The FASEB Journal • Research Communication

Generation of transplantable, functional satellite-like cells from mouse embryonic stem cells

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ABSTRACT **Satellite cells are myogenic stem cells responsible for the postnatal regeneration of skeletal muscle. Here we report the successful *in vitro* induction of Pax7-positive satellite-like cells from mouse embryonic stem (mES) cells. Embryoid bodies were generated from mES cells and cultured on Matrigel-coated dishes with Dulbecco's modified Eagle medium containing fetal bovine serum and horse serum. Pax7-positive satellite-like cells were enriched by fluorescence-activated cell sorting using a novel anti-satellite cell antibody, SM/C-2.6. SM/C-2.6-positive cells efficiently differentiate into skeletal muscle fibers both *in vitro* and *in vivo*. Furthermore, the cells demonstrate satellite cell characteristics such as extensive self-renewal capacity in subsequent muscle injury model, long-term engraftment up to 24 wk, and the ability to be secondarily transplanted with remarkably high engraftment efficiency compared to myoblast transplantation. This is the first report of transplantable, functional satellite-like cells derived from mES cells and will provide a foundation for new therapies for degenerative muscle disorders.—Chang, H., Yoshimoto, M., Umeda, K., Iwasa, T., Mizuno, Y., Fukada, S., Yamamoto, H., Motohashi, N., Yuko-Miyagoe-Suzuki, Takeda, S., Heike, T., Nakahata, T. Generation of transplantable, functional satellite-like cells from mouse embryonic stem cells. *FASEB J.* 23, 1907–1919 (2009)**

Key Words: long-term engraftment · secondary transplantation · high engraftment efficiency · self-renewal

DUCHENNE MUSCULAR DYSTROPHY (DMD; ref. 1) is a progressive, lethal muscular disorder (2) with no effective cure despite extensive research efforts. DMD results from mutations in the X-linked *dystrophin* gene (3). Dystrophin and its associated proteins function to link the intracellular actin cytoskeleton of muscle fibers to laminin in the extracellular matrix (4), thereby protecting myofibers from contraction-induced damage (5). Skeletal muscle fibers are continuously regenerated following exercise and injuries when satellite cells (6) are induced to differentiate into myoblasts that form myotubes and replace the damaged myofibers (7, 8). This muscular regeneration is observed at a much higher frequency in DMD patients (9). Continuous damage to myofibers and constant activation of resident satellite cells due to loss of dystrophin leads to the exhaustion of the satellite cells (10, 11), and the eventual depletion of satellite cells is primarily responsible for the onset of DMD symptoms.

Successful transplantation of normal satellite cells into the skeletal muscle of DMD patients may enable *in situ* production of normal muscle tissue and create a treatment option for this otherwise fatal disease. A recent report has shown that the transplantation of satellite cells collected from mouse muscle tissues can produce muscle fibers with normal dystrophin expression in mdx mice (12-14), a model mouse for DMD (15). This study suggests that stem cell transplantation may be a viable therapeutic approach for the treatment of DMD (16).

Satellite cells are monopotent stem cells that have the ability to self-renew and to differentiate into myoblasts and myotubes to maintain the integrity of skeletal muscle (17). Satellite cells lie dormant beneath the basal lamina and express transcription factors such as Pax3 (13, 18) and Pax7 (19). Pax7, a paired box transcription factor, is particularly important for satellite cell function. A recent study of *Pax7-null* mice revealed that Pax7 is essential for satellite cell formation (19) and that the *Pax7-null* mice exhibit a severe deficiency in muscle fibers at birth and premature mortality with complete depletion of the satellite cells. Surface markers such as M-cadherin and c-met (20) are also expressed by satellite cells. However, these markers are not specific to satellite cells because they are also expressed in the cerebellum (21) and by hepatocytes (22). To specifically identify quiescent satellite cells, a

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doi: 10.1096/fj.08-123661

novel monoclonal antibody, SM/C-2.6, has recently been established (23). Satellite cells purified with this antibody regenerate muscle fibers on implantation into mdx mice (15).

The use of satellite cells for clinical therapies would require the establishment of a reliable source of these cells. Embryonic stem (ES) cells are totipotent stem cells that are able to differentiate into various types of somatic cells *in vitro*. While mouse embryonic stem (mES) cells can be readily induced to differentiate into muscle fibers (24, 25) and the myogenicity of human ES cells was recently validated (26), the induction of mES cells into functional satellite cells has not been reported. Here we have successfully induced mES cells to generate cells expressing Pax7 *in vitro* by forming embryoid bodies (EBs). These ES cell-derived (ES-derived) Pax7-positive cells can be enriched using the SM/C-2.6 antibody (23) and possess a great potential for generating mature skeletal muscle fibers both *in vitro* and *in vivo*. The Pax7-positive cells display a self-renewal ability that can repopulate Pax7-positive cells *in vivo* in the recipient muscles following an injury. Furthermore, these ES-derived Pax7-positive cells could engraft in the recipient muscle for long periods, up to 24 wk, and could also be serially transplanted. These results indicate that ES-derived Pax7-positive cells possess satellite cell characteristics. This is the first report of effective induction of functional satellite cells from mES cells, and these novel findings may provide a new therapeutic approach for treatment of DMD.

MATERIALS AND METHODS

Cell culture

D3 cells, mES cells (27) that ubiquitously express the *EGFP* gene under the *CAG* promoter (28) (a gift from Dr. Masaru Okabe, Osaka University, Osaka, Japan), were used in this study. ES cells were maintained on tissue culture dishes (Falcon) coated with 0.1% gelatin (Sigma, Oakville, CA, USA), in DMEM (Sigma) supplemented with 15% fetal bovine serum (FBS; Thermo Trace, Melbourne, Australia), 0.1 mM 2-mercaptoethanol (Nakalai Tesque, Japan), 0.1 mM nonessential amino acids (Invitrogen, Burlington, CA, USA), 1 mM sodium pyruvate (Sigma), penicillin/streptomycin (50 μg/mL), and 5000 U/ml leukemia inhibitory factor (Dainippon Pharmaceutical Co., Japan).

In vitro differentiation of ES cells into a muscle lineage

To induce EB formation, undifferentiated ES cells were cultured in hanging drops for 3 d at a density of 800 cells/20 μl of differentiation medium, which consisted of DMEM supplemented with penicillin/streptomycin, 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptonethanol, 5% horse serum (HS), and 10% FBS. EBs were transferred to suspension cultures for an additional 3 d (d 3+3). Finally, the EBs were plated in differentiation medium in 48-well plates (Falcon) coated with Matrigel (BD Bioscience, Bedford, MA, USA). The medium was changed every 5 d.

Immunofluorescence and immunocytochemical analysis

Immunostaining of cultured cells and recipient mouse tissues were carried out as described previously (29). Briefly, the left tibialis anterior (LTA) muscle of the recipient mouse was fixed with 4% paraformaldehyde and cut into 6 μm cross sections using a cryostat, and samples were fixed for 5 min in 4% paraformaldehyde (PFA) in PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min. After incubation in 5% skim milk for 10 min at room temperature to block nonspecific antibody binding, cells were incubated for 12 h at 4°C with anti-mouse monoclonal antibodies. Antibodies used in this study were mouse anti-Pax7, which was biotinylated using a DSB-X Biotin Protein Labeling Kit (D20655; Molecular Probes, Eugene, OR, USA), mouse anti-Pax3 (MAB1675, MAB2457; R&D Systems, Minneapolis, MN, USA), rabbit anti-mouse Myf5 (sc-302; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-mouse M-cadherin (205610; Calbiochem, San Diego, CA, USA), mouse anti-myosin heavy chain (MHC; 18-0105; Zymed Laboratories, San Francisco, CA, USA; reacts with human, rabbit, rat, mouse, bovine, and pig skeletal MHC), mouse anti-mouse myogenin and mouse anti-mouse Myo-D1 (M3559, M3512; Dako, Carpinteria, CA, USA), monoclonal rabbit anti-mouse laminin (LB-1013; LSL, Tokyo, Japan), and mouse anti-mouse dystrophin (NCL-DYS2; Novocastra Laboratories, Newcastle-upon-Tyne, UK). Cy3-labeled antibodies to mouse or rabbit IgG, fluorescein isothiocyanate-labeled antibodies to mouse or rabbit IgG (715-005-150, 711-165-152; Jackson ImmunoResearch Laboratory, Bar Harbor, ME, USA), or Alexa 633-labeled goat anti-rabbit IgG (A21070; Invitrogen, Molecular Probes) were applied as secondary antibodies. Hoechst 33324 (H3570; Molecular Probes) was used for nuclear staining. The samples were examined with a fluorescence microscope (Olympus, Tokyo, Japan) or an AS-MDW system (Leica Microsystems, Wetzlar, Germany). Micrographs were obtained using an AxioCam (Carl Zeiss Vision, Hallbergmoos, Germany) or the AS-MDW system (Leica Microsystems). In sections of muscles transplanted with ES-derived satellite cells, the number of GFP-positive muscle fascicles and GFP/Pax7-double-positive cells were counted, per field, at ×100. More than 10 fields in each tissue sample were observed. To prevent nonspecific secondary antibody binding to Fc receptors, all immunostaining of frozen sections used the Vector® M.O.M™ Immunodetection Kit (BMK-2202; Vector Laboratories, Burlingame, CA, USA).

PCR analysis

Total RNA was isolated from cultured cells in 48-well plates, using TRIzol reagent (Invitrogen). The following specific primers were used for PCR:

Pax3, sense, 5′-AACACTGGCCCTCAGTGAGTTCTAT-3′, and antisense, 5′-ACTCAGGATGCCATCGATGCTGTG-3′; Pax7, sense, 5′-CATCCAGTGCTGGTACCCCACAG-3′, and antisense, 5′-CTGTGGATGTCACCTGCTTGAA-3′; Myf5, sense, 5′-GAGCTGCTGAGGGAACAGGTGG-3′, and antisense, 5′-GTTCTTTCGGGACCAGACAGGG-3′; MyoD, sense, 5′-AGGCTCTGCTGCGCGACCAG-3′, and antisense, 5′-TGCAGTCGATCTCTCAAAGC-3′; myogenin, sense, 5′-TGAGGGAGAAGCGCAGGCTCAAG-3′, and antisense, 5′-ATGCTGTCCACGATGGACGTAAGG-3′; M-cadherin, sense, 5′-CCACAAACGCCTCCCCTACCC-3′, and antisense, 5′-GTCGATGCTGAAGAACTCAGGGC-3′; C-met, sense, 5′-GAATGTCGTCCTACACGGCCAT-3′, and antisense, 5′-CACTACACAGTCAGGACACTGC-3′; GAPDH, sense, 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′, and antisense, 5′-TGTTGGGGGCCGAGTTGGGATA-3′. AmpliTaqGold (Applied

Biosystems, Foster City, CA, USA) was used for PCR amplification. The amplification program used was 35 cycles of 30 s at 94°C, 30 s at 64°C, and 40 s at 72°C, with a final incubation of 7 min at 72°C.

Flow cytometry and cell sorting

Cultured cells were incubated with enzyme-free Hank's-based Cell Dissociation Buffer (Invitrogen) for 30 min at 37°C and gently dissociated into single cells. The cells were then washed with PBS twice, probed with biotinylated-SM/C-2.6 (23) antibody for 15 min at room temperature, and stained with phycoerythrin-conjugated strepavidin (12-4312; eBioscience, San Diego, CA, USA) for 15 min at room temperature. Dead cells were excluded from the plots based on propidium iodide staining (Sigma), and SM/C-2.6-positive cells were collected using a FACS Vantage instrument (Becton Dickinson, San Jose, CA, USA). Sorted cells were plated (1×10^4 cells/well) with differentiation medium in 96-well plates (Falcon) coated with Matrigel (008504; BD Bioscience). The medium was changed every 5 d, and 7 d after plating the cultured cells were analyzed.

