本研究は、疾患を有する患者さんから検体を頂きiPS細胞を作成して行う研究であるため、患者さんの同意・協力を必要とする研究である。また、作成するiPS細胞を用いた疾患解析においては、遺伝子解析が必須であり、個人情報の取り扱いの配慮を必要とする研究である。この２点に対して、我々は、疾患特異的iPS細胞研究を行うにあたり、京都大学医学部医の倫理委員会に、ヒトを対象とした医学の研究および臨床応用実施申請書として「ヒト疾患特異的iPS細胞の作成とそれを用いた疾患解析に関する研究」およびヒト遺伝子解析申請書として「ヒト疾患特異的iPS細胞を用いた遺伝子解析研究」の２申請を行った。その後、京都大学医学部医の倫理委員会での審査を頂き、平成２０年６月４日付けで、実施に関して承認を頂いた。今後の研究においては、その内容を忠実に順守して行う。

C．研究結果

本年度は、脊髄性進行性筋委縮症、筋委縮性側索硬化症、脊髄小脳変性症、多発性硬化症、パーキンソン病、後縦靭帯骨化症、進行性骨化性線維異形成症、細網異形成症、CINCA症候群、多発性嚢胞腎などを対象とし、アウトグロース法によって各疾患毎に数本から十数本の線維芽細胞アンプルを作成した。線維芽細胞株樹立まではいずれも5-6週を要したが、マイコプラズマ感染が確認されたものは本年度採取したものの中にはなかった.

樹立された筋委縮性側索硬化症、後縦靭帯骨化症、CINCA症候群、多発性嚢胞腎の線維芽細胞株については、レトロウィルスベクター法によりOct3/4、SoxⅡ、Klf4、cMycの４遺伝子、またはOct3/4、SoxⅡ、Klf4の３遺伝子を導入し、疾患特異的iPS細胞株をそれぞれ数クローンずつ樹立し、完全気相型液体窒素タンクに保管するとともに、各疾患の分担研究者に分配した。

D．考察

本年度樹立した患者由来の線維芽細胞株においてはいずれもマイコプラズマ感染は確認されず、他の微生物の発生も見られなかった。またいずれの場合も６週以内で10本前後のストックが得られたことから、皮膚の採取方法および線維芽細胞株の樹立方法は、現時点の方法で特に問題ないと考える。

疾患特異的iPS細胞の樹立については各線維芽細胞株毎にiPS細胞株樹立までの期間が異なるが、材料となる皮膚片に含まれる他の組織や、年齢、性別も様々で、線維芽細胞の性状自体が樹立に影響している可能性も考えられる。今後線維芽細胞株自体の詳細な解析が必要と考える。

E．結論

脊髄性進行性筋委縮症、筋委縮性側索硬化症、脊髄小脳変性症、後縦靭帯骨化症、多発性嚢胞腎等の患者から線維芽細胞株が樹立され、後縦靭帯骨化症、CINCA症候群、多発性嚢胞腎等の疾患特異的iPS細胞が樹立された。

F．研究発表

１．論文発表

該当なし

２．学会発表

該当なし

G．知的財産権の出願・登録状況（予定も含む）

１．特許取得

該当なし

２．実用新案登録

該当なし

３．その他

該当なし

Ⅳ. 班会議

平成21年度厚生労働科学研究費補助金
「疾患特異的iPS細胞を用いた難治性疾患の
画期的診断・治療法の開発に関する研究」班会議 議事録

第1回班会議

1．日時　　平成 21 年 12 月 1 日(火)　15:00～17:00
2．場所　　京都大学　物質細胞統合システム拠点西館　2階 会議室
3．演題名

個別発表
座長　中畑龍俊

演題名		演者
1	研究総括、疾患特異的 iPS 細胞作成、疾患特異的 iPS 細胞バンク事業	中畑龍俊
2	原発性免疫不全症候群･Fanconi 貧血･骨髄異形成症候群･iPS 細胞作成及び解析	斎藤潤
3	網膜変性疾患 iPS 細胞作成、網膜細胞検証	高橋政代
4	パーキンソン病疾患 iPS 細胞の解析	高橋淳
5	難治性骨軟骨疾患特異的 iPS 細胞作製と病態解明	戸口田淳也
6	パーキンソン病関連特異的 iPS 細胞作製と解析、バンク作製	高橋良輔
7	ALS・ｱﾙﾂﾊｲﾏｰ病・脊髄性筋萎縮症関連疾患特異的 iPS 細胞作製と解析、バンク作製	井上治久
8	疾患特異的 iPS 細胞作成（多発性嚢胞腎、急速進行性糸球体腎炎、ウェゲナー肉芽腫症、アレルギー性肉芽腫性血管炎）	長船健二
9	原発性免疫不全症候群･拡張型心筋症･疾患特異的 iPS 細胞作成及び解析	平家俊男
10	疾患特異的 iPS 細胞の創薬探索系への活用	中西淳
11	疾患特異的 iPS 細胞の標準化	高橋和利
12	患者線維芽細胞の保存･疾患 iPS 細胞ﾊﾞﾝｸ事業	浅香勲

