1. Abstract

CD31-negative CD45-negative (CD31(-) CD45(-)) side population (SP) cells are a minor SP subfraction that have mesenchymal stem cell-like properties in uninjured skeletal muscle, but that expand upon muscle injury. To clarify the role of CD31(-) CD45(-) SP cells in muscle regeneration, we injected GFP-positive myoblasts with or without CD31(-) CD45(-) SP cells into TA muscles of immunodeficient *NOD*/*scid* mice or dystrophin-deficient *mdx* mice. More GFP-positive fibers were formed after cotransplantation than after transplantation of GFP-positive myoblasts alone in both *mdx* and *NOD*/*scid* muscles. Moreover, grafted myoblasts were more widely spread after co-transplantation than after transplantation of myoblasts alone. Immunohistochemistry with anti-phosphorylated histone H3 antibody revealed that CD31(-) CD45(-) SP cells stimulated the cell division of co-grafted myoblasts. Genome-wide gene expression analysis showed that CD31(-) CD45(-) SP cells specifically express a variety of extracellular matrix proteins, membrane proteins, and cytokines. We also found that CD31(-) CD45(-) SP cells express high levels of matrix metalloproteinase (MMP)-2 mRNAs and gelatinase activities. Furthermore, MMP-2 derived from CD31(-) CD45(-) SP cells promoted migration of myoblasts *in vivo*. Our results suggest that CD31(-) CD45(-) SP cells support muscle regeneration by promoting proliferation and migration of myoblasts.

2. Introduction

Regeneration of skeletal muscle is a complex but well-organized process involving activation, proliferation, and differentiation of myogenic precursor cells, infiltration of macrophages to remove necrotic tissues, and remodeling of the extracellular matrix[1-3]. Muscle satellite cells are myogenic precursor cells that are located between the basal lamina and the sarcolemma of myofibers in a quiescent state, and are largely responsible for muscle fiber regeneration in adult muscle[4]. Recent studies also demonstrated that a fraction of satellite cells self-renew and behave as muscle stem cells *in vivo*[5, 6]. On the other hand, several research groups reported multipotent stem cells derived from skeletal muscle. These include muscle-derived stem cells (MDSC)[7], multipotent adult precursor cells (MAPC)[8], myogenic-endothelial progenitors[9], CD34(+) Sca-1(+) cells[10], CD45(+) Sca-1(+) cells[11], mesoangioblasts[12], and pericytes[13], and all were demonstrated to contribute to muscle regeneration as myogenic progenitor cells.

Side population (SP) cells are defined as the cell fraction that efficiently effluxes Hoechst 33342 dye and therefore shows a unique pattern on FACS analysis[14]. Muscle SP cells are proposed to be multipotent[15, 16] and are clearly distinguished from satellite cells[17]. Previous reports showed that muscle SP cells participated in regeneration of dystrophic myofibers after systemic delivery[15] and gave rise to muscle satellite cells after intramuscular injection into cardiotoxin-treated muscle[17]. Muscle SP cells adapted to myogenic characteristics after co-culture with proliferating satellite cells/myoblasts *in vitro*[17], and expressed a satellite cell-specific transcription factor, Pax7, after intra-arterial transplantation[18]. However, the extent to which muscle SP cells participate in muscle fiber regeneration as myogenic progenitor cells is

still largely unknown. Importantly, Frank *et al*. recently showed that muscle SP cells secret BMP4 and regulate proliferation of BMP receptor1α (+) Myf5 high myogenic cells in human fetal skeletal muscle[19], raising the possibility that SP cells in adult muscle play regulatory roles during muscle regeneration.

Previously we showed that skeletal muscle-derived SP cell fraction are heterogeneous and contain at least three subpopulations: CD31(+) CD45(-) SP cells, CD31(-) CD45(+) SP cells, and CD31(-) CD45(-) SP cells[20]. These three SP subpopulations have distinct origins, gene expression profiles, and differentiation potentials[20]. CD31(+) CD45(-) SP cells account more than 90% of all SP cells in normal skeletal muscle, take up Ac-LDL, and are associated with the vascular endothelium. CD31(+) CD45(-) SP cells did not proliferate after cardiotoxin-induced muscle injury. Bone marrow transplantation experiments demonstrated that CD31(-) CD45(+) SP cells are recruited from bone marrow into injured muscle. A few of them are thought to participate in fiber formation[21]. Cells of the third SP subfraction, CD31(-) CD45(-), constitute only 5-6% of all SP cells in adult normal skeletal muscle, but they actively expand in the early stages of muscle regeneration and return to normal levels when muscle regeneration is completed. Although CD31(-) CD45(-) SP cells are the only SP subset that exhibited the capacity to differentiate into myogenic, adipogenic, and osteogenic cells *in vitro*[20], their myogenic potential *in vivo* is limited compared with satellite cells. Therefore, we hypothesized that CD31(-) CD45(-) SP cells might play critical roles during muscle regeneration other than as myogenic stem cells.

In the present study, we demonstrate that the efficacy of myoblast transfer is markedly improved by co-transplantation of CD31(-) CD45(-) SP cells in both regenerating

immunodeficient *NOD*/*scid* and dystrophin-deficient *mdx* mice. We also show that CD31(-) CD45(-) SP cells increased the proliferation and migration of grafted myoblasts *in vivo* and *in vitro*. We further show that CD31(-) CD45(-) SP cell-derived MMP-2 greatly promotes the migration of myoblasts *in vivo*. Our findings would provide us insights into the molecular and cellular mechanisms of muscle regeneration, and also help us develop cell therapy for muscular dystrophy.

3. Materials and Methods

1) Animals

All experimental procedures were approved by the Experimental Animal Care and Use Committee at the National Institute of Neuroscience. Eight- to 12-*week*-old C57BL/6 mice and *NOD*/*scid* mice were purchased from Nihon CLEA (Tokyo, Japan). MMP-2-null mice were obtained from Riken BioResource Center (Tsukuba, Japan)[22] . GFP-transgenic mice (GFP-Tg) were kindly provided by Dr. M. Okabe (Osaka University, Japan). C57BL/6-background *mdx* mice were generously given by Dr. T. Sasaoka (National Institute for Basic Biology, Japan) and maintained in our animal facility.

2) Isolation of muscle SP cells

To evoke muscle regeneration, cardiotoxin (CTX, 10 *μM* in saline, Sigma, St. Louis, MO, USA) was injected into the tibialis anterior (TA) (50*μl*), gastrocnemius (150 *μl*), and quadriceps femoris muscles (100 *μl*) of 8- to 12-*week*-old GFP-Tg mice, C57BL/6 mice, MMP-2-null mice, and their wild-type littermates. Three days later, SP cells were isolated from the muscles as described by Uezumi *et al.*[20] . In brief, limb muscles were digested with 0.2% type II collagenase (Worthington Biochemical, Lakewood, NJ, USA) for 90 *minutes* at 37℃. After elimination of erythrocytes by treatment with 0.8% NH_4Cl in Tris-buffer (pH 7.15), mononucleated cells were suspended at 10^6 cells per *ml* in Dulbecco's modified Eagle's medium (DMEM, Wako) containing 2% FBS (JRH Biosciences, Inc., Kansas City, KS, USA), 10 *mM* Hepes, and 5 *μg*/*ml* Hoechst 33342 (Sigma), incubated for 90 *minutes* at 37°C in the presence or the absence of 50 *μM* Verapamil (Sigma), and then incubated with PE-conjugated anti-CD31

antibody (1:200, clone 390; SouthernBiotech, Birmingham, AL, USA) and PE-conjugated anti-CD45 (1:200, clone 30-F11; BD Pharmingen) for 30 *minutes* on ice. Dead cells were eliminated by propidium iodide staining. Analysis and cell sorting were performed on an FACS VantageSE flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA). APC-conjugated anti-CD90, Sca-1, CD34, CD49b, CD14, CD124, c-kit, CD14 (BD Pharmingen), CD44 (Southern Biotechnology Associates, Birmingham, AL, USA), and CD133 (eBioscience, San Diego, CA, USA) were used at 1:200 dilution.

3) Preparation of satellite cell-derived myoblasts and macrophages

Satellite cells were isolated from GFP-Tg mice or C57BL/6 mice by using SM/C-2.6 monoclonal antibody[23] and expanded *in vitro* in DMEM containing 20% FBS and 2.5 *ng/ml* bFGF (Invitrogen, Carlsbad, CA, USA) for 4 *days* prior to transplantation.

Macrophages were isolated from C57BL/6 mice 3 *days* after CTX injection. Mononucleated cells were stained with anti-Mac-1-PE (1:200, clone M1/70; BD PharMingen) and anti-F4/80-APC (1:200, clone CI: A3-1; Serotec, Oxford, UK). Mac-1(+) F4/80(+) cells were isolated by cell sorting as macrophages.

4) *Cell transplantation*

To induce muscle regeneration, 100 *μl* of 10 *μM C*TX was injected into the TA muscle of *NOD/scid* muscles, and 24 *hours* later, 30 *μl* of cell suspensions containing $3x10^4$ myoblasts, $3x10^4$ CD31(-) CD45(-) SP cells, or $3x10^4$ GFP (+) myoblasts plus $2x10^4$ CD31(-) CD45(-) SP cells were directly injected into the TA muscles of 8-*week*-old *NOD/scid* or *mdx* mice. At

several time points after transplantation, the muscles were dissected, fixed in 4% paraformaldehyde for 30 *minutes*, immersed in 10% sucrose/PBS and then in 20% sucrose/PBS, and frozen in isopentane cooled with liquid nitrogen.

5) Retrovirus transduction in vitro analyses

Red fluorescent protein (DsRed) cDNA (BD Biosciences San Diego, CA, USA) was cloned into a retrovirus plasmid, pMXs, kindly provided by Dr. T. Kitamura of the University of Tokyo [24]. Viral particles were prepared by introducing the resultant pMXs-DsRed into PLAT-E retrovirus packaging cells[25], and the filtered supernatant was added to the myoblast culture. The next day, DsRed (+) myoblasts were collected by flow cytometry.

