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Supplemental Information

Induced Fetal Human Muscle Stem Cells with High Therapeutic Potential in a Mouse Muscular Dystrophy Model

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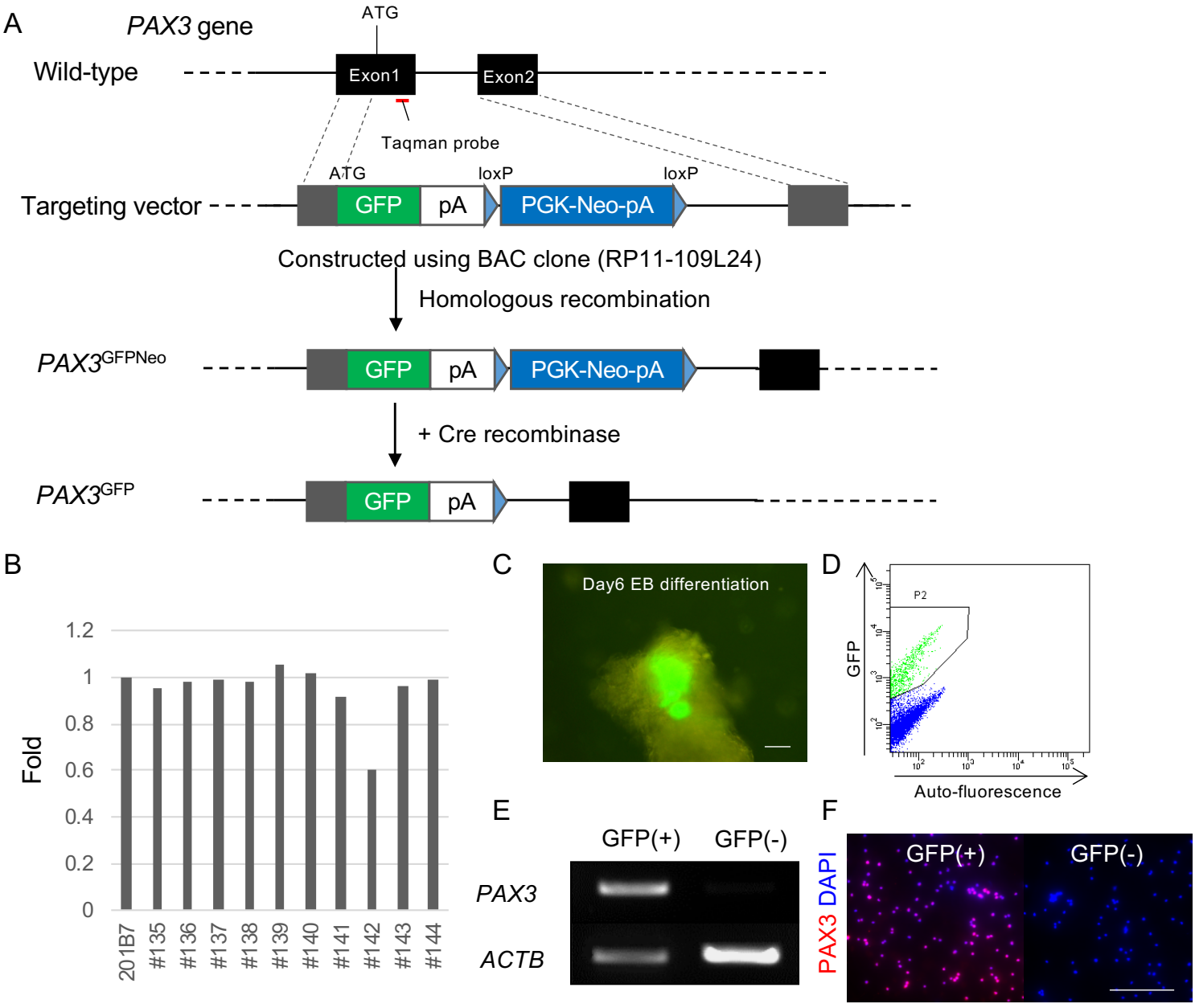
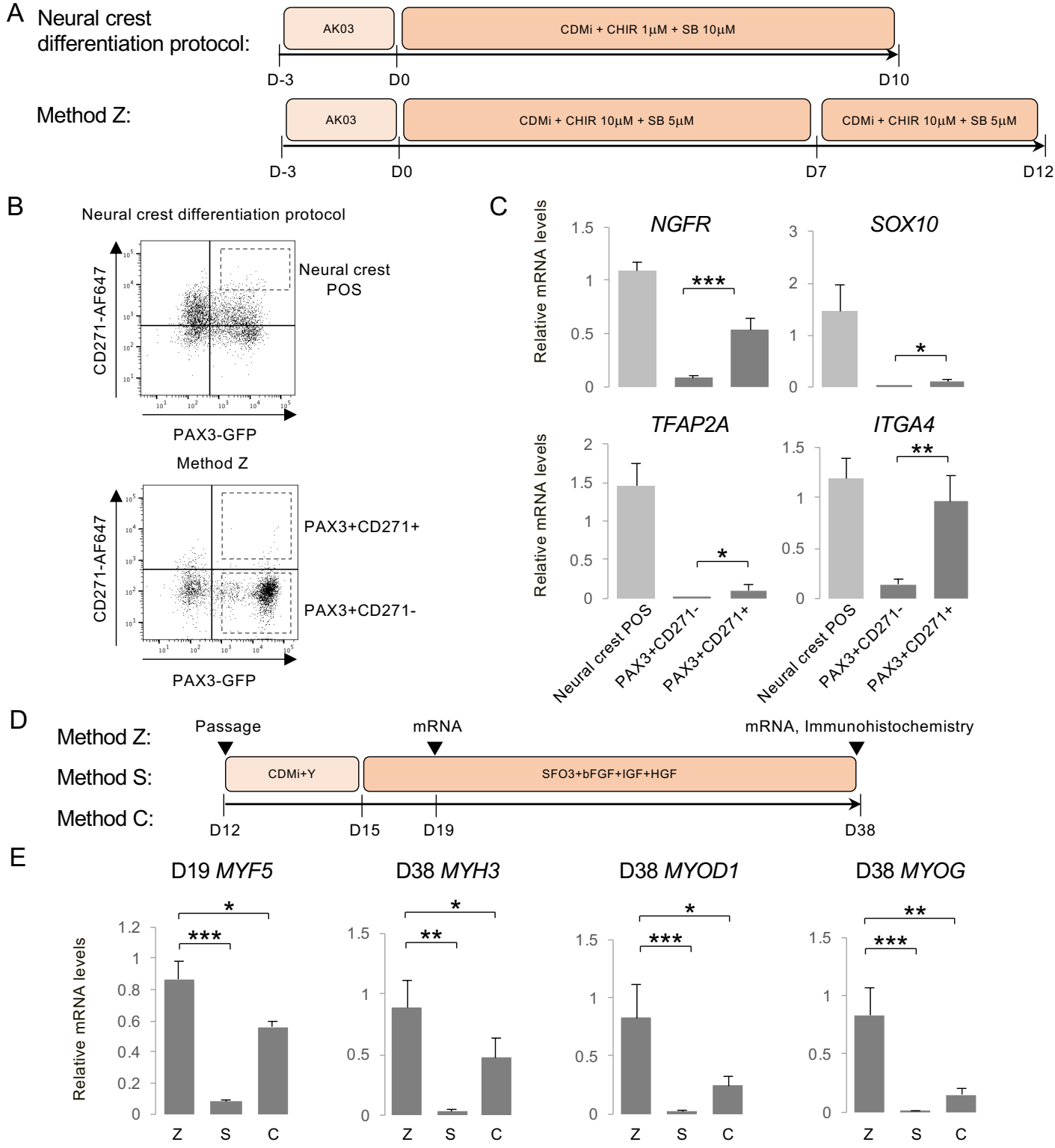
Figure. S1



Supplemental Fig S1. Generation of PAX3-GFP Knock-in (KI) hiPSC line (201B7). Related to Figure1.

(A) Schematic picture demonstrates homologous recombination (HR) using BAC-based vectors to generate the PAX3 GFP knock-in hiPSC line. Knock