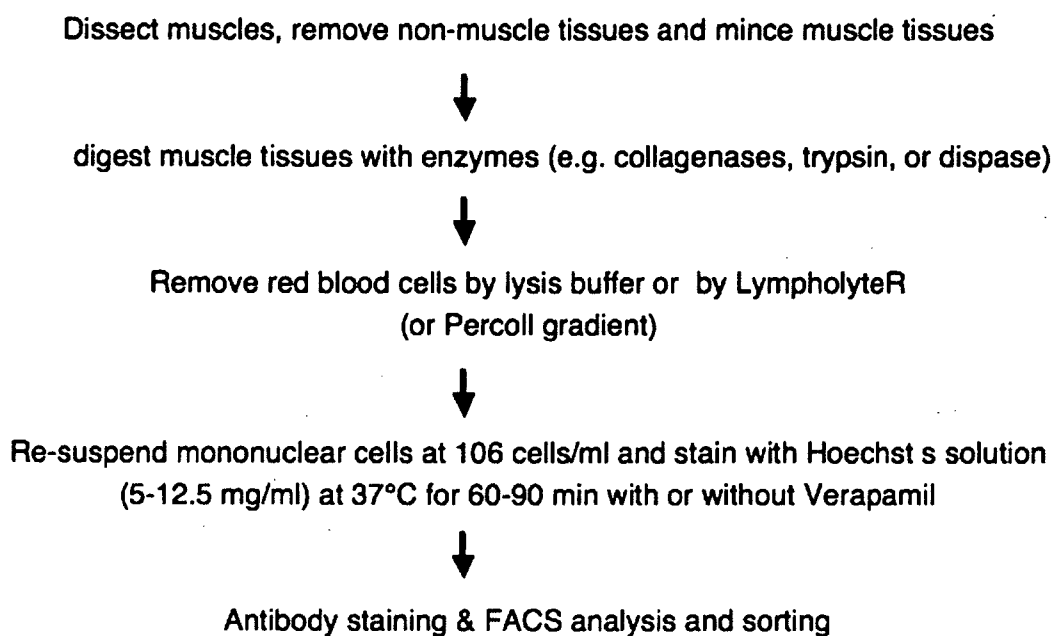
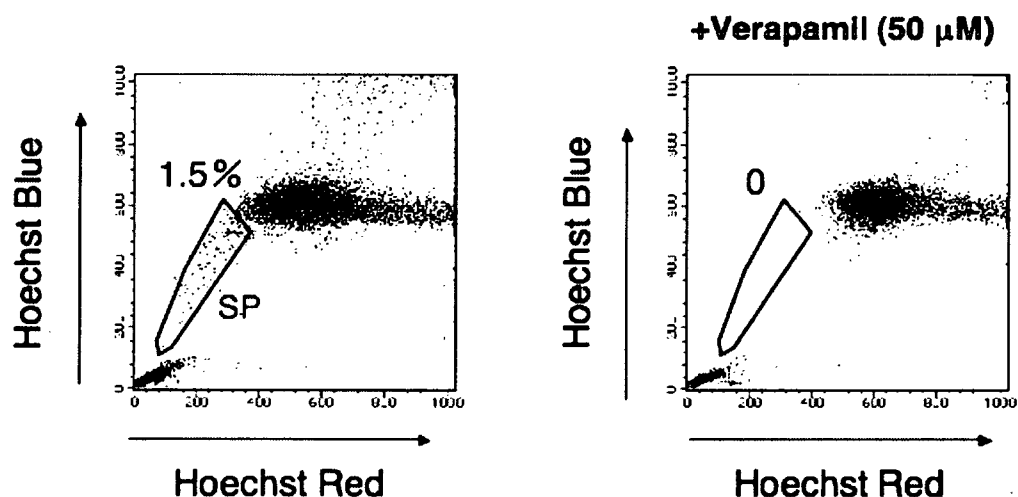


## Preparation of SP cells from mouse skeletal muscle



**Figure 1.** Preparation of SP cells from mouse skeletal muscle.



**Figure 2.** SP cells in adult skeletal muscle. Left panel: Muscle mononuclear cells were isolated from C57Bl/6 mice, stained with 5 mg/ml Hoechst 33342 dye, and analyzed on a dual laser FACS Vantage SE (Becton-Dickinson). Right panel: A calcium channel blocker verapamil was added to Hoechst solution to confirm the SP fraction.