6) Immunohistochemistrical analyses

We cut the entire TA muscle tissues on a cryostat into 6 *μm*-cross sections, and observed all serial sections under fluorescence microscopy. We then selected two or three sections in which GFP (+) cells were found most frequently. The sections were then blocked with 5% goat serum (Cedarlane, Hornby, ON, Canada) in PBS for 15 *minutes*, and then reacted with anti-GFP antibody (Chemicon International, Temecula, CA, USA), anti-laminin α2 antibody (4H8-2, Alexis, San Diego, CA, USA), anti-phospho-histone H3 antibody (Upstate, Lake Placid, NY, USA), or anti-DsRed antibody (Clontech, Palo Alto, CA, USA) at 4°C overnight. Dystrophin was detected using a monoclonal antibody, Dys-2 (Novocastra, Newcastle upon Tyne, UK), and an M.O.M. Kit (Vector, Burlingame, CA, USA). The sections were then incubated with appropriate combinations of Alexa 488-, 568-, or 594-labeled secondary antibodies (Molecular

Probes, Eugene, OR, USA) and TOTO-3 (Molecular Probes), and photographed using a confocal laser scanning microscope system TCSSP (Leica, Heidelberg, Germany). The area occupied by GFP (+) cells or myofibers was measured by using Image J software (NIH, Bethesda, MD, USA) on cross-sections from three independent experiments, and defined as the "distribution area".

7) RNA isolation and real-time PCR

Total RNA was isolated from muscles using TRIzol (Invitrogen). First strand cDNA was synthesized using a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). The levels of GFP mRNA and 18S rRNA were quantified using SYBR Premix Ex Taq (Takara, Otsu, Shiga, Japan) on a MyiQ Single-Color system (BioRad Laboratories, Richmond, CA, USA) following the manufacturer's instructions. Primer sequences for real-time PCR were: 18s rRNA, forward: 5'-TAC CCT GGC GGT GGG AT TAAC-3', reverse: 5'-CGA GAG AAG ACC ACG CCA AC-3' and EGFP, forward: 5'- GAC GTA AAC GGC CAC AAG TT-3', reverse: 5'-AAG TCG TGC TGC TTC ATG TG-3'. The expression levels of *MMP-2* and *MMP-9* were evaluated by conventional RT-PCR using following primers. MMP-2, forward: 5'-TGC AAG GCA GTG GTC ATA GCT-3', reverse: 5'-AGC CAG TCG GAT TTG ATG CT-3'.

8) Cell proliferation assay

CD31(-) CD45(-) SP cells or 10T1/2 cells were cultured in DMEM containing 20% FBS for 5 *days*, and the supernatants were collected as "conditioned medium". Myoblasts were plated on 96-well culture plates at a density of 5000 cells/well and cultured in conditioned

medium for 3 *days*. BrdU was then added to the culture medium (final concentration 10 μM). Twenty-four hours later, BrdU uptake was quantified by Cell Proliferation ELISA, a BrdU Kit (Roche Diagnostics, Meylan, France), and lumi-Image F1 (Roche).

9) Gene expression profiling

Total RNAs were extracted from CD31(-) CD45(-) SP cells, macrophages, or myoblasts using an RNeasy RNA isolation kit (Qiagen). cDNA synthesis, biotin-labeled target synthesis, MOE430A GeneChip (Affymetrix, Santa Clara, CA, USA) array hybridization, staining, and scanning were performed according to standard protocols supplied by Affymetrix. The quality of the data presented in this study was controlled by using the Microarray Suite MAS 5.0 (Affymetrix). The MAS-generated raw data were uploaded to GeneSpring™ software version 7.0 (Silicon Genetics, Redwood City, CA, USA). The software calculates signal intensities, and each signal was normalized to a median of its values in all samples or the 50*th* percentile of all signals in a specific hybridization experiment. Fold ratios were obtained by comparing normalized data of CD31(-) CD45(-) SP cells and macrophages or myoblasts.

10) In situ zymographical analyses

CD31(-) CD45(-) SP cells, myoblasts, and macrophages were isolated from regenerating muscles 3 *days* after CTX injection by cell sorting and collected by a Cytospin3 centrifuge (ThermoShandon, Cheshire, UK) on DQ-gelatin-coated slides (Molecular Probes). The slides were then incubated for 24 *hours* at 37°C in the presence or absence of GM6001 (a broad-spectrum inhibitor of MMPs ; 50 μM; Calbiochem, San Diego, CA, USA) or E-64 (a cysteine

protease inhibitor ; 50 μM ; Calbiochem). Fluorescence of FITC was detected with excitation at 460-500 *nm* and emission at 512-542 *nm*.

11) Statistics

Statistical differences were determined by Student's unpaired *t*-test. For comparison of more than two groups, one-way ANOVA was used. All values are expressed as means ± standard errors (SE). A probability of less than 5% ($p < 0.05$) or 1% ($p < 0.01$) was considered statistically significant.

4. Results

1) Marker expression on muscle-derived CD31(-) CD45(-) SP cells

When incubated with 5 *μg/ml* of Hoechst 33342 dye at 37°C for 90 *minutes*, 1-3% of muscle mononuclear cells show the SP phenotype (Figure 1A). Previously, we reported that muscle SP cells can be further divided into three subpopulation, CD31(-) CD45(-) cells, CD31(-) CD45(+) cells, and CD31(+) CD45(-) SP cells[20] (also see Figure 1B). The CD31(-) CD45(-) SP cells did not express Pax3, Pax7, or Myf5[20], indicating that they are not yet committed to the muscle lineage. RT-PCR suggested that CD31(-) CD45(-) SP cells have mesenchymal cell characteristics[20]. To further clarify the properties of CD31(-) CD45(-) SP cells, we analyzed their cell surface markers. CD31(-) CD45(-) SP cells were negative for CD124, CD133, CD14, c-kit (Figure 1B), and CD184 (data not shown), weakly positive for CD34 and CD49b, and strongly positive for Sca-1, CD44, and CD90 (Figure 1). The FACS patterns shown in Figure 1B suggested that CD31(-) CD45(-) SP cells are a homogeneous cell population. CD14 is an exception. A small fraction of CD31(-) CD45(-) SP cells were strongly positive for CD14, but the majority weakly expressed this marker. The function of CD14 high CD31(-) CD45(-) SP cells remains to be determined.

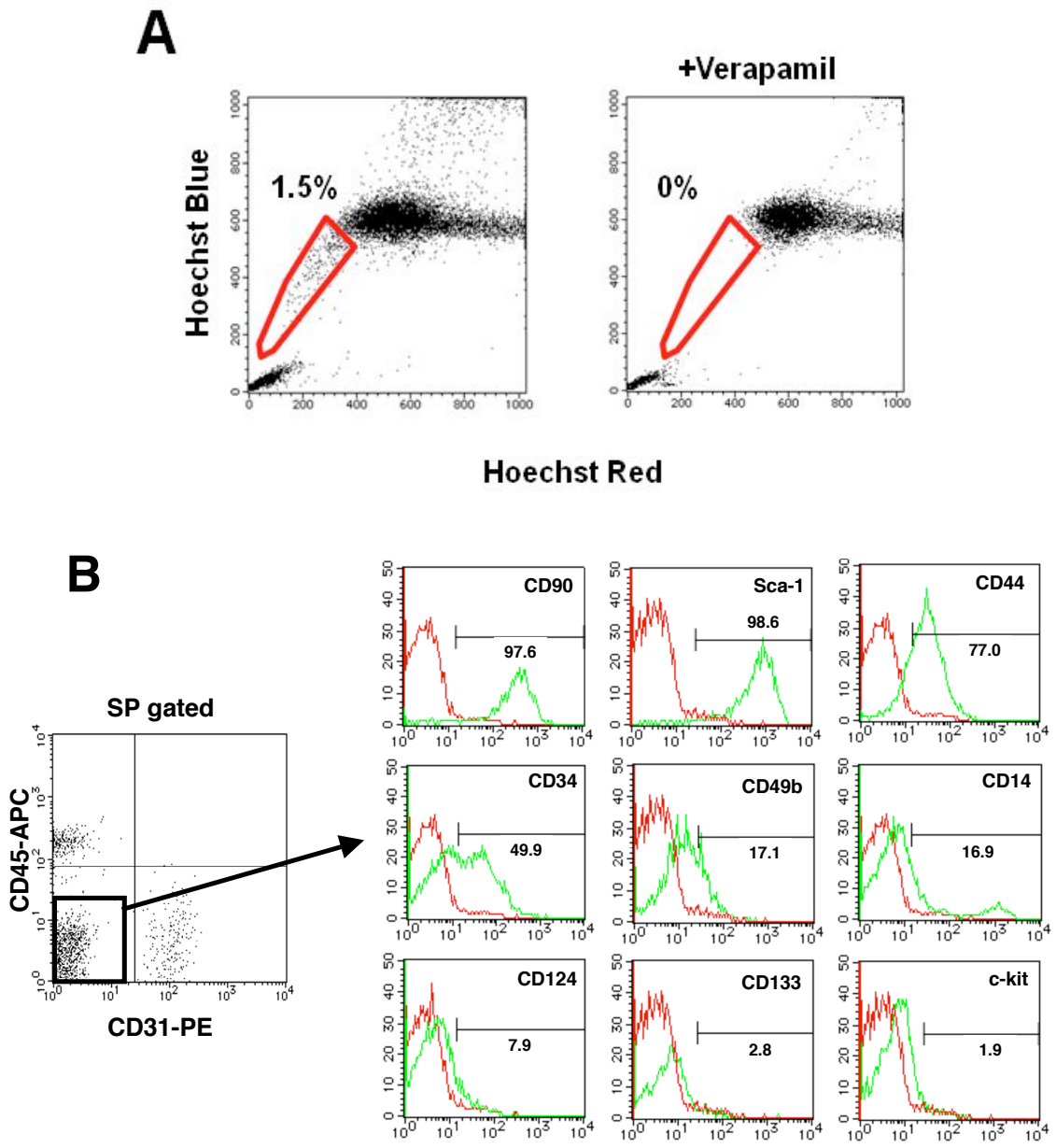

Figure 1 Cell surface markers on CD31(-) CD45(-) SP cells from regenerating muscle

A) Mononuclear cells were prepared from limb muscles of C57BL/6 mice at 3 *days* after cardiotoxin (CTX) injection, incubated with 5 μM Hoechst 33342 with (right) or without (left) Verapamil, and analyzed by a cell sorter. SP cells are shown by red polygons. The numbers indicate the percentage of SP cells in all mononuclear cells. B) (left) Expression of CD45 and CD31 on muscle SP cells. (right) The expression of surface markers (CD90, Sca-1, CD44, CD34, CD49b, CD14, CD124, CD133, and c-kit) on CD31(-) CD45(-) SP cells was further analyzed by FACS. The X axis shows the fluorescence intensity, and the y axis indicates cell numbers. Green lines are with antibodies; red lines are negative controls.

