要な大量培養が困難であるなどの問題もある．同時に ES 細胞，あるいは胎児の細胞から筋肉細胞を誘導する方法が検討されてきており，細胞移植治療に大きな可能性が見出されつつあるが，倫理問題や安全性，細胞数確保などの問題もある．

今回，著者らの研究グループは骨髄間葉系細胞から骨格筋系譜の細胞を誘導する方法を開発した．この細胞のもつメリットや問題点などに焦点を当て，筋細胞移植治療の可能性について考察したい．

骨髄間葉系細胞とは

骨髄には造血系細胞とは別に間葉系の細胞がある．造血系の細胞は浮遊細胞であるので，培養皿に接着しないが，骨髄間葉系細胞はよく接着するため，骨髄液を培養すると，この性質の違いによって接着性細胞として容易に採取することが可能である[7]．ここで念頭におかなくてはならないのは，

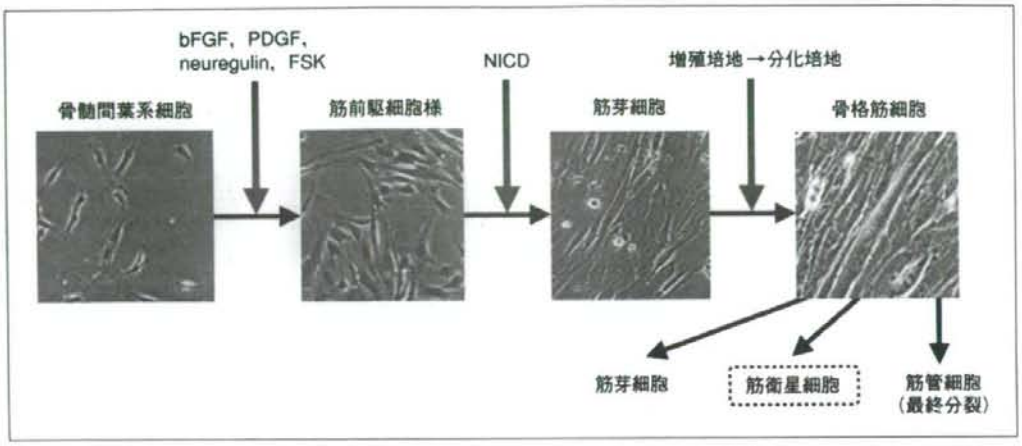

図 1　ヒト骨髄間葉系細胞からの骨格筋系細胞の誘導法

このようにして採取された細胞は間葉系細胞ではあるものの，遺伝子発現や細胞表面抗原などにおいてはけっして均質な細胞群ではなく，ヘテロな集団であるということである．実際に FACS 解析を行うと，間葉系のマーカーとされている CD29, CD90 などはほとんどの細胞に出ているが，一方，CD34，c-Kit など造血系マーカーは数パーセントであるとはいえ混在しており，けっしてゼロではない．おそらく分化転換能においてもヘテロな集団であり，ポテンシャルは細胞によって差があるものと推定される．

骨髄間葉系細胞は増殖力が非常に旺盛であり，短期間で移植にもっていけるだけの細胞数を確保することが可能である．たとえば，数十 ml の骨髄液を培養すれば数週間で約 1,000 万個の細胞を獲得することができる．なによりも患者本人の細胞を利用できるので，倫理問題のハードルが低く，また免疫拒絶の問題も回避できるなど，利点の多い細胞である．

また，骨髄間葉系細胞には骨・軟骨・脂肪・心筋などに分化する能力があることが以前より示唆されてきた[6-8]．静脈注射などの方法で生体に投与するとさまざまな臓器に分布し，肝，神経系細胞などをはじめ多様な細胞に分化転換すると報告されている．しかし，このような生体内での自発的な分化転換の効率はきわめて低いようである．一方で，培養下で成長因子や薬剤，メチル化剤などを投与し，特定の細胞に分化させ生体に移植しようという試みもされてきた[8]．

