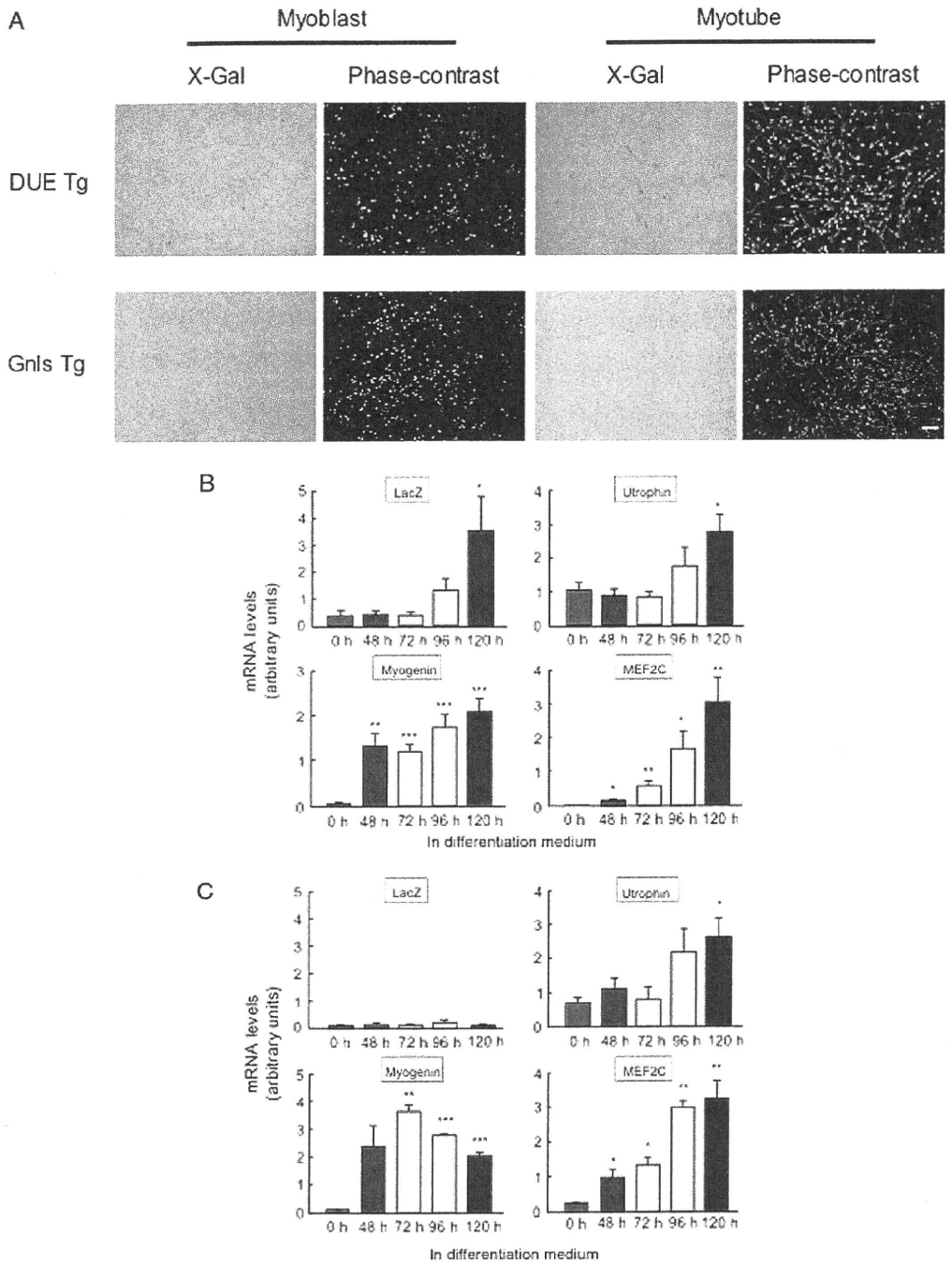

Figure 6. Transgene expression in primary cultured myoblasts and myotubes from DUE Tg mice. (A) Primary cultured myoblasts and myotubes from DUE Tg mice and Gnls Tg mice were stained with X-Gal. Image of myotube after 3 days in differentiation medium. Scale bar = 200 μm. (B, C) Quantification of q-RT-PCR products for transgene, A-utrophin, myogenin, and MEF2C optimized to expression of 18s rRNA in primary myoblasts and myotubes from DUE Tg mice and Gnls Tg mice. The ratios of the transgene, A-utrophin, myogenin, and MEF2C to 18s rRNA are shown as the mean ± SEM of four independent experiments performed in triplicate. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (versus 0 time)

Discussion

We previously showed that a 5385-bp 5′-flanking region of the utrophin gene containing the A-utrophin core promoter drives high levels of transgene expression in liver, testis, colon, submandibular gland, and small intestine, but not in heart and skeletal muscle [24]. In the present study, we demonstrated that addition of DUE to the 5385-bp 5′-flanking region enabled transgene expression in a pattern that was almost identical to that endogenous utrophin expression (Tables 1 and 2). Moreover, β-gal-expressing nuclei were basically located in the vicinity of the endogenous utrophin expression in heart and skeletal muscle.

In regenerating muscle of DUE Tg mice and skeletal muscle of DUE Tg/*mdx* mice, which lack dystrophin, the transgene expression was considerably

Table 1. Cells that express β-gal in Gnls Tg and DUE Tg mice and comparison with endogenous utrophin expression

Tissue	Endogenous utrophin	β-Gal expression		
		Gnls	DUE line 1	DUE line2
Liver	Surface of hepatocyte	Hepatocyte	Hepatocyte	Hepatocyte
Testis	BM of seminiferous tubule	Sertoli cell	Sertoli cell	Sertoli cell
	Leydig cell	Leydig cell	Leydig cell	Leydig cell
Colon	BM of large intestinal gland	Goblet cell	Goblet cell	Goblet cell
	Muscularis mucosa	ND	ND	ND
Submandibular gland	BM of serous & mucous acinus	Serous & mucous secretory cell	Serous & mucous secretory cell	Serous & mucous secretory cell
Small intestine	BM of villus & crypt	Paneth cell, goblet cell	Paneth cell, Goblet cell	Paneth cell, Goblet cell
	Muscularis mucosa	ND	ND	ND
Kidney	BM of cortical renal tubule	Epithelial cell of cortical renal tubule	Epithelial cell of cortical renal tubule	Epithelial cell of cortical renal tubule
	BM of collecting duct in renal medulla			
	Glomerulus	ND	ND	ND
Lung	Alveolus	Alveolar cell	Alveolar cell	Alveolar cell
	Terminal bronchiole epithelium	ND	ND	ND
Cerebrum	Choroid plexus	ND	Ependymal cell of choroid plexus	ND
	Pia mater		Fibroblastic cell of pia mater	
Cerebellum	Pia mater	ND	Stellate cell and basket cell of molecular layer	ND
Heart	Intercalated disk	ND	Peripheral cell of intercalated disk	ND
	T tubule			
Skeletal muscle	Neuromuscular junction	ND	Peripheral cell of neuromuscular junction	ND
	Myotendinous junction			
	Regenerating muscle fiber			

The localization of endogenous utrophin is based on this study and previous studies [11,24]. BM, basement membrane; ND, not detected.

Table 2. Summary of β-gal expression in Gnls Tg mice and DUE Tg mice

	Gnls	DUE line 1	DUE line 2
Liver	++	++	±
Testis	+++	+++	+
Colon	++	++	+
Submandibular gland	+++	+++	+
Small intestine	+	+	±
Kidney	±	+	+
Lung	+	++	±
Cerebrum	-	++	-
Cerebellum	-	++	-
Heart	-	++	-
TA muscle	-	+	-

Tg mice were sacrificed at 4–7 weeks, and β-gal expression was examined in several tissues. No β-gal positive nuclei were found in nontransgenic littermates. β-gal expression levels: −, none; ±, trace; +, weak; ++, moderate; +++, strong.

up-regulated. Another study [10] also reported that utrophin transcription was controlled by DUE activity in regenerating muscle and that its activity was dependent on an AP-1 binding site. Injection of marcain into TA muscles of CD1 mice demonstrated elevation of members of the AP-1 factor, *c-fos, fosB, fra-1, fra-2, c-jun, junB* and *junD* [10]; however, we also found distinct elevation of *c-fos* and *fra-1* mRNA in our regeneration system (unpublished observations).