2) Efficiency of myoblast transplantation is increased by co-transplantation of muscle CD31(-) CD45(-) SP cells in NOD/scid mice

To clarify the functions of CD31(-) CD45(-) SP cells during muscle regeneration, we isolated myoblasts from GFP-transgenic mice (GFP-Tg) and injected them (3 x 10^4 cells/muscle) with or without CD31(-) CD45(-) SP cells (2 x 10^4 cells/muscle) into TA muscles of immunodeficient *NOD/scid* mice (depicted in Figure 2A). CTX was injected into recipient muscles 24 *hours* before cell transplantation to induce muscle regeneration. Two weeks after transplantation, the contribution of grafted myoblasts to muscle regeneration was investigated by immunodetection of GFP (+) myofibers. Co-transplantation of GFP (+) myoblasts with non-labeled CD31(-) CD45(-) SP cells produced a higher number of GFP (+) myofibers than transplantation of GFP (+) myoblasts alone (Figure 2B and C). Furthermore, the average diameter of GFP (+) myofibers was significantly larger in co-transplanted muscles than in muscles transplanted with myoblasts alone (Figure 2D). These results suggest that more myoblasts participated in myofiber formation after co-transplantation than after single transplantation, injected SP cells promoted growth of regenerating myofibers, or both.

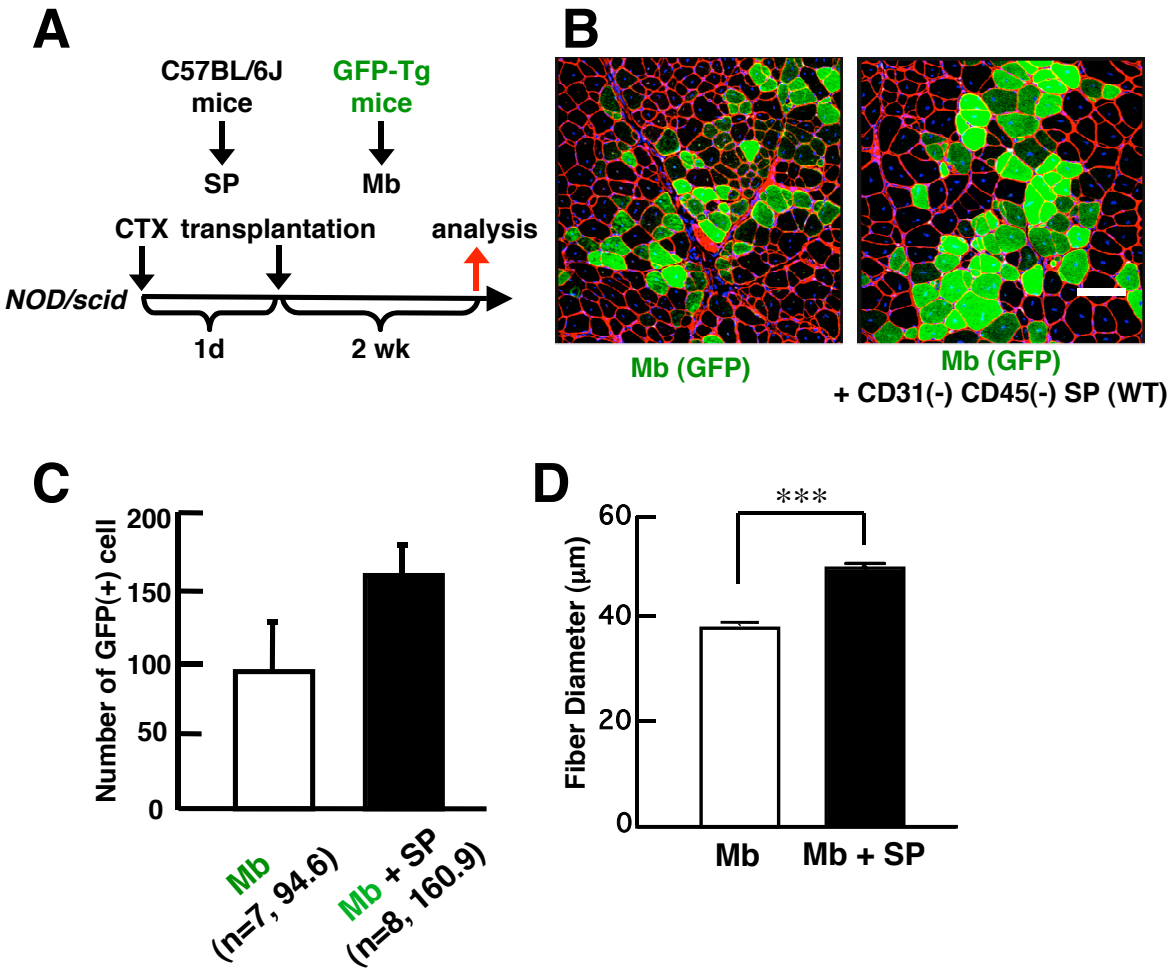

Figure 2 Co-transplantation of myoblasts and CD31(-) CD45(-) SP cells into skeletal muscle of immunodeficient *NOD/scid* mice promotes myofiber formation by transplanted myoblasts

A) Schematic protocol of co-transplantation experiments. CTX was injected into TA muscle 1 *day* prior to transplantation. Then, GFP (+) myoblasts (Mb) alone or with a mixture of GFP (+) myoblasts and CD31(-) CD45(-) SP cells derived from wild-type (WT) mice were transplanted to CTX-injected TA (tibialis anterior) muscles of 8- to 12-*week*-old *NOD/scid* mice, and sampled 2 *weeks* after transplantation. B) Cross-sections of transplanted TA muscles stained with anti-GFP (green) and anti-laminin-α2 chain (red) antibodies. Nuclei were stained with TOTO3 (blue). Scale bar : 80 *μm*. C) The number of GFP (+) fibers per cross-section of transplanted TA muscle. Values are means with SE (7 to 8 mice in each group). ** $p < 0.01$. D) Average diameters of GFP (+) fibers in the TA muscles transplanted with myoblasts (Mb) or myoblasts plus CD31(-) CD45(-) SP cells (Mb+SP). Values are means with SE. *** $p < 0.001$.

3) ***Co-transplantation of myoblasts with muscle CD31(-) CD45(-) SP cells significantly increased efficiency of myoblast transplantation in mdx mice***

Next, co-transplantation experiments were performed using 8-*week*-old dystrophin-deficient *mdx* mice as a host. Three kinds of transplantations were performed: 3 x 10^4 myoblasts derived from GFP-Tg mice, 3 x 10^4 CD31(-) CD45(-) SP cells derived from GFP-Tg mice, or a mixture of GFP (+) 3 x 10^4 myoblasts and 2 x 10^4 CD31(-) CD45(-) SP cells derived from C57BL/6 mice (Figure 3A).

When analyzed at 2 *weeks* after transplantation, a much higher number of GFP (+) myofibers were detected on cross-sections after co-transplantation of myoblasts and CD31(-) CD45(-) SP cells than after transplantation of GFP (+) myoblasts alone (Figure 3B and C). On the other hand, transplantation of GFP (+) SP cells alone resulted in formation of few GFP (+) myofibers. This observation is consistent with the recent report[20]. Co-transplantation of myoblasts and CD31(-) CD45(-) SP cells also gave rise to more myofibers expressing dystrophin at the sarcolemma in dystrophin-deficient *mdx* muscles than transplantation of myoblasts alone (data not shown). Again, the diameter of GFP (+) myofibers was significantly larger in co-transplanted muscles than in muscles transplanted with myoblasts or CD31(-) CD45(-) SP cells alone (Figure 3D).

The transplantation efficiency of myoblasts in *mdx* mice was 40-60% lower than that in *NOD*/*scid* mice. In the present study, *mdx* mice were not treated with any immunosuppressant. Although cellular infiltration was not evident when examined 2 *weeks* after transplantation (data not shown), some immune reaction might be evoked and eliminate myoblasts transplanted into *mdx* muscle.

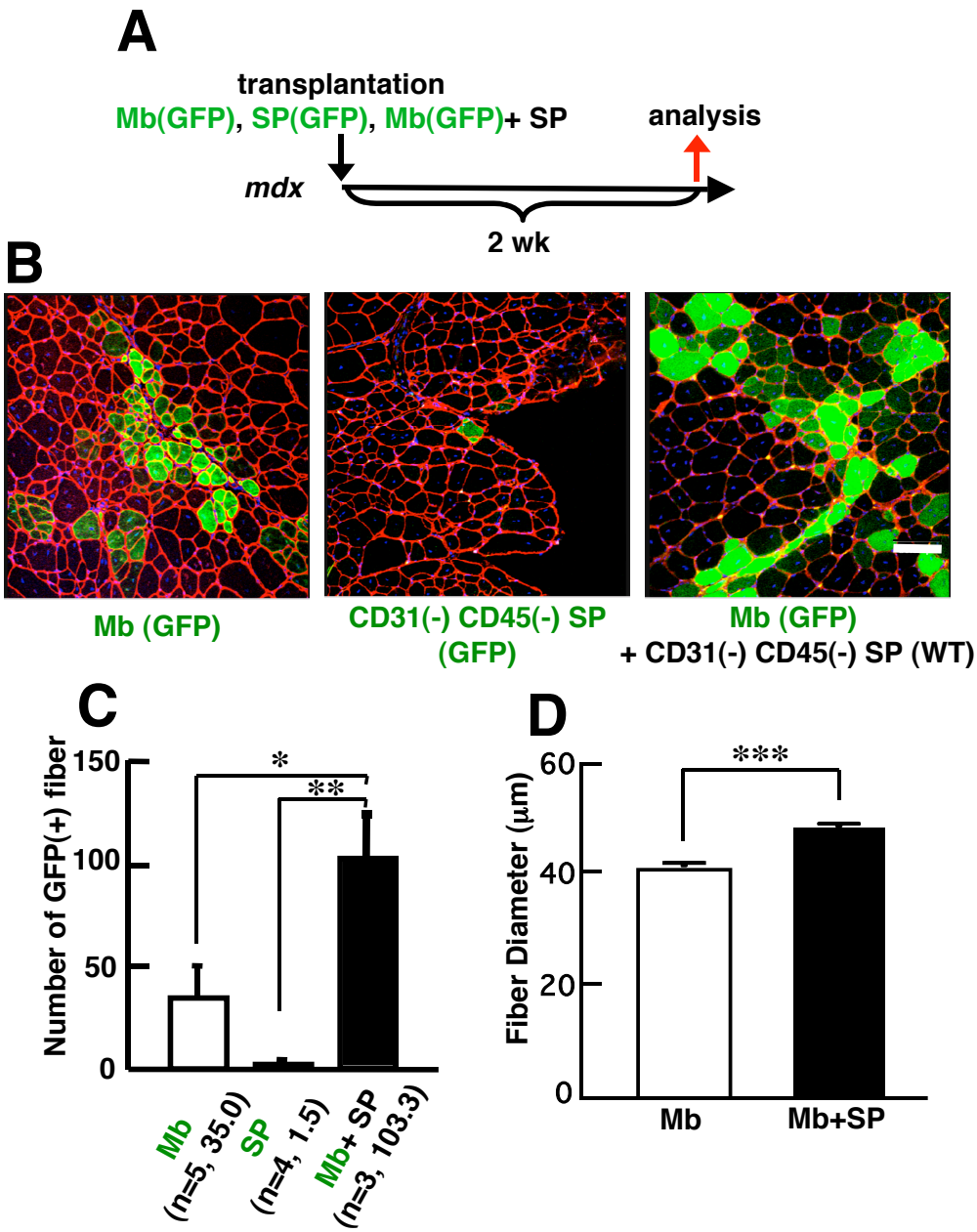

Figure 3 Co-transplantation of CD31(-) CD45(-) SP cells and myoblasts improves efficiency of myoblast transfer in dystrophin-deficient *mdx* mice