Intramuscular cell transplantation (primary transplantation)

Recipient mice were injected with 50 μl of 10 μM cardiotoxin (CTX; Latoxan, Valence, France) (30) in the LTA muscle 24 h before transplantation (31). CTX is a myotoxin that destroys myofibers, but not satellite cells, and leaves the basal lamina and microcirculation intact. Since proliferation of host myogenic cells may prevent the incorporation of transplanted cells, recipient mdx mice (15) received 8 cGy of systemic irradiation (32) 12 h before transplantation to block muscle repair by endogenous cells. An average of 4.53×10^4 ES-derived SM/C-2.6-positive or -negative cells were washed twice with 500 μl of PBS, resuspended in 20 μl of DMEM, and injected into the LTA muscle of recipient mdx mice using an allergy syringe (Becton Dickinson). Mdx mice, which are derived from the CL/B16 strain, were used as the recipient mice in all experiments. Similarly, D3 ES cells, which are derived from the 129X1/SvJ ES cells, were used in all experiments. The major histocompatibility complex (MHC) of mdx mouse and D3 cells are very similar, both possessing type *b* MHC H2 haplotypes. All animal-handling procedures followed the Guild for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the Guidelines of the Animal Research Committee of the Graduate School of Medicine, Kyoto University.

Secondary transplantation

The LTA muscles of recipient mice were collected 8 wk after the primary transplantation. The muscles were minced and digested into single cells with 0.5% collagenase type I (lot S4D7301; Worthington Biochemical Corp., Lakewood, NJ, USA). After washing with PBS and filtration through a 100 μm filter, Pax7-positive cells were sorted by FACS using the SM/C-2.6 antibody. SM/C-2.6-positive cells (200 cells/mouse) were injected into preinjured LTA muscles of secondary recipient mice. The LTA muscles were analyzed 8 wk after transplantation.

Isolation and immunostaining of single fibers

To detect muscle satellite cells attaching to single fibers with Pax7, muscle fibers from the LTA muscle of recipient mice were prepared essentially according to the method of Bischoff in Rosenblatt *et al.* (33). Briefly, dissected muscles were incubated in DMEM containing 0.5% type I collagenase (Worthington) at 37°C for 90 min. The tissue was then transferred to prewarmed DMEM containing 10% FBS. The tissue was gently dissociated into single fibers by trituration with a fire-polished wide-mouth Pasteur pipette. Fibers were transferred to a Matrigel-coated 60 mm culture dish (Falcon) and fixed in 4% PFA for 5 min at room temperature. Fibers were permeabilized with 0.1% Triton X-100 in PBS for 10 min, and nonspecific binding was blocked by incubation in 5% skim milk for 10 min at room temperature. Primary mouse monoclonal antibodies against mouse Pax7 were applied for 12 h at 4°C. Antibodies were detected using the secondary antibodies described above.

Statistics

Data are presented as means ± SD. For comparison of the numbers of MHC and Pax7-positive cells in the sorted SM/C-2.6-positive and -negative fractions and the numbers of GFP-positive muscle fascicles and GFP/Pax7-double-positive cells in reinjured and noninjured groups, the unpaired Student's *t* test was used, and a value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Myogenic lineage cells are effectively induced from mES cells *in vitro*

EBs were formed in hanging drop cultures for 3 d followed by an additional 3 d in suspension cultures (**Fig. 1*A***). These EBs were then plated onto Matrigel-coated 48-well plates in differentiation medium, which contained 5% HS. This culture method is a modified version of the classical ES cell differentiation method (25) and the skeletal muscle single fiber culture method (33). After plating, EBs quickly attached to the bottom of the coated dishes, and spindle-shaped fibers appeared surrounding the EBs by the seventh day of plating (d 3+3+7; Fig. 1*B*). As these spindle fibers grew, they began to fuse with each other, forming thick multinucleated fibers resembling skeletal myofibers (Fig. 1*C, D*). At the same time we observed spontaneous contractions by the fibers (Supplemental Videos 1 and 2), a trait commonly seen in cultured skeletal muscle fibers. Immunostaining showed that these fused fibers were positive for skeletal-muscle-specific MHC (Fig. 1*E*). Furthermore, cells expressing muscle regulatory factor (MRF) proteins, including Pax7 (Fig. 1*F*), Myf5 (Fig. 1*G*), MyoD (Fig. 1*H*), and myogenin (Fig. 1*I*) were observed. On d 3 + 3 + 14, the average number of MHC-positive wells was 73.6 ± 5.8% (n=144). In all the MHC-positive wells, cells expressing Pax7, an essential transcription factor in satellite cells, were also observed. Double staining for Pax7 and MyoD confirmed the existence of cells staining for Pax7 alone, indicating the presence of quiescent-state satellite cells (34) within the culture (Supplemental Fig. 1). Next, the time course of MRF expression was examined by RT-PCR (Fig. 1*J*). Expression of Pax3 and Pax7 both peaked on d 3 + 3 +

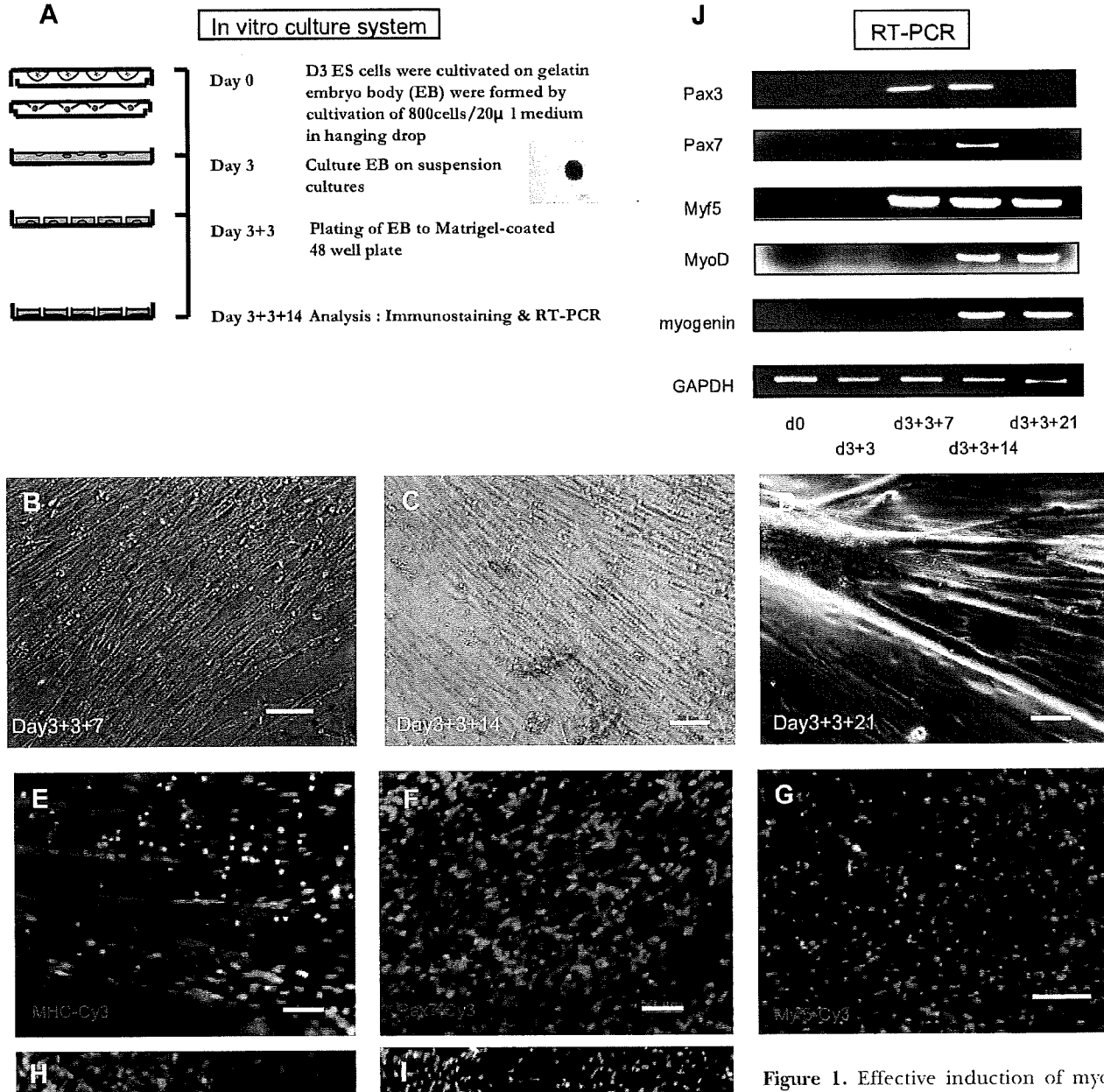

Figure 1. Effective induction of myogenic lineage cells from mES cells *in vitro*. *A*) *In vitro* culture system for ES cells. *B–E*) Skeletal myofibers observed within the culture system (*B–D*), which were positive for the mature myofiber marker MHC (*E*). *F, G*) Commonly recognized markers for satellite cells, Pax7 (*F*) and Myf5 (*G*), were observed in the cultures. *H, I*) MyoD-positive (*H*) and myogenin-positive fibers (*I*) were also observed in the cultures. White boxes indicate multinucleated myotubes (*I*). *J*) RT- PCR expression of MRFs including Pax3, Pax7, Myf5, MyoD, and myogenin in ES cells in our novel culture system at d 0, 3 + 3, 3 + 3 + 7, 3 + 3 + 14, and 3 + 3 + 21. Scale bars = 50 μm (*A–F*); 100 μm (*G–I*).

14, but Myf5, MyoD, and myogenin continued to be expressed after d 3 + 3 + 14.

Thus, using Matrigel plates and differentiation medium containing HS, myogenic lineages including Pax7-positive satellite-like cells were successfully induced from mES cells.

A novel antibody, SM/C-2.6, can enrich for Pax7-positive satellite-like cells derived from ES cells

To examine the characteristics of ES-derived Pax7-positive satellite-like cells, we needed to isolate these cells from the culture. Since Pax7 is a nuclear protein rather than a

surface marker, anti-Pax7 antibodies cannot be used for living cell separation by FACS. Therefore, a novel antibody, SM/C-2.6 (23), was used to detect satellite cells. SM/C-2.6 detects quiescent adult mouse satellite cells, as well as satellite cells in neonatal muscle tissue, as determined by immunostaining (Supplemental Fig. 2). RT-PCR confirmed that sorted SM/C-2.6-positive cells expressed Pax3, Pax7, Myf5, and c-met, whereas sorted SM/C-2.6-negative cells did not (Supplemental Fig. 3). Thus, the SM/C-2.6 antibody was shown to be useful for isolating living satellite cells by FACS.

