reported that SP cells in muscle are CD45-negative, c-kit-negative, and Sca-1-positive [6]. On the other hand, Jackson et al. reported that muscle-derived SP cells are Sca-1-positive (79 %), c-kit-positive (75 %), and CD45-negative [32]. Using a 5 μg/ml solution of Hoechst 33342, Asakura et al. reported that most muscle-derived SP cells are CD45-negative Sca-1-positive, but more than 10% of SP cells are CD45-positive [33]. Using an almost identical isolation procedure and Hoechst staining conditions, we reported that muscle-derived SP cells isolated from uninjured adult skeletal muscle have three phenotypically distinct SP subpopulations [29]. The report shows that approximately 90 % of muscle SP cells are CD31-positive CD45-negative. This fraction strongly expresses Bcrp-1 on the cell surface. About 5 % of muscle SP cells are CD45-negative CD31-negative, and approximately 3-5 % of muscle SP cells are CD45-positive.

Cell surface markers and origin of muscle SP cells

Interestingly, the difference in cell surface markers on SP subfractions is clearly related to their developmental origins. CD45-positive SP cells are thought to originate from BM and home into skeletal muscle via the circulation [29, 34]. Further, the HSC activity found in skeletal muscle was proven to be almost completely limited to the CD45-positive SP fraction [34]. On the other hand, the majority, but not all, of the CD45-negative SP cells in limb muscles were shown to be derived from the hypaxial somite and to have higher myogenic potentials than CD45-positive SP cells [35]. Interestingly, the study also revealed that a certain percentage of CD45-negative SP cells are not likely derived from the somite and are less myogenic than SP cells of somitic origin.

4. Gene expression in skeletal muscle SP cells

No expression of myogenic regulators in SP cells

Asakura et al. showed that the muscle SP fraction expresses no desmin or Pax7. Furthermore, muscle SP cells prepared from Myf-5-nLacZ mice do not express β-galactosidase [33]. Using sensitive RT-PCR analysis, we also showed that muscle SP cells prepared from non-injured or regenerating muscles do not express Pax7, Pax3, Myf-5, or MyoD [29]. These observations suggest that muscle SP cells are not committed to a myogenic lineage.

Molecular signatures of SP cells revealed by microarray analysis

Genome-wide gene expression analyses of SP fractions have been performed to elucidate the molecular regulation of SP cells. Meeson et al. examined the gene expression profiles of muscle SP cells isolated from uninjured and regenerating muscles, bone marrow-SP cells, and embryonic stem (ES) cells [30]. The results showed that skeletal muscle SP cells express Abcg2 (Bcrp-1) and endothelial and hematopoietic transcripts. They concluded

that muscle SP and BM SP cells have distinct molecular programs. Liadaki et al. reported the gene expression of muscle SP and BM SP cells. The analysis revealed that BM SP and muscle SP cells share a transcriptome signature but at the same time express tissue-specific markers [36]. When compared with MP cells within the same tissues, SP cells were found to underexpress genes reflecting tissue-specific functions [36]. Rochon et al. reported gene expression analysis of SP cells isolated from adult mouse bone marrow, adult male germinal cells, muscle primary culture, and mesenchymal cells [37]. These four types of SP cells are proposed to be a "stem cell-like" population. Transcriptional profiles for SP and the more differentiated non-SP cells isolated from these four tissues were compared by microarray analysis. The authors reported that the genes commonly upregulated in SP cells are implicated in the quiescent status of cells, maintenance of their pluripotency, and capacity to undergo asymmetric division, and that the repression of lineage-affiliated genes in SP cells is responsible for their undifferentiated state.

5. Location of SP cells in skeletal muscle

SP cells are defined by FACS analysis, but their location in muscle is ill-defined. Because Bcrp-1 is largely responsible for the SP phenotype [12,13], it is informative to stain muscle tissues sections with an anti-Bcrp-1 antibody. Using a polyclonal antibody against Bcrp-1, we showed that CD31-positive SP cells on glass slides are strongly stained with the antibody after sorting. Interestingly, CD31-positive Bcrp-1-positive cells are found in capillaries and venous endothelium, suggesting that the majority of muscle SP cells are associated with blood vessels [29]. Meeson et al. also showed that Abcg2 (Bcrp-1)-expressing cells are closely associated with the vasculature [30]. However, Bcrp-1 positive cells are also found in the MP fraction [29]. Therefore, not all Bcrp-1-positive cells found on muscle cross sections have the SP phenotype [29]. On the other hand, CD45-positive SP cells express quite low levels of Bcrp-1 [29]. Currently CD45-positive SP cells lack a distinctive marker available for use on tissue sections. Therefore, it is difficult to see BM-derived CD45-posotive SP cells on muscle cross sections. Likewise, CD45-negative CD31-negative SP cells on glass slides are hardly stained with anti-Bcrp-1 antibody after cell sorting, so it is also difficult to find CD45-negative CD31-negative SP cells in muscle sections [29].