We cultured primary myogenic cells from DUE Tg mice and found that transgene expression was up-regulated during the differentiation process. Moreover, these transgene expression patterns corresponded to the endogenous utrophin expression profile. This result indicates that the participation of DUE in utrophin expression during muscle regeneration might depend largely on DUE activity in the later stage of muscle differentiation. It is intriguing to note that transgene and endogenous utrophin expression patterns coincided with the expression profile of MEF2C, but not that of myogenin. It has been already reported that MEF2C mediates the promoter activity of c-jun [33]. The MEF2C-c-jun pathway is one of the candidates for regulation of utrophin expression via DUE. Analysis of the transcriptional factors that interact with DUE sequences, particularly the AP-1 site, would be very intriguing and should be clarified by a future study.

In the present study, we also demonstrated that the addition of DUE augmented transgene expression not only in the heart and skeletal muscle, but also in other tissues, such as the cerebral pia mater and choroid plexus and the cerebellar choroid plexus and molecular layer. In addition, transgene expression was elevated in the kidney and lung of DUE Tg mice compared to that of Gnls Tg mice, although it is necessary to consider the difference in transgene copy numbers. These results suggest that DUE activity is not muscle specific, consistent with the data of Galvagni *et al.* [26]. In their study, a construct of DUE added to the utrophin promoter was transiently transfected to various cells and revealed that DUE enhanced utrophin promoter activity not only in C2C12 myoblasts, but also in HeLa cells and RD cells.

However, the addition of DUE cannot fully explain the transcriptional regulation of utrophin. In the cerebrum and cerebellum, endogenous utrophin was expressed in the pia mater and choroid plexus. We found β-gal-positive

nuclei in the cerebral pia mater along the basal lamina, but did not find many β-gal-positive nuclei in the cerebellum. There are several possibilities to explain this discrepancy. The first possibility is that the domain that regulates utrophin expression in the pia mater of the cerebellum is different from that for the pia mater in the cerebrum. The second possibility is that transcription of utrophin might be less active in fibroblastic cells of the pia mater of cerebellum compared to those in the cerebrum. However, a fundamental difference between fibroblastic cells in the cerebrum and those in the cerebellum has not been reported; further experiments are required to explain this discrepancy.

We demonstrated that DUE is necessary for utrophin expression in skeletal muscle, but the increase in the utrophin expression level was much larger than the transgene expression in regenerated muscle. Another study [11] also detected the increase in the abundance of A-utrophin protein in muscle from *mdx* mice but could not find any parallel elevation in the levels of utrophin transcripts. Therefore, A-utrophin expression may also be regulated at the post-transcriptional level. Indeed, recent studies have shown that distinct cis-acting elements within the utrophin 3′-UTR were important not only for controlling the stability of utrophin transcripts in muscle cells, but also for targeting them to specific subcellular locations [34,35].

Post-translational levels are also important for utrophin expression through stabilization of the protein. DAPs such as dystrophin, β-dystroglycan, α-dystroglycan, and α-sarcoglycan have been linked to regulation by protein degradation mechanisms including the ubiquitin-proteasome pathway [36] and calpain-mediated proteolysis [37]. Inhibition of the proteasomal degradation pathway was found to rescue the expression levels of several DAPs in *mdx* mice [36]. Treatment of normal and DMD human myotubes with glucocorticoid induced utrophin protein without elevations in transcripts, and this was suggested to involve calpain inhibition [38].

It is likely that extrasynaptic expression of utrophin in skeletal muscle of DMD patients would ameliorate the dystrophic pathology, at least to some extent [17,18]. The results of the present study demonstrate that DUE is indispensable to utrophin expression in skeletal muscle and heart. To further investigate the up-regulation mechanisms of utrophin in both tissues, we need to search for transcription factors bound to DUE. In addition, we established primary myogenic cell cultures from DUE Tg mice and found that utrophin up-regulation depends on the DUE motif during muscle differentiation. These cells provide a high through-put screening system for drugs that can up-regulate utrophin expression in myogenic cells.

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Musculoskeletal Pathology

Muscle CD31(−) CD45(−) Side Population Cells Promote Muscle Regeneration by Stimulating Proliferation and Migration of Myoblasts

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CD31(−) CD45(−) side population (SP) cells are a minor SP subfraction that have mesenchymal stem cell-like properties in uninjured skeletal muscle but that can expand on muscle injury. To clarify the role of these SP cells in muscle regeneration, we injected green fluorescent protein (GFP)-positive myoblasts with or without CD31(−) CD45(−) SP cells into the tibialis anterior muscles of immunodeficient *NOD/scid* mice or dystrophin-deficient *mdx* mice. More GFP-positive fibers were formed after co-transplantation than after transplantation of GFP-positive myoblasts alone in both *mdx* and *NOD/scid* muscles. Moreover, grafted myoblasts were more widely distributed after co-transplantation than after transplantation of myoblasts alone. Immunohistochemistry with anti-phosphorylated histone H3 antibody revealed that CD31(−) CD45(−) SP cells stimulated cell division of co-grafted myoblasts. Genome-wide gene expression analyses showed that these SP cells specifically express a variety of extracellular matrix proteins, membrane proteins, and cytokines. We also found that they express high levels of matrix metalloproteinase-2 mRNA and gelatinase activity. Furthermore, matrix metalloproteinase-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. Future studies to further define the molecular and cellular mechanisms of muscle regeneration will aid in the development of cell therapies for muscular dystrophy. ***(Am J Pathol 2008, 173:781–791; DOI: 10.2353/ajpath.2008.070902)***

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 primarily 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,[7] multipotent adult precursor cells,[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 fluorescence-activated cell sorting (FACS) analysis.[14] Muscle SP cells are proposed to be multipotent[15,16] and are clearly distinguished from satellite

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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 (CTX)-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 primarily unknown. Importantly, Frank and colleagues[19] recently showed that muscle SP cells secret BMP4 and regulate proliferation of BMP receptor1α (+) Myf5^{high} myogenic cells in human fetal skeletal muscle, 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 for 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 CTX-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 to 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 matrix metalloproteinase (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.

Materials and Methods

Animals

All experimental procedures were approved by the Experimental Animal Care and Use Committee at the National Institute of Neuroscience. Eight- to twelve-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, Osaka, Japan). C57BL/6-background *mdx* mice were generously given by Dr. T. Sasaoka (National Institute for Basic Biology, Aichi, Japan) and maintained in our animal facility.

Isolation of Muscle SP Cells

To evoke muscle regeneration, CTX (10 μmol/L in saline; Sigma, St. Louis, MO) 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; 3 days later, SP cells were isolated from the muscles as described by Uezumi and colleagues.[20] In brief, limb muscles were digested with 0.2% type II collagenase (Worthington Biochemical, Lakewood, NJ) for 90 minutes at 37°C. 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 (Wako, Richmond, VA) containing 2% fetal bovine serum (JRH Biosciences, Inc., Kansas City, KS), 10 mmol/L Hepes, and 5 μg/ml Hoechst 33342 (Sigma), incubated for 90 minutes at 37°C in the presence or the absence of 50 μmol/L Verapamil (Sigma), and then incubated with phycoerythrin (PE)-conjugated anti-CD31 antibody (1:200, clone 390; Southern Biotechnology, Birmingham, AL) and PE-conjugated anti-CD45 (1:200, clone 30-F11; BD Pharmingen, Franklin Lakes, NJ) 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). APC-conjugated anti-CD90, Sca-1, CD34, CD49b, CD14, CD124, c-kit, CD14 (BD Pharmingen), CD44 (Southern Biotechnology Associates), and CD133 (eBioscience, San Diego, CA) were used at 1:200 dilution.

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 Dulbecco's modified Eagle's medium containing 20% fetal bovine serum and 2.5 ng/ml of basic fibroblast growth factor (Invitrogen, Carlsbad, CA) for 4 days before 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.

Cell Transplantation

To induce muscle regeneration, 100 μl of 10 μmol/L CTX was injected into the TA muscle of *NOD/scid* muscles.

and 24 hours later, 30 μl of cell suspensions containing 3×10^4 myoblasts, 3×10^4 CD31(−) CD45(−) SP cells, or 3×10^4 GFP(+) myoblasts plus 2×10^4 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/phosphate-buffered saline (PBS) and then in 20% sucrose/PBS, and frozen in isopentane cooled with liquid nitrogen.

Retrovirus Transduction in Vitro

Red fluorescent protein (DsRed) cDNA (BD Biosciences, San Diego, CA) was cloned into a retrovirus plasmid, pMXs, kindly provided by Dr. T. Kitamura of the University of Tokyo, Tokyo, Japan.[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.