このように，骨髄間葉系細胞は移植治療の候補としてはポテンシャルの高い細胞であるが，効率のよい分化誘導系の確立が必要な段階にあった．

筋肉細胞の誘導法

骨髄液から接着性の骨髄間葉系細胞を培養し，数代の継代培養を経て，細胞がある程度安定して増殖するようになってから誘導を開始する．RT-PCR レベルでは，これら誘導前の骨髄間葉系細胞は胎児期での筋肉発生初期に認められる Pax7 や骨格筋に特有のマーカー MyoD, Myogenin などは発現していないが，筋発生初期でみられる Pax3 は弱いながら発現が認められている[9]．

1,700～1,900cells/cm^{2}の密度で細胞をまき，細胞分化の誘導因子でもある線維芽細胞増殖因子(bFGF：10 ng/ml)のほかに，ニューレグリン(neuregulin：200 ng/ml)および血小板由来増殖因子(PDGF：5 ng/ml)，細胞内 cAMP 上昇作用をもつフォルスコリン(forskloin：5 μM)などを含んだ培地(10%仔ウシ血清の入った α-MEM という培養液)で数日間培養する．この段階で Pax7 が発現されるようになり，骨髄間葉系細胞は筋肉の前駆細胞様(未分化な筋肉細胞)に分化したと考えられる(図 1)．

このように変化した細胞に Notch1 の細胞質ドメイン(Notch intracellular domain：NICD)(動物個体の発生過程で細胞の分化や増殖を制御する重要

な遺伝子)を含むプラスミドベクター(pCI-neo vector の CMV promoter の下流に組み込んだもの)をリポフェクションにて導入し，G418 選択を行う．この細胞を数日培養すると，細胞は骨格筋細胞へと分化転換し，MyoD，myogenin などの骨格筋特有のマーカーの発現が認められるようになる．さらに，培養液を成熟した骨格筋に分化させる培地(2%ウマ血清を含む DMEM 培地，あるいは無血清培地)に切り替えることによって，一部の細胞が融合を開始して成熟した多核の筋管細胞に分化し，Myosin-heavy chain などの細胞骨格蛋白とともに MRF4/Myf6 の成熟マーカーの発現も認められる．

最終的に誘導される細胞群には，増殖可能な，①単核の筋芽細胞(MyoD 陽性)，および②筋衛星細胞様細胞(Pax7 陽性)，そして③最終分裂を終えた成熟した多核の筋管細胞(Myosin-heavy chain などが陽性)，の 3 種類の細胞が含まれる(図 1)[9]．さらに，増殖可能な①筋芽細胞，および②筋衛生細胞様細胞をクローン培養することによって，誘導過程で混在していた骨格筋に分化する能力をもたない細胞を除去することができる[9]．

骨髄には，わずかではあるが骨格筋に分化する能力を有する幹細胞があることが報告されている[6]．それらの幹細胞は間葉系ではなくむしろ c-kit，CD45，CD34 陽性の細胞で造血系細胞に含まれると考えられている[6]．しかし，培養した骨髄間葉系細胞にごくわずかに骨格筋系の幹細胞が混在し，それらの細胞が上記の筋誘導を担っている可能性もある．著者らはこの点を検証するために，FACS を用いて c-kit，CD45，CD34 陰性の骨髄間葉系細胞だけを集め，上記の方法で骨格筋誘導を行った．その結果，同じ時間経過で 3 種の骨格筋系譜の細胞が誘導することができた．このことから，本現象は培養細胞に含まれるわずかな骨格筋幹細胞によるものではなく，間葉系細胞のシステム的な分化転換によるものであると推定している[9]．