6. Origin of muscle SP cells

Satellite cells originate in somite

Repair of mature skeletal muscle is largely mediated by the muscle progenitor cells referred to as satellite cells [2]. Satellite cells reside beneath the basal lamina of adult skeletal muscle juxtaposed against skeletal muscle

fibers and account for 2-5 % of sublaminal nuclei in adult muscle. The developmental origin of satellite cells is reported to be the somite [35, 38, 39].

Satellite cells and muscle SP cells are distinct populations

In the beginning, the relationship between muscle SP cells and satellite cells was controversial. Seale et al. reported that Pax7-null mice have severely reduced numbers of satellite cells, but show normal levels of muscle SP cells [40]. Asakura et al. showed that satellite cells and muscle SP cells have distinct differentiation potentials both *in vitro* and *in vivo* [33]. Fukada et al. established an antibody, named SM/C-2.6, that can purify quiescent satellite cells from muscle efficiently [41], and directly showed that almost all satellite cells are found in the MP fraction [41]. Furthermore, it was shown that muscle SP cells are negative for Pax7 or Pax3 and hardly differentiate into myotubes *in vitro* without co-culturing with myoblasts [29, 33]. Taken together, satellite cells and muscle SP cells seem to be distinct populations. Some reports, however, suggest that when transplanted into skeletal muscle, a portion of muscle SP cells differentiate into Pax7-positive cells [6, 33] and express Pax7. Although a recent study demonstrated that satellite cells vigorously self-renew, denying the existence of muscle stem cells that replenish the satellite cell pool [42], muscle SP cells might slowly supply severely damaged muscle with myogenic precursor cells.

Majority of limb muscle SP cells are derived from hypaxial somite

The source of muscle SP cells has long been debated. Schienda et al. explicitly tested and quantified the contribution of embryonic somitic cells to side populations [35]. Chick somitic cells were labeled by using replication-defective retroviruses or quail/chick chimeras, and mouse cells were labeled by crossing somite-specific, Pax3-derived Cre driver lines with a Cre-dependent reporter line. The results showed that a significant number, but not all, of limb muscle SP cells are derived from the hypaxial somite. Notably, the developmental origin of SP cells is related to their potentials; somitically derived CD45-negative SP cells are intrinsically more myogenic than CD45-negative SP cells from other sources. As mentioned above, CD45-positive SP cells are thought to be derived from bone marrow [29, 34].

7. Differentiation potential of muscle SP cells

The differential potentials of muscle SP cells have long been controversial. However, their cell surface markers reveal their developmental origin and their differential potentials.

Hematopoietic activities of muscle SP cells

Skeletal muscle-derived cells have the potential to repopulate the major peripheral blood lineages of lethally irradiated mice and thus behave like HSC

[6, 32]. To further clarify the properties of muscle-derived HSC, skeletal muscle-derived cells were fractionated based on the expression of CD45 and c-kit and Hoechst 33342 efflux, and examined for HSC activity in vivo [34]. The results revealed that muscle-derived HSC activities fall exclusively in the c-kit (dim) CD45 (pos) compartment of the muscle side population (msSP). Furthermore, it was shown that the CD45-positive msSP compartment of skeletal muscle is derived from whole bone marrow HSC. CD45-positive muscle SP cells are, however, shown to be much less potent in HSC activity than bone marrow HSC cells in competitive repopulation assays [34].

Muscle SP cells contain vascular progenitors

Muscle SP cells have also been shown to contribute to vascular regeneration after local injection into chemically damaged regenerating muscle [27]. Majka et al. showed that more than 70 % of muscle SP cells are CD45-positive and derived from bone marrow. The higher percentage of CD45-positive cells in the muscle-derived SP fraction, compared with those reported by other laboratories, may be due to pre-fractionation by a Percoll gradient of crude muscle-derived mononuclear cells prior to Hoechst staining (Table 1).

Myogenic potential of muscle SP cells

Gussoni et al. reported that muscle SP cells fail to settle on the plate during the first two weeks after cell sorting and that they then differentiate as a mixture of myoblasts and fibroblasts [6]. In contrast, Asakura et al. showed that skeletal muscle SP cells cultured in myoblast growth medium do not give rise to myogenic progenitors unless cultured with primary myoblasts [33]. We also observed that muscle SP cells alone hardly form myotubes *in vitro* [29]. After transplantation into muscle, however, muscle-SP cells generate both satellite cells [6, 33] and mature myofibers [6, 29, 33].

Mesenchymal potential of muscle SP cells

We showed that a minor subset of muscle SP cells (the CD31-negative CD45-negative fraction) differentiate into adipocytes or osteocytes *in vitro* upon induction. Furthermore, when these cells are transplanted into irradiated muscle, muscle fiber regeneration is severely impaired, and transplanted CD31-negative CD45-negative SP cells differentiate into many adipocytes and fibrotic cells (our published data). This observation suggests that they might be a source of the adipogenesis seen in advanced muscular dystrophy. CD31-positive CD45-negative SP cells uptake 1,1'-dioctadecyl- 3,3,3',3'-tetramethylindo-carbocyanine perchlorate-acetylated-low density lipoprotein (Dil Ac-LDL), suggesting that they possesses endothelial cell-like properties [29], but their functions *in vivo* remain to be determined.