We collected all the differentiated ES cells (1×10^6 cells) from cultures on d 3 + 3 + 14. FACS analysis using the SM/C-2.6 antibody showed that 15.7% of the cells were SM/C-2.6 positive (**Fig. 2*A***). RT-PCR analysis revealed that sorted SM/C-2.6-positive cells strongly expressed Pax3, Pax7, Myf5, c-met, and M-cadherin (Fig. 2*B*). Using a cytospin preparation of sorted SM/C-2.6-positive cells, we also confirmed the expression of M-cadherin (Fig. 2*C*) and Pax7 (Fig. 2*D;* 70.7±16.5% and 59.9±1.1% positive, respectively); only 2.3 ± 0.49% of the sorted SM/C-2.6-negative cells expressed M-cadherin, and 2.7 ± 0.1% expressed Pax7. Thus, the SM/C-2.6 antibody could enrich for satellite-like cells derived from mES cells *in vitro.*

ES-derived satellite-like cells have strong myogenic potential *in vitro*

To evaluate the myogenic potential of ES-derived SM/C-2.6-positive satellite-like cells *in vitro,* both SM/C-2.6-positive and -negative cells were sorted by FACS and plated in 96-well Matrigel-coated plates (see Fig. 4A). One week after cultivation, the number of muscle fibers in the wells was assessed. Although there were fibroblast-like and endothelium-like cells, MHC-positive fibers (787.3±123.7/well, 10.7±0.8% of the total cells per well, n=3) and Pax7-positive cells (222±81.4/well, 2.9±1.1% of the total cells per well, n=9) were observed in the SM/C-2.6-positive wells. In contrast, very few MHC-positive fibers (8.75±32.6/well, n=15; 0.12±0.46%) or Pax7-positive cells (2.6±2.0/well, n=8; 0.03±0.01%) were seen in the SM/C-2.6-negative wells

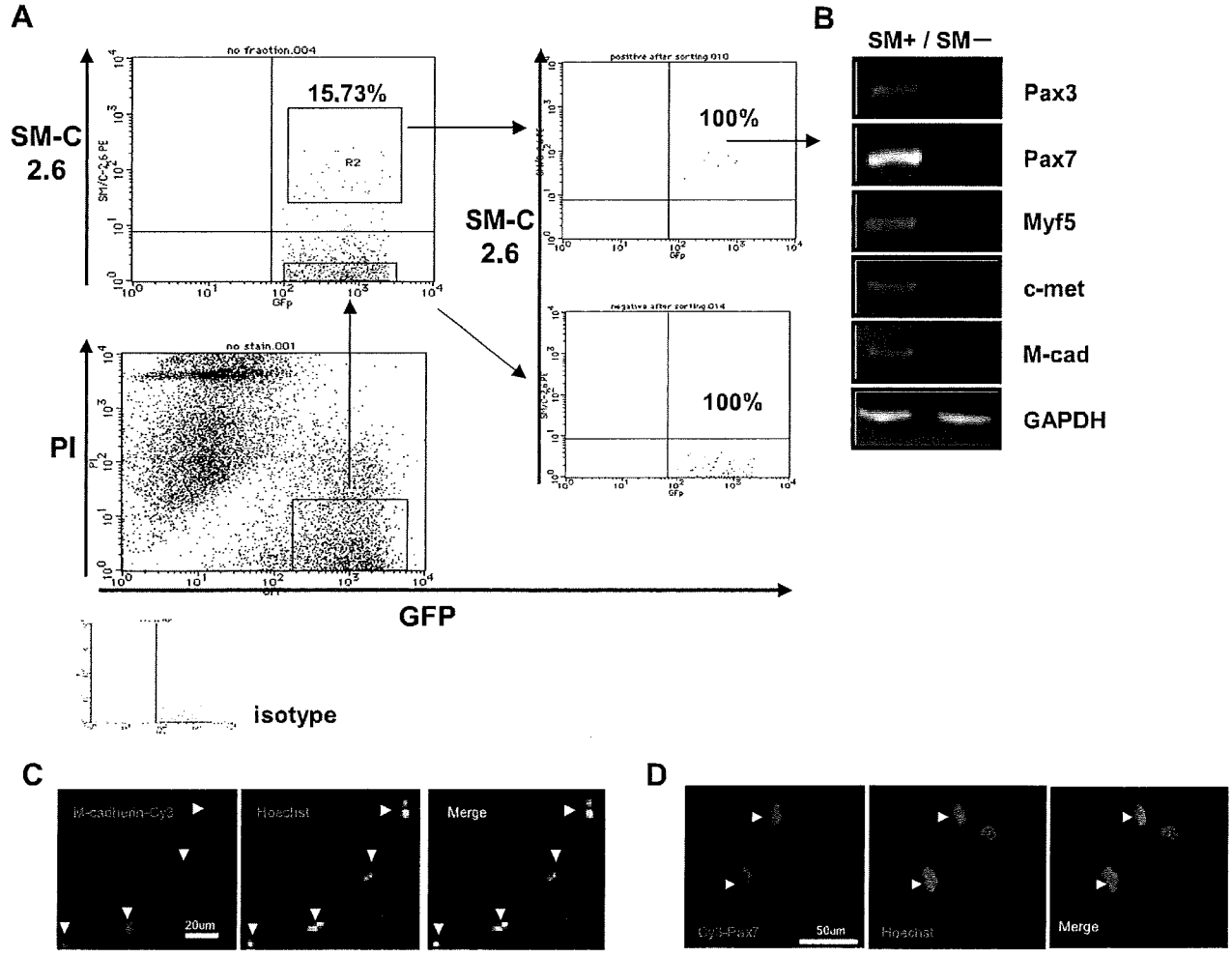

Figure 2. A novel antibody, SM/C-2.6, can enrich Pax7-positive satellite-like cells derived from ES cells. *A*) FACS data of cultured ES cells at d 3 + 3 + 14 indicate that 15.7% of total cultured cells are SM/C-2.6-positive cells. *B*) RT-PCR of the SM/C-2.6-positive fraction showed strong expression of Pax3, Pax7, Myf5, c-met, and M-cadherin. Immunostaining of a cytospin preparation of the sorted SM/C-2.6-positive cells showed that these cells were positive for M-cadherin (*C*), and Pax7 (*D*) (white arrowheads). Scale bars = 20 μm (*C*); 50 μm (*D*).

(both $P<0.05$; **Fig. 3**). Thus, ES-derived satellite-like cells isolated using the SM/C-2.6 antibody possess strong myogenic potential *in vitro*.

Damaged muscle can be repaired by transplantation of ES-derived satellite-like cells

To examine the myogenic potential of ES-derived satellite-like cells *in vivo*, SM/C-2.6-positive and -negative cells were transplanted into conditioned mdx mice (15). The LTA muscles of recipient mdx mice were preinjured with CTX (primary injury; ref. 30) 24 h prior to transplantation, and mice were exposed to 8 cGy of γ-irradiation (whole body) 12 h prior to transplantation (**Fig. 4A**). GFP-positive ES cells were used as donor cells in this experiment. GFP$^+$ES-derived SM/C-2.6-positive and -negative cells were directly injected into the predamaged LTA muscles. The recipient mice were analyzed 3 wk post-transplantation. By fluorescence stereomicroscopy, GFP-positive tissues were clearly observed within the LTA muscles injected with SM/C-2.6-positive cells (Fig. 4*B* and **Table 1**). In contrast, no GFP-positive tissue was observed in muscles injected with SM/C-2.6-negative cells (Fig. 4*C*). These GFP-positive tissues were further confirmed by diaminobenzidine staining using anti-GFP and a peroxidase-conjugated secondary antibody (Supplemental Fig. 4) to exclude the possibility of autofluorescence of the muscle tissues. Immunostaining with anti-MHC confirmed that these GFP-positive tissues were mature skeletal myofibers (Fig. 4*D*). In addition, GFP/Pax7 double-positive cells were observed within the LTA muscles of the recipient mice (Fig. 4*E* and Supplemental Fig. 5) and in isolated single fibers (Fig. 4*F* and Table 1). The GFP-positive cells were also confirmed to be positive for other satellite cell markers such as Myf5 and M-cadherin (Supplemental Figs. 6 and 7). These GFP/Pax7-double-positive cells were located along the periphery of the muscle fascicle. With laminin immunostaining we verified that the location of the GFP-positive mononuclear cells was between the basal lamina and the muscle cell plasma membrane, a location consistent with the anatomical definition of satellite cells

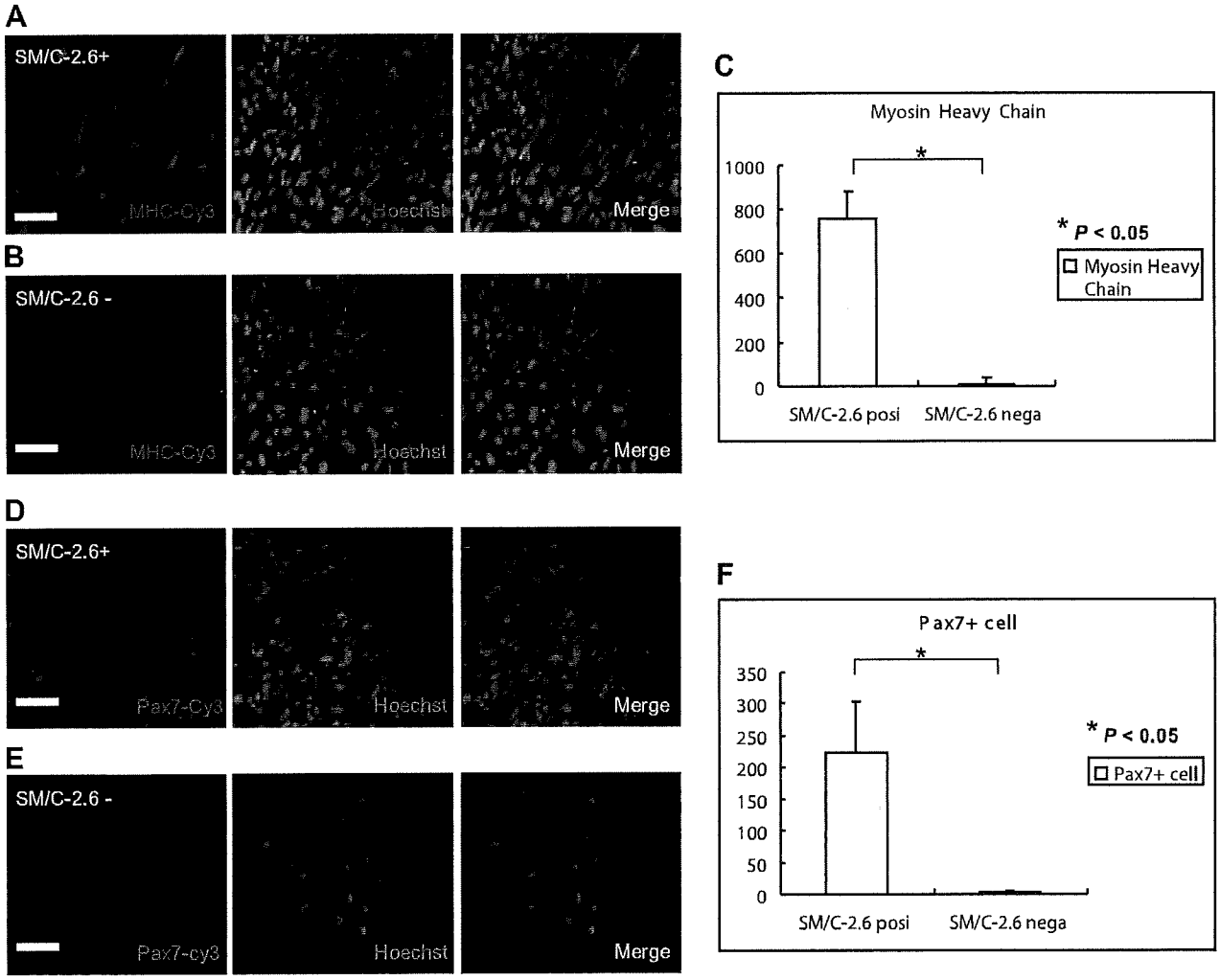

Figure 3. ES-derived satellite-like cells have strong myogenic potential *in vitro*. Immunostaining detected an abundant number of MHC-positive fibers and Pax7-positive cells in SM/C-2.6-positive cell culture (*A*, *D*) but not SM/C-2.6-negative cells (*B*, *E*) after 1 wk in culture. Scale bars = 50 μm. Significant differences were observed in the number of MHC-positive fibers and Pax7-positive cells per well between sorted SM/C-2.6-positive and -negative cell cultures (*C*, *F*).