骨格筋への移植

誘導細胞が実際に生体で機能することを確認するために，上記の①筋芽細胞，および②筋衛星細胞様細胞を，cardiotoxin によって変性させたラットの前脛骨筋に移植した．移植細胞は GFP で標識し，10^6の細胞を変性筋肉組織に局所注入，または尾静脈からの静脈注射により投与した．いずれの投与方法でも 2 週後には移植細胞は変性筋組織に生着し，ヒト dystrophin を発現しており，また局所注入のほうが静脈投与に比べやや高い生着率を示していた．筋ジストロフィーのモデルマウスである mdx-nude マウスに移植しても，ほぼ同様の結果が確認された[9]．

通常，筋肉が変性・崩壊すると，筋幹細胞である筋衛星細胞が刺激を受けて増殖を開始し，筋細胞に分化すると同時に一部が筋衛星細胞自身として残るので，繰り返し再生が可能となっている．このようにわれわれの筋肉は日々維持されており，筋衛星細胞はその筋再生の鍵である．

今回，著者らは骨髄間葉系細胞から Pax7 陽性の筋衛星細胞様細胞を誘導しているが，この細胞が本来の筋衛星細胞と同様の特性をもつかということを検討した．最初の移植 4 週後に移植細胞の生着を組織生検によって確認し(筋管細胞の多くは辺縁核で成熟した筋管であることを確認した)，ふたたび細胞を移植することなく cardiotoxin を投与し，2 度目の筋変性を誘導した．移植された細胞から形成された多核の筋管細胞は筋変性によって崩壊し，ふたたび筋細胞になることはできない．しかし，もし移植された細胞の一部が筋衛星細胞の性質を保持していれば，筋細胞変性に伴って増殖誘導が起こり，筋管細胞を形成することができる．そこで 2 度目の筋変性を誘導し，2 週後に筋組織を調べたところ，最初に移植したヒト由来の GFP 陽性の細胞から分化した“中心核の筋管細胞”の再生を確認することができた．筋衛星細胞から筋管細胞が形成される過程ではまず中心核の成熟過程にある筋管細胞がつくられ，ついで辺縁核の筋管(筋線維)へと成熟する．“中心核の筋管細胞”が観察されたことは，2 度目の筋変性後に筋衛星細胞からあらたに形成された筋管細胞であることを示している．すなわち，本方法で誘導された筋衛生細胞を移植すれば，2 度 3 度と筋肉が崩壊することがあっても生着した筋衛星細胞が増殖し，崩壊した筋肉を補うことが可能であ

る[9].

筋ジストロフィーの治療においては，移植された正常な機能をもつ筋管細胞は長期にわたって生着し，機能することが期待できるが，1回の移植ですべての細胞を置換することは困難である．残念ながら残った患者本人の筋肉細胞は日々，変性・崩壊が進行すると推定される．ところが，本方法では移植された正常な筋衛星細胞が繰り返し増殖刺激を受け，増殖と分化を繰り返すと推定される．すなわち，徐々に異常な筋管細胞から正常な筋管細胞への置き換えが起こることが期待できる．この性質は筋ジストロフィーの治療においては利点であると考えられる．

骨格筋発生において，Notch は筋芽細胞の分化を抑制すると報告されている[10]．今回の研究結果では骨髄間葉系細胞への Notch 遺伝子が用いられているが，これまで知られている筋肉発生における Notch の作用からすれば，逆の作用をもたらしたように見受けられる．従来より Notch の下流で Hes1，Hes5 が機能し，細胞分化を抑制していると考えられているが，今回のシステムでは NICD の導入による Hes1，Hes5 の誘導は認められないなど，骨髄間葉系細胞に特有のシステムがあると推定している．

さらに安全面という観点からすれば，遺伝子導入ではなく蛋白導入などの方法を用いることによって汎用性が広がると思われる．

おわりに

骨髄間葉系細胞には，ES 細胞には匹敵しないまでもいろいろな細胞に分化するポテンシャルがあり，これまで骨，軟骨，脂肪のほかに，心筋，肝細胞，インスリン産生細胞，気道系上皮細胞が誘導されると報告されている[8,11,12]．重要なことは，骨髄間葉系細胞が生体内において仮に自発的に分化転換を起こすとしても，その効率はきわめて低く，細胞移植治療に活用できるものではないということである．胚葉を超えた分化転換を引き起こし，目的とする細胞を一定量以上得るためには，順序立てた分化誘導操作を step-by-step に行う必要があると考えている．