A) Schematic protocol of experiments. GFP (+) myoblasts alone ($3x10^4$), GFP (+) CD31(-) CD45(-) SP cells alone ($3x10^4$ cells), or a mixture of GFP (+) myoblasts ($3x10^4$) and CD31(-) CD45(-) SP cells ($2x10^4$) were directly injected into TA muscles of 8-*week*-old *mdx* mice, and the muscles were sampled 2 *weeks* after transplantation. B) Cross-sections of transplanted TA muscles stained with anti-GFP (green) and anti-laminin-α2 chain (red) antibodies. Nuclei were stained with TOTO3 (blue). Scale bar : 80 *μm*. C) The number of GFP (+) fibers per cross-section. Myoblasts gave rise to more myofibers when co-transplanted with CD31(-) CD45(-) SP cells (Mb + SP) than when transplanted alone (Mb). Transplantation of only GFP (+) SP cells resulted in formation of few myofibers (SP). Values are means with SE (n = 3-5 mice). * $p < 0.05$, ** $p < 0.01$. D) Average diameters of GFP (+) fibers in the TA muscles transplanted with myoblasts (Mb) or with myoblasts plus CD31(-) CD45(-) SP cells (Mb + SP). Values are means with SE. *** $p < 0.001$.

4) Localization of transplanted myoblasts and CD31(-) CD45(-) SP cells after intramuscular injection

To examine the interaction between grafted myoblasts and CD31(-) CD45(-) SP cells during muscle regeneration, we labeled C57BL/6 myoblasts with a retrovirus vector expressing a red fluorescent protein, DsRed. CD31(-) CD45(-) SP cells were isolated from GFP-Tg mice. We then injected a mixture of DsRed (+) myoblasts and GFP (+) CD31(-) CD45(-) SP cells into CTX-injected *NOD/scid* TA muscles. At 24 *hours* after transplantation, DsRed (+) myoblasts and GFP (+) CD31(-) CD45(-) SP cells were observed clearly (Figure 4A). At 48 *hours* after transplantation, immunohistochemistry revealed that grafted CD31(-) CD45(-) SP cells expanded, and surrounded both grafted myoblasts and damaged myofibers, but rarely fused with myoblasts (Figure 4B).

5) CD31(-) CD45(-) SP cells promote proliferation of myoblasts in vivo and in vitro

Next, to clarify the mechanism by which co-transplanted CD31(-) CD45(-) SP cells increased the contribution of grafted myoblasts to myofiber regeneration, we investigated the survival of grafted myoblasts after transplantation (Figure 5). GFP (+) myoblasts were injected into TA muscles of *NOD/scid* mice with or without unlabeled CD31(-) CD45(-) SP cells. At 24, 48, and 72 *hours* after transplantation, injected TA muscles were dissected, and the GFP mRNA level in injected muscles was evaluated by using real time PCR (Figure 5A). There was a decline of the GFP mRNA level of injected muscles from 24 to 72 *hours* post-injection (Figure 5B) with no differences in survival rates between single transplantation and co-transplantation.

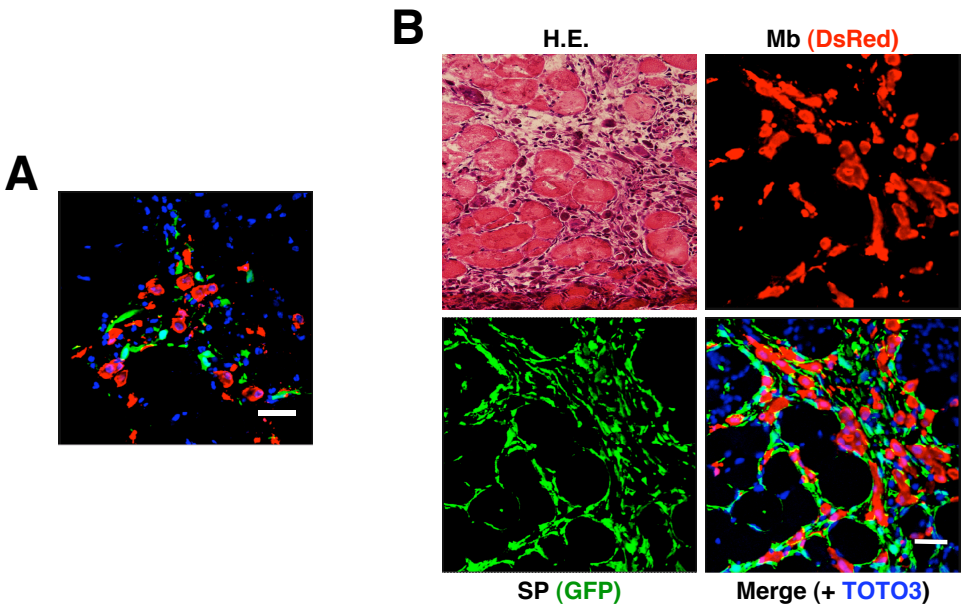

Figure 4 Behavior of GFP⁺ CD31(-) CD45(-) SP cells and DsRed-labeled myoblasts after transplantation

A) *NOD/scid* TA muscles were injected with CTX 24 *hours* before transplantation. Then, myoblasts transduced with a retrovirus vector expressing DsRed were injected together with GFP (+) CD31(-) CD45(-) SP cells into the muscles. The muscles were dissected 24 *hours* after the transplantation, sectioned, and stained with anti-DsRed (red) and anti-GFP antibodies (green). Nuclei were stained with TOTO3 (blue). Scale bar : 40 *μm*. B) Representative image of DsRed (+) myoblasts and GFP (+) SP cells 48 *hours* after co-transplantation. One serial section was stained with hematoxylin and eosin (H.E.). Scale bar : 40 *μm*.

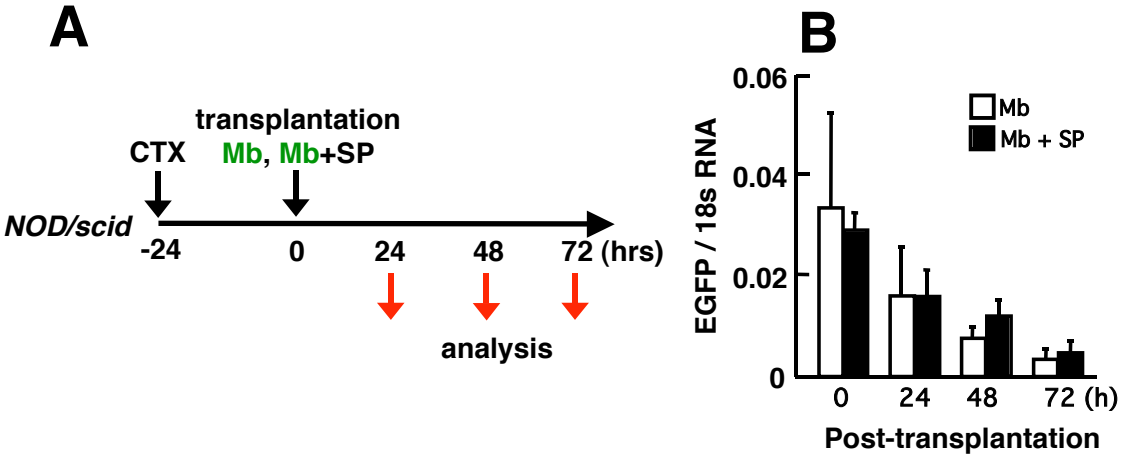

Figure 5 Survival of injected myoblasts in *NOD/scid* mice

A) Experimental design. GFP (+) myoblasts alone (3 x 10^4 cells) or a mixture of GFP (+) myoblasts (3 x 10^4 cells) and non-labeled CD31(-) CD45(-) SP cells (2 x 10^4 cells) were injected into previously CTX-injected TA muscles of *NOD/scid* mice. The muscles were then sampled at 0, 24, 48, and 72 *hours* after transplantation. B) The mRNA level of GFP at each time point was quantified by real time PCR. The Y axis shows GFP mRNA levels normalized to 18s RNA with SE (n = 4-5).

At 48 and 72 *hours* after transplantation, however, GFP mRNA levels were slightly higher in co-injected muscle than in muscle injected with myoblasts alone (Figure 5B). Therefore, we directly counted the number of GFP (+) myoblasts at 72 *hours* after transplantation. As shown in Figure 6A and B, many more GFP (+) myoblasts were detected in co-transplanted muscles than in myoblast-transplanted muscles (Figure 6A and B). In addition, GFP (+) cells were more widely spread in the co-injected muscles than in muscles transplanted with myoblasts alone (Figure 6C).

To determine whether CD31(-) CD45(-) SP cells promote proliferation of implanted myoblasts, we dissected the muscles at 48 *hours* after transplantation, and stained the cross-sections with anti-phosphorylated histone H3 antibody, a marker of the mitotic phase of the cell cycle. Co-transplantation of myoblasts with CD31(-) CD45(-) SP cells significantly increased the percentage of mitotic GFP (+) cells compared with transplantation of myoblasts alone (Figure 7A). These observations suggest that co-injection of CD31(-) CD45(-) SP cells promoted proliferation of grafted myoblasts.