Immunohistochemistry

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, Canada) in PBS for 15 minutes, and then reacted with anti-GFP antibody (Chemicon International, Temecula, CA), anti-laminin α2 antibody (4H8-2; Alexis, San Diego, CA), anti-phospho-histone H3 antibody (Upstate Biotechnology, Lake Placid, NY), or anti-DsRed antibody (Clontech, Palo Alto, CA) at 4°C overnight. Dystrophin was detected using a monoclonal antibody, Dys-2 (Novocastra, Newcastle on Tyne, UK), and a M.O.M. Kit (Vector Laboratories, Burlingame, CA). The sections were then incubated with appropriate combinations of Alexa 488-, 568-, or 594-labeled secondary antibodies (Molecular Probes, Eugene, OR) 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 (National Institutes of Health, Bethesda, MD) on cross sections from three independent experiments, and defined as the distribution area.

RNA Isolation and Real-Time Polymerase Chain Reaction (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 (Bio-Rad Laboratories, Richmond, CA) following the manufacturer's instructions. Primer sequences for real-time PCR were: 18s rRNA, forward: 5′-TACCCTGGCGGTGGGATTAAC-3′, reverse: 5′-CGAGAGAAGACCACGCCAAC-3′ and EGFP, forward: 5′-GACGTAAACGGCCACAAGTT-3′, reverse: 5′-AAGTCGTGCTGCTTCATGTG-3′. The expression levels of *MMP-2* and *MMP-9* were evaluated by conventional reverse transcriptase (RT)-PCR using the following primers: MMP-2, forward: 5′-TGCAAGGCAGTGGTCATAGCT-3′, reverse: 5′-AGCCAGTCGGATTTGATGCT-3′.

Cell Proliferation Assay

CD31(−) CD45(−) SP cells or 10T1/2 cells were cultured in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum 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 μmol/L). Twenty-four hours later, BrdU uptake was quantified by a cell proliferation enzyme-linked immunosorbent assay, a BrdU kit (Roche Diagnostics, Meylan, France), and Lumi-Image F1 (Roche).

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) 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). The software calculates signal intensities, and each signal was normalized to a median of its values in all samples or the 50th 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.

In Situ *Zymography*

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 μmol/L; Calbiochem, San Diego, CA) or E-64 (a cysteine protease inhibitor, 50 mmol/L; Calbiochem). Fluorescence of fluorescein isothiocyanate was detected with excitation at 460 to 500 nm and emission at 512 to 542 nm.

Statistics

Statistical differences were determined by Student's unpaired *t*-test. For comparison of more than two groups,

one-way analysis of variance was used. All values are expressed as means ± SE. A probability of less than 5% ($P < 0.05$) or 1% ($P < 0.01$) was considered statistically significant.

Results

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 to 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 (Figure 1B).[20] The CD31(−) CD45(−) SP cells did not express Pax3, Pax7, or Myf5, indicating that they are not yet committed to the muscle lineage.[20] 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.

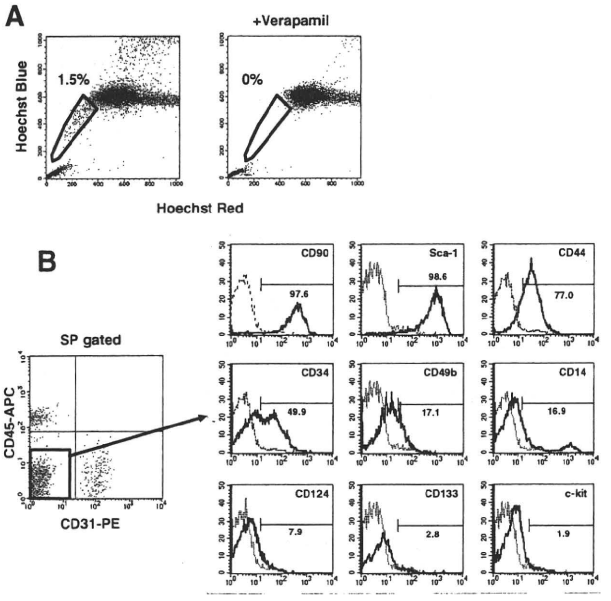

Figure 1. Cell surface markers on CD31(−) CD45(−) SP cells from regenerating muscle. **A:** Mononuclear cells were prepared from limb muscles of C57BL/6 mice at 3 days after CTX injection, incubated with 5 μmol/L Hoechst 33342 with (**right**) or without (**left**) Verapamil, and analyzed by a cell sorter. SP cells are shown by 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. Solid lines are with antibodies; dotted lines are negative controls.

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 (3×10^4 cells/muscle) with or without CD31(−) CD45(−) SP cells (2×10^4 cells/muscle) into TA muscles of immunodeficient *NOD/scid* mice (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 nonlabeled CD31(−) CD45(−) SP cells produced a higher number of GFP(+) myofibers than transplantation of GFP(+) myoblasts alone (Figure 2, B 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.

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: 3×10^4 myoblasts derived from GFP-Tg mice, 3×10^4 CD31(−) CD45(−) SP cells derived from GFP-Tg mice, or a mixture of GFP(+) 3×10^4 myoblasts and 2×10^4 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 3, B and C). On the other hand, transplantation of GFP(+) SP cells alone resulted in formation of few GFP(+) myofibers. This observation is consistent with our previous 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).

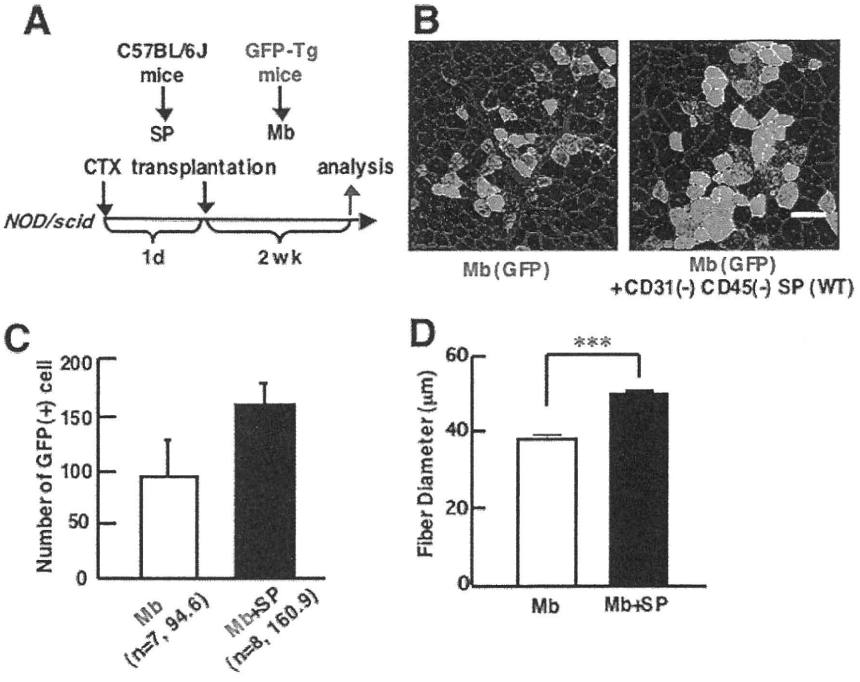

Figure 2. Co-transplantation of myoblasts and CD31(−) CD45(−) SP cells into skeletal muscle of immunodeficient *NOD/scid* mice promotes myofiber formation by transplanted myoblasts. **A:** Schematic protocol of co-transplantation experiments. CTX was injected into TA muscle 1 day before transplantation. 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 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). **C:** The number of GFP(+) fibers per cross section of transplanted TA muscle. Values are means with SE (seven to eight 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. $^{***}P < 0.001$. Scale bar = 80 μm.

The transplantation efficiency of myoblasts in *mdx* mice was 40 to 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.

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).

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 after injection (Figure 5B) with no differences in survival rates between single transplantation and co-transplantation.

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 6, A and B, many more GFP(+) myoblasts were detected in co-transplanted muscles than in myoblast-transplanted muscles (Figure 6, A 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 6D). These observations suggest that co-injection of CD31(−) CD45(−) SP cells promoted proliferation of grafted myoblasts.