**Table 1.** Variations in isolation protocols and properties of muscle SP cells.

Authors (year)	Preparation	Markers on SP cells	Properties	SP abundance
Gussoni <i>et al.</i> , 1999	Hoechst 12.5 µg/ml	Sca-1 <sup>+</sup> , lin <sup>-</sup> , c-kit <sup>-</sup> , CD45 <sup>-</sup> , CD43 <sup>-</sup>	hematopoietic stem cell-like activity produce myofibers after i.v. injection	1-1.5%
Jackson <i>et al.</i> , 1999	Hoechst 5 µg/ml	Sca1 <sup>+</sup> , c-kit <sup>+</sup> , CD45 <sup>-</sup>	HSC activity (+)	1%
Asakura <i>et al.</i> , 2002	Hoechst 5 µg/ml	Sca1 <sup>+</sup>	CD45 <sup>-</sup> fraction: hematopoietic differentiate into muscle cells in co-culture differentiate into satellite cells and muscle fibers <i>in vivo</i>	CD45-SP: 0.3-0.5% CD45-SP: 2-3%
Majka <i>et al.</i> , 2003	Percoll gradient Hoechst 5 µg/ml	c-met <sup>+</sup> , CD45 <sup>-</sup> , Sca-1 <sup>-</sup> , PE-CAM <sup>+</sup> , Tie-2 <sup>+</sup>	bone marrow-derived vascular progenitor	0.2%
Meeson <i>et al.</i> , 2004	Percoll gradient Hoechst 12.5 µg/ml	Sca-1 <sup>+</sup> , c-kit <sup>+</sup> , CD31 <sup>-</sup> , Abcg2 <sup>+</sup>	increase during muscle regeneration	0.2%
Uezumi <i>et al.</i> , 2006	Hoechst 5 µg/ml	heterogeneous	CD31-SP: Bcrp-1 <sup>-</sup> , vessel-associated CD45-SP: bone marrow origin CD31-CD45-SP: differentiate into adipocyte, osteocyte, and myocyte	1-3% < 0.1% < 0.1%

have a much higher ability to exclude Hoechst 33342 than BM-SP cells. The study also points out that Hoechst 33342 is toxic to cells. This fact is important, because the cell toxicity of Hoechst dye makes it difficult to directly compare the biological properties of SP and non-SP cells.

In our opinion, one further parameter that has an effect on the heterogeneity of muscle SP cells is pre-fractionation by a Percoll gradient before Hoechst staining [27] (Table 1). In our experience, this procedure eliminates CD45-negative SP cells, resulting in enrichment of CD45-positive SP cells (our unpublished data).

The definition of SP cells for non-hematopoietic tissues is not clear. Therefore, it is difficult to determine which SP cells are the true muscle SP cells. We also think that Hoechst staining alone is insufficient to collect a homogeneous cell population. This approach should be combined with identification of other cell surface markers.

## 2. SP phenotype and ABC transporters

SP cells are generally defined as a cell population that actively and efficiently expels Hoechst 33342 dye. This property is thought to be mediated mainly by ATP-binding cassette (ABC) transporters. ABC transporters bind ATP as an energy source to transport endogenous or exogenous molecules, in most cases unidirectionally, across the cell membrane. Various tissues and cells express different combinations of ABC transporters, therefore, it is no wonder that SP cells from different tissues are heterogeneous in phenotypes and functions [10].

### Muscle SP phenotype is not simply determined by Bcrp-1 expression

The ABCG2/Bcrp1 transporter is most often related to the SP phenotype. Bcrp-1-null mice show reduced numbers of SP cells in bone marrow and

skeletal muscle [13], but expression of this transporter is often found in non-SP cells [12]. Furthermore, isolation of human hematopoietic stem cells using an anti-ABCG2 antibody does not work [28]. These observations suggest that Bcrp-1 expression is not sufficient to endow the cell with the SP phenotype. In addition, it seems that a certain percentage of muscle SP cells are not Bcrp-1-dependent. When stained with an anti-Bcrp-1 antibody, the CD31-positive SP fraction is found to strongly express Bcrp-1, whereas two fractions, CD45-negative CD31-negative SP cells and CD45-positive SP cells reacted weakly with the anti-Bcrp-1 antibody [29]. Thus, transporters other than Bcrp-1 likely efflux Hoechst dye in a subset of muscle SP cells.