Next, to examine whether CD31(-) CD45(-) SP cells directly promote proliferation of myoblasts or not, we performed *in vitro* proliferation assay using primary myoblasts and conditioned medium (CM) of CD31(-) CD45(-) SP cells and CM of 10T1/2 cells. BrdU uptake analysis showed that SP-CM more strongly stimulated the proliferation of myoblasts than 10T1/2-CM did (Figure 7B). The results suggest that CD31(-) CD45(-) SP cells promote proliferation of injected myoblasts at least in part by producing soluble factors.

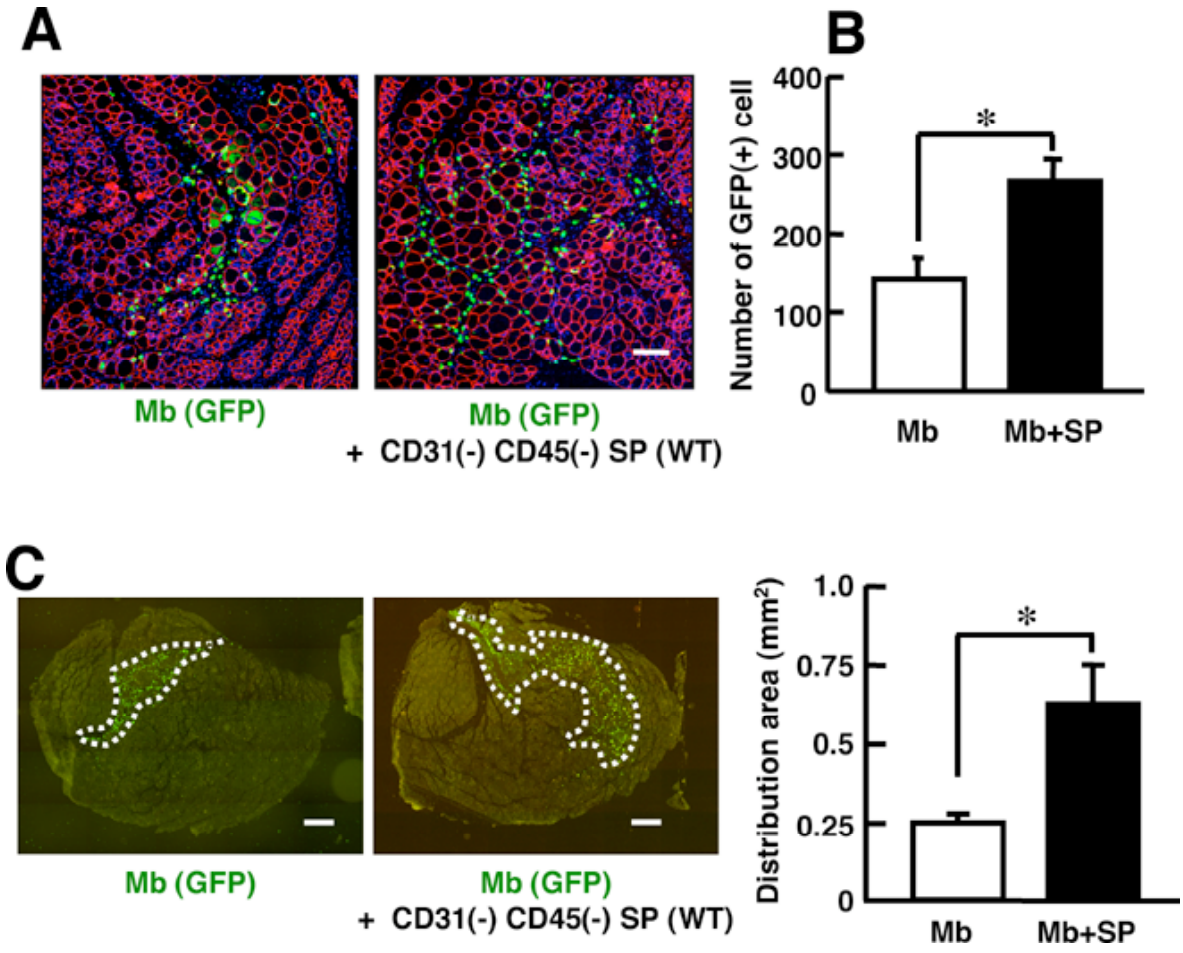

Figure 6 CD31(-) CD45(-) SP cells promote proliferation and migration of myoblasts *in vivo*

A) Representative images of cross-sections of 72 *hours* samples stained with anti-GFP (green) and anti-laminin-α2 chain (red) antibodies. GFP (+) myoblasts are more widely scattered in injected muscle when co-transplanted with CD31(-) CD45(-) SP cells, compared with single transplantation. Scale bar : 100 *μm*. B) The number of GFP (+) cells per cross-section of TA muscles injected with myoblasts or myoblasts and CD31(-) CD45(-) SP cells. Values were means with SE (n = 4-5). *$p < 0.05$ C) (left) Representative distributions of GFP (+) myoblasts/myotubes 72 *hours* after transplantation. Scale bar : 200 *μm*. (right) Distribution area (marked by white dotted lines in left panels) was measured by Image J software. Values were means with SE (n = 4-5). *$p < 0.05$

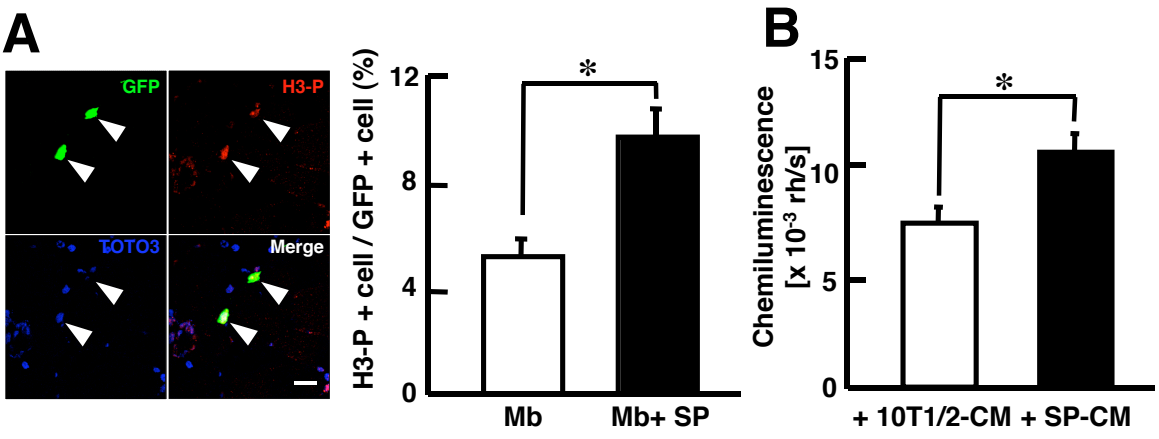

Figure 7　CD31(-) CD45(-) SP cells promote proliferation of myoblasts *in vitro*

A) GFP (+) myoblasts were transplanted into CTX-injected TA muscles of *NOD/scid* mice with (Mb + SP) or without CD31(-) CD45(-) SP cells (Mb). Forty-eight *hours* after transplantation, the muscles were dissected, sectioned, and stained with anti-phosphorylated histone-H3 (H3-P) (red) and anti-GFP (green) antibodies. Arrow heads indicate H3-P(+) GFP (+) cells. The right graph shows the percentage of H3-P(+) cells in GFP (+) myoblasts in single-transplanted muscle (Mb) or in co-transplanted muscle (Mb+SP). The values are means with SE (n = 3). Scale bar : 80 μm. *$p < 0.05$　B) Myoblasts were cultured for 3 *days* in conditioned medium of either CD31(-) CD45(-) SP cells (SP-CM) or 10T1/2 cells (10T1/2-CM) and then cultured for an additional 24 *hours* in the presence of BrdU. The vertical axis shows BrdU uptake by myoblasts. Values are means with SE (n = 6). *$p < 0.05$.

6) Gene expression profiling of CD31(-) CD45(-) SP cells

To identify the growth factor produced by CD31(-) CD45(-) SP cells that promotes proliferation of myoblasts, we extracted total RNAs from CD31(-) CD45(-) SP cells, myoblasts, and macrophages isolated from regenerating muscles 3 *days* after CTX injection, and examined the gene expression in these three cell populations by microarray. Eventually, we identified 192 genes that were expressed at more than 10-fold higher levels in CD31(-) CD45(-) SP cells than in either macrophages or myoblasts. We categorized the 192 genes based on gene ontology, and found that CD31(-) CD45(-) SP cells preferentially express extracellular matrix proteins and cytokines and their receptors (Table 1). We found numerous genes involved in wound healing and tissue repair on the gene list, suggesting that CD31(-) CD45(-) SP cells play a regulatory

role in the muscle regeneration process. Interestingly, the gene list contained both muscle proliferation or differentiation-promoting (follistatin) [26], and inhibitory factors (e.g., insulin-like growth factor binding proteins[27], Nov[28]). The list also contains regulators of TGF-beta (e.g. thrombospondins[29], Prss11[30], Ltbp3[31]), which would consequently attenuate or stimulate proliferation and differentiation of myoblasts.

7) CD31(-) CD45(-) SP cell-derived MMP-2 promotes the migration of myoblasts

Genome-wide gene expression analysis revealed that CD31(-) CD45(-) SP cells highly express matrix metalloproteinases (Table 1 and Figure 8). MMPs are a group of zinc-dependent endopeptidases that degrade extracellular matrix (ECM) components, thereby facilitating cell migration and tissue remodeling[32, 33]. Furthermore, MMPs are known to release growth factors stored within the extracellular matrix (ECM) and process growth factor receptors, resulting in stimulation of cell proliferation[34-36]. Among the MMPs upregulated in CD31(-) CD45(-) SP cells, we paid special attention to MMP-2 (also called gelatinase A or 72-kDa type IV collagenase). In CTX-injected muscle, MMP-2 activity was shown to be increased concomitantly with the transition from the regeneration phases characterized by the appearance of young myotubes to maturation of the myotubes into multinucleated myofibers[37, 38]. MMP-2 was also activated in the endomysium of regenerating fibers in dystrophin-deficient muscular dystrophy dogs[39]. Furthermore, MMP-2 transcripts were found in the areas of fiber regeneration, and were localized to mesenchymal fibroblasts in DMD skeletal muscle[40].