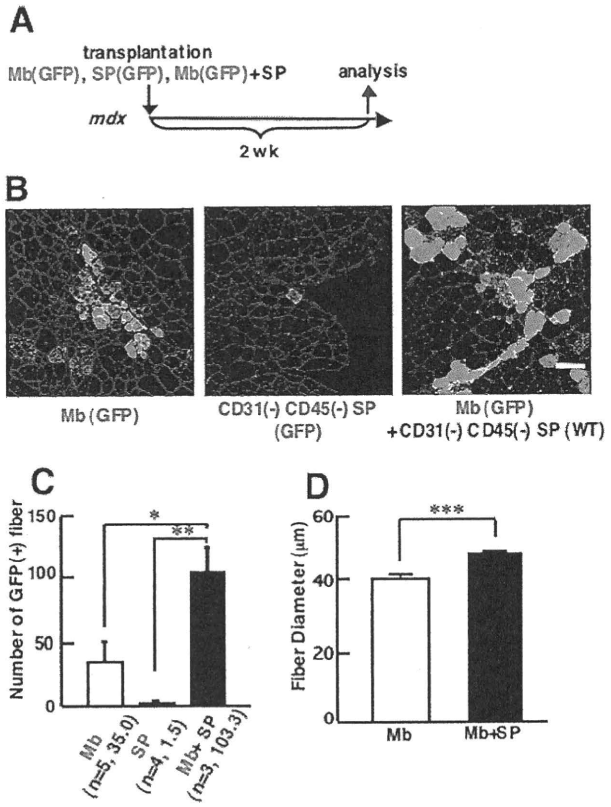

Figure 3. Co-transplantation of CD31(−) CD45(−) SP cells and myoblasts improves efficiency of myoblast transfer in dystrophin-deficient *mdx* mice. **A:** Schematic protocol of experiments. GFP(+) myoblasts alone (3×10^4), GFP(+) CD31(−) CD45(−) SP cells alone (3×10^4 cells), or a mixture of GFP(+) myoblasts (3×10^4) and CD31(−) CD45(−) SP cells (2×10^4) 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). **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 to 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. $^{***}P < 0.001$. Scale bar = 80 μm.

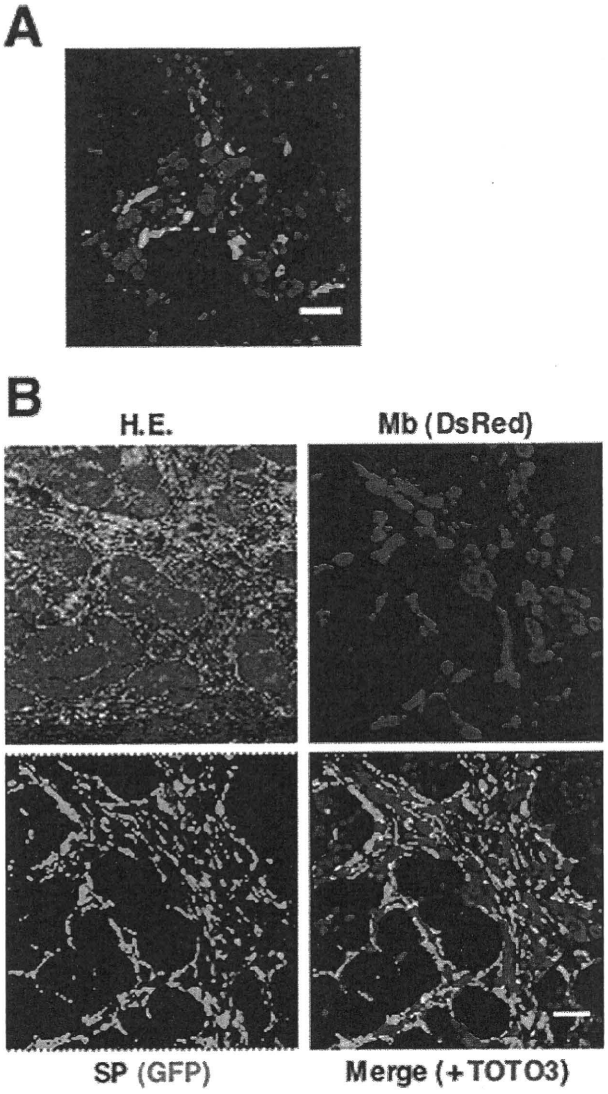

Figure 4. Behavior of GFP$^+$ CD31(−) CD45(−) SP cells and DsRed-labeled myoblasts after transplantation. **A:** *NOD/scid* TA muscles were injected with CTX 24 hours before transplantation. Then, myoblasts transduced with a retrovirus vector expressing DsRed were injected together with GFP(+) CD31(−) 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). **B:** Representative image of DsRed(+) myoblasts and GFP(+) SP cells 48 hours after co-transplantation. One serial section was stained with H&E. Scale bars = 40 μm.

Next, to examine whether CD31(−) CD45(−) SP cells directly promote proliferation of myoblasts or not, we performed an *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 6E). The results suggest that CD31(−) CD45(−) SP cells promote proliferation of injected myoblasts at least in part by producing soluble factors.

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 (see Supplementary Table S1 at *http://ajp.amjpathol.org*). 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 prolif-

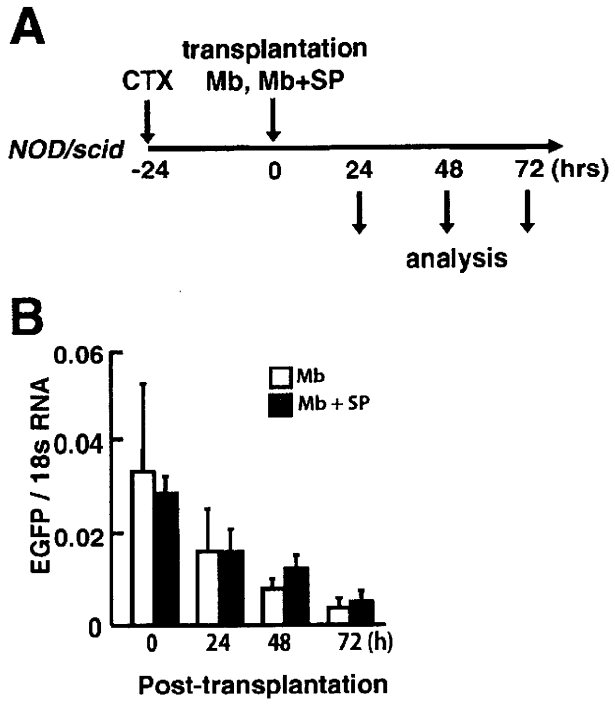

Figure 5. Survival of injected myoblasts in *NOD/scid* mice. **A:** Experimental design. GFP(+) myoblasts alone (3×10^4 cells) or a mixture of GFP(+) myoblasts (3×10^4 cells) and nonlabeled CD31(−) CD45(−) SP cells (2×10^4 cells) were injected into previously CTX-injected TA muscles of *NOD/scid* mice. 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 ($n = 4$ to 5).

eration or differentiation-promoting (follistatin),[26] and inhibitory factors (eg, insulin-like growth factor binding proteins,[27] Nov[28]). The list also contains regulators of TGF-β (eg, thrombospondins,[29] Prss11,[30] Ltbp3[31]), which would consequently attenuate or stimulate proliferation and differentiation of myoblasts.

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 (see Supplementary Table S1 and Supplementary Figure S1 at *http://ajp.amjpathol.org*). MMPs are a group of zinc-dependent endopeptidases that degrade extracellular matrix components, thereby facilitating cell migration and tissue remodeling.[32,33] Furthermore, MMPs are known to release growth factors stored within the extracellular matrix and process growth factor receptors, resulting in stimulation of cell proliferation.[34–36] Among the MMPs up-regulated 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]

We confirmed that the mRNA level of MMP-2 was much higher in CD31(−) CD45(−) SP cells than in macrophages or myoblasts (Figure 7A). 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 7B). 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.

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 7C). In contrast, there was no difference in the number of GFP(+) myoblasts between two groups (Figure 7D). 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.