On the other hand, Meeson et al. examined the muscle SP fraction on a fluorescence-activated cell sorter (FACS) using both verapamil and fumitremorgin C (FTC), and confirmed that muscle-SP cells are highly sensitive to both of them [30]. Verapamil is a calcium channel blocker widely used to confirm the SP fraction on FACS profile, but shows no specificity toward a single transporter. FTC was shown to be a specific inhibitor of Abcd2, where it functions to inhibit Abcg2-associated ATPase activity [31]. Thus, the study showed that muscle SP cells are dependent on Abcg2/Bcrp-1 for the SP phenotype. SP preparation by Litman et al. employed a pre-fractionation of the muscle mononuclear cells using a Percoll gradient and a high concentration of Hoechst dye (12.5  $\mu\text{g/ml}$ ) for 90 min. The percentage of SP cells (0.19 %) was low, compared with other reports (see Table 1). Therefore, this result does not exclude the contribution of ABC transporters other than ABCG2/Bcrp-1 to the SP phenotype.

### **3. Other properties of muscle-derived SP cells**

#### **Most muscle SP cells are in quiescent stage**

Freshly isolated SP cells from muscle are often reported to be non-adherent in culture, and are characterized by small cell size, stages G0/G1 of the cell cycle, and low metabolic activity, elements common to stem cells. Indeed, small size ( $6.6 \pm 0.1$   $\mu\text{m}$ ) and a high nucleus-to-cytoplasm ratio were reported for muscle-derived SP cells [30]. However, when we analyzed muscle SP cells during muscle regeneration, they discovered CD31-negative CD45-negative SP cells, which are large in size and Ki-67-positive. Thus, at least one subset of SP cells is not small and is cycling.

#### **Cell surface markers on muscle SP cells**

Reflecting the variety of experimental protocols for SP preparation, there are discrepancies in reported cell surface markers of SP cells (Table 1). In 1999, Gussoni et al. described the isolation of SP cells from skeletal muscle for the first time [6]. They used a higher concentration of Hoechst dye (12.5  $\mu\text{g/ml}$ ) than the original protocol (5  $\mu\text{g/ml}$ ) described by Goodell et al. [9], and

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  $\mu$ g/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  $\beta$ -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-positive 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. 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'-tetramethylindocarbocyanine perchlorate-acetylated-low density lipoprotein (Dil Ac-LDL), suggesting that they possess 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.