Table 1 Extracellular matrix proteins, membrane components, or cytokines highly expressed in CD31(-) CD45(-) SP cells compared with macrophages or myoblasts

Extracellular matrix

Synonyms	Genebank	Description	Fold change (vs. Macrophage)	Fold change (vs. Myoblast)
Adams family				
Adamts1	D67076	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1	27.9	28.9
Adamts5	NM_011782	a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 5 (aggrecanase-2)	24.2	26.2
Adam12	BI964347	a disintegrin and metalloproteinase domain 12 (meltrin alpha)	12.3	13.7
Procollagens				
Col1a1	U08020	procollagen, type I, alpha 1	125.7	128.9
Col14a1	AJ131395	procollagen, type XIV, alpha 1	98.2	104.4
Col5a3	NM_016919	procollagen, type V, alpha 3	36.4	37.6
Col3a1	AW550625	procollagen, type III, alpha 1	34.6	35.0
Pcolce	NM_008788	procollagen C-proteinase enhancer protein	30.4	31.1
Col6a2	BI455189	procollagen, type VI, alpha 2	28.0	30.5
Col5a1	NM_015734	procollagen, type V, alpha 1	26.5	27.7
Col6a2	BI455189	procollagen, type VI, alpha 2	24.0	29.4
Col6a3	AF064749	procollagen, type VI, alpha 3	21.6	23.2
Col6a1	NM_009933	procollagen, type VI, alpha 1	18.2	19.6
P4ha2	NM_011031	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha II polypeptide	16.5	16.9
Col1a2	AW545978	procollagen, type I, alpha 2	15.7	16.3
Col5a2	AV229424	procollagen, type V, alpha 2	13.5	13.7
Col4a2	BC013560	procollagen, type IV, alpha 2	12.0	12.5
Plod2	BC021352	procollagen lysine, 2-oxoglutarate 5-dioxygenase 2	11.6	11.6
Microfibrilar components				
Efemp1	BC023060	epidermal growth factor-containing fibulin-like extracellular matrix protein 1	250.2	384.3
Mfap4	BC022666	microfibrillar-associated protein 4	247.6	265.8
Mfap5	NM_015776	microfibrillar associated protein 5	70.5	74.2
Fbln2	BF228318	fibulin 2	55.5	57.4
Mfap2	NM_008546	microfibrillar-associated protein 2	38.9	51.8
Fbn1	NM_007993	fibrillin 1	22.6	24.1
Emilin1	NM_133918	elastin microfibril interfacer 1	12.2	14.1
Matrix metalloproteinase				
Mmp23	NM_011985	matrix metalloproteinase 23	48.0	48.3
Mmp2	NM_008610	matrix metalloproteinase 2	32.1	41.3
Other ECM components				
Postn	NM_054077	periostin, osteoblast specific factor	359.3	360.8
Prelp	NM_019759	proline arginine-rich end leucine-rich repeat	107.3	107.6
Scara3	U69176	scavenger receptor class A, member 3	98.2	99.1
Sparcl1	NM_010097	SPARC-like 1 (mast9, hevin)	50.9	69.7
Aspn	BI110565	asporin	46.8	48.8
C1qtnf5	AK014312	C1q and tumor necrosis factor related protein 5	23.5	23.9
Lama4	NM_025711	laminin, alpha 4	23.3	24.1
Ltbp3	NM_010917	latent transforming growth factor beta binding protein 3	20.4	21.3
Lum	BC023068	lumican	13.7	15.3
Nid1	BC026446	nidogen 1	13.5	17.7
Dpt	NM_008520	dermatopontin	13.4	14.5

Membrane components

Synonyms	Genebank	Description	Fold change	
			(vs. Macrophage)	(vs. Myoblast)
Tmem45a	NM_019631	transmembrane protein 45a	190.4	36.7
Cd248	NM_054042	CD248 antigen, endosialin	180.3	173.3
Fap	NM_007986	fibroblast activation protein	53.6	65.9
Cdh11	NM_009866	cadherin 11	37.2	25.1
Tmeff2	NM_019790	transmembrane protein with EGF-like and two follistatin-like domains 2	35.9	10.2
Copz2	NM_019877	coatomer protein complex, subunit zeta 2	28.0	26.7
Entpd2	NM_009849	ectonucleoside triphosphate diphosphohydrolase 2	16.8	13.6
Plxdc2	NM_026162	plexin domain containing 2	15.2	12.4
Prnp	NM_011170	prion protein	11.3	12.2

Receptor proteins

Synonyms	Genebank	Description	Fold change	
			(vs. Macrophage)	(vs. Myoblast)
Osmr	NM_011019	oncostatin M receptor	126.6	13.5
Il1rl1	NM_010743	interleukin 1 receptor-like 1	81.9	18.1
Pdgfra	AW537708	platelet derived growth factor receptor, alpha polypeptide	47.8	40.7
Fzd2	NM_020510	frizzled homolog 2 (Drosophila)	31.3	11.2

Cytokine & chemokines

Synonyms	Genebank	Description	Fold change	
			(vs. Macrophage)	(vs. Myoblast)
Wisp2	NM_016873	WNT1 inducible signaling pathway protein 2	332.7	110.1
Thbs3	NM_013691	thrombospondin 3	270.8	34.0
Fstl	NM_008047	follistatin-like	182.4	21.1
Slit3	BM570006	slit homolog 3 (Drosophila)	179.9	65.7
C1s	BC022123	complement component 1, s subcomponent	178.8	86.3
Fbln5	NM_011812	fibulin 5	154.1	32.9
Svep1	NM_022814	sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1	142.7	32.4
Thbs2	NM_011581	thrombospondin 2	130.1	22.4
Steap4	NM_054098	STEAP family member 4	95.6	55.5
Cxcl5	NM_009141	chemokine (C-X-C motif) ligand 5	94.9	24.0
Fst	NM_008046	follistatin	92.5	35.3
Nov	X96585	nephroblastoma overexpressed gene	79.6	15.2
Cxcl14	AF252873	chemokine (C-X-C motif) ligand 14	77.7	16.5
Smoc2	NM_022315	SPARC related modular calcium binding 2	76.5	73.4
Igfbp6	NM_008344	insulin-like growth factor binding protein 6	66.9	12.4
Igfbp7	AI790558	insulin-like growth factor binding protein 7	46.3	31.8
Gdf10	L42114	growth differentiation factor 10	43.1	127.8
Cxcl12	BC006640	chemokine (C-X-C motif) ligand 12	37.5	37.6
Prss11	NM_019564	protease, serine, 11 (Igf binding)	29.0	11.9
Inhba	NM_008380	inhibin beta-A	25.7	48.5
Tnfaip6	NM_009398	tumor necrosis factor alpha induced protein 6	22.4	34.0
Plat	NM_008872	plasminogen activator, tissue	19.5	10.5
Igfbp4	AA119124	insulin-like growth factor binding protein 4	11.3	30.1
Ctgf	NM_010217	connective tissue growth factor	9.0	10.9

Genes more highly expressed (more than 10-fold, raw signal > 1000) in CD31(-) CD45(-) SP cells than in macrophages and myoblasts were categorized into subgroups based on Gene Ontology annotations by using Gene Spring software. Fold changes are relative to CD31(-) CD45(-) SP cells.

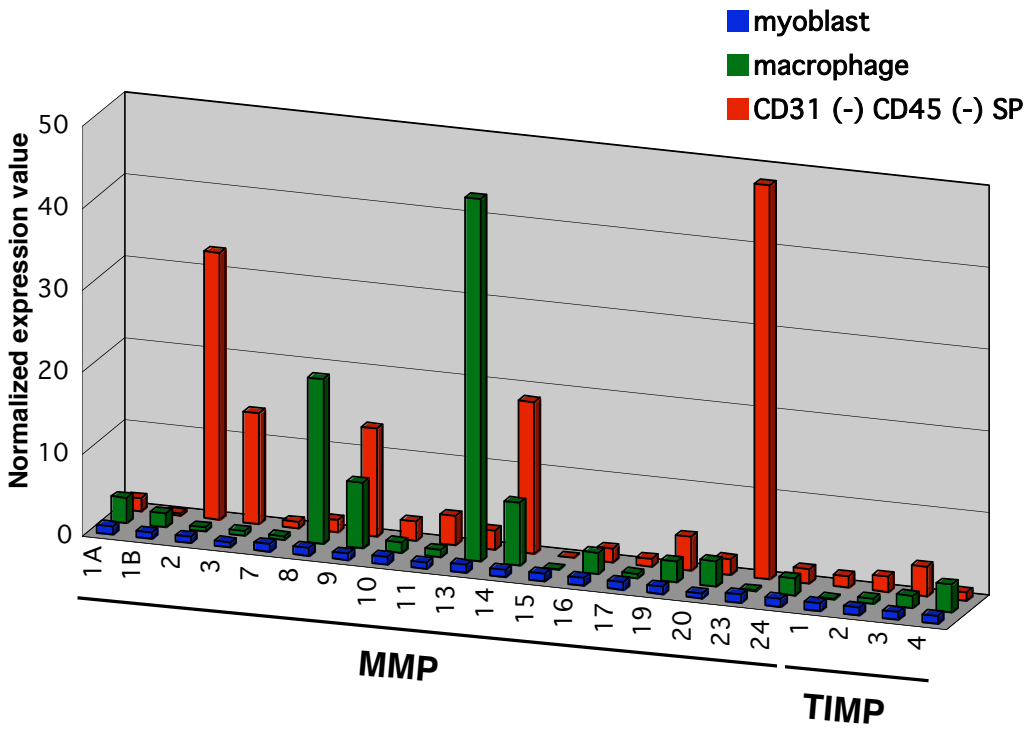

Figure 8 Relative expression levels of members of MMP family and TIMP family in CD31(-) CD45(-) SP cells, macrophages, and myoblasts

Expression levels of genes were analyzed by GeneChip microarray and GeneSprings software (Agilent Technologies, Wilmington, DE, USA).

We confirmed that the mRNA level of MMP-2 was much higher in CD31(-) CD45(-) SP cells than in macrophages or myoblasts (Figure 9A). Next, we examined the gelatinolytic activity in CD31(-) CD45(-) SP cells, macrophages, and myoblasts by DQ-gelatin zymography. The cells were directly isolated from regenerating muscle. High gelatinolytic activity was detected in CD31(-) CD45(-) SP cells, compared to myoblasts or macrophages (Figure 9B). Importantly, the signal in MMP-2-null SP cells was considerably weak, compared with wild-type SP cells. The results indicate that DQ-gelatin was degraded mainly (but not exclusively) by MMP-2 in the assay. We hardly detected the green fluorescence in wild-type SP cells in the presence of a broad-spectrum inhibitor of MMPs, GM6001, but not a potent inhibitor of

cysteine proteases, E-64, suggesting that other MMPs contribute to gelatin degradation to some extent in the assay. Collectively, these results indicate that CD31(-) CD45(-) SP cells have high MMP-2 activity.