骨髄間葉系細胞は骨髄移植の際にすでに移植されている細胞であり，安全，かつ容易に採取可能で，旺盛な増殖力をもっている．患者本人や家族のほうからの採取も可能であり，既存の骨髄バンクの登録者から提供を受ける方法もある．とくに患者本人の細胞を用いた場合には，免疫拒絶のない自分の細胞を用いた細胞移植治療，すなわち“自己細胞移植治療”が可能となる．ただし筋ジストロフィーではジストロフィン遺伝子など機能を欠失している遺伝子を何らかの方法で補う必要がある．

実際に治療に応用するためには，安全性，有効性など解決しなければならない多くの課題が残されている．とくに Notch の遺伝子導入が操作に入っていること，また本来の間葉系細胞から大きく性質を転換させた骨格筋を移植するとなれば，腫瘍形成の有無などを大型哺乳類を用いて慎重に検証しなくてはならない．

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Review Article

Induction system of neuronal and muscle cells from bone marrow stromal cells and applications for degenerative diseases

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Bone marrow stromal cells (MSCs) 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 neuronal cells and skeletal muscle cells have been found in MSCs. These induced cells were transplanted into animal models of spinal cord injury, 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 neuro- and muscle-degenerative diseases.

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Introduction

While solid organ transplantation such as liver, heart and kidney has already been performed on thousands of patients, the nervous systems and general muscle tissue have faced a great many limitations. Cell therapy is expected to be one of solutions, and thus it is hoped that effective therapeutic strategies will be developed.

Bone marrow contains a category of nonhematopoietic multipotent cells that can be cultivated *in vitro* as plastic adherent cells, namely bone marrow stromal cells (MSCs)[1]. MSCs are mesenchymal elements normally providing structural and functional support for hemopoiesis, express mesenchymal markers such as CD29 (beta1-integrin), CD90 (Thy-1), CD54 (ICAM-1), CD44 (H-CAM), CD71 (transferrin receptor), CD105(SH2), SH3, Stro-1 and CD13 but lack the hematopoietic surface markers such as CD34, CD3, CD117(c-kit)[2,3]. They are easily accessible through the aspiration of the bone marrow, can be isolated from patients without touching serious ethical problems, and can easily be expanded in a large scale for autotransplantation. Thus, they are hopeful candidate for use in cell-based therapy.

According to a hierarchical paradigm, MSCs differentiate into mesenchymal lineage cells such as osteocytes, chondrocytes and

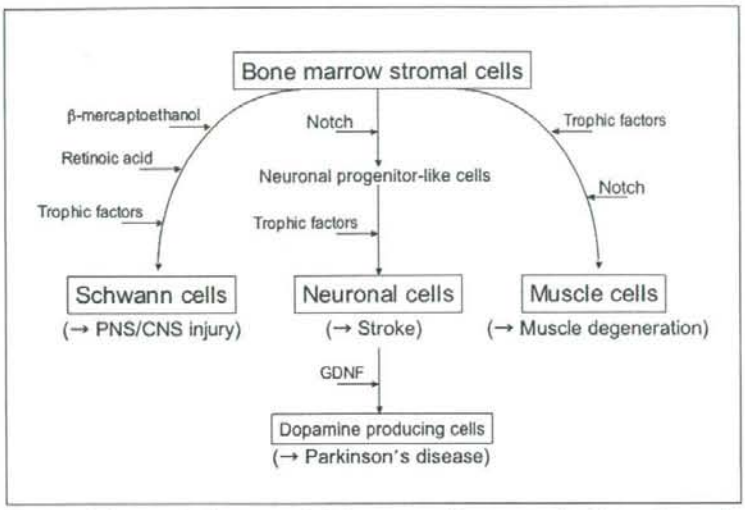

Fig.1 Schematic diagram of Schwann cell, neuronal cells and muscle cell-induction from MSCs, and their application for neuro- and muscle-degeneration