8. Relationship to other muscle stem cells

Several types of stem cells have been isolated from skeletal muscle, including SP cells, muscle-derived stem cells (MDSC) [43], multipotent adult precursor cells (MAPC) [44], myogenic-endothelial progenitors [45], CD45-positive Sca-1-positive cells [46], mesoangioblasts [7], and pericytes [47]. Although all these stem cells were isolated from skeletal muscle and showed multi-lineage differentiation potential, use of different cell isolation techniques, different culture conditions, and partial characterization make it difficult to clarify the interrelationships among them. For establishment of effective and safe cell therapy for muscular dystrophies, we must establish a standard protocol for isolation of muscle stem cells.

9. Physiological role of SP cells in muscle regeneration

Interestingly, the number of muscle SP cells increases during muscle regeneration [29,30] (Figure 3). In particular, CD45-negative, CD31-negative SP cells actively proliferate during muscle regeneration. In the later stage of muscle regeneration, however, the double-negative SP cells decrease in number and return to the normal level [29]. Although double-negative SP cells barely differentiate into muscle cells when cultured alone [29, 33], they form myofibers *in vivo* after injection into regenerating muscle of immunocompetent mice, suggesting that endogenenous muscle SP cells participate in muscle fiber repair. Intriguingly, CD31-negative CD45-negative SP cells express the genes that are expected to regulate muscle regeneration [29]. These results suggest that CD31-negative CD45-negative SP cells not only act as myogenic progenitors but also regulate muscle regeneration by acting on satellite cells or inflammatory cells. Contrary to expectation, CD31-positive SP cells (the major SP subset in muscle) do not expand in response to muscle injury. Their roles in muscle regeneration remain to be determined. Figure 4 shows our model of the roles for muscle SP cells in muscle regeneration.

10. Muscle SP cells as a tool for cell therapy of muscular dystrophy

Cell-based therapy continues to be a promising avenue for the treatment of DMD, an X-linked skeletal muscle-wasting disease. However, myoblast transplantation into animal models results in local restoration of dystrophin expression [48], and human clinical studies using myoblast transfer performed on DMD patients failed to improve the muscle strength of the treated group [49-51]. An alternative to myoblast transfer is a systemic delivery of precursor cells with myogenic potential. Recently, it was demonstrated that skeletal

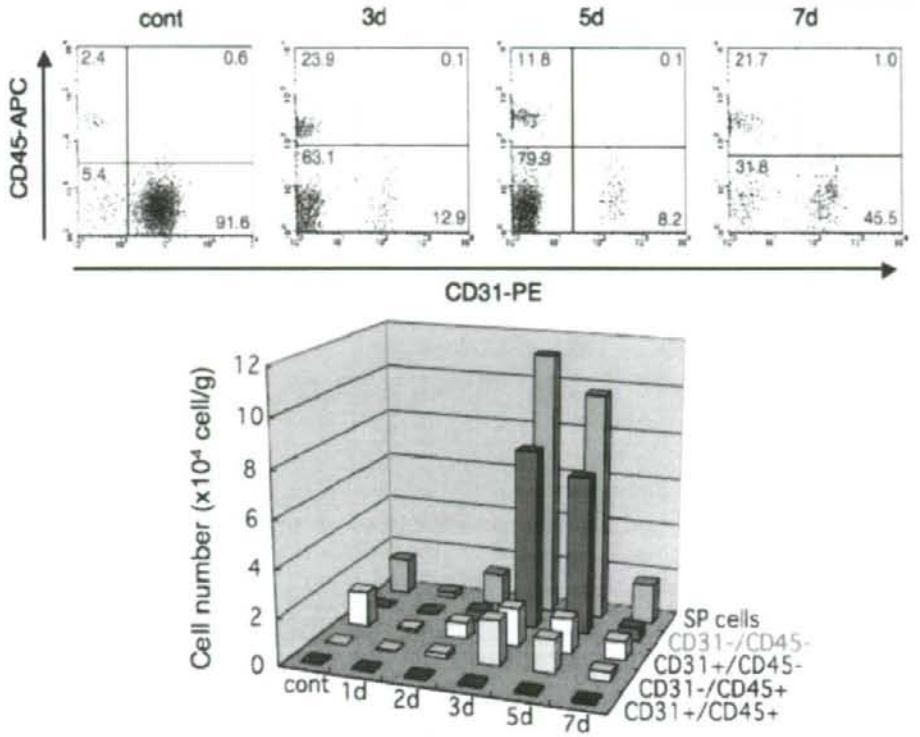

Figure 3. Number of CD31-negative CD45-negative SP subpopulation cells increases during muscle regeneration. Cardiotoxin was injected into hind limb muscles of 8-week-old C57BL/6 mice, muscles were dissected, and SP fractions were analyzed by FACS 1, 2, 3, 5, and 7 days after injection. Upper panel: Muscle SP fraction was further analyzed by CD31 and CD45 expression. Lower panel: The cell numbers of each SP fraction were counted and plotted. Note that CD45-negative CD31-negative SP cells are a minor SP subset, but are significantly increased in response to muscle injury. Reproduced from [29] by permission.