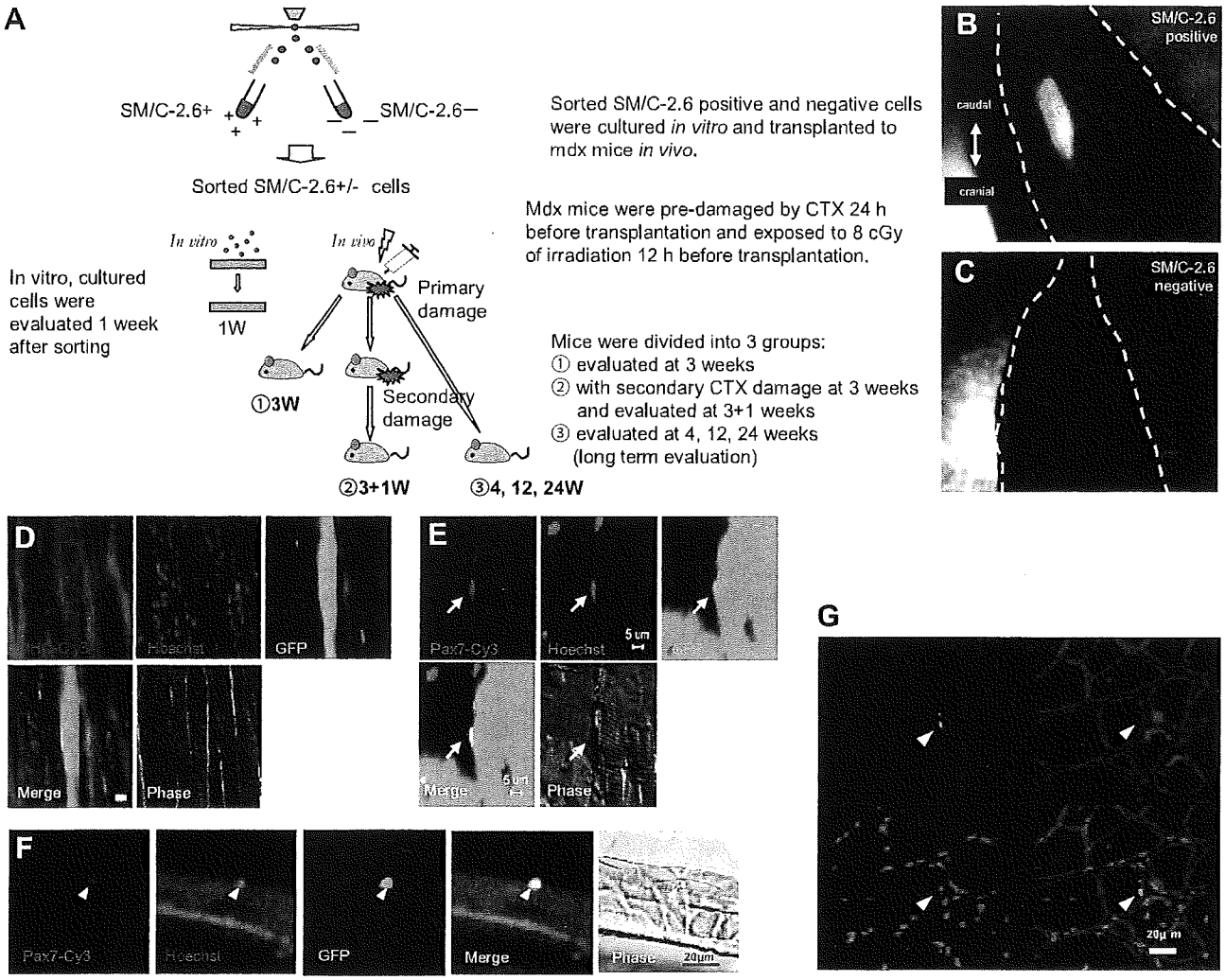

Figure 4. ES-derived satellite-like cells can repair damaged muscle *in vivo*. *A*) Methods for *in vitro* and *in vivo* analysis of sorted SM/C-2.6-positive and -negative cells derived from mES cells. *B*, *C*) ES-derived GFP-positive tissue engrafted to the LTA muscle of a recipient mouse that received SM/C-2.6-positive cells (*B*) but SM/C-2.6-negative cells (*C*). *D*) Grafted GFP-positive tissues were histologically MHC positive. *E*) GFP/Pax7-double-positive cells were observed in mice that received SM/C-2.6-positive cells by anti-Pax7 immunostaining. *F*) GFP/Pax7-double-positive cells were also confirmed by immunostaining of isolated single fibers. *G*) Laminin immunostaining indicated that the GFP-positive cells were located between the basal lamina and the muscle cell plasma membrane, which is consistent with the anatomical definition of muscle satellite cells. Scale bars = 1 mm (*B*, *C*); 15 μm (*D*); 5 μm (*E*); 20 μm (*F*, *G*).

(Fig. 4*G*). In contrast, in mice transplanted with SM/C-2.6-negative cells, GFP-positive tissues were rarely observed, and none of the GFP-positive cells were positive for skeletal MHC. H&E staining indicated that these GFP-positive tissues were surrounded by inflammatory cells (Supplemental Fig. 8), suggesting that these nonmyogenic tissues may undergo phagocytosis. These results demonstrate that ES-derived SM/C-2.6-positive satellite-like cells could be engrafted *in vivo* and repair damaged muscle tissues of the host.

Engrafted ES-derived satellite-like cells function as satellite cells following muscle damage

Muscle satellite cells are generally considered to be self-renewing monopotent stem cells that differentiate into myoblasts and myofibers to repair damaged skeletal muscles. To determine whether these engrafted GFP$^+$ES-derived satellite-like cells are functional stem cells, we injured the LTA muscle of primary recipient mice 3 wk after primary transplantation with GFP$^+$SM/C-2.6-positive cells. This experiment let us assess the ability of satellite-like cells to repair damaged muscle fibers and self-renew *in vivo* (14). The LTA muscles were removed and analyzed 1 wk after the secondary injury (reinjured group). Mice that were initially injected with GFP$^+$SM/C-2.6-positive cells without a second injury were used as a control (nonreinjured group). These control mice were analyzed 3 or 4 wk after transplantation (Fig. 4*A*). GFP-positive muscle fascicles were counted in sections of both reinjured and nonreinjured muscle (**Fig. 5*A*, *B***). In the reinjured group 461.7 ± 117.4 (n=6; per view, ×100) GFP-positive muscle fascicles were observed. In comparison, only 136.7 ± 27.9 (n=4) and 168.7 ± 72.9 (n=6; per view, ×100) GFP-positive muscle fascicles were evident in

TABLE 1. *Transplantation of reinjured and nonreinjured mice and long-term evaluation*

Group	TA with GFP$^+$ fascicles [n(%)][a]	Mouse	Cells/TA injected (n)	GFP$^+$ fascicles/TA (avg)[b]	GFP$^+$/Pax7$^+$ cells/TA (avg)[c]	Engraftment efficiency
SM/C-2.6$^+$						
3W	4/8 (50%)	1	1.75×10^4	125.3	5.3	
		2	3.5×10^4	111.1	7.1	
		3	5×10^4	134.2	5.1	
		4	8×10^4	176.1	4.2	
Mean			$4.5 \pm 2.6 \times 10^4$	136.7 ± 27.0	5.4 ± 1.2	0.30%
4W	6/9 (66.67%)	1	2×10^4	77.3	6.1	
		2	1.3×10^5	153.2	4.6	
		3	5×10^4	163.1	6.8	
		4	3.5×10^4	168.9	5.1	
		5	8×10^4	281.1	7.2	
		6	1.75×10^4	169.4	6.2	
Mean			$3.6 \pm 2.5 \times 10^4$	168.7 ± 72.9	6 ± 1	0.47%
3 + 1W	6/8 (75%)	1	2×10^4	581.2	11.2	
		2	1.3×10^5	370.3	11.5	
		3	5×10^4	586.6	10.1	
		4	3.5×10^4	486.6	5.9	
		5	8×10^4	347.1	15.3	
		6	1.75×10^4	542.9	10.8	
Mean			$5.5 \pm 4.3 \times 10^4$	461.7 ± 117.3	10.8 ± 3	0.84%
12W	3/5 (60%)	1	2×10^4	391.5	9.7	
		2	5×10^4	266	9.3	
		3	8×10^4	280.2	6	
Mean			$5 \pm 3 \times 10^4$	312.6 ± 68.7	8.3 ± 2	0.59%
24W	1/2 (50%)	1	2×10^4	58.62	3.45	
Mean			2×10^4	58.62	3.45	0.20%
SM/C-2.6$^-$						
3W	0/8 (0%)	1–8	$1–8 \times 10^4$	0	0	0%
4W	0/9 (0%)	1–9	$1.3–8 \times 10^4$	0	0	0%
3 + 1W	0/8 (0%)	1–8	$1.75–13 \times 10^4$	0	0	0%
12W	0/5 (0%)	1–8	$2–8 \times 10^4$	0	0	0%
24W	0/2 (0%)	1–2	2×10^4	0	0	0%

Serial transplantation

	Primary transplantation		Secondary transplantation		
Mouse	Cells injected	Collected GFP$^+$ cells/TA	Cells injected	GFP$^+$ fascicles/TA	Engraftment efficiency
1	2×10^4	3253	200	29.3	14.7%
2	2×10^4	2277	200	28.6	14.3%
Mean	2×10^4	2765	200	29 ± 0.5	14.5%

TA, tibialis anterior; 3W, nonreinjured group analyzed 3 wk after cell transplantation; 4W, nonreinjured group analyzed 4 wk after cell transplantation; 3 + 1W, reinjured group reinjured 3 wk after cell transplantation and analyzed 1 wk after reinjury; 12W, long-term engraftment evaluation analyzed 12 wk after cell transplantation; 24W, long-term engraftment evaluation analyzed 24 wk after cell transplantation. [a]Percentage of TA that had engrafted with GFP$^+$ fibers was calculated as number of TAs with GFP$^+$ fibers/total TAs injected with cells. [b]Average determined from number of GFP$^+$ muscle fascicles counted per field at ×100 in 10 fields. [c]Average determined from number of GFP$^+$/Pax7+ cells counted per field at ×100 in 10 fields.

the nonreinjured groups at 3 and 4 wk, respectively, after transplantation (Fig. 5*B* and Table 1). Furthermore, we also observed that many GFP-positive muscle fibers had a typical central nucleus in the reinjured group (Fig. 5*C*), indicating regenerating muscle fibers. Taken together, these results suggest that these GFP-positive muscle tubes were freshly regenerated by the engrafted GFP$^+$ ES-derived satellite-like cells in response to the second injury. Surprisingly, immunostaining with anti-Pax7 revealed an increase in number of GFP/Pax7-double-positive cells in the reinjured group (10.8±3.0/view compared to 5.4±1.2, and 6.0±1.0 in the nonreinjured group; Fig. 5*D* and Table 1). This result strongly suggests that engrafted ES-derived satellite-like cells not only self-renewed but also expanded in number, possibly replacing the recipient satellite cells lost because of excessive repair of skeletal muscle in response to the second injury.