Ⅴ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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雑誌

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中畑龍俊	さまざまな幹細胞を用いた今後の再生医療	血液フロンティア	19	17-18	2009
高橋良輔	パーキンソン病の神経 細胞移植治療	日本医事新報	4445	79-80	2009
長船健二	iPS細胞作製の最先端と作製されたiPS細胞株間の特性差異についての最新の知見	実験医学増刊 (再生医療の最前線2010)	28	55-61	2010
中西　淳	iPS細胞を用いた創薬研究－薬効・副作用評価系への活用	Medical Science Digest	35	13-16	2009

VI. 研究成果の刊行物・印刷物

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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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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 +

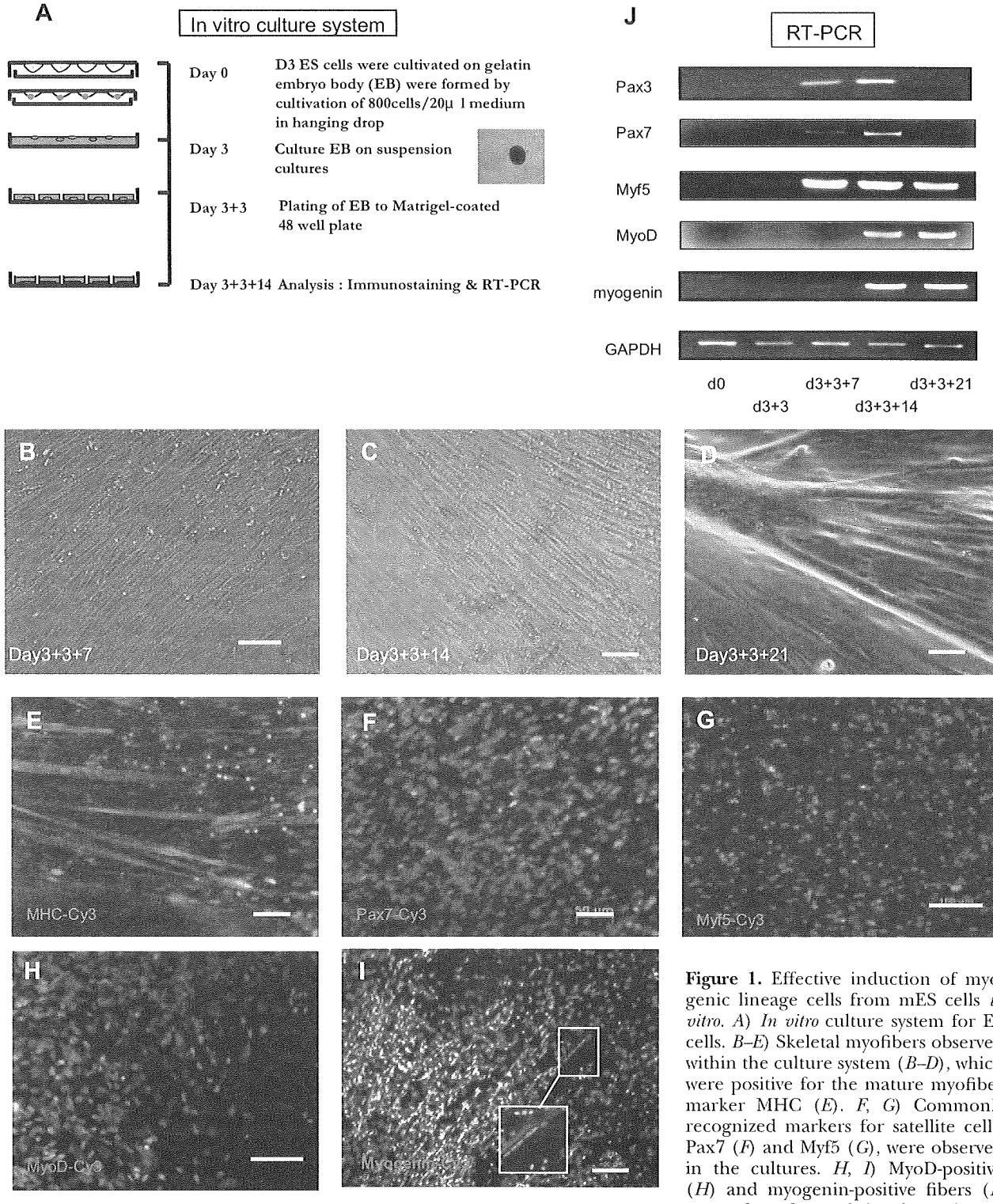

Figure 1. Effective induction of myogenic lineage cells from mES cells *in vitro*. *A*) *In vitro* culture system for ES cells. *B–E*) Skeletal myofibers observed within the culture system (*B–D*), which were positive for the mature myofiber marker MHC (*E*). *F, G*) Commonly recognized markers for satellite 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

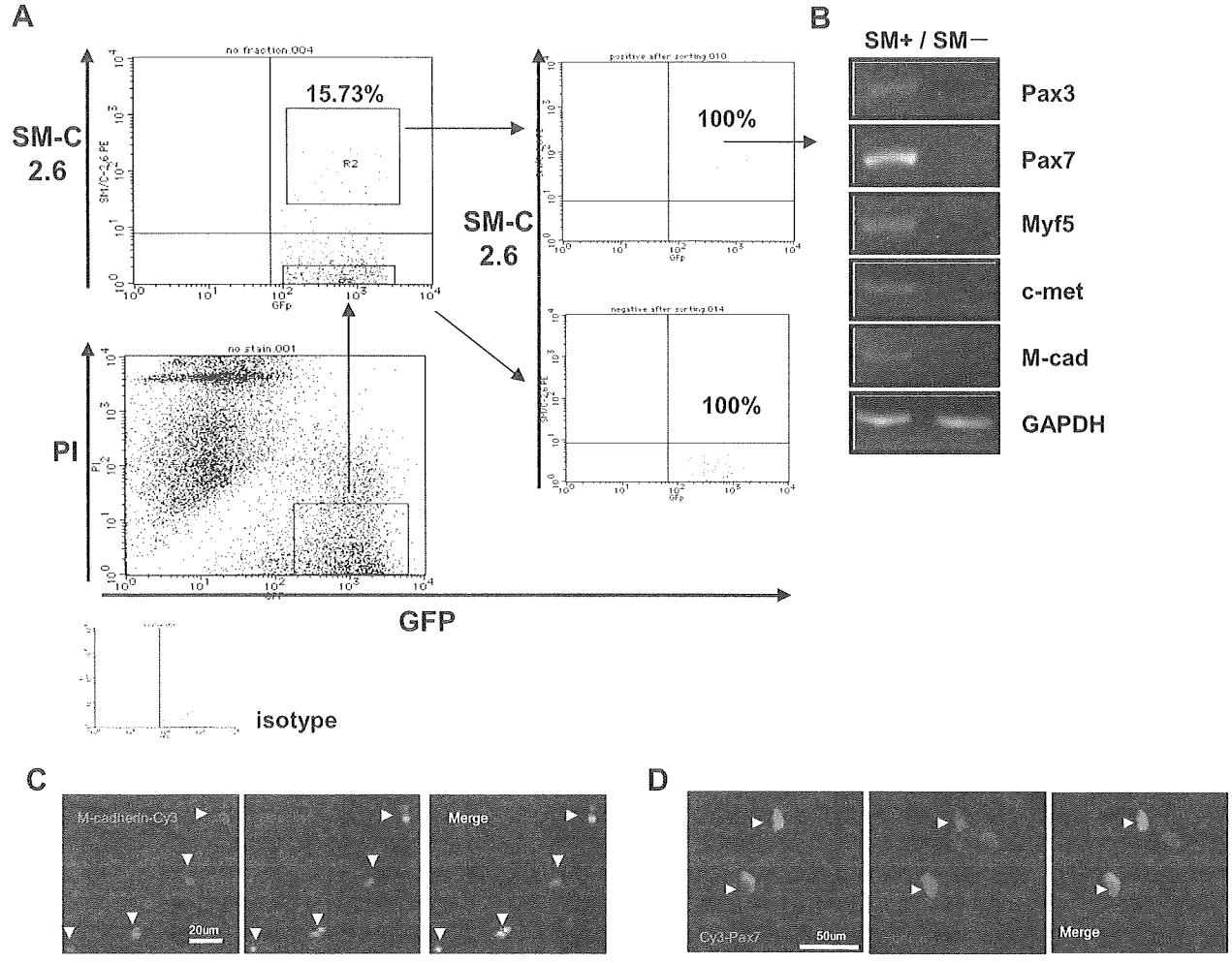

Figure 2. A novel antibody, SM/C-2.6, can enrich Pax7-positive satellite-like cells derived from ES cells. *A*) FACS data of cultured ES cells at d 3 + 3 + 14 indicate that 15.7% of total cultured cells are SM/C-2.6-positive cells. *B*) RT-PCR of the SM/C-2.6-positive fraction showed strong expression 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. 4*A***). 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

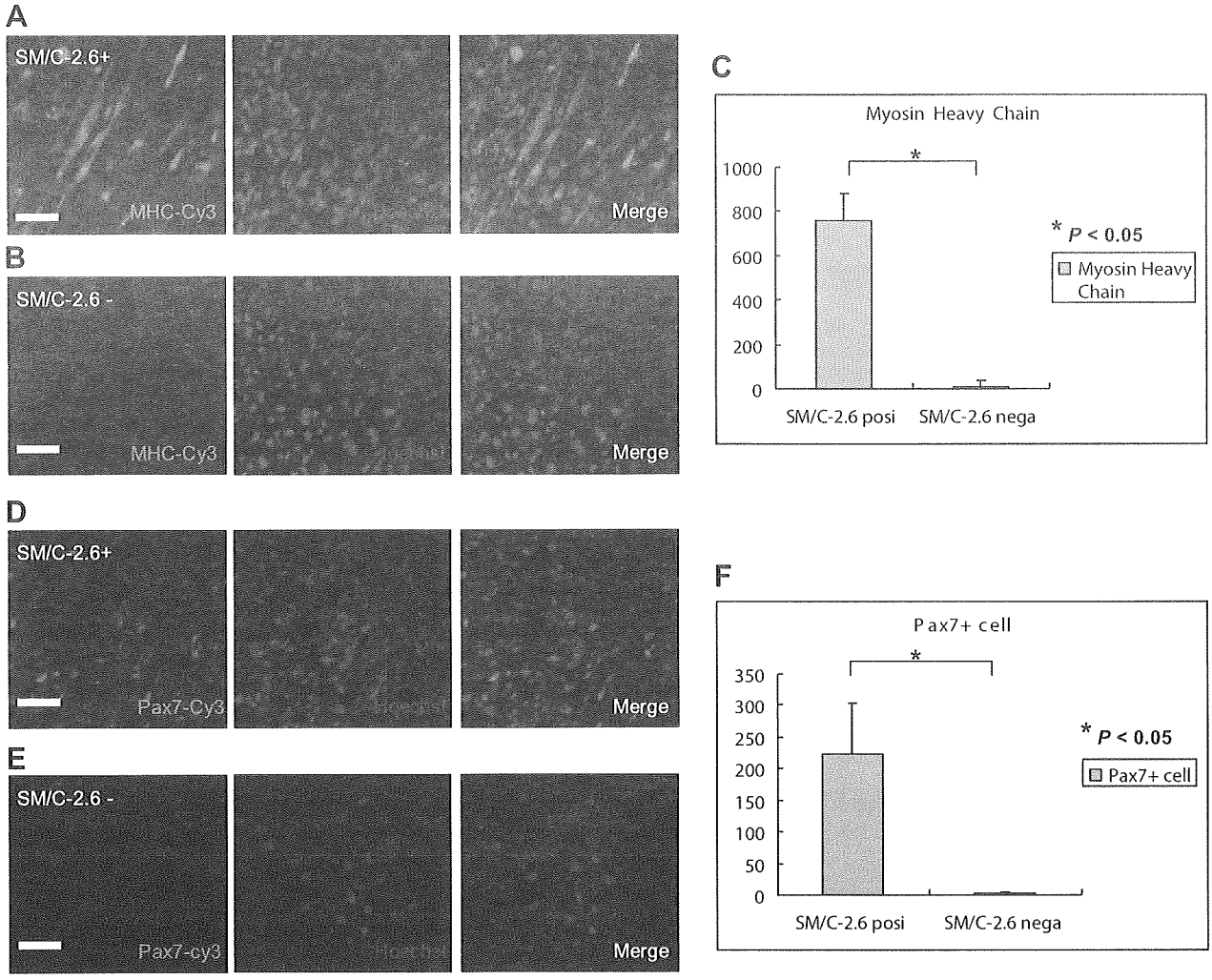

Figure 3. ES-derived satellite-like cells have strong myogenic potential *in vitro*. Immunostaining detected an abundant number of MHC-positive fibers and Pax7-positive cells in SM/C-2.6-positive cell culture (*A*, *D*) but not SM/C-2.6-negative cells (*B*, *E*) after 1 wk in culture. Scale bars = 50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

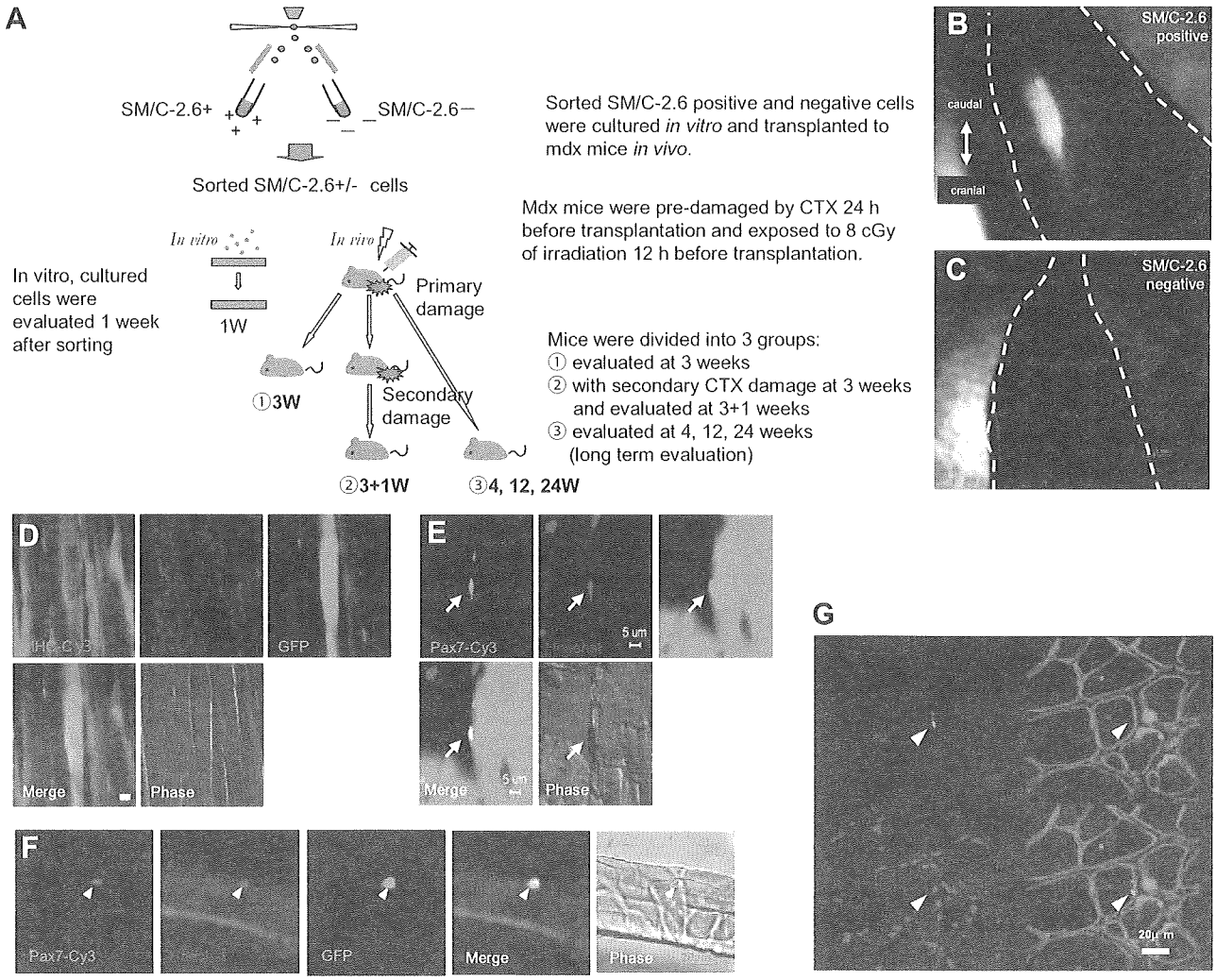

Figure 4. ES-derived satellite-like cells can repair damaged muscle *in vivo*. *A*) Methods for *in vitro* and *in vivo* analysis of sorted SM/C-2.6-positive and -negative cells derived from mES cells. *B, C*) ES-derived GFP-positive tissue engrafted to the LTA muscle of a recipient mouse that received 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-