adipocytes[1]. Recently, however, unorthodox plasticity of MSCs have been described in that they have an ability to cross oligolineage boundaries which were previously thought to be uncrossable. Makino et al. showed that rhythmically contracting cardiomyocytes with cardiac muscle markers and electrophysiological characters could be induced from MSCs[4]. Furthermore, hepatocytes, insulin-producing cells and airway epithelial cells are reported to be inducible from MSCs, while some of these reports contain lower induction efficiency[5-7]. Accordingly, the potential of MSCs to transdifferentiate from mesenchymal lineages to other lineages is now of great interests. While they offer great potential for cell transplantation therapy, their practical application to human degenerative diseases is dependent on the ability to control their differentiation into certain functional cells with high efficiency and purity.

Recently, we have found a method to systematically induce Schwann cells, neurons and skeletal muscle cells from human and rat MSCs in therapeutic scale[8-10](Fig.1). In this review, we focus on the potentials, benefits and drawbacks of MSCs and discuss the possibility of clinical application in neurodegenerative, neurotraumatic and muscle degenerative diseases.

Schwann cell induction from MSCs

One kind of glial cells, namely Schwann cells which constitute peripheral nervous system (PNS), are known to support axonal regeneration by providing various kinds of trophic factors, cytokines, cell adhesion molecules and extracellular matrix, and finally reconstruct myelin of regenerated axons[11-13]. Thus in PNS, damaged nerves are known to successfully regenerate. Schwann cells are also known to support axonal regeneration and finally reconstruct myelin in central nervous system (CNS). From these reasons, they are "cells with a purpose" and represent one of the good candidates for implantation to support regeneration both of PNS and CNS.

Although Schwann cells have a strong ability to induce nerve regeneration in nervous system, there is a difficulty for clinical use to obtain sufficient amount of cells. For the cultivation of Schwann cells for clinical application, another peripheral nerve must be sacrificed. There also remain technical difficulties in harvesting and expanding Schwann cells. Accordingly, it would be more desirable to establish cells of Schwann cell characteristic from sources other than PNS which are easy to access, capable of rapid expansion, amenable to survive and integrate into host tissue eliciting axonal regeneration and re-myelination. We have reported that MSCs can be transdifferentiated into cells with Schwann cell characteristics, capable of eliciting regeneration and re-myelination of PNS and CNS nerve fibers[8].

MSCs were treated with beta-mercaptoethanol (BME) followed by retinoic acid (RA) and cultured in the presence of forskolin (known to up-regulate intracellular cAMP; FSK), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and neuregulin[8]. Such differentiated cells were mor-

phologically different from the original untreated MSCs but were quite similar to Schwann cell morphology, and expressed p75, GFAP, S-100, O4 and P0, known as markers of Schwann cells[8]. Immunocytochemistry showed higher ratio (approximately 90-95%) of the induced cells were positive for S-100 and p75. Omission either of BME, RA, FSK or neuregulin from the induction procedure resulted in incomplete differentiation both in morphology and immunoreactivity, showing that pre-treatment with reducing agent of BME and the subsequent RA and trophic factor treatment are critical and required for MSC-derived cells to acquire competence for Schwann cell characteristics.

1) Axonal regeneration in spinal cord injury by induced Schwann cells

PNS could successfully be regenerated in morphologically and functionally by transplantation of bone marrow stromal cell-derived Schwann cells (M-Schs), suggesting a great potential of M-Schs to develop an alternative therapeutic approach for the difficult reconstruction of a long distant gap in the peripheral nerve[14]. M-Schs are also effective in promoting axonal regeneration and functional recovery in completely transected adult rat spinal cord[15].