muscle SP cells engraft into dystrophic fibers of nonirradiated mdx(5cv) mice after intravenous or intraarterial delivery [52, 53]. SP cells prepared from *mdx*(5cv) muscle were transduced with a recombinant lentivirus encoding microdystrophin or GFP, and transplanted into the femoral artery of non-injured *mdx*(5cv) mice. Sections of the recipient muscles demonstrated that 5-8 % of skeletal muscle fibers expressed donor-derived transgenes. Further, donor muscle SP cells, which did not express any myogenic markers prior to transplant, expressed a satellite cell transcription factor, Pax7, and a muscle-specific intermediate filament, desmin, after extravasation into host muscle. These results indicate that systemic autologous transplantation of SP cells genetically corrected *ex vivo* could be an option for cell therapy of DMD.

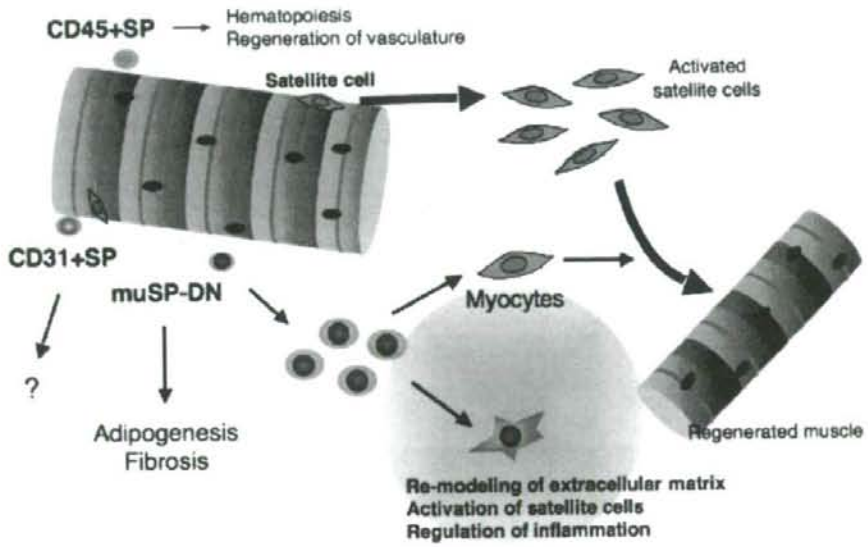

Figure 4. Roles of SP cells in muscle regeneration. Muscle-derived SP cells stained with Hoechst (5 μg/ml) are divided into three distinct subpopulations based on the expression of CD31 and CD45. The main population is CD31-positive. Their roles in muscle remain unclear. CD45-positive SP cells (less than 10% of total muscle SP cells) are shown to be bone marrow-derived and account for hematopoietic activity found in muscle. They are also reported to re-constitute the vasculature after muscle injury. The CD31-negative CD45-negative SP subset actively proliferates during muscle regeneration. These cells differentiate into adipocytes, osteocytes, and myocytes *in vitro*. Their roles in muscle regeneration are just beginning to be elucidated.

III. SP fraction in cultures of primary myoblasts and myogenic cell line C2C12 cells

One can identify a subset of cells with the SP phenotype in both primary myobasts and cells of a myogenic cell line, C2C12. Benchaouir et al. stained myoblasts and C2C12 cells with a variety of concentrations of Hoechst (5-30 μg/ml) and analyzed them on a dual-laser MoFlo flow cytometer; they found that 2-3 % of the cells show SP-like phenotypes [54]. Interestingly, gene expression analysis of C2C12-SP cells suggested that mdr1a, an ABC transporter is likely responsible for the SP phenotype of C2C12-derived SP cells. Cell cycle analysis by propidium iodide staining revealed that more than 90 % of SP cells in C2C12 cells are in the G0/G1 phase. Electron microscopy showed that they have a high nucleus-to-cytoplasm ratio. C2C12 SP cells express reduced levels of MyoD and exhibit delayed differentiation compared to main population cells in differentiation-promoting conditions. Because forced expression of mdr1a

increases the number of SP cells and blocks the differentiation of C2C12 cells, mdr1a may regulate the differentiation of C2C12 cells. One possible mechanism for this regulation is that mdr1a excludes certain differentiation-promoting substances from the cells. Interestingly, the study also demonstrated that SP and MP cells in C2C12 cells are in a dynamic equilibrium and largely reversible. Moreover, addition of FGF6 to the culture medium increased the ratio of SP cells [55]. C2C12 SP cells might be a useful *in vitro* model to study a reserve pool of muscle progenitor cells in skeletal muscle.

IV. Others

Roles for ABC transporters in SP cells

SP cells in several tissues represent immature, undifferentiated cell fractions [10]. SP-like cells are also found in cancer cells, and are considered to be cancer stem cells [11]. Although not all SP cells are stem cells, the Hoechst efflux phenomenon correlates well with stem cell activities in several tissues or organs. ABC transporters would protect stem/progenitor cells from toxic substances, hypoxia, or other stresses, ensuring long-term survival of the cells, and keeping stem/progenitor cells in an undifferentiated state.