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

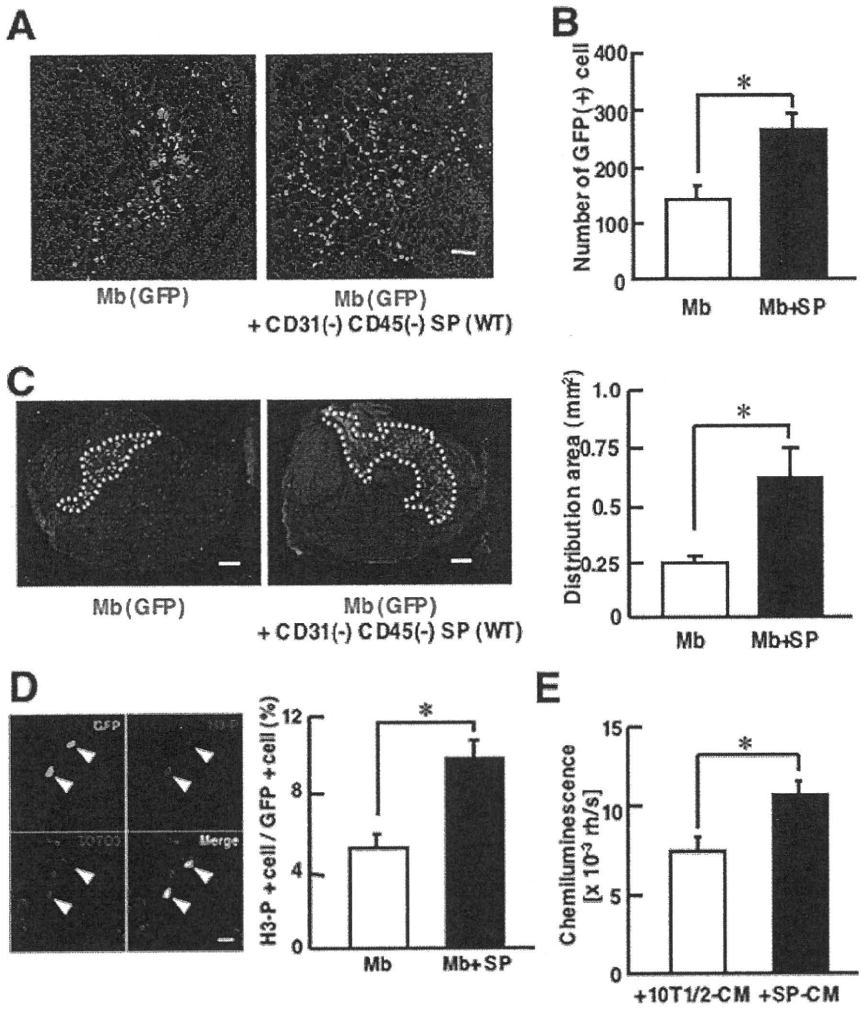

Figure 6. CD31(−) CD45(−) SP cells promote proliferation of myoblasts *in vitro* and *in vivo*. **A:** Representative images of cross sections of 72-hour samples stained with anti-GFP (green) and anti-laminin-α2 chain (red) antibodies. GFP(+) myoblasts are more widely scattered in injected muscle when co-transplanted with CD31(−) CD45(−) SP cells, compared with single transplantation. **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 to 5). $^*P < 0.05$. **C: Left:** Representative distributions of GFP(+) myoblasts/myotubes 72 hours after transplantation. **Right:** Distribution area (marked by white dotted lines in **left** panels) was measured by Image J software. Values were means with SE (n = 4 to 5). $^*P < 0.05$. **D:** 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. **Arrowheads** 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). $^*P < 0.05$. **E:** 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). $^*P < 0.05$. Scale bars: 100 μm (**A**); 200 μm (**C**); 80 μm (**D**).

findings suggest that endogenous CD31(−) CD45(−) SP cells support muscle regeneration by stimulating proliferation and migration of myoblasts.

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.

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 attributable 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 prolifera-

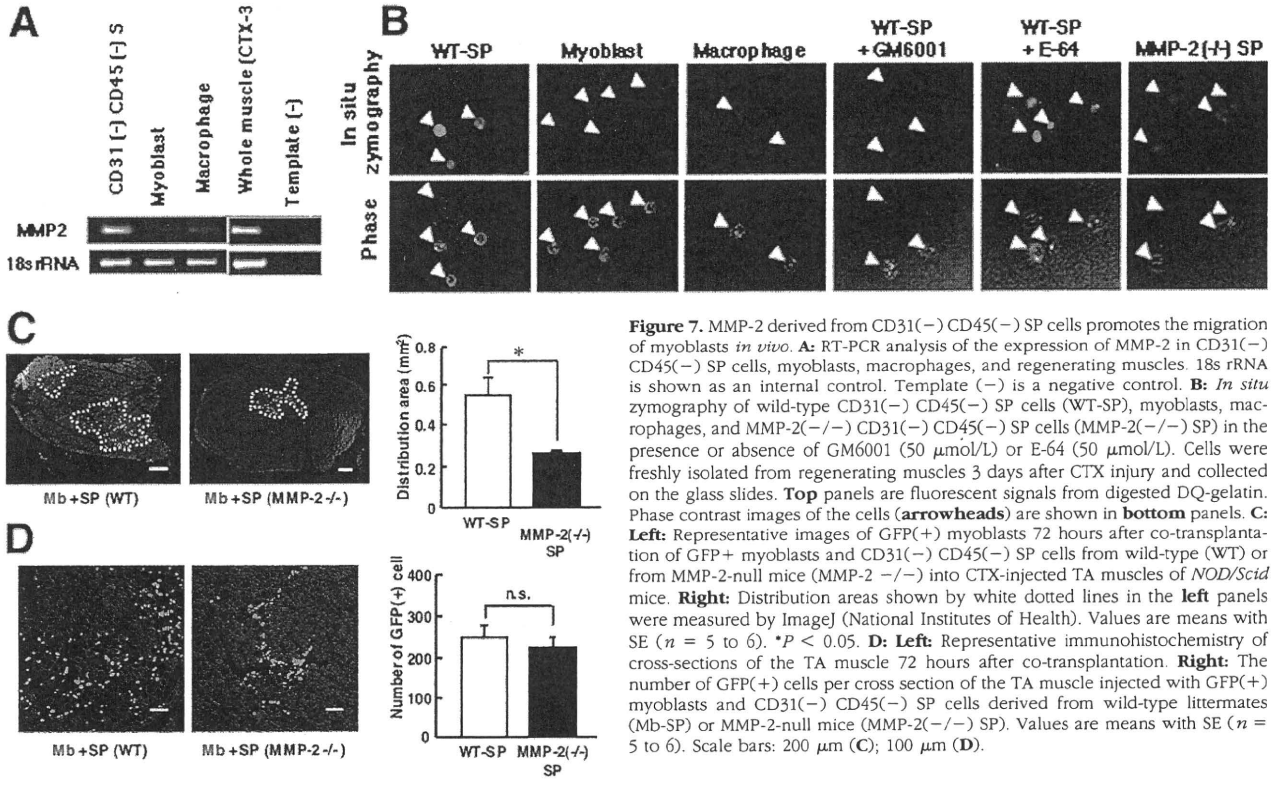

Figure 7. MMP-2 derived from CD31(−) CD45(−) SP cells promotes the migration of myoblasts *in vivo*. **A:** RT-PCR analysis of the expression of MMP-2 in CD31(−) CD45(−) SP cells, myoblasts, macrophages, and regenerating muscles. 18s rRNA is shown as an internal control. Template (−) is a negative control. **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 μmol/L) or E-64 (50 μmol/L). Cells were freshly isolated from regenerating muscles 3 days after CTX injury and collected on the glass slides. **Top** panels are fluorescent signals from digested DQ-gelatin. Phase contrast images of the cells (**arrowheads**) are shown in **bottom** panels. **C: 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. **Right:** Distribution areas shown by white dotted lines in the **left** panels were measured by ImageJ (National Institutes of Health). Values are means with SE (n = 5 to 6). $^*P < 0.05$. **D: Left:** Representative immunohistochemistry of cross-sections of the TA muscle 72 hours after co-transplantation. **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 to 6). Scale bars: 200 μm (**C**); 100 μm (**D**).

tion 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 (see Supplementary Table S1 at *http://ajp.amjpathol.org*). These molecules may directly or indirectly stimulate proliferation of myoblasts.

MMP-2 Derived from CD31(−) CD45(−) SP Cells Promotes 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 that promotes migration of myoblasts. MMP-2 plays a critical role in myogenesis[44] and is up-regulated in muscle regeneration (see Supplementary Figure S2 at *http://ajp.amjpathol.org*).[38] MMP-2 expression is also detected in regenerating areas of dystrophic muscles.[39,40] Importantly, El Fahime and colleagues[45] reported that forced expression of MMP-2 in normal myoblasts significantly increased migration of myoblasts *in vivo*. In the present study, we demonstrated that CD31(−) CD45(−) SP cells highly express MMP-2 (see Figure 7A and Supplementary Table S1 at *http://ajp.amjpathol.org*). Gelatin zymography confirmed that CD31(−) CD45(−) SP cells have high gelatinolytic activities (Figure 7B). 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 7C). 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 (see Supplementary Figures S1 and S2 and Supplementary Table S1 at *http://ajp.amjpathol.org*). 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.[34,35,46] In the present study, however, MMP-2 derived from CD31(−) CD45(−) SP cells did not stimulate the proliferation of myoblasts *in vivo* (Figure 7D). 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.