ES-derived satellite-like cells are capable of long-term engraftment in recipient muscles

Long-term engraftment is an important characteristic of self-renewing stem cells. If these ES-derived satellite-

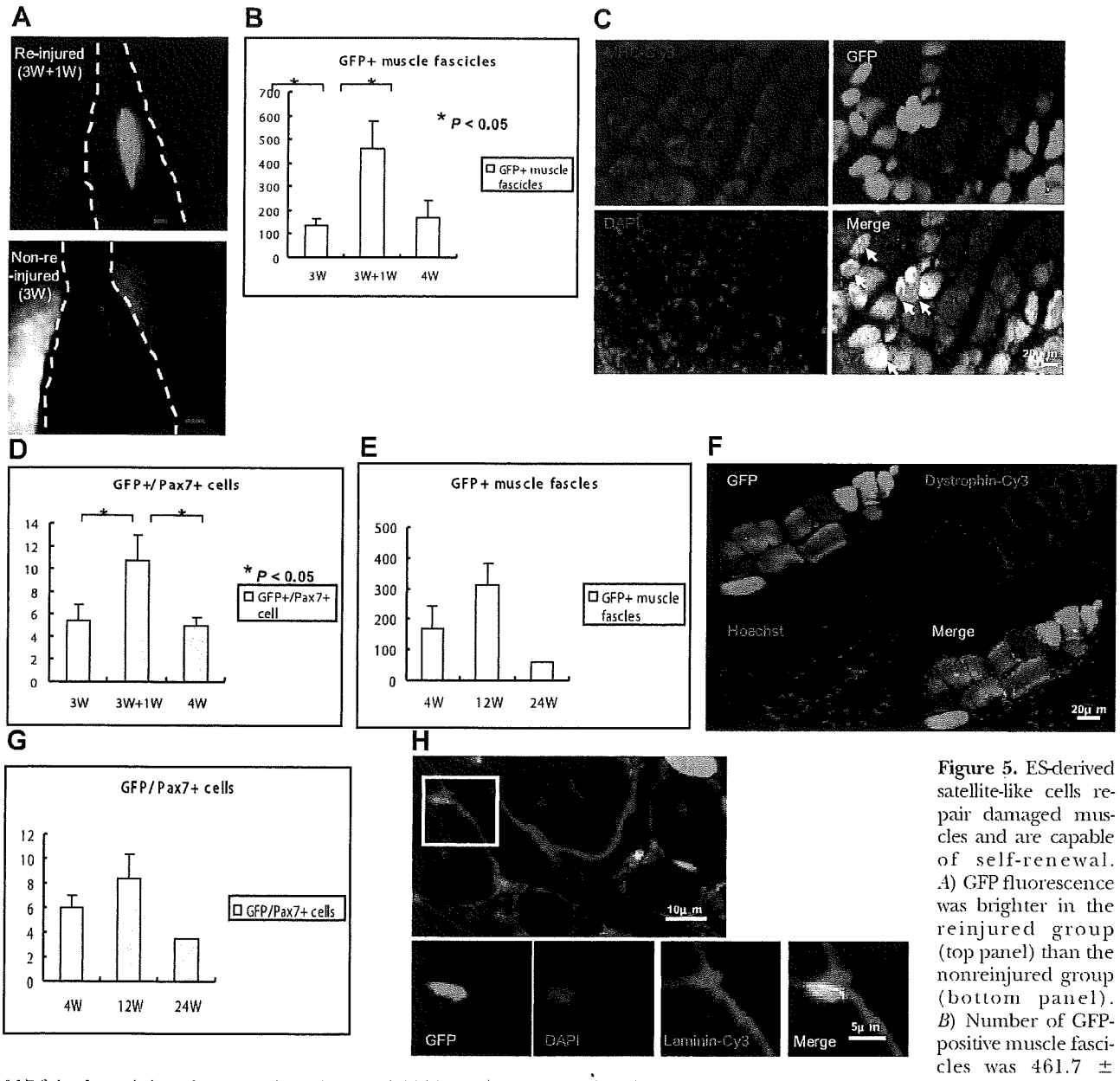

Figure 5. ES-derived satellite-like cells repair damaged muscles and are capable of self-renewal. *A*) GFP fluorescence was brighter in the reinjured group (top panel) than the nonreinjured group (bottom panel). *B*) Number of GFP-positive muscle fascicles was 461.7 ± 117.3 in the reinjured group (3W+1W) and 136.7 ± 27.9 and 168.7 ± 72.9 in the nonreinjured group at 3 wk (3W) and 4 wk (4W), respectively. *C*) GFP-positive fibers were confirmed to be MHC positive and contained central nuclei (arrows). *D*) Number of GFP/Pax7-double-positive cells also increased significantly in the reinjured group (10.8±3.0 cells at 3W+1W) compared to the nonreinjured group (5.4±1.2 and 6.0±1.0 at 3W and 4W, respectively). *E*) In long-term evaluations, number of GFP-positive muscle fascicles at 12 wk (12W) increased relative to number at 4 wk after transplantation [312.6±68.7 (n=3) *vs.* 168.7±72.9]. However, a decrease was observed at 24 wk (58.6; n=1). *F*) Immunostaining showed dystrophin (red) surrounding the donor-derived GFP-positive fibers (green), 24 wk after transplantation of SM/C-2.6-positive cells. *G*) Results similar to *E* were observed with the number of GFP/Pax7-double-positive cells. *H*) A GFP-positive cell beneath the basal lamina was observed. Scale bars = 1 mm (*A*); 20 μm (*C*); 20 μm (*F*); 10 μm (*H*, top panel); 5 μm (*H*, bottom panels).

like cells function as normal stem cells in skeletal muscle, they should be able to reside within the tissue for long periods of time and undergo asymmetric cell divisions to maintain the number of satellite cells and to generate muscle fibers. To examine this stem cell function, we analyzed the recipient mice at 4, 12, and 24 wk after transplantation. Intriguingly, in the LTA muscle of mdx mice transplanted with SM/C-2.6-positive cells, the number of GFP-positive fascicles at 12 wk increased over that at 4 wk [12.6±68.7 (n=3) *vs.* 168.7±72.9; Fig. 5*E*] but decreased by 24 wk (58.6; n=1). These engrafted GFP-positive tissues were confirmed to be MHC positive through immunostaining (Supplemental Fig. 9), and surrounding these GFP-positive fibers, dystrophin was observed (Fig. 5*F*). The numbers of GFP/Pax7-double-positive cells were maintained from week 4 to week 24 (Fig. 5*G*, Table 1, and Supplemental Fig. 10) and the location of GFP-positive cells under the basal lamina meets the anatomical definition of satellite cells (Fig. 5*H*). No teratomas were

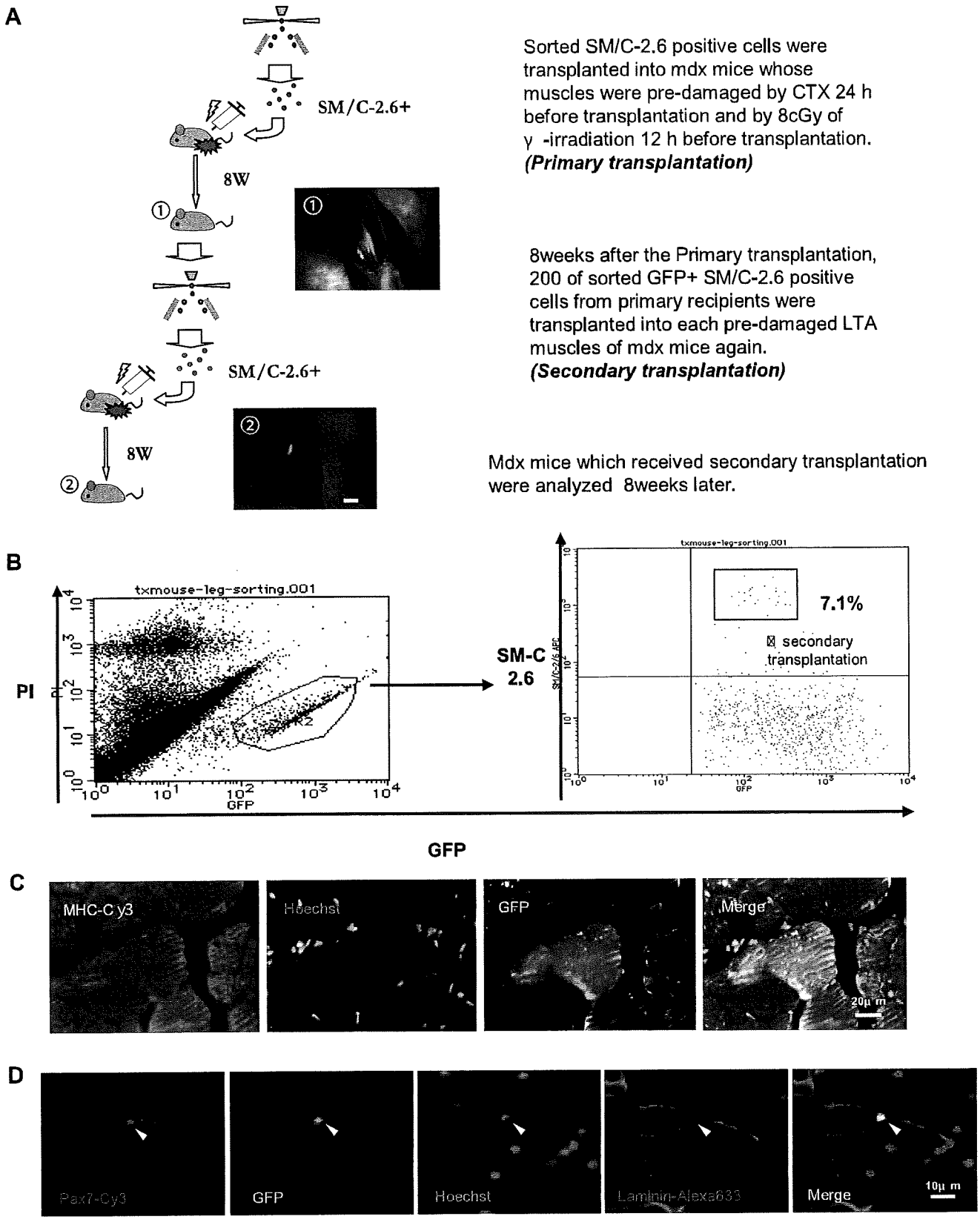

Figure 6. ES-derived satellite-like cells can be secondarily transplanted. *A*) SM/C-2.6-positive cells (2.5×10^4) were transplanted into the LTA muscle of recipient mice in primary transplantation, and as few as 200 SM/C-2.6-positive cells collected from the primary recipients were retransplanted (secondary transplantation) into the LTA muscle of secondary recipient mice. *B*) FACS data of primary transplantation indicated that 7.1% of engrafted (GFP-positive) cells were SM/C-2.6-positive. *C*) Eight weeks after secondary transplantation, immunostaining of LTA muscle for MHC showed that engrafted ES-derived GFP-positive tissues formed mature skeletal muscle fibers. *D*) GFP/Pax7-double-positive cells (arrowhead) located beneath the basal lamina were observed within GFP-positive LTA muscle of secondary recipient mice. Scale bars = 2 mm (*A*); 20 μm (*C*); 10 μm (*D*).

found in recipient mice transplanted with SM/C-2.6-positive cells. Thus, ES-derived satellite-like cells effectively engrafted and provided long-term stem cells, which played an important role in maintenance of the integrity of the surrounding muscle tissue.

ES-derived satellite-like cells can be secondarily transplanted

For a more thorough characterization of the ES-derived satellite-like cells, we performed serial transplantations. Eight weeks after the primary cell transplantation with 2×10^4 SM/C-2.6-positive cells, the LTA muscles of the primary recipient mice were dissected to isolate the engrafted ES-derived cells, 2765 ± 685.9 (n=2; **Fig. 6*A***). The GFP$^+$/SM/C-2.6-positive cells within the engrafted cells were sorted by FACS (204±33.9; n=2), and only 200 GFP+/SM/C-2.6-positive cells/mouse were transplanted into predamaged LTA muscles of mdx mice (Fig. 6*B*). Eight weeks later (16 wk after the primary transplantation), the recipient mice were analyzed. GFP-positive tissue in the LTA muscle of the secondary recipient mice was observed (Fig. 6*A*). The GFP-positive tissues were confirmed to be MHC-positive mature skeletal muscle (Fig. 6*C*), and surrounding these engrafted GFP-positive skeletal muscle fascicles, dystrophin was observed (Supplemental Fig. 11). GFP/Pax7-double-positive cells located beneath the basal lamina were also detected in the engrafted tissue (Fig. 6*D*). Thus, with only 200 GFP$^+$SMC/2.6-positive cells, injured skeletal muscle and Pax7+ cells were successfully restored in the secondary recipients. These findings demonstrate that stem cell fraction contained within SM/C-2.6-positive cells was enriched *in vivo* through transplantation.