Conclusions

Muscle SP cells are thought to be enriched in stem cells, but they are highly heterogeneous. In addition, variations in techniques for isolation of the muscle SP fraction make the properties of muscle SP cells elusive. To further characterize the origins, phenotypes, the functions of muscle SP cells in physiological and pathological conditions and their therapeutic potential, it might be necessary to find more reliable SP-specific markers and use limited numbers of SP cells in a functional assay to equate muscle SP cells with stem cells.

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5

Transdifferentiation system in bone marrow stromal cells and its application to muscle dystrophy: Insights into cell-based therapy

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Abstract

Many kinds of cells, including embryonic stem cells and tissue stem cells, have been considered candidates for cell transplantation therapy for 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

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technical problems. Recently, new methods for the highly efficient and specific induction of functional skeletal muscle cells have been found in MSCs. Induced cells differentiate into muscle fibers upon transplantation into degenerated muscles of rats and mdx-nude mice. Furthermore, the induced population contained Pax7-positive cells that contribute to subsequent regeneration of muscle upon repetitive damage without additional transplantation of cells. Here we describe the discovery of these induction systems and focus on the potential use of MSC-derived cells for cell-based therapy in muscle-degenerative diseases.

Introduction

Muscle degenerative diseases, such as muscle dystrophy, are responsible for a decline in muscular function, which limits life span. While transplantation of the liver, kidney, and bone marrow has already been performed on thousands of patients, transplantation of the general muscle tissue has faced many limitations. Thus, it is hoped that effective therapeutic strategies will be developed. As for muscle tissue, satellite cells are considered stem cells in adult muscle tissue, although the difficulty in isolating a sufficient number of pure satellite cells has precluded their use in cell-based tissue repair [1-3]. Furthermore, there is a need to establish cell therapies based on healthy donors since muscle dystrophies are inheritable diseases.

Recently, 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 skeletal muscle cells, either by spontaneous differentiation or by certain induction methods [4, 5].

Tissue specific stem cells are identified in various tissues of more advanced developmental stages. Stem cells and satellite cells isolated from adult and prenatal muscle tissue [1-3] and myogenic stem cells from the bone marrow [6, 7] are considered to be sources of cell replacement, and there have been several attempts to ameliorate muscle degeneration by transplantation of these muscle stem cells [6]. Although tissue stem cells have great potential, they face limitations inherent in procurement from fetal tissue, including problems of histocompatibility and of ethical concerns. Recently mesangioblast, one type of adult mesenchymal stem cell, has generated particular interest and expectation since it offers sufficient myogenic cells for use in therapy [8].

The bone marrow contains a category of nonhematopoietic mesenchymal cells that can be cultivated *in vitro* as plastic adherent cells, namely bone marrow stromal cells (MSCs) [9]. MSCs are mesenchymal elements that normally provide structural and functional support for hematopoiesis and express mesenchymal markers [10, 11]. 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". Other than this, MSCs with same HLA subtype is obtainable from

healthy donors in marrow bank or from relatives. They are also easily expanded in a large scale; for example, 20-100 ml of bone marrow aspirate provides 10^7 cells within two to three weeks, a plentiful number of cells for transplantation.

At the present time, the efficacy of MSCs for transplantation therapy is twofold. First, the transient trophic effect of MSCs can delay cell death and restore the tissues [12-14]. 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 [9, 15, 16]. 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 into muscle cells was initially described in *in vivo* experiments, where transplanted donor bone marrow-derived cells integrated into the recipient tissue and supported regeneration [6]. While this study suggested the plasticity of MSCs because of the expression of donor markers and cell specific markers, however, the clonality and functions of these transdifferentiated cells were not clearly estimated in some cases. Moreover, these phenomena have been suspected to be based on cell fusion or spontaneous trans-differentiation with extremely rare frequency [17, 18].

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 lower induction efficiency [19-22]. Indeed, 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 skeletal muscle cells from human and rat MSCs on a therapeutic scale [23]. This review describes the process of discovery of systemic induction, the properties of induced cells, and finally their potential, advantages and disadvantages for clinical application in muscle-degenerative diseases.

I. The process of discovery

The finding of muscle induction system from MSCs owes its properties to 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. As described previously, induction of Schwann cells was finally established using a reducing reagent, retinoic acid, and trophic factors related to Schwann cell development (see other review) [24, 25] (Fig. 1).

However, Dezawa et al first tried to induce Schwann cells from MSCs by introducing glial instructive factor Notch gene. The Notch gene encodes a 300 KD single transmembrane cell-surface receptor protein that is activated by Delta/Serrate/Lag-1 ligands presented by neighboring cells [26]. 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 [26-28]. In fact, a series of studies have shown that when Notch signaling is activated, astrocytes and Schwann cells differentiate from neural stem cells (NSCs) and neural crest stem cells, respectively [27, 28]. However, it was very surprising to see neuronal cells induced in the final product by introducing Notch gene followed by trophic factor treatment related to neurogenesis such as basic fibroblast growth factor (bFGF), ciliary neurotrophic factor (CNTF) and forskolin known to upregulate intracellular camp [29]. While it was quite accidental, this method was found to induce functional post-mitotic neurons without containing glial cells from MSCs (Fig.1).