The spinal cord was completely transected with microsurgical scissors at the T7 and T8 levels, and the T7 spinal cord segment was removed. A2 mm diameter permeable tube filled with a mixture of Matrigel containing was transplanted into the gap between the rostral and caudal spinal cord stumps. After the transplantation, the T6 and T9 spinous processes were tied together to obtain contact between the graft and spinal cord stumps[15].

Grafts had integrated well into the host spinal cords in the M-Schs transplanted group. In contrast to control group that had received only matrigel, the number of neurofilament-positive nerve fibers was significantly larger in M-Sch group. Large majority of these nerve fibers were revealed to be tyrosine-hydroxylase positive fibers, while some of CGRP- and serotonin-positive fibers were also contained[15].

Hind limb function recovered in M-Sch group from 4 weeks after transplantation, and a significant difference comparing to the control group was recognized in BBB score up to 6 weeks after transplantation. The average recovery score in the M-Sch group 6 weeks after transplantation was 7.0 (minimum 5 to maximum 10), which indicates all three joints of hind limbs had extensive movement. The best recovery score in the M-Sch group was 10, which indicates occasional weight supporting plantar steps, but no forelimb-hind limb coordination. In contrast, the average recovery score in the control group was 3.6 (minimum 2 to maximum 5), showing only two joints of hind limbs had extensive movement. Re-transection of the grafts at their mid-point in M-Sch group was performed 6 weeks after transplantation, which completely abolished the recovered hind limb function and no significant recovery was observed even 4 weeks after re-transection[15]. Accordingly, these results exclude the possibility that transplanted cells enhanced the activity of a locomotor pattern generator in the spinal cord, but rather emphasize axonal regeneration induced by transplanted M-Schs contributed to functional recovery.

Induction of neuronal cells from MSCs

Recently we established a method to systematically induce neuronal cells 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 Notch intracellular domain followed by the administration of certain combination of trophic factors[9].

The Notch signaling pathway has been known to influence cell fate determination during development, from maintenance of a pool of uncommitted precursors to the terminal specification of cells[16]. The mouse Notch1 intracellular domain (NICD) cDNA was subcloned into a pCI-neo, a cytomegalovirus (CMV) promoter-containing mammalian expression vector, and transfected into MSCs by lipofection followed by G418 selection. After transfection with constitutive active form of NICD, MSCs substantially up-regulated markers related to neural stem cells (NSCs) and/or neuronal progenitor cells (NPCs), such as glutamate transporter GLAST, 3-PDGH and nestin[17,18] suggesting that MSCs may acquire some of the characters of NSCs/NPCs when NICD is introduced.

Cells were then subcultured once with administration of trophic factors (bFGF, FSK and ciliary neurotrophic factor (CNTF)) for five days, resulted in a highly efficient and specific induction of post-mitotic neuronal cells, showing that approximately 96% of cells were immunopositive to MAP-2ab. The outstanding character of those MSC-derived neuronal cells (MSC-Ns) is that they are devoid of glial development in the final population. In fact, few positive cells either to GFAP (marker for astrocytes) and galactocerebroside and O4 (markers for oligodendrocytes) were detected in MSC-Ns.

1) Application for stroke

MSC-Ns were transplanted into the infarction area in middle cerebral artery occlusion (MCAO) rat model. Transplanted rats showed significant recovery, compared to controls in Beam balance (vestibulomotor function), Limb placing (sensorimotor function) and Morris water maze (cognitive function) test ($p<$

0.01). Histologically, GFP-labeled transplanted cells migrated from the injection site into the ischemic boundary area, expressed neuronal markers of neurofilaments, MAP-2ab and beta3-tubulin, integrated into the hippocampus and resulted in extended neurites while only a small number of cells (approximately 1%) were positive for GFAP. These results showed that induced neuronal cells are effective in the ameriolation of rat brain ischemic injury model.