CD31(−) CD45(−) SP Cells Are the 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 such as 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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Reduced proliferative activity of primary POMGnT1-null myoblasts *in vitro*

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ABSTRACT

Protein O-linked mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1) is an enzyme that transfers N-acetylglucosamine to O-mannose of glycoproteins. Mutations of the *POMGnT1* gene cause muscle–eye–brain (MEB) disease. To obtain a better understanding of the pathogenesis of MEB disease, we mutated the *POMGnT1* gene in mice using a targeting technique. The mutant muscle showed aberrant glycosylation of α-DG, and α-DG from mutant muscle failed to bind laminin in a binding assay. *POMGnT1*$^{-/-}$ muscle showed minimal pathological changes with very low-serum creatine kinase levels, and had normally formed muscle basal lamina, but showed reduced muscle mass, reduced numbers of muscle fibers, and impaired muscle regeneration. Importantly, *POMGnT1*$^{-/-}$ satellite cells proliferated slowly, but efficiently differentiated into multinuclear myotubes *in vitro*. Transfer of a retrovirus vector-mediated *POMGnT1* gene into *POMGnT1*$^{-/-}$ myoblasts completely restored the glycosylation of α-DG, but proliferation of the cells was not improved. Our results suggest that proper glycosylation of α-DG is important for maintenance of the proliferative activity of satellite cells *in vivo*.

1. Introduction

POMGnT1 is the glycosyltransferase that catalyzes the transfer of *N*-acetylglucosamine (GlcNAc) to O-mannose of glycoproteins, the second step of Ser/Thr O-mannosylation (Yoshida et al., 2001; reviewed in Endo and Toda, 2003). Mutations in the *POMGnT1* gene cause muscle–eye–brain (MEB) disease, a rare autosomal recessive disorder characterized by congenital muscular dystrophy with elevated serum creatine kinase (CK) levels, severe visual failure, and gross mental retardation (Yoshida et al., 2001).

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α-Dystroglycan (α-DG) is a heavily glycosylated glycoprotein and a well-known substrate of POMGnT1. Dystroglycan is encoded by a single gene (*DAG1*) and is cleaved into two proteins, α-dystroglycan (α-DG) and β-dystroglycan (β-DG), by posttranslational processing (Ibraghimov-Beskrovnaya et al., 1992). DGs are central components of the dystrophin–glycoprotein complex (DGC) at the sarcolemma, and α-DG was shown to serve as a cell surface receptor for laminin (Ibraghimov-Beskrovnaya et al., 1992), agrin (Gee et al., 1994; Campanelli et al., 1994), perlecan (Peng et al., 1998; Kanagawa et al., 2005), and neurexin (Sugita et al., 2001). In skeletal muscle, the laminin-α-DG linkage is thought to be critical for plasma membrane stability (recently reviewed in Kanagawa and Toda 2006). In MEB muscle, the α-DG core protein is preserved but hypo-glycosylated, and α-DG prepared from the muscle fails to bind laminin *in vitro* (Michele et al., 2002). Therefore, it is proposed that the disruption of the α-DG-laminin linkage is the main pathomechanism of dystrophic changes seen in MEB muscle.

To further elucidate the molecular pathogenesis of MEB disease, we generated *POMGnT1*-knockout mice using a gene targeting technique, and examined the mutant skeletal muscle. During our experiments, Liu et al. reported the generation of POMGnT1-deficient mice (Liu et al., 2006). The report showed severe muscle pathology, but the mechanism by which POMGnT1 deficiency causes muscle phenotype was not clearly shown. In this report, we report that POMGnT1-deficient mice show remarkably minimal signs of muscle degeneration and regeneration, but also show small muscle mass, reduced numbers of muscle fibers, and impaired muscle regeneration. POMGnT1-deficient myoblasts proliferate poorly *in vitro*. The proliferation was not improved by retrovirus vector-mediated *POMGnT1* expression in *POMGnT1*$^{-/-}$ myoblasts, suggesting that α-DG–laminin interaction *in vivo* is important for maintenance of the proliferative activity of satellite cells.

2. Results

2.1. *Inactivation of the POMGnT1 gene in mice*

We mutated the *POMGnT1* gene by replacing exon 18 with a neomycin-resistance gene in mouse ES cells (depicted in Fig. 1). Two ES clones successfully entered the germline. Although there was no evidence of embryonic lethality, more than 60% of the homozygotes died within 3 weeks of birth. Survivors were smaller than their wild-type littermates (Fig. 3A) throughout life, but most of them had a normal life span. We confirmed that the *POMGnT1*$^{-/-}$ mice completely lacked the POMGnT1 enzyme activity (Fig. 2A). A monoclonal antibody, VIA4-1, that reacts with the suger moiety of α-DG gave no signal in either *POMGnT1*$^{-/-}$ brain (Fig. 2B) or muscle (data not shown). A polyclonal antibody against α-DG core protein revealed that the *POMGnT1*$^{-/-}$ brain expresses approximately 80 kDa α-DG protein, which is much smaller than that seen in the wild-type brain (ca. 110 kDa) (Fig. 2C). We next examined whether α-DG in *POMGnT1*$^{-/-}$ brain binds laminin. Wheat germ agglutinin (WGA)-enriched brain protein from control and *POMGnT1*$^{-/-}$ mice was separated on SDS–PAGE gels, blotted onto a PVDF membrane, incubated with EHS laminin, and then bound laminin was detected by an anti-laminin antibody. α-DG in *POMGnT1*$^{-/-}$ brain failed to bind to laminin (Fig. 2D).

2.2. *POMGnT1*$^{-/-}$ *muscle shows very mild dystrophic changes*

Immunohistochemistry of cross-sections of tibialis anterior (TA) muscles showed that dystrophin and other members of the DGC complex were normally expressed at the sarcolemma of *POMGnT1*$^{-/-}$ muscle (Fig. 3 and Table 1). Laminin α2 chain was detected around *POMGnT1*$^{-/-}$ muscle fibers. On H.E.-stained cross-sections, surprisingly, the *POMGnT1*$^{-/-}$ muscle showed almost normal morphology. Central nucleation of myofibers indicates regeneration events in the past. In the TA muscles of 4-week-old wild-type mice, 0.25% of myofibers were centrally nucleated. In *POMGnT1*$^{-/-}$ mice, 0.28% of the myofibers had central nuclei. In contrast, ca. 40–50% of myofibers of age-matched mdx mice were centrally nucleated. Even at 24 months of age, the percentage of centrally nucleated myofibers was lower (3.6%) in *POMGnT1*$^{-/-}$ TA muscle, compared with age-matched wild-type TA muscle (9.0%). *POMGnT1*$^{-/-}$ TA muscle also lacked other signs of degeneration and regeneration. Electrophoresis of muscle extracts on glycerol SDS–PAGE gels showed no difference in MyHC isoform composition of quadriceps and gastrocnemius muscles between *POMGnT1*$^{-/-}$ and wild-type littermates (data not shown). In both wild-type and *POMGnT1*$^{-/-}$ muscle, the muscle basal lamina was normally formed (Supplementary Fig. 1). Electron microscopy also showed that the sarcomere structures are almost normal in *POMGnT1*$^{-/-}$ mice. We next examined the serum creatine kinase (CK) levels, an index of on-going muscle damage, in wild-type, *POMGnT1*$^{-/-}$, and age-matched mdx mice (Fig. 5). The serum CK levels of 5- to 20-week-old *POMGnT1*$^{-/-}$ mice were slightly higher (av. 586 U/L, $n = 10$) ($p < 0.05$) than those of wild-type littermates (less than 100 U/L, $n = 4$), but were much lower than those of mdx mice (more than 5000 U/L, $n = 3$, $p < 0.01$). The serum CK levels of 2-year-old *POMGnT1*$^{-/-}$ mice were still low (less than 300 U/L, $n = 4$).