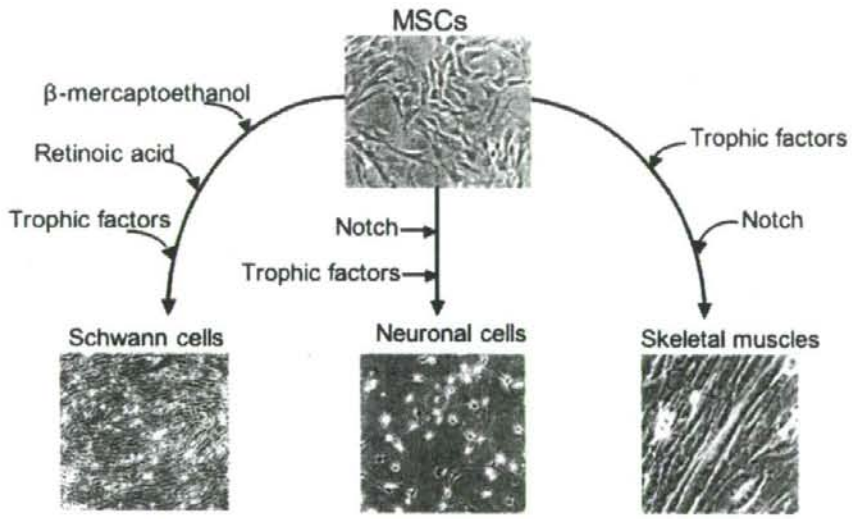

Figure 1. Schematic diagram of induction system from MSCs. Schwann cells could be induced by treatment with beta-mercaptoethanol, retinoic acid followed by trophic factor administration of bFGF, forskolin, PDGF and neuregulin. In the final step, MSCs became similar to Schwann cells, and express Schwann cell markers of p75. Neurons are induced by Notch intracellular domain gene transfer followed by trophic factor administration of bFGF, FSK and CNTF. The final population is consisted mostly of neurons immunopositive to neuronal markers such as neurofilament. Skeletal muscle lineage cells could be obtained by trophic factor treatment of bFGF, FSK, PDGF and neuregulin, followed by Notch gene transfer.

During the experiment of neural induction, the order of treatment was just reversed for the control experiment (Fig.1). Again, the surprising phenomenon of muscle differentiation, small number of slender cells containing two to three nuclei, could be recognized in the culture dish. Considering the advantages of MSCs, this phenomenon was expected to develop the large-scale induction system of skeletal muscle cells from patient's own MSCs. Thus, the induction experiment was repeated, and finally a new method to systematically and efficiently induce skeletal muscle lineage cells with high purity from large population of MSCs was established [23].

II. Induction systems of skeletal muscle cells from MSC

Human and rat MSCs were passaged at least for three times, and then plated on plastic dishes at 1,700~1,900 cells/cm^2. They were first treated with the trophic factors bFGF, FSK, platelet-derived growth factor (PDGF) and neuregulin for three days. After this treatment (C-MSCs), Pax7 expression could be recognized in MSCs (Fig.2). They were then transfected with a plasmid expression vector containing constitutive active form of Notch gene (The mouse Notch1 intracellular domain (NICD) cDNA was subcloned into

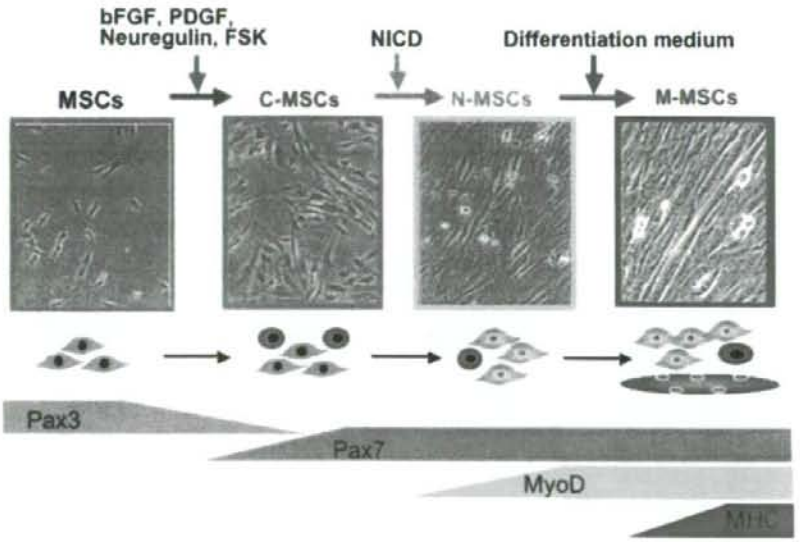

Figure 2. Induction of skeletal muscle cells from MSCs (human). MSCs originally express Pax3 become positive to Pax7 after trophic factor stimulation (C-MSCs). NICD transfection induced MyoD- and myogenin expression in N-MSCs. These N-MSCs fuse to form multinucleated myotubes by differentiation medium, expressing the marker of maturity, such as myosin heavy chain (MHC).