2) Application for Parkinson's disease

For Parkinson's disease, transplantation of dopaminergic neurons is believed to be effective[19]. However, cells committed positive for tyrosine hydroxylase (TH), a marker for dopaminergic neurons, accounted for lower ratios (~ 3 %) in MSC-Ns[9]. As GDNF is known to be involved in the generation and development of midbrain dopaminergic neurons[20], GDNF was administered to MSC-Ns to increase the proportion of cells immunopositive for TH and resulted in nearly 40 % of MSC-Ns became TH-positive cells. These GDNF treated MSC-Ns were referred to as TH-MSC-Ns. The dopamine release upon depolarization *in vitro* was measured by HPLC, showing that TH-MSC-Ns released dopamine to the culture media in response to high K+ depolarizing stimuli. These results indicate that functional dopamine producing neuronal cells could effectively be induced from MSCs[9].

Rat TH-MSC-Ns were transplanted into the striatum of Parkinson's disease model rat induced by 6-hydroxy dopamine (6-OHDA). Apomorphine-induced rotational behavior (mean rotation index = the mean rotation number in post-/pre-grafting) was examined every two weeks until 10 weeks following cell implantation. Rats grafted with TH-MSC-Ns demonstrated substantial recovery from rotation behavior up to 10 weeks[9]. In addition, non-pharmacological behavior tests, adjusting step and paw-reaching tests were performed and showed significant improvement in both experiments. In immunohistochemistry, the grafted striatum showed migration of GFP-positive transplanted cells that express marker of neurofilaments, TH and dopamine transporter (DAT). In contrast, most of GFP-labeled cells were negative to GFAP and O4, consistent with in vitro data. Animals grafted were followed up to 16 weeks and there was no tumor formation observed in the brain.

Human TH-MSC-Ns were similarly transplanted into the striatum of Parkinson's model rats. Animals were immunosuppressed with FK 506 daily, and rotational behavior was recorded at four weeks after cell transplantation. Grafting resulted in significant improvement in rotational behavior as well[9].

In summary, the additional administration of GDNF to MSC-Ns can efficiently induce TH positive, dopamine-producing cells. Striatal implantation of these cells reversed the rotatory behavior and improved stepping behavior and paw reaching tests in Parkinson model rats.

Muscle cell induction from MSCs

A method was developed to systematically and efficiently induce skeletal muscle lineage cells with high purity from large population of MSCs[10]. The induced population effectively differentiated into mature myotubes with some cells persisting as satellite cells that continued to function in host muscle to restore degenerating muscles in the absence of repetitive transplantations[10].

Human and rat MSCs were firstly treated with trophic factors of bFGF, FSK, PDGF and neuregulin, followed by transfection with a NICD expression plasmid by lipofection and selection, and allowed to recover to 100% confluency. At this stage, a large majority of MSCs developed to mononucleated myogenic cells expressing MyoD. Cells were then supplied either with 2% horse serum or ITS (Insulin-Transferrin-Selenite)-serum free medium, both of which are known to promote differentiation of myoblasts to myotubes[21]. After treatment, MSC-derived muscle lineage cells (M-MLCs) were obtained. This final population contained 3 kinds of muscle-lineage cells; (1) post-mitotic multinucleated myotubes expressing Myf6/MRF4 (a marker for mature skeletal muscle) and contractile proteins (2) mononucleated myoblasts: expressing MyoD, and (3) satellite-like cells: immunopositive for Pax7, marker for muscle satellite cells[22].

1) Application to muscle degeneration models

To estimate how workable these induced muscle lineage cells are in the repair of degenerated muscles, human M-MLCs were transplanted into immunosuppressed rats whose gastrocnemius muscles were damaged with cardiotoxin pretreatment[23]. Cells were labeled by means of a GFP-encoding retrovirus and then transplanted by local injection (L.I.) into degenerated muscles. Two weeks after transplantation, GFP-labeled cells incorporated into newly formed immature myofibers, exhibited centrally located nuclei in treated animals. Four weeks after transplantation, GFP-positive myofibers exhibited mature characteristics with peripheral nuclei just beneath the plasma membrane. Functional differentiation of grafted human cells was also confirmed by the detection of human dystrophin in GFP-labeled myofibers[10].