DISCUSSION

Many attempts have been made to induce mES cells into the skeletal muscle lineage, with hanging drop cultures for EB formation being the most widely applied method (25). However, although EBs contain cells derived from all 3 germ layers, effective induction of mES cells into the myogenic lineage, including myogenic stem cells (satellite cells), has not yet been achieved. Because of the lack of adequate surface markers, purifying ES-derived myogenic precursor/stem cells from differentiated mES cells *in vitro* has been difficult. To overcome these problems, we modified the classic EB culture system by combining it with aspects of the single-fiber culture method. Single-fiber culture (33) has been used for functional evaluation of satellite cells. When a single myofiber is plated on a Matrigel-coated plate with DMEM containing HS, satellite cells migrate out of the fiber and differentiate into myoblasts to form myofibers *in vitro*. Matrigel allows the migrating satellite cells to proliferate before differentiating and fusing into large multinucleated myotubes (35). We hypothesized that this Matrigel environment might be suitable for ES cell differentiation into satellite cells and myoblasts. Therefore, we introduced Matrigel and HS into the classic EB culture system and established an efficient induction system for myogenic lineage cells, including cells expressing Pax7, a commonly recognized marker for skeletal muscle stem cells. Furthermore, we also successfully enriched ES-derived Pax7-positive myogenic precursor/stem cells using the SM/C-2.6 antibody.

The steps in ES cell induction are thought to be homologous to normal embryogenesis. During normal skeletal myogenesis, the initial wave of myogenic precursor cells in the dermomyotome express Myf5/MRF4 and Pax3, followed by a wave of Pax3/Pax7 expression (36). These waves of myogenesis act upstream of the primary myogenic transcription factor MyoD (37-39). In myotome formation skeletal myogenesis begins with myoblasts, termed somitic myoblasts, which appear at approximately E8.5, followed by the appearance of embryonic myoblasts (E11.5), fetal myoblasts (E16.5), and, ultimately, satellite cells, which are responsible for postnatal muscle regeneration (40). Our RT-PCR results (Fig. 1*J*) showed an earlier appearance of Pax3 expression, on d 3 + 3, followed by Pax3/Pax7 expression on d 3 + 3 + 7 and stronger expression of Pax3 than Pax7. These results resemble normal myogenesis, in which the primary wave of myogenesis is followed by a secondary wave of Pax3/Pax7-dependent myogenesis (41). Considering that in the time course of myogenesis satellite cells emerge during late fetal development, ES-derived Pax7-positive cells were collected on d 3 + 3 + 14 in an attempt to acquire cells that correspond to those of the late fetal to neonatal period. However, RT-PCR results of myogenic factors in SM/C-2.6-positive cells (Fig. 2*B*) indicated that these ES-derived SM/C-2.6-positive cells are a heterogeneous population, because they express not only Pax3 and Pax7 but also Myf5 and c-met. Although further confirmation is needed, we hypothesize that both embryonic/fetal myoblasts expressing Myf-5 and/or c-met and satellite/long-term stem cells expressing Pax3/Pax7 are present.

To confirm that the ES-derived SM/C-2.6-positive cell population contained functional satellite cells, the muscle regeneration and self-renewal capacities were examined. Recently Collins *et al.* established an excellent system in which sequential damage to the muscle of a recipient mouse was applied, to evaluate both muscle regeneration and self-renewal (14) Using their experimental approach, a significant increase in numbers of both ES-derived GFP-positive muscle fascicles and GFP/Pax7-double-positive cells was observed in mice that received a second injury. This result not only demonstrates the myogenic ability of ES-derived cells but also strongly supports the idea that these cells undergo self-renewal *in vivo*.

Analysis of long-term engraftment is an important method to verify self-renewal ability, for 2 reasons. First, ES-derived satellite cells must be able to engraft for long periods of time in order to provide the amount of progeny needed for repairing damaged tissue for an

extended period. In our study the ES-derived GFP-positive skeletal muscle tissues and Pax7-positive cells engrafted up to 24 wk and were located beneath the basal lamina, which is consistent with the anatomical definition of satellite cells. Although the number of GFP-positive fascicles at 24 wk decreased compared to 12 wk, this diminution may be due to the heterogeneity of ES-derived SM/C-2.6-positive cells as we mentioned. Because myoblasts cannot support myogenesis in the long term, we believe that GFP-positive fascicles at 24 wk are products of ES-derived satellite-like cells. Second, one of the potential risks of ES cell transplantation is teratoma formation. Considering clinical applications, it is extremely important to prevent formation of teratomas in the recipients. In our study more than 60 transplanted mice were evaluated through gross morphological and histological examination. There were no teratomas formed in mice that received SM/C-2.6-positive cells, and only 1 teratoma was found among the mice that received SM/C-2.6-negative cells. This result suggests that the risk of tumor formation by the ES cells was eliminated by using sorted SM/C-2.6-positive cells.

In addition to the sequential damage model and the long-term engraftment evaluation, we performed serial transplantations to further confirm the stem cell properties of these ES-derived SM/C-2.6-positive cells. Serial transplantation enables the identification and separation of long-term stem cells from short-term progenitors (42). To eliminate myoblast involvement, we designed a serial transplantation protocol of 8 + 8 wk (*i.e.*, a second transplantation 8 wk after the primary transplantation and an analysis of recipient mice 8 wk after the second transplantation). Strikingly these recollected ES-derived SM/C-2.6-positive cells showed significantly higher engraftment efficiency compared to the primary transplantation. In the previous reports engraftment efficiencies of myoblasts transplantation was ~0.1-0.2%, with the highest reported value being 2% (43-45). This engraftment efficiency is similar to our primary transplantation (0.2-0.8%) as well as the plating efficiency of SM/C-2.6-positive cells *in vitro* (0.07%). In our study as few as 200 recollected ES-derived SM/C-2.6-positive cells were transplanted in the second transplantation, and 29.0 ± 0.47 (n=2) fascicles were observed, which indicates 14.7% of higher engraftment efficiency. Thus, through the serial transplantation, ES-derived stem cell fraction was purified. A comparison of these SM/C-2.6-positive cells before and after injection might help to characterize the stem cell fraction derived from ES cells.

There have been few reports describing transplantation of ES-derived myogenic cells into injured muscles, and the report of engraftable skeletal myoblasts derived from human ES cells represents significant progress (26). Recently Darabi *et al.* (46) have reported that by introducing Pax3 into mouse embryoid bodies, autonomous myogenesis was initiated *in vitro*, and Pax3-induced cells regenerated skeletal muscles *in vivo* by sorting the PDGF-a+Flk-1- cells. The Pax3 expression was not observed until 7 d of differentiation culture, but introduced Pax3 expression pushed EBs to myogenic differentiation. Interestingly, we observed Pax3 expression at d 3 + 3 weakly and d 3 + 3 + 7 strongly, and gene expression process in our culture is very similar to theirs. In prolonged culture using Matrigel and HS, EBs were able to initiate myogenesis without gene modification in our system.

In conclusion, we successfully generated transplantable myogenic cells, including satellite-like cells, from mES cells. The ES-derived myogenic precursor/stem cells could be enriched using a novel antibody, SM/C-2.6. These ES-derived SM/C-2.6-positive cells possess a high myogenic potential, participate in muscle regeneration, and are located beneath the basal lamina where satellite cells normally reside. The self-renewal of these ES-derived satellite-like cells enabled them to survive long-term engraftment, up to 24 wk. Through serial transplantation, these ES-derived SM/C-2.6-positive cells were further enriched and produced a high engraftment efficiency of 14.7%.

Our success in inducing mES cells to form functional muscle stem cells, the satellite-like cells, will provide an important foundation for clinical applications in the treatment of DMD patients. FJ

This work was supported by a Grant-in-Aid for Scientific Research (S) (19109006) and a Grant-in-Aid for Scientific Research (B) (18390298) from the Ministry of Education, Science, Technology, Sports, and Culture of Japan.

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Received for publication October 21, 2008.
Accepted for publication January 8, 2009.

福山型先天性筋ジストロフィー

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要旨

日本に特異的な福山型筋ジストロフィーや muscle-eye-brain 病，Walker-Warburg 症候群は，先天性筋ジストロフィーに滑脳症と眼奇形を伴う類縁疾患である．われわれは福山型原因遺伝子を同定し，原因蛋白質をフクチンと命名した．近年，これらの筋ジストロフィーに共通した病態として，αジストログリカンの糖鎖修飾異常が注目され，「αジストログリカノパチー」という新しい疾患概念が提唱されている．本稿では福山型の病態を中心にαジストログリカノパチーについて解説する．

Key words：福山型筋ジストロフィー，フクチン，αジストログリカノパチー，LARGE，糖鎖

Ⅰ 福山型先天性筋ジストロフィー（FCMD）の特徴―骨格筋-眼-脳を中心に侵す一系統疾患

福山型先天性筋ジストロフィー（Fukuyama-type congenital muscular dystrophy：FCMD）は1960年福山らにより発見された常染色体劣性遺伝疾患である．わが国の小児期筋ジストロフィー中 Duchenne 型の次に多く，日本人の約90人に1人が保因者と計算され，日本に1,000～2,000人くらいの患者が存在すると推定され，日本人特有の疾患とされていたが，近年海外からの報告が相次いでいる（後述）．本症は重度の筋ジストロフィー病変とともに，多小脳回を基本とする高度の脳奇形（敷石（2型）滑脳症）が共存し，さらに最近は近視，白内障，視神経低形成，網膜剝離などの眼症状も注目されている．すなわち本症は遺伝子異常により骨格筋-眼-脳を中心に侵す一系統疾患である[1]．

患児は生後から乳児早期に筋緊張低下，筋力低下で発症する．運動障害は重症で，2歳前後で座位まで獲得するものは多いが，歩行まで獲得するものはまれである．また同時に脳奇形による中枢神経症状も伴い，全例に精神発達遅延を認め，約半数にけいれんを認める．筋力低下，全身関節拘縮により10歳前後に完全臥床状態となり，多くは20歳までに死亡する[1]．

福山型は1960年の発表後，日本国内ではスムーズに認知されたが，海外ではなかなか認知されなかった．理由は，発表された雑誌が private なものであったことと，海外に患者が存在しなかったためである．海外の総説では，1979年でさえ，「先天性筋ジストロフィーという疾患自体やがては分類から消えていくであろう」とあるくらいである．しかし福山らの不断の活動や幾つかの影響力のある論文を通して，徐々に本症の知識は世界に広がっていった．そして1986年には Mendelian Inheritance in Man（いわゆる McKusick のカタログ）に登録された．しかし国際疾病分類（神経）に独立した疾患として登場したのが1991年であることは，われわれ日本人からみれば大変意外である．

Ⅱ 福山型遺伝子フクチン同定のヒント，幸運

このように日本に多く重度な疾患であるにもかかわらず，Duchenne 型筋ジストロフィーに比べ本態はほとんどわかっていなかった．筆者

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は1989年に筋ジストロフィー専門病院で筋ジストロフィーの臨床に携わっていた．当時Duchenne型はジストロフィンが発見され病態が明らかになりつつあったが，福山型に関しては，常染色体劣性遺伝疾患という以外，遺伝子の位置も不明であり，確実なマーカー蛋白となり得る物質もなく，分子的には何もわかっていなかった．

筆者はこの原因不明の疾患の診療に取り組みながら，「日本人の名前のついた病気は日本人の手で何とかしたい」と思っていたが，そのきっかけは，1990年偶然行った班会議で，「近親婚の症例10人くらい患者さんだけで連鎖解析できる」と，自分とは無関係な演題への質問コメントを聞いたことによる．この「ホモ接合性マッピング」[2]の理論は当然知るはずもなく休憩時間にその先生に体当たりしてお伺いした．

その原理を**図1**に示す．図のように，常染色体劣性遺伝病の場合，いとこ婚家系で，仮にある共通祖先の片方の染色体にFCMD変異（ここではfとし，正常をプラスとする）をもつとすると，その1個のfが両側に伝わり，患者ではじめてf,fのホモになり病気が出現する場合が多く考えられる．言い換えれば患者は共通祖先の1個の染色体の2つのコピーをもつことになり，これを「同祖ホモ」という．その疾患遺伝子にごく近いDNA多型マーカーのアレルが仮に1である場合，近くて組み換えが起きにくいので，この1はfとともに動いて患者において同祖ホモになる．逆にいえば近親婚により生まれた患者において，常にホモとなるようなDNA多型を探せば，それは原因遺伝子に近いことが示唆されるわけである[2]．