pCI-neo, a cytomegalovirus promoter-containing mammalian expression vector) by lipofection followed by G418 selection, and allowed to recover to 100% confluency. At this stage (N-MSCs), a large majority of MSCs developed into mononucleated myogenic cells expressing MyoD and myogenin, while a small population of Pax7 (+) satellite-like cells also existed (Fig.2). Cells were then supplied with a differentiation medium of either 2% horse serum, Insulin-Transferrin-Selenite (ITS)-serum free medium or the supernatant of the original untreated MSCs [23], and the final muscle lineage population (M-MSCs) was acquired (Fig.2). M-MSCs contained three kinds of muscle-lineage cells. The first population included post-mitotic multinucleated myotubes, which expressed myogenin, Myf6/MRF4 (a marker for mature skeletal muscle) and contractile proteins of skeletal myosin, myosin heavy chain, and troponin, all related to skeletal muscle characteristics. In fact, some multinucleated cells exhibited spontaneous contraction *in vitro*. They are also positive for p21, a marker for post-mitotic muscle lineage cells. The second group was mononucleated myoblasts which expressed MyoD and myogenin. The third group was composed of satellite-like cells and were immunopositive for Pax7 and c-Met, both markers for muscle satellite cells [23].

However, it is critical to determine if these MSC-derived skeletal muscle cells integrate into the host tissue and are genuine muscle cells. In the following sections, the effectiveness of these induced cells is verified by a transplantation experiment using animal models of muscle degeneration and dystrophy.

III. Mechanism of induction

To examine the induction events leading from MSCs to M-MSCs, we investigated the expression of genes related to myogenesis in these cells by RT-PCR [23]. Before trophic factor treatment, MSCs expressed Pax3, Six1 and Six4 while Pax7, MyoD and myogenin were not. After treatment with trophic factors bFGF, FSK, PDGF and neuregulin (C-MSCs), Pax3 was down-regulated instead Pax7 expression was recognized which persisted after NICD introduction (N-MSCs) and final population of M-MSCs. Expression of MyoD and myogenin was firstly detectable in N-MSCs and persisted in the M-MSCs. These results were also confirmed by Western analyses. Myf6/MRF4, a marker for mature skeletal muscle, was detectable only in the final MSC-M population. While expression of Six1 and Six4 persisted for the entire period, another myogenic factor, myf5 was not detected in any induction step. In this way, the induction process mimicked some aspects of conventional skeletal muscle development since Pax3, Pax7, MyoD, Myogenin and Myf6/MRF4, all of which are related to muscle development [30-33], could be detected in a sequential manner. However, as MSCs used in this induction system possess different characteristics from the conventional myogenic progenitor cells, it is

possible that some of mechanisms should differ, especially in the initial step converting MSCs to MyoD-positive N-MSC population. For this initial step, cytokine pre-treatment and the subsequent NICD transfection are critical and required for MSC-derived cells to acquire competence for myogenic induction. In fact, when we reversed the order of cytokine treatment and NICD transfection, muscle-lineage markers were not detected nor were multinucleated cells observed.

It is well established that Notch signaling inhibits myogenic differentiation; Delta1/Jagged1 inhibits MyoD expression, blocks the differentiation of myoblasts, and prevents the formation of myotubes [34, 35]. Hes 1/5, downstream effectors of Notch, are reported unrelated to the inhibition of the myogenic pathway in C2C12 myoblasts, while others report that Hes1 up-regulation results in the prevention of myogenesis [36, 37].

We examined the expression of Hes family members to judge whether conventional Notch pathway was activated in our induction process [38-40]. The expression of Hes 1/5 was not significantly upregulated by NICD transfection (N-MSCs). The forced expression of Hes 1/5 in place of NICD failed to induce skeletal muscle lineage cells, suggesting that Hes 1/5 signaling is not involved in the muscle induction event in MSCs. Hes 6, another Hes family member known to induce the myogenic differentiation program, was slightly up-regulated, while muscle induction by the forced expression of Hes6 in place of NICD could barely elicit muscle lineage cells.

In our induction system, NICD transfection up-regulated MyoD while it has been shown to inhibit myogenic differentiation in cultured muscle cells and in the embryo [34, 35]. We re-expressed NICD in rat N-MSCs and analyzed MyoD expression. N-MSCs were transfected with pCI-neo-NICD by lipofection, followed by G418 selection, and were brought to RT-PCR. Interestingly, the down-regulation of MyoD was recognized after re-expression of NICD in N-MSCs as well as in C2C12 cells. Furthermore, after the re-expression of NICD, cells were subjected to differentiation medium containing 2% horse serum to analyze myotube formation. The differentiation into multinucliated myotubes was significantly suppressed by re-expression of NICD in N-MSCs as well as C2C12 cells. These results collectively suggest that cellular response to NICD in MSCs is different from that of conventional myogenic progenitor cells, but once they differentiate into myogenic lineage cells by this induction system, they behave like real myogenic cells such as C2C12 cells [34, 35].