2.3. *Repetitive muscle injury causes more fibrosis and fatty infiltration in POMGnT1*$^{-/-}$ *than in WT TA muscle*

Dystroglycans expressed on the cell membrane of satellite cells are proposed to play an important role in muscle regeneration (Cohn et al., 2002). In addition, the average size of *POMGnT1*$^{-/-}$ myofibers was smaller than those of wild-type myofibers (Fig. 4). Moreover, the number of myofibers is reduced in *POMGnT1*$^{-/-}$ skeletal muscle of neonatal and adult POMGnT1 mice, suggesting proliferation defect of *POMGnT1*$^{-/-}$ myoblasts (Fig. 4). To test the hypothesis, we damaged *POMGnT1*$^{-/-}$ TA muscle by cardiotoxin (CTX) and examined their regeneration. After single cardiotoxin injection, *POMGnT1*$^{-/-}$ muscle regenerated well like wild-type (data not shown). Next, we injected CTX into TA muscles of *POMGnT1*$^{-/-}$ and heterozygous littermates three times at intervals of 2 weeks, or 1 week interval, and examined the muscle. We summarized the results in Fig. 6. *POMGnT1*$^{-/-}$ muscle showed more fibrosis and

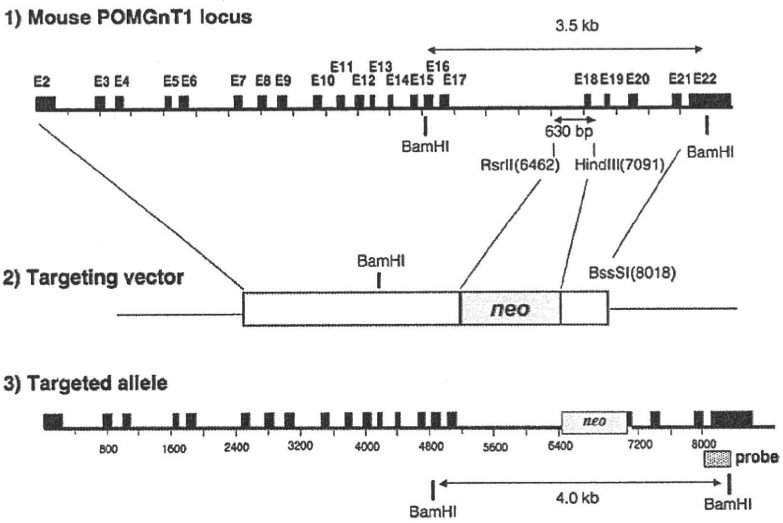

Fig. 1 – Targeted disruption of the mouse *POMGnT1* gene in embryonic stem (ES) cells. The successfully targeted allele lacks a 630 bp-genome fragment containing exon 18, and instead has a *neo* resistance gene. Recombination in ES cells was confirmed by Southern blotting with the probe shown by a shaded box. The nucleic acid numbers are from AB053221 in GenBank.

fatty infiltration, which is a sign of inefficient muscle regeneration, than *POMGnT1*$^{+/-}$ muscle. Together with reduced numbers of myofibers in muscle, the results suggest that the function of satellite cells in *POMGnT1*$^{-/-}$ skeletal muscle is impaired.

2.4. *Defective proliferative activity of POMGnT1*$^{-/-}$ *myoblasts*

We next tested activation and proliferation of satellite cells on living myofibers isolated from wild-type and *POMGnT1*$^{-/-}$

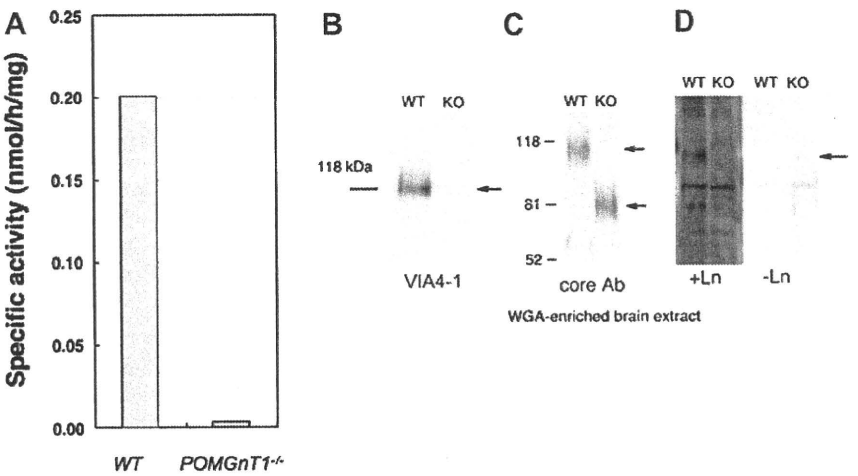

Fig. 2 – *POMGnT1*$^{-/-}$ mice show undetectable POMGnT1 enzyme activity and aberrant glycosylation of α-dystroglycan (α-DG) in *POMGnT1*$^{-/-}$ mice. (A) The amount of POMGnT1 activity is based on the amount of [^{3}H]GlcNAc transferred from UDP-GlcNAc to mannosyl peptide. The reaction product was purified by reverse-phased HPLC, and the radioactivity was measured. (B) Wheat germ agglutinin (WGA) agarose-enriched brain extracts from wild-type (WT) or *POMGnT1*$^{-/-}$ (KO) mice were resolved on a 7.5% SDS–PAGE gel, transferred to a PVDF membrane, and probed with anti-α-DG antibody, VIA4-1, which recognizes glycosylated α-DG. (C) The blot was incubated with polyclonal antibodies specific for α-DG core protein. The antibody detected ~110 kDa bands in wild-type brain extract, and 80 kDa bands in the brain extract of *POMGnT1*$^{-/-}$ mice. (D) Laminin overlay assay showing that α-DG in *POMGnT1*$^{-/-}$ brain does not bind laminin *in vitro*. +Ln, laminin was incubated with the blotted membrane. –LN, without laminin.

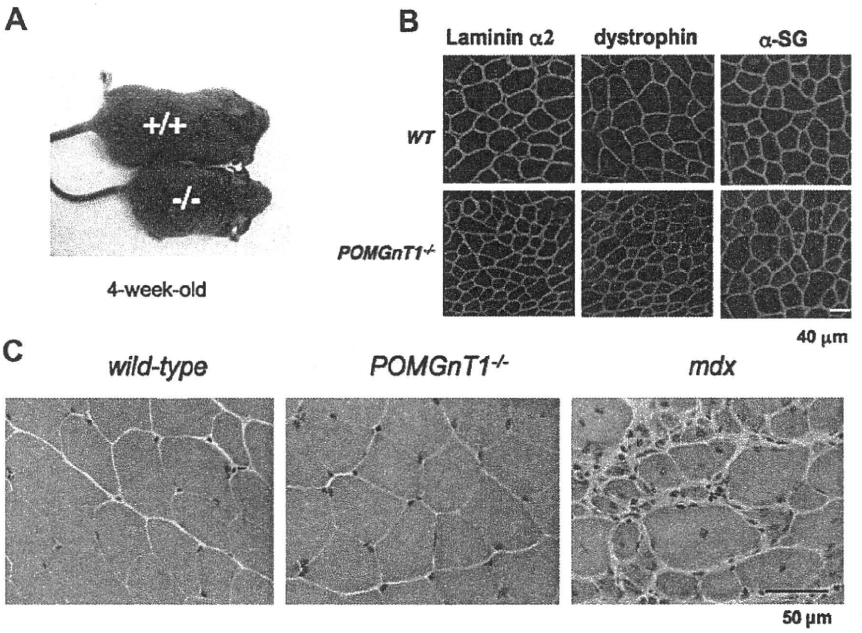

Fig. 3 – Remarkably mild dystrophic phenotypes of POMGnT1$^{-/-}$ muscle. (A) A photo of representative 4-week-old wild-type (+/+) and POMGnT1$^{-/-}$ (−/−) mice. POMGnT1$^{-/-}$ mice are smaller than wild-type littermates. (B) Immunohistochemistry of wild-type (+/+) and POMGnT1-knockout (−/−). Laminin α2 chain, dystrophin, and α-sarcoglycan are expressed normally on the sarcolemma of POMGnT1$^{-/-}$ muscle. (C) Representative H.E. staining of cross-sections of the TA muscles from POMGnT1$^{-/-}$, wild-type, and age-matched dystrophin-deficient *mdx* mice. POMGnT1$^{-/-}$ muscle shows minimal signs of degeneration and regeneration.