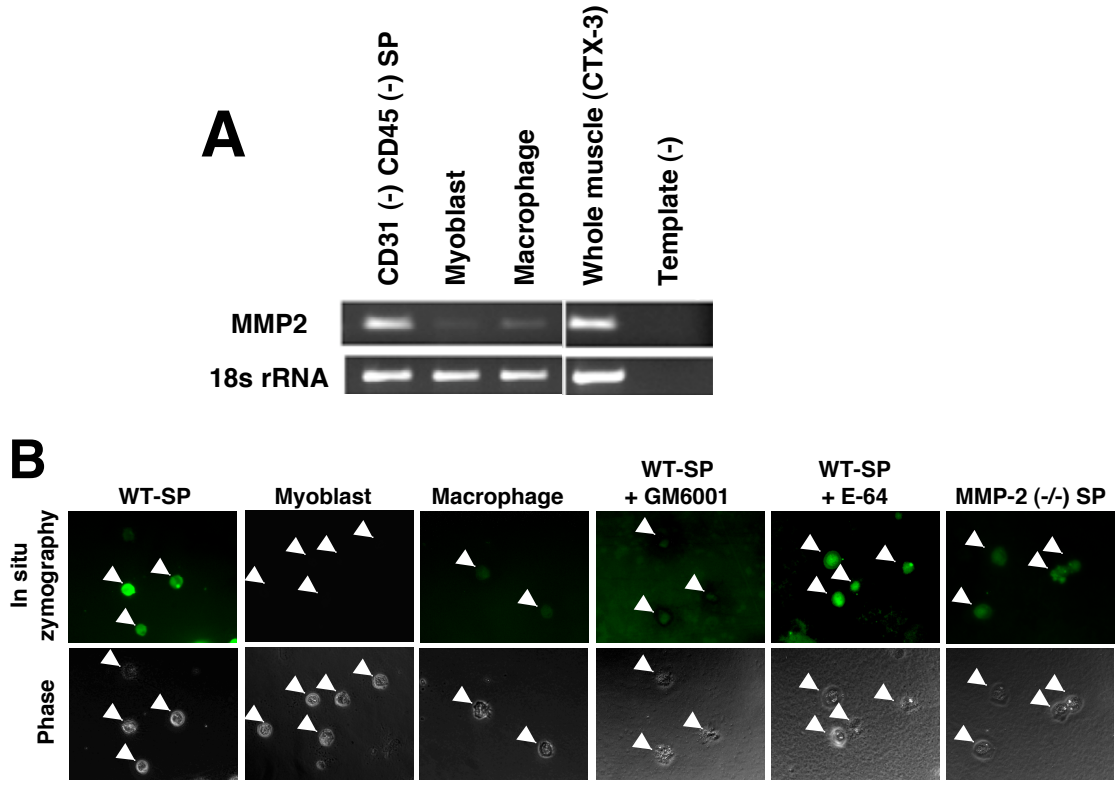

Figure 9 The expression of MMP-2 in CD31(-) CD45(-) SP cells

A) RT-PCR analysis of the expression of MMP-2 in CD31(-) CD45(-) SP cells, myoblasts, macrophages, and regenerating muscles. 18s rRNA is shown as an internal control. Template (-) is a negative control. B) In situ zymography of wild-type CD31(-) CD45(-) SP cells (WT-SP), myoblasts, macrophages, and MMP-2 (-/-) CD31(-) CD45(-) SP cells (MMP-2(-/-) SP) in the presence or absence of GM6001 (50 μM) or E-64 (50 μM). Cells were freshly isolated from regenerating muscles 3 *days* after CTX injury and collected on the glass slides. Upper panels are fluorescent signals from digested DQ-gelatin. Phase contrast images of the cells (arrowheads) are shown in lower panels.

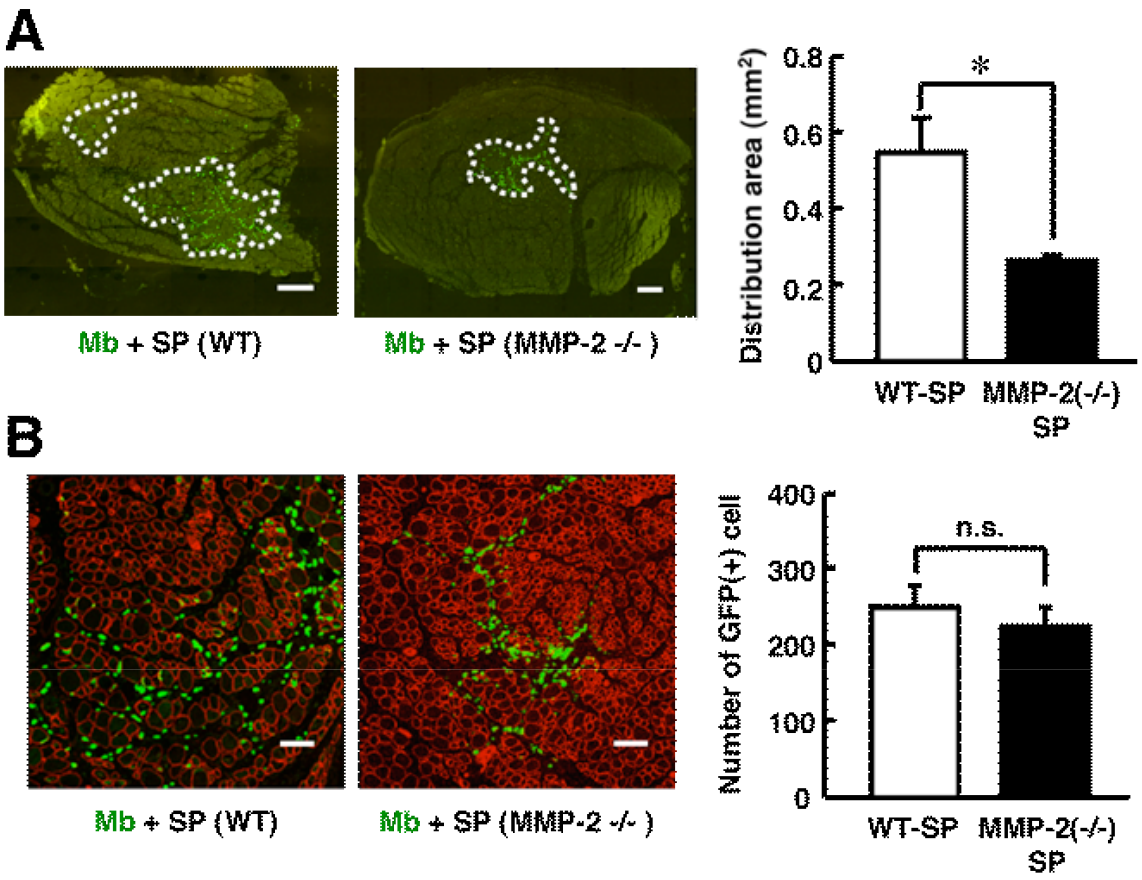

Figure 10 MMP-2 derived from CD31(-) CD45(-) SP cells promotes the migration of myoblasts *in vivo*

A) (left) Representative images of GFP (+) myoblasts 72 *hours* after co-transplantation of GFP+ myoblasts and CD31(-) CD45(-) SP cells from wild-type (WT) or from MMP-2-null mice (MMP-2 -/-) into CTX-injected TA muscles of *NOD/Scid* mice. Scale bar : 200 *μm*. (right) Distribution areas shown by white dotted lines in the left panels were measured by ImageJ (NIH). Values are means with SE (n = 5-6). *$p < 0.05$. B) (left) Representative immunohistochemistry of cross-sections of the TA muscle 72 *hours* after co-transplantation. Scale bar : 100 *μm*. (right) The number of GFP (+) cells per cross-section of the TA muscle injected with GFP (+) myoblasts and CD31(-) CD45(-) SP cells derived from wild-type littermates (Mb-SP) or MMP-2-null mice (MMP-2 (-/-) SP). Values are means with SE (n = 5-6).

MMP-2 is reported to mediate cell migration and tissue remodeling[32, 33]. To directly investigate the effects of MMP-2 on the migration and proliferation of transplanted myoblasts, we injected GFP (+) myoblasts with CD31(-) CD45(-) SP cells prepared from wild-type mice or from MMP-2-null mice into CTX-injected TA muscles of *NOD/scid* mice. There was no difference in the yield of CD31(-) CD45(-) SP cells from regenerating muscle between wild-

type and MMP-2-null mice (data not shown). Consistent with this observation, MMP-2-null CD31(-) CD45(-) SP cells proliferated as vigorously as wild-type *in vitro* (data not shown).

At 72 *hours* after transplantation, GFP (+) myoblasts were more widely spread in the muscle co-injected with wild-type CD31(-) CD45(-) SP cells than in the muscles co-injected with MMP-2-deficient CD31(-) CD45(-) SP cells (Figure 10A). In contrast, there was no difference in the number of GFP (+) myoblasts between two groups (Figure 10B). These results strongly suggest that MMP-2 derived from CD31(-) CD45(-) SP cells significantly promotes migration of myoblasts, but does not influence the proliferation of myoblasts.

5. Discussion

We previously reported a novel SP subset: CD31(-) CD45(-) SP cells[20]. They are resident in skeletal muscle and are activated and vigorously proliferate during muscle regeneration. RT-PCR analysis suggested that CD31(-) CD45(-) SP cells are of mesenchymal lineage, and indeed they differentiated into adipocytes, osteogenic cells, and muscle cells after specific induction *in vitro*[20]. In the present study, we further characterized CD31(-) CD45(-) SP cells and found that co-transplantation of CD31(-) CD45(-) SP cells markedly improves the efficacy of myoblast transfer to dystrophic *mdx* mice. Our findings suggest that endogenous CD31(-) CD45(-) SP cells support muscle regeneration by stimulating proliferation and migration of myoblasts.

1) Are CD31(-) CD45(-) SP cells mesenchymal stem cells?

Analysis of cell surface antigens on CD31(-) CD45(-) SP cells suggests that they are a homogeneous population. Several reports showed that mesenchymal stem cells (MSCs) express CD44, CD90, but not CD31, CD45, or CD14[41, 42]. The expression patterns of these markers on CD31(-) CD45(-) SP cells and their differentiation potentials into osteogenic cells, adipocytes, and myogenic cells suggest that CD31(-) CD45(-) SP cells are closely related to MSCs[20]. On the other hand, the expression of PDGFRβ[20], CD44, CD49b, CD90, and the lack of CD133 expression on CD31(-) CD45(-) SP cells are similar to those of human pericytes[13]. Unlike human pericytes, however, CD31(-) CD45(-) SP cells have limited myogenic potential *in vivo*[13, 20]. The relationship between CD31(-) CD45(-) SP cells and MSCs or pericytes remains to be determined in a future study.