M-MLCs contained satellite-like cells those developed into satellite cells in the host muscle. In general, muscle satellite cells are known to contribute to regenerating myofiber formation upon muscle damage[24]. Therefore, we tested whether transplanted satellite-like cells were able to contribute to muscle regeneration as satellite cells *in vivo*. Four weeks after transplantation of

human M-MLCs, cardiotoxin was re-administered into the same muscles without additional transplantation. Two weeks after the second cardiotoxin treatment (6weeks after initial transplantation), many regenerating GFP-positive myofibers with centrally-located nuclei were observed. This implies that, upon transplantation of M-MSCs to muscles of patients, those retained as satellite cells should be able to continue to contribute to future muscle regeneration[10].

Compared to the various muscle stem cell systems that have been reported, this system offers several important advantages. Since our induction system does not depend on a rare stem cell population, but can utilize the general population of adherent MSCs, which can be easily isolated and expanded, functional skeletal muscle cells can be obtained within a reasonable time course on a therapeutic scale. In case of MSCs derived from inherited muscle dystrophy patients, genetic manipulation is possible after the isolation and expansion of MSCs. Moreover, transplantation of MSC-derived cells should encounter fewer ethical problems.

General conclusions

While ES cells and tissue stem cells have great potential, MSCs also provide hopeful possibilities for clinical application, since they can be efficiently expanded *in vitro* and we could acquire a therapeutic scale of induced cells. In addition, transplantation of MSC-derived cells should pose fewer ethical problems by preventing stem cell controversy, since bone marrow transplantation has already been widely performed. As MSCs are easily obtained from patients or marrow banks, autologous transplantation of induced cells or transplantation of induced cells with the same HLA subtype from a healthy donor may minimize the risks of rejection. Needless to say, bone marrow should at least be 'normal and healthy' for transplantation.

Although we showed the high ratio and specific induction of Schwann cells, neurons and skeletal muscle cells, we still have to solve the following problems[8-10]. Although there have been so far few reports referring to tumor formation after transplantation of untreated MSCs, further studies are needed to ensure safety, tumor formation and efficacy of manipulated MSCs over a long-term period using primates. In fact, recent reports raised the possibility of transformation in the long term cultivation of MSCs[25,26]. Second, as the potential of differentiation would differ by age, individual, race, and sexes, each of these must be investigated in the future. Third, MSCs have been shown to be heterogeneous in terms of growth kinetics, morphology, phenotype and plasticity. With the development of specific markers and detailed characterization of heterogeneous general adherent MSCs, their properties and plasticity can be studied and defined with more certainty.

Notch-Hes signaling are known to inhibit neuronal and myogenic differentiation in conventional development[16]. However, in our system, NICD introduction accelerated the induction of neuronal and skeletal muscle cells from MSCs. Although our results appear inconsistent with previous work, they do not refute the known role of Notch-Hes signals during development. In the previous report, JAK/STAT inhibitor administration and constitutive active STAT1/3 transfection showed that down regulation of STATs was tightly associated with NICD-mediated neuronal induction, whereas Hes, down stream of Notch, was not involved in the induction event[9]. Skeletal muscle induction was also revealed to be independent of Hes1/5. Thus, our results suggest the distinct cellular responses to Notch signals; for example, the repertoire of second messengers and active factors may well be different between conventional neural stem cells and/or neural progenitor cells and MSCs. It might be possible that unknown signaling pathway downstream of Notch may be involved in these events, and thus further studies are needed to identify the factor involved in this phenomenon.

Since MSCs can be obtained from patients, it is possible to establish a "self-regenerative system" using MSCs. To realize this ideal, it is necessary to develop the regulatory system of differentiating MSCs into cells with a purpose. Our method would be one of possible way to regulate MSC differentiation into functional Schwann cells, neurons and skeletal muscle cells which will be applicable to neurodegenerative and muscle degenerative diseases.

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