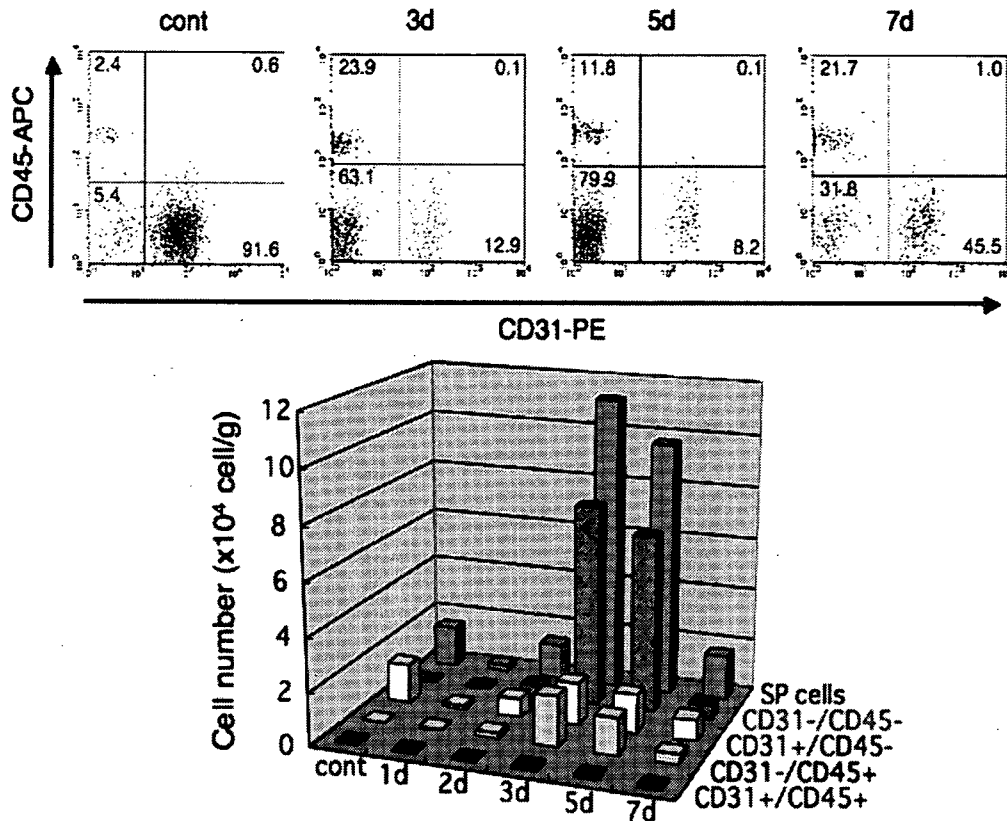
## 9. Physiological role of SP cells in muscle regeneration

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 endogenous 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

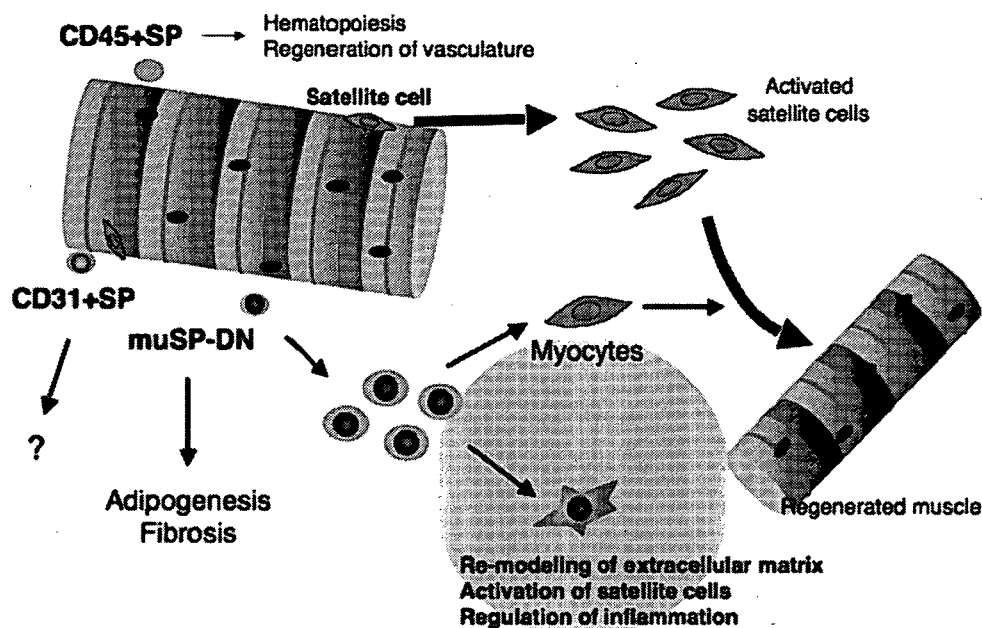
Cell-based therapy continues to be a promising avenue for the treatment of DMD, an X-linked skeletal muscle-wasting disease. However, myoblast transplantation into animal models results in local restoration of dystrophin expression [48], and human clinical studies using myoblast transfer performed on 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 FACS1, 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  $\mu\text{g/ml}$ ) 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 myoblasts and cells of a myogenic cell line, C2C12. Benchaour et al. stained myoblasts and C2C12 cells with a variety of concentrations of Hoechst (5-30  $\mu\text{g/ml}$ ) 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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