2) CD31(-) CD45(-) SP cells promote proliferation of myogenic cells

In the present study, we demonstrated that the efficiency of myoblast transfer is greatly improved by co-transplantation of CD31(-) CD45(-) SP cells. Transplanted CD31(-) CD45(-) SP cells proliferated in the injection site and surrounded both engrafted myoblasts and damaged myofibers, but rarely fused with myoblasts (Figure 4). Transplantation of CD31(-) CD45(-) SP cells alone contributed little to myofiber formation. Therefore, the improvement in efficiency of myoblast transfer by co-transplantation is not due to differentiation of CD31(-) CD45(-) SP cells into muscle fibers.

Because the conditioned medium from CD31(-) CD45(-) SP cells modestly stimulated the proliferation of myoblasts *in vitro,* when compared with CM of 10T1/2 cells, it is possible that CD31(-) CD45(-) SP cells stimulated proliferation of myoblasts by secreting growth factors. CD31(-) CD45(-) SP cells are found in close vicinity to myoblasts 48 *hours* after transplantation. Therefore, even low levels of growth factors produced by CD31(-) CD45(-) SP cells may effectively stimulate the proliferation of myoblasts. Importantly, several reports showed that MSCs secrete a variety of cytokines and growth factors, which suppress the local immune system, inhibit fibrosis and apoptosis, enhance angiogenesis, and stimulate mitosis and differentiation of tissue-specific stem cells[43)] . On the gene list, we found a variety of cytokines/chemokines and their regulators (Table 1). These molecules may directly or indirectly stimulate proliferation of myoblasts.

3) MMP-2 derived from CD31(-) CD45(-) SP cells promote the migration of myoblasts

Transplanted GFP (+) myoblasts were more widely spread in injected muscle when co-

injected with CD31(-) CD45(-) SP cells than when transplanted alone (Figure 6C). MMP-2 is a candidate molecule which promotes migration of myoblasts. MMP-2 plays a critical role in myogenesis[44] and is up-regulated in muscle regeneration[38] (also see Figure 11). MMP-2 expression is also detected in regenerating areas of dystrophic muscles[39, 40]. Importantly, El Fahime *et al.* reported that forced expression of MMP-2 in normal myoblasts significantly increased migration of myoblasts *in vivo*[45]. In the present study, we demonstrated that CD31(-) CD45(-) SP cells highly express MMP-2 (Figure 9A and Table 1). Gelatin zymography confirmed that CD31(-) CD45(-) SP cells have high gelatinolytic activities (Figure 9B). Importantly, CD31(-) CD45(-) SP cells prepared from wild-type mice promoted the migration of transplanted myoblasts, but those from MMP-2 null mice did not (Figure 10A). Our results suggest that CD31(-) CD45(-) SP cells promote the migration of myoblasts via MMP-2 secretion. CD31(-) CD45(-) SP cells highly express MMP-2, 3, 9, 14, and 23 during regenerating muscle (Figures 8 and 11, and Table 1). Therefore, it remains to be determined whether MMPs other than MMP-2 also promote the migration of myoblasts. MMPs are reported to promote cell proliferation by releasing local growth factors stored within the extracellular matrix and process growth factor receptors[34, 35]. In the present study, however, MMP-2 derived from CD31(-) CD45(-) SP cells did not stimulate the proliferation of myoblasts *in vivo* (Figure 10B). The factors that stimulate the proliferation of myoblasts remain to be determined in a future study. MMP-3, 9, 14, and 23 are candidates that play a role in stimulating the proliferation of myoblasts.

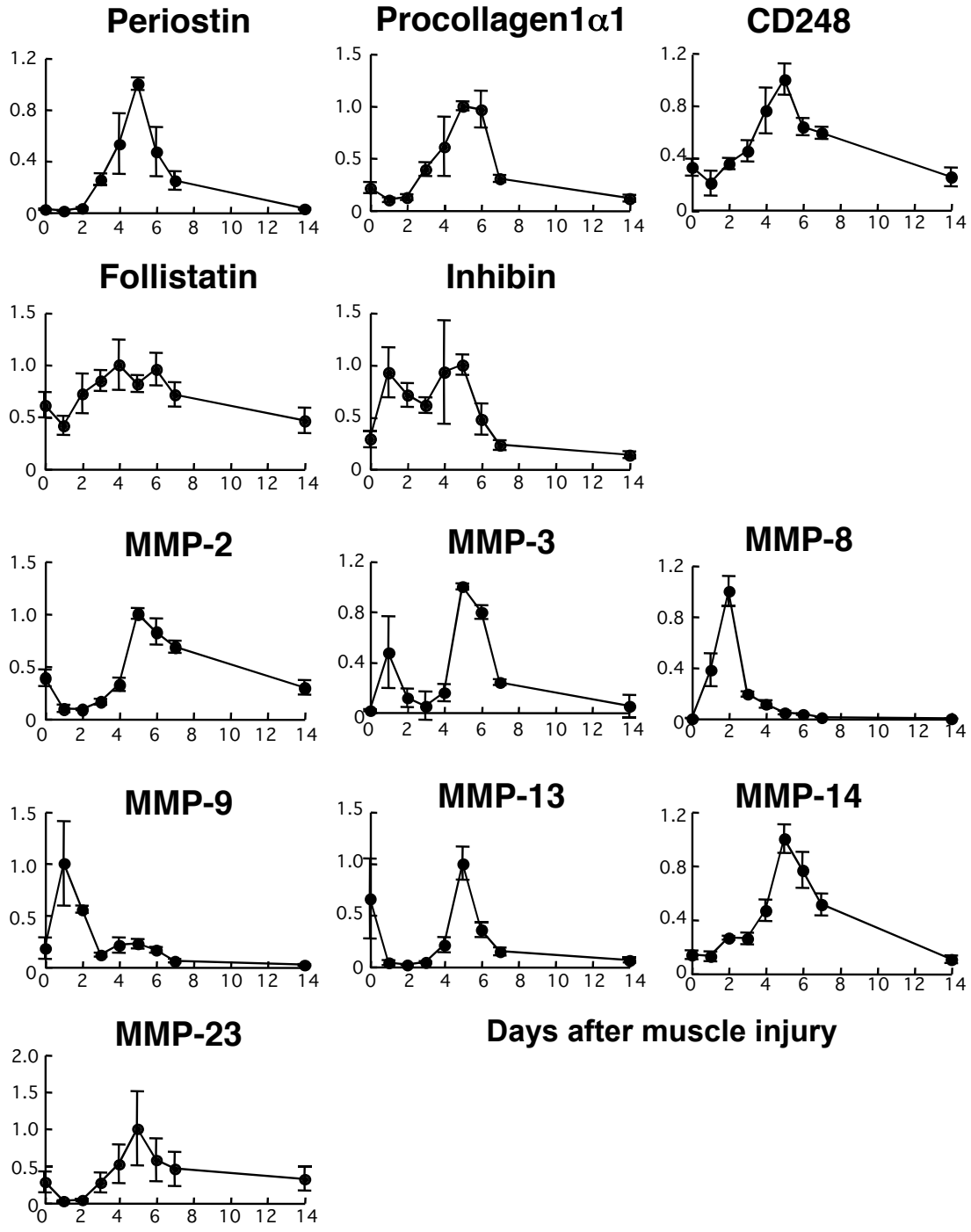

Figure 11 Real-time PCR analyses on CD31(-) CD45 (-) SP cell-specific genes and MMPs during muscle regeneration

TA muscles of C57BL/6 mice (n = 32) were injured by injection of cardiotoxin (CTX) and 1 *day*, 2 *days*, 3 *days*, 4 *days*, 5 *days*, 6 *days*, 7 *days*, and 14 *days* after muscle injury (4 mice for each time point: n = 4). Then total RNA was extracted from muscle tissues and analyzed by real-time PCR (Bio-Rad). All data were normalized to 18s rRNA. The value of day 5 is shown as 1.0. Sequences of PCR primers are available upon request. The expressions of periostin, procollagen1α1, CD248/endosialin/TEM1, MMP-2, MMP-3, MMP-13 MMP-14, and MMP-23 peaked at 5 *days* after CTX injection, confirming that CD31(-) CD45 (-) SP cells specifically express these genes.

4) CD31(-) CD45(-) SP cells are third cellular component of muscle regeneration

Our results suggest that transplanted CD31(-) CD45(-) SP cells stimulate myogenesis of co-transplanted myoblasts by supporting their proliferation and migration. Our results also suggest that endogenous CD31(-) CD45(-) SP cells promote muscle regeneration by the same mechanisms. Muscle regeneration is a complex, highly coordinated process in which not only myogenic cells but also inflammatory cells like macrophages play critical roles[3)]. Based on our finding that CD31(-) CD45(-) SP cells regulate myoblast proliferation and migration, we propose that CD31(-) CD45(-) SP cells are a third cellular component of muscle regeneration. In addition, gene expression analysis on CD31(-) CD45(-) SP cells revealed that CD31(-) CD45(-) SP cells express a wide range of regulatory molecules implicated in embryonic development, tissue growth and repair, angiogenesis, and tumor progression, suggesting that CD31(-) CD45(-) SP cells are a versatile player in regeneration of skeletal muscle. Future studies of ablation of endogenous CD31(-) CD45(-) SP cells in the mouse will likely further clarify the mechanisms by which CD31(-) CD45(-) SP cells promote muscle regeneration.

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7. Publication List

1. Shirato K, Motohashi N, Tanihata J, Tachiyashiki K, Tomoda A and Imaizumi K: Effects of two types of inactivity on the number of white blood cells in rats. ***European Journal of Applied Physiology***, 2006, 98(6), 590-600
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8. Oral Publication List

1. Motohashi N, Tanihata J, Tachiyashiki K and Imaizumi K: *In vivo* effect of hydrocortisone on plasma energy substrate levels in rats. ***Japanese Journal of Physiology***, 54 (*Supplement*), S233 (2004)
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3. Motohashi N, Tachiyashiki K, Tanihata J, Ichinose T and Imaizumi K: Muscle atrophy-induced changes of chatepsin and dipeptide levels in rats. ***Japanese Journal of Physiology***, 55 (*Supplement*), S225 (2005)
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