Our results showing that NICD introduction accelerates the induction of skeletal muscle cells from MSCs are surprising from the viewpoint of conventional Notch signaling in myogenesis. We consider our results do not refute the known role of Notch-Hes signals in myogenesis, but rather reflecting the distinct cellular responses of MSCS to Notch signals; for example, the

repertoire of proteins, second messengers and other active factors may well be quite different between conventional myogenic progenitor cells and MSCs. Notably, as described above, we observed the induction of neuronal cells from MSCs by NICD introduction. A yet unknown signaling pathway downstream of Notch may be involved in these events. Further studies are nevertheless needed to identify the factor involved in this phenomenon.

Bone marrow (mostly hematopoietic cells) contains a small population of myogenic stem cells known to express c-Kit, CD45 and CD34 [1-3, 7, 41, 42]. Hematopoietic cells are generally non-adherent and cells we used were adherent MSCs. However, even though we used adherent MSCs, several percent of cells are positive to above markers. To exclude the possibility that the production of muscle-lineage cells was due to the vast proliferation of myogenic stem cells contained in MSCs, human MSCs negative for c-Kit, CD45 and CD34 were isolated by FACS and subjected to the induction process [43]. We confirmed that isolated cells could also be driven to be muscle-lineage cells as efficiently as the unsorted MSCs. Therefore, in our system, it appears that it is not a small fraction of bone-marrow-derived myogenic stem cells, but rather the major population of MSCs contribute to the production of muscle lineage cells.

IV. Application of M-MSCs to muscle degenerative disease model

As induced multinucleated myotubes in M-MSCs are already post-mitotic, single cells of MyoD-positive myoblasts and Pax7-positive satellite cells were subjected to clonal culture (clonal M-MSCs) to exclude non-muscle cells and transplanted into muscle degenerative disease models [43]. To estimate how workable these clonally-cultured M-MSCs are in the repair of degenerated muscles, human cells were transplanted into immunosuppressed rats whose gastrocnemius muscles were damaged with cardiotoxin pretreatment [43]. Cells were labeled by means of a GFP-encoding retrovirus and then transplanted by local injection (L.I.) into muscles or by intravenous injection (I.V.). Two weeks after transplantation, GFP-labeled cells incorporated into newly formed immature myofibers, exhibited centrally located nuclei in both L.I. and I.V. treated animals. The ratio (%) of GFP (+) fibers in total fibers (1500 fibers with centrally located nuclei were counted for each sample) was 37.1±9.9 % in L.I. and 22.6±7.9 % in I.V. 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. 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 [43].

Clonal M-MSCs contained Pax7-positive satellite cells which integrated into the satellite cell position after transplantation, namely the plasma membrane and the basal lamina inbetween [43]. The ratio of Pax7/GFP (+) cells in total Pax7-positive cells at 2 weeks was 17.2±4.2 % in L.I. and 5.9±2.8 % in I.V. In general, muscle satellite cells are known to contribute to the regeneration of myofiber formation upon muscle damage [44]. To confirm the contribution of transplanted satellite cells to muscle regeneration as *in vivo* satellite cells, the following experiment was performed. Four weeks after the initial transplantation of human clonal-M-MSCs intraveneously, cardiotoxin was re-administered into the same muscles without additional transplantation. Two weeks after the second cardiotoxin treatment (6 weeks after initial transplantation), many regenerating GFP-positive myofibers with centrally-located nuclei were observed. This implies that, upon transplantation of clonal-M-MSCs to the muscles of patients, those retained as satellite cells should be able to contribute to future muscle regeneration [23].

Transplantation of muscle lineage cells is a potential therapeutic approach for muscle degenerative disorders such as Duchenne muscular dystrophy (DMD), a severe progressive muscle wasting disease that results from a mutation in the dystrophin gene. The *mdx*-mouse, an animal model for DMD, was used for this experiment. The *mdx*-mouse is characterized by the absence of the muscle membrane associated protein, dystrophin. We locally injected GFP-labeled human clonal-M-MSCs into cardiotoxin-pretreated muscles of *mdx*-nude mice. Immunohistochemistry revealed the incorporation of transplanted cells into newly formed myofibers which expressed human dystrophin after transplantation as same as in case of above rat experiment [23].

V. Perspective

Cell transplantation therapy also offers hope for the treatment of intractable muscle degenerative disorders. Indeed, ES cells, stem cells derived from adult and prenatal muscle tissues, and myogenic stem cells from bone marrow are powerful candidates for transplantation therapy [1-5, 41]. Compared to these sources, the MSC system offers several important advantages. Firstly, 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. MSCs provide hopeful possibilities for clinical application, since they can efficiently expand *in vitro* and a therapeutic scale of induced cells are available. Thus functional skeletal muscle cells can be obtained within a reasonable time course on a therapeutic scale. Secondary, transplantation of MSC-derived cells should pose fewer ethical problems than ES cells and other kinds of stem cells, since bone marrow transplantation has already been widely performed. Hopefully, this MSC differentiation system may contribute substantially to eventual cell-based therapies for muscle disease.