Table 1 – Summary of immunohistochemistry of hind-limb muscles of wild-type (WT) POMGnT1$^{-/-}$, and mdx mice.

	WT	POMGnT1$^{-/-}$	*mdx*
Laminin α2 chain	+	+	+
Dystrophin	+	+	−
α-Dystroglycan (VIA4-1)	+	−	±
Dystroglycan (core protein)	+	+	±
β-Dystropglycan	+	+	±
α-Sarcoglycan	+	+	±
α-Syntrophin	+	+	±
nNOS	+	+	±
Aquaporin 4	+	+	±
Integrin α7	+	+	++
Integrin β1	+	+	++

+, expressed; −, absent; ±, down-regulated; ++, up-regulated.

mice (Fig. 7). Three days after plating of single myofibers on Matrigel-coated 24-well plates in growth medium, the numbers of detached satellite cells (activated and proliferating satellite cells) were counted. In both extensor digitorum longus (EDL) (fast twitch muscle) and soleus (slow twitch muscle) muscles, the numbers of activated satellite cells and proliferating satellite cells (myoblasts) around the parental myofiber were more numerous in wild-type than in POMGnT1$^{-/-}$ (Fig. 7). Furthermore, wild-type satellite cells migrate a little faster than POMGnT1$^{-/-}$ satellite cells on transwells (data not shown), although the difference was little. Therefore, our results suggest that POMGnT1$^{-/-}$ satellite cells are activated more slowly or proliferate more slowly than wild-type. We next isolated satellite cells from hind limb muscles of wild-type and POMGnT1$^{-/-}$ mice by a monoclonal antibody, SM/C-2.6, and flow cytometry (Fukada et al., 2007), and examined their proliferation rate. The total yield of satellite cells per gram of POMGnT1$^{-/-}$ muscle tissue was nearly the same as those of wild-type muscle (data not shown). The percentage of Ki67-positive satellite cells (cycling cells) was less than 1% in both wild-type and POMGnT1$^{-/-}$ mice, indicating that they are in the quiescent stage (data not shown). However, after plating wild-type and POMGnT1$^{-/-}$ satellite cells onto Matrigel-coated 6-well plates at the same density, we found that POMGnT1$^{-/-}$ satellite cells grew poorly in growth medium (Fig. 7B). The timing of activation (i.e. enlargement of the cytoplasm and MyoD expression) was the same with that of wild-type satellite cells (data not shown). Next, we cultured satellite cells on Matrigel-coated 24-well-plates in growth medium, and the cells growth was evaluated by MTT assay 1, 2, 3, 4, 5, 6, and 7 days after plating (Fig. 8). The assay revealed that wild-type myoblasts proliferated more rapidly than POMGnT1$^{-/-}$ myoblasts *in vitro*. POMGnT1$^{-/-}$ myoblasts fused normally to form multinucleated myotubes in differentiation conditions like the wild-type (data not shown), and there was no significant difference in the fusion index between wild-type (45%) and POMGnT1$^{-/-}$ myoblasts (40%) ($p > 0.05$).

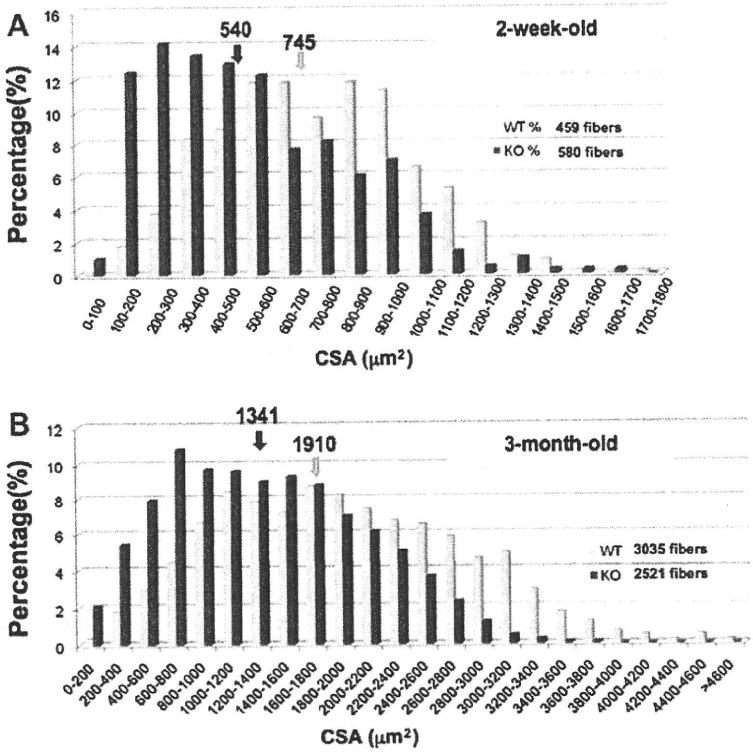

Fig. 4 – Cross-sectional area (CSA) of myofibers of POMGnT1$^{-/-}$ and wild-type mice. (A) A representative frequency graph of CSA of rectus femoris muscles from 2-week-old POMGnT1$^{-/-}$ (blue) and wild-type (light blue) littermates. The cross-sections were stained with anti-laminin α2 chain antibody. CSA of 459 POMGnT1$^{-/-}$ fibers and 580 wild-type fibers were measured and plotted. X-axis indicates CSA (μm^2), and Y-axis indicates percentages. Arrows indicate the averages. The total number of myofibers was also reduced in POMGnT1$^{-/-}$ mice (4169 vs. 3510). (B) The CSA of myofibers in TA muscles from 3-month-old POMGnT1$^{-/-}$ (blue) and wild-type (light blue) male mice was plotted as in (A)). In (B), almost all myofibers were measured (3035 fibers in wild-type TA and 2521 fibers in POMGnT1$^{-/-}$ TA).

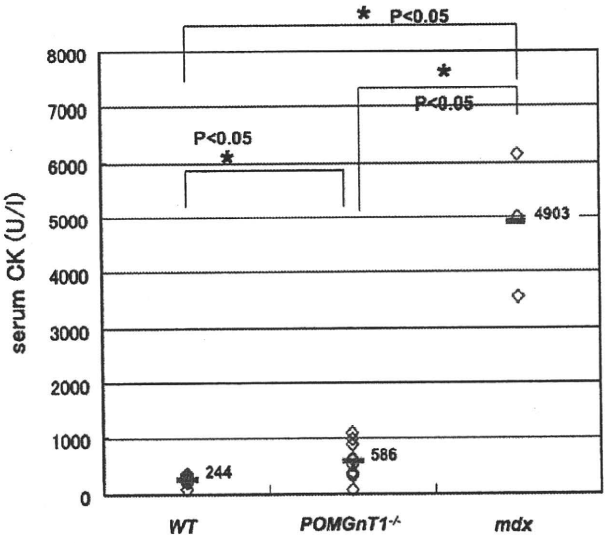

Fig. 5 – Serum CK levels of POMGnT1$^{-/-}$, wild-type, and *mdx* mice. Serum CK levels of 7–20 weeks old POMGnT1$^{-/-}$ mice (5 males and 5 females), wild-type littermates (3 males and 1 female), and three male *mdx* mice were measured and plotted on the graph with average. $^*p < 0.05$.

Next, we examined whether restoration of the expression of the POMGnT1 gene in mutant myoblasts improved their proliferation. To this end, we prepared a retrovirus vector, (pMX-POMGnT1-IRES-GFP) expressing human POMGnT1 and GFP. The recombinant retrovirus successfully restored O-mannosyl glycosylation of α-DG (Fig. 7A), but the proliferation rate was not changed (Fig. 8B).

2.5. *Cell growth signaling in POMGnT1$^{-/-}$ myoblasts*

It was previously reported that enhanced expression of α7β1 integrin ameliorates the development of muscular dystrophy and extends longevity in α7BX2-*mdx/utr*($^{-/-}$) transgenic mice (Burkin et al., 2001; Burkin et al., 2005), suggesting that integrin compensates for the function of α-DG in skeletal muscle to some extent. Therefore, we next examined the expression of β1-integrin in wild-type and POMGnT1$^{-/-}$ myoblasts (Supplementary Fig. 1). Western blotting, however, showed no difference between the β1-integrin protein levels in wild-type and POMGnT1$^{-/-}$ myoblasts (Supplementary Fig. 1A). Furthermore, FACS analysis showed similar levels of β1 integrin expression on the surfaces of myoblasts (Supplementary Fig. 1B). We then examined the activation levels of Akt and GSK-3β, both of which are involved in the