当時このような劣性遺伝疾患で通常の連鎖解析をするのは何年かかるかわからず不可能といわれており，この理論があったから猛然と近親婚症例の集積を開始した．「近親婚症例いらっしゃいませんでしょうか？」と全国数百カ所に手紙を書き，1991年より山中まで症例を集めに

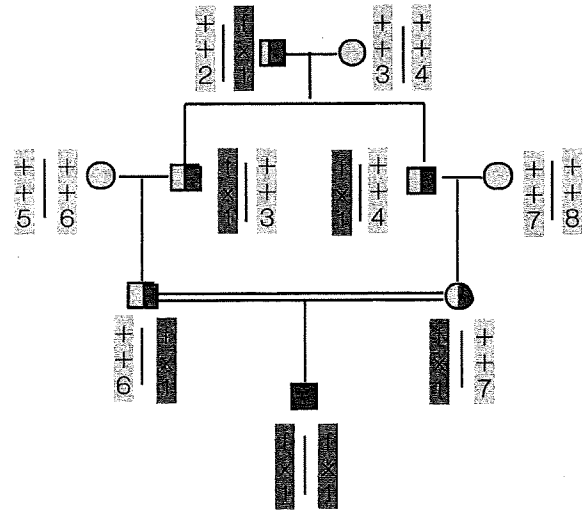


図1　ホモ接合性マッピングの原理

f：FCMD遺伝子変異，x：XPA遺伝子変異．患者は曽祖父の1個の染色体断片の2つのコピーをもつ「同祖ホモ」であり，FCMD遺伝子にごく近いDNA多型もホモ（ここでは1,1）になる．また同様の理由でXPA遺伝子変異もホモになり，FCMD以外にXPAも発症する．

出かけた．まさに班会議に行っていなければ，この仕事は行わなかったであろうと思われる．

またその後1991年症例検討会で福山型と色素性乾皮症A群（xeroderma pigmentosum A：XPA，常染色体劣性遺伝，9q22.3-31に遺伝子同定された直後であった）を合併した近親婚の症例が存在するとの情報を得，これも両遺伝子がそばになければありえないことに気付いた．その理由は，両親ともに2つの無関係な常染色体劣性遺伝病の保因者である可能性は少ない，むしろ曽祖父（母）が福山型とXPAの変異保因者であり，両遺伝子は近くに存在し組み換えが起きずにともに患者の父方，母方へ伝わり，患者で両変異ともホモとなって両者が合併した（図1，XPA変異をxとする），という状況を考えたわけである[2]．そしてまさにその周辺から連鎖解析を行い，FCMD遺伝子が9q31-33領域に存在することを，1993年に報告した[3]．

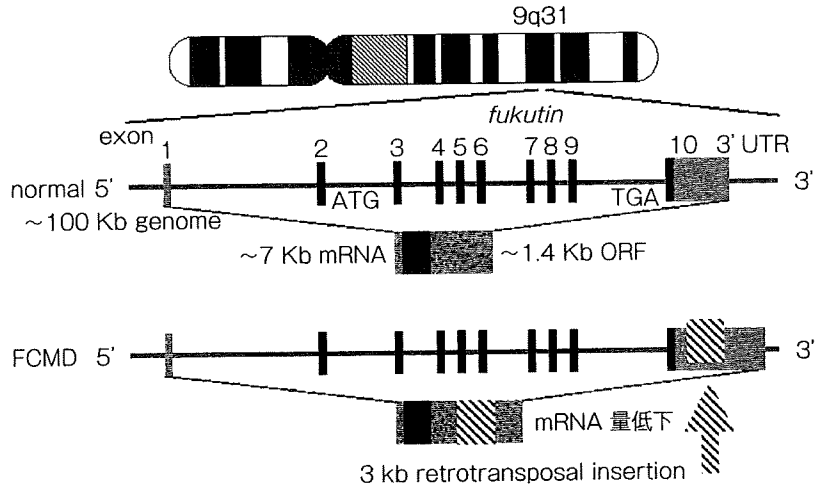


図2　フクチン遺伝子の模式図

フクチン遺伝子はゲノム上で約100 Kbにわたり，10個のエクソンからなる．大部分のFCMD染色体には，この遺伝子の3'非翻訳領域内にレトロトランスポゾン挿入変異がある．遺伝子産物フクチンは461個のアミノ酸（分子量53.7 kD）からなる糖鎖修飾に関与する新規蛋白質である．

III　FCMD（フクチン）遺伝子とレトロトランスポゾン

その後もわれわれのグループは遺伝子の存在範囲を狭める解析を続けた．まず組み換え家系の解析により約数cMに限局化したあと，その範囲の一つのマイクロサテライト多型に連鎖不平衡を見出した．その多型周辺のYACならびにコスミド整列化クローンを作製し，独自にマイクロサテライト多型を単離し，連鎖不平衡マッピングを行い，存在範囲を100 kb以下と大幅に狭めた[4]．この領域の整列化コスミド各クローンをプローブとしてサザン法で解析し，患者ゲノムDNAのほとんどに約3 kbの挿入配列が入っていることを見出し，次に挿入配列のまわりの断片を起点としてcDNAをスクリーニングし続け1種のcDNAを得た．そして1998年福山型原因遺伝子をつきとめることに成功し，遺伝子産物をフクチンと命名した[5]．

正常型のフクチンcDNAは約7 Kbで，骨格筋，心筋，脳で優位に発現している．ほとんどの患者ゲノムDNAでみられた約3 Kbの挿入配列は，この遺伝子の3'非翻訳領域に挿入されていた「利己的」な動く遺伝因子であるSVA型レトロトランスポゾンであった．mRNAの発現が検出できない機能喪失型変異である．(**図2**)[5]．

フクチン遺伝子を挟んで存在する，近傍の4つの多型性マーカーを用いて多型解析を行うと，レトロトランスポゾン挿入をもつ染色体のすべては同じハプロタイプを示す．これは日本人のFCMD患者は共通した一人の祖先（この人は保因者）から由来していることを意味する．遺伝的に隔離された集団である日本人のなかで，その一本のレトロトランスポゾン挿入型染色体が全国に広がっていった．この『創始者』は約2,500年前［約102世代（95%信頼区間86～117世代）前］に存在したと考えられる．日本ではこの時代はちょうど縄文から弥生時代へと移行する頃である．古代から伝わったレトロトランスポゾンがヒトの遺伝性疾患の原因で

あることがわかったのは世界で初めてである[5].

遺伝子産物フクチンは，461個のアミノ酸からなる分子量53.7 kDの蛋白であり，N末に膜貫通部位をもつ．抗フクチン抗体は発現量の少ない内在性フクチンを検出できないが，細胞に強制発現させるとフクチンはゴルジ体に認められる[5]．相同性を示す既知の蛋白やモチーフ検索から，糖鎖修飾に関与する蛋白である可能性が示唆されている．

Ⅳ 福山型の幅広い臨床スペクトラム—胎生致死から心筋症まで，超軽症FCMDの存在

日本人患者のほとんどすべては，約3 Kbのレトロトランスポゾン挿入型染色体のホモ接合体，またはレトロトランスポゾン挿入型染色体と他の変異（フレームシフト，ノンセンスなど）の複合ヘテロ接合体である．複合ヘテロ接合の患者は挿入変異ホモ接合の患者よりも水頭症，小眼球などを示すなど重症である[6]．点変異を2個もつ症例は日本にはいまだ報告がなく，海外の2報告例ではさらに重度の症状を呈し，それぞれ10日と6カ月で死亡している[7]．点変異は表現型を重症化し，点変異2個の患者が見つからないのは，胎生致死と考えられた．すなわち，レトロトランスポゾンDNAの挿入された染色体からは微量ながらフクチン遺伝子の発現が認められるため生存してFCMDとなるが，点変異2個の患者ではフクチン遺伝子はまったく機能しなくなるため生存し得ないと考えられる．西欧では点変異2個であり（したがって胎生致死），これこそが本症が西欧に存在しにくい理由であり，これはフクチン遺伝子のノックアウトマウスが胎生致死であることとよく一致していた[8]．

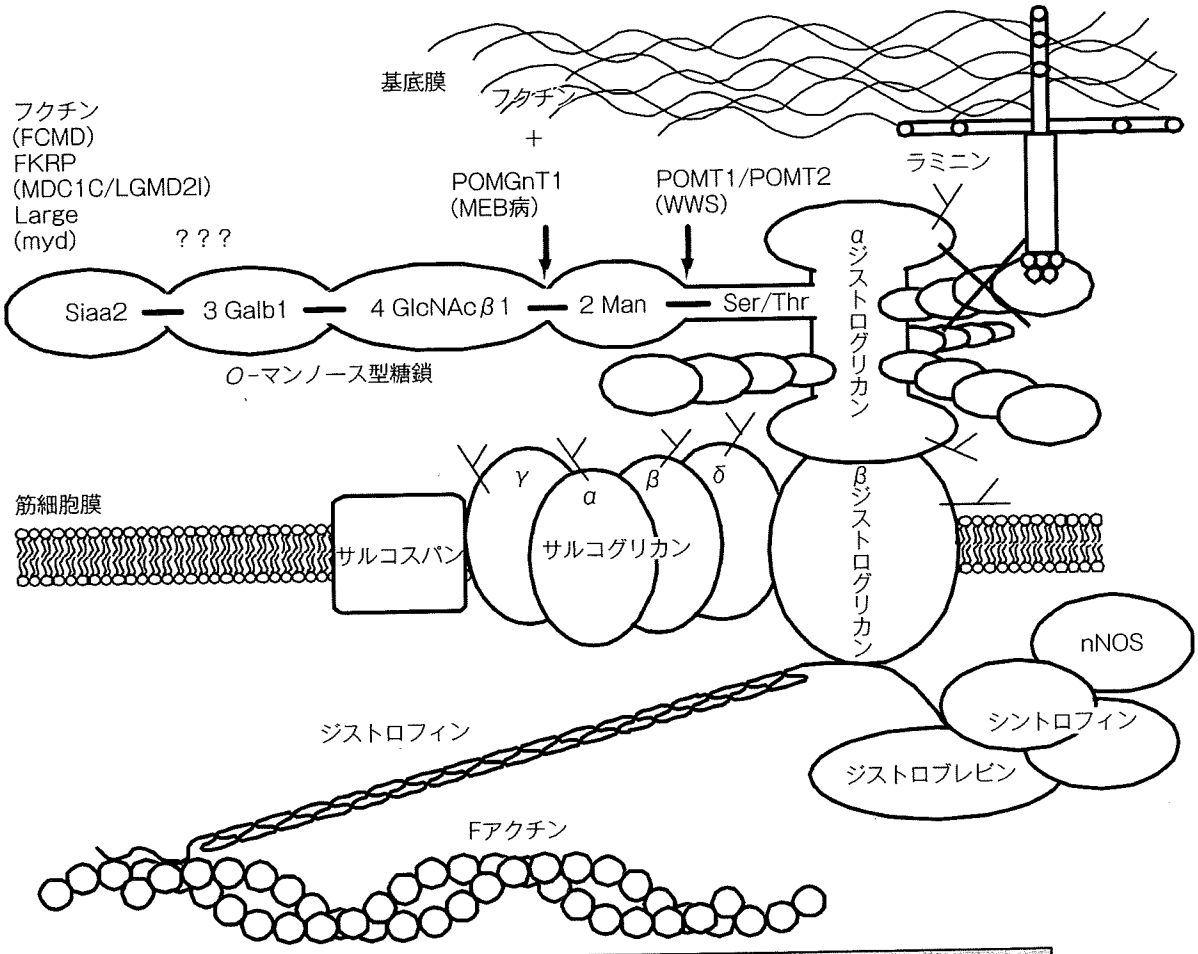
しかしながら2006年日本から心筋症と軽度筋力低下，知能正常の臨床的には肢帯型の成人6例が報告された[9]．これはレトロトランスポゾン挿入型染色体とミスセンス変異の複合ヘテロ接合であった．また同様にユダヤ人で肢帯型例3例が報告され，点変異2個のホモ例であった．これらは従来の福山型の先天性のイメージを変え，福山型のさらなる広い臨床スペクトラムを考えさせる．また同様の報告も続き，フクチン遺伝子変異はさまざまな臨床型を引き起こすことが明らかになった[10]．点変異もその位置によっては，軽症型変異が存在すると思われる．

Ⅴ 福山型筋ジストロフィーとαジストログリカンの糖鎖修飾異常

ジストロフィン関連糖蛋白質複合体の中のαジストログリカン（α-DG）は高度に糖鎖修飾を受け，全体が細胞外に存在し基底膜構成成分ラミニンと*O*-マンノース型糖鎖 Siaa2-3Galb1-4GlcNAcb1-2Man-Ser/Thr）で結合しており，一連のつながりは，骨格筋の収縮弛緩による機械的負荷に対して筋形質膜の保護をしている（**図3**）．

先天性筋ジストロフィーに，Ⅱ型滑脳症，眼奇形を伴うmuscle-eye-brain（MEB）病やWalker-Warburg症候群（WWS）は，その病変部位，症状の類似性からFCMDと類縁疾患と考えられている．われわれと東京都老人研究所の遠藤らは類縁疾患MEB病が，このα-DGとラミニンの連結部の*O*-マンノースにGlcNAcを付加する新規の糖転移酵素POMGnT1の異常により発症する疾患であることを見出した．筋ジストロフィーの原因遺伝子として糖転移酵素が同定され，実際に患者で変異が確認されたのは初めてのことである[11]．

続けて，一部のWWSに*O*-マンノース転移酵素POMT1とPOMT2の変異が発見された．さらにフクチンもアミノ酸の相同性などから，糖鎖修飾にかかわる酵素と推測されており，また先天性筋ジストロフィー1C型や肢帯型筋ジ



疾患名	原因遺伝子産物	染色体座位
福山型先天性筋ジストロフィー（FCMD）	fukutin	9q31
muscle-eye-brain 病（MEB 病）	POMGnT1[§]	1p33-34
Walker-Warburg 症候群（WWS）	POMT1[*]	9q34. 1
先天性筋ジストロフィー 1C 型（MDC1C）	FKRP（fukutin-related protein）	19q13. 3
肢帯型筋ジストロフィー 2I 型（LGMD2I）	FKRP	19q13. 3
myodystrophy マウス（myd）	Large	8（マウス）
先天性筋ジストロフィー 1D 型（MDC1D）	LARGE	22q12. 3

§：protein *O*-mannose β1, 2-*N*-acetylglucosamyltransferase 1

*：protein *O*-mannosyltransferase 1

図3　筋細胞膜のジストロフィン糖蛋白質複合体（DGC）とαジストログリカンに対する糖鎖修飾の異常によるとされている疾患群「αジストログリカノパチー」

細胞外基底膜から細胞内骨格につながる DGC 内の α-DG はラミニン α2 鎖と *O*-マンノース型糖鎖で結合している．この糖鎖修飾に異常をきたすと，ラミニンなどのリガンド結合能が低下し，αジストログリカノパチーを発症すると考えられている．確実な活性が明らかなのは，POMGnT1（MEB），POMT1（WWS）のみ．フクチンは POMGnT1 と複合体を形成し，その活性を修飾していると考えられている．

ストロフィー2I型の原因がフクチンと相同性のあるfukutin-related protein (FKRP) という糖鎖修飾酵素と考えられる蛋白質であることが報告された．ほかに，myodystrophyマウスや先天性筋ジストロフィー1D型の原因がLargeという糖転移酵素と推定されている蛋白質の異常が原因であることが報告された．これらすべての疾患においてα-DGの糖鎖認識抗体による免疫組織染色性が低下する異常がみられている[12]．筋ジストロフィーや神経細胞移動異常に，α-DGの糖鎖の異常という新たな病態メカニズムが示唆され，大変興味深い（図3）．

さらにMEB, FCMD, myodystrophyマウス，フクチンキメラマウスの大脳，骨格筋でαジストログリカンの糖鎖の異常の存在がより強く確認された．α-DGは筋細胞膜表面に残ってはいるが，糖鎖修飾異常により分子量が小さくなっており，ラミニンなどのリガンドとの結合能が低下していた[13]．つまりFCMDではフクチンの欠損によって，α-DGの糖鎖修飾に異常をきたし，ラミニンなどの細胞外基質との結合能が低下し，基底膜と細胞骨格のつながりが破綻するために筋ジストロフィー，脳表奇形が発症すると考えられる．

すなわち前述のα-DGの糖鎖付加異常，ラミニンとの結合能の低下はこれらの疾患群で共通しており，基底膜異常という発症要因も共通していると考えられ，これら疾患群を総称して「αジストログリカノパチー」という概念ができた（図3）[12]．またその後も世界中から報告が続き，現在FKRP, POMT1, POMT2, POMGnT1, フクチン, LARGE遺伝子変異は，WWS, MEB, FCMD，肢帯型などさまざまな臨床型を引き起こすことが明らかになっている[10]．

VI フクチンの機能とαジストログリカノパチーの病態

しかしαジストログリカノパチーの中で，その酵素活性がわかっているのは，POMGnT1 (MEB) とPOMT1/2 (WWS) だけである．われわれは，フクチンにはα-DGとラミニンの結合にかかわる糖鎖に関して明らかな糖転移活性が検出されないこと，フクチンとPOMGnT1は両者ともゴルジ体に局在し，フクチンの膜貫通領域を通して結合していること，フクチン欠損細胞にPOMGnT1を発現してもPOMGnT1活性が出ないこと，さらにFCMDモデルマウスではPOMGnT1活性が減少していることを示した．フクチンはPOMGnT1と複合体を形成し，その活性に影響すると思われる[14]（図3）．

またマイクロアレイによる発現解析，免疫組織学的，形態学的解析から，FCMDだけでなくαジストログリカノパチーに神経筋接合部の形態異常とα-DGの集積障害を見出した．αジストログリカノパチーの主要病態として，筋ジストロフィー以外に，神経筋接合部由来の筋分化シグナルが不完全となり，筋線維の成熟障害を起こすことが考えられる[15]．

VII FCMDおよびαジストログリカノパチーの治療へのヒント

多くの神経・筋変性疾患と同様，現在のところ福山型を含むαジストログリカノパチーに治療法はないが，治療的アプローチにむけて研究が緒についたところである．

アデノウイルスベクターを用いてmyodystrophyマウスの骨格筋に原因遺伝子である*LARGE*遺伝子を発現させると，α-DGの糖鎖異常が回復し，筋ジストロフィーが改善することが報告された．さらに興味深いことに，FCMD，MEB病およびWWS患者由来の細胞

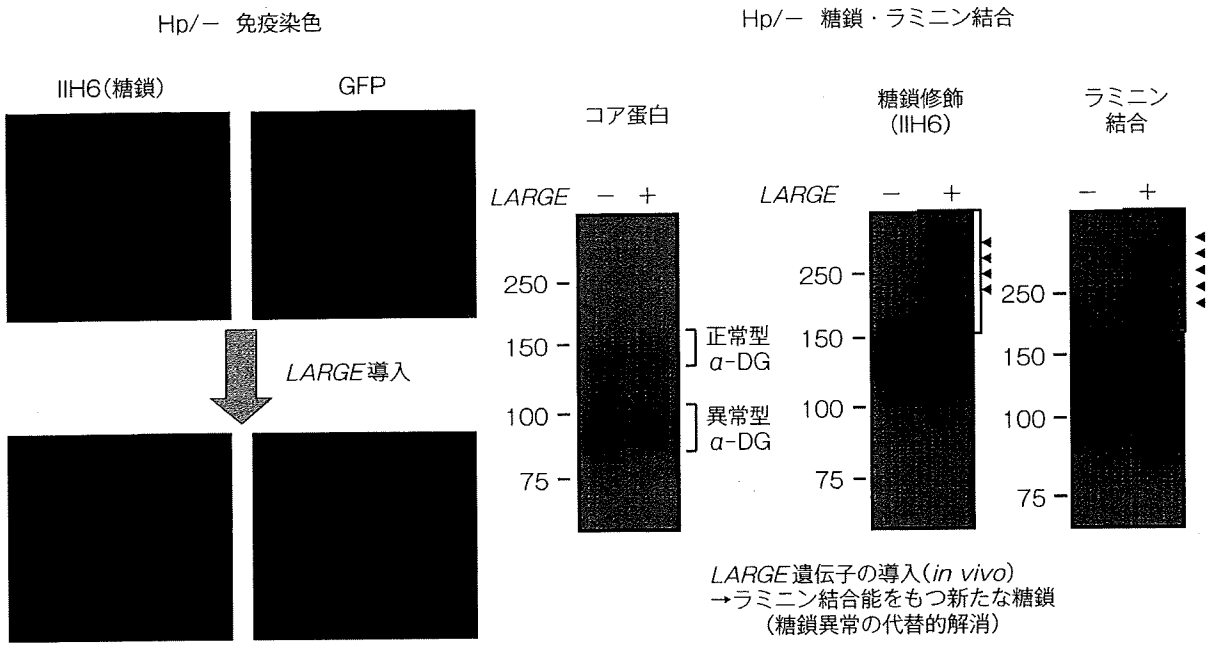


図4　FCMDモデルマウスへの*LARGE*遺伝子導入

アデノウィルスベクターを用いた*LARGE*遺伝子導入により，FCMDノックインマウスのα-DGの糖鎖異常が*in vivo*で解消できることが明らかになった．糖鎖抗体IIH6の免疫染色やラミニン結合能も回復している．同様の所見はPOMGnT1ノックアウトマウスでも得られた．

(筋芽細胞，線維芽細胞)においても，*LARGE*の過剰発現によりα-DGの糖鎖異常に改善を認め，リガンドとの結合能が回復した[16]．*LARGE*が付加する糖鎖がMEB病などで欠損する通常のラミニン結合O-マンノース型糖鎖である保証はないが，遺伝的に異なるこれらの疾患群に対して，共通の病態である「α-DGの糖鎖異常」を標的とした治療法の開発につながる可能性があり，大変興味深い．

FCMDの病態解明および治療法の構築には，モデルマウスを用いた研究が不可欠であるが，フクチン欠損マウスは胎生致死であり[8]，フクチン欠損キメラマウスは個体ごとに組織ごとにキメラ率が異なり[17]，モデルとしては有効ではない．そこで，われわれは，レトロトランスポゾン挿入変異をもつノックインモデルマウスを作製し，その解析を行った．レトロトランスポゾン挿入変異ホモ接合，より重症と思われる複合ヘテロ接合，いずれのモデルマウスにおいても筋ジストロフィー症状は認められなかった．いずれのマウスにおいてもα-DG糖鎖に異常が生じていたが，正常糖鎖型のα-DG分子種の残存も検出された．複合ヘテロ接合モデルにおけるラミニン結合能は，50%程度残存していた．一方FCMD類縁疾患のモデルで顕著な病態を示すLarge^{myd}マウスでは，糖鎖正常型α-DG分子種もラミニン結合能も，ほとんど検出されない[18]．

FCMDノックインマウス，Large^{myd}マウスの結果から，正常糖鎖型のα-DGが少し残存していれば，筋ジストロフィー発症を抑制できる可能性がある．さらに，アデノウィルスベクターを用いた*LARGE*遺伝子導入により，FCMDノックインマウス，POMGnT1ノックアウトマウスのα-DGの糖鎖異常が*in vivo*で解消できることが明らかになった(**図4**)[18]．つまり，糖鎖異常を部分的にでも解消できれば，膜脆弱化や筋再生異常を抑制し，FCMDを含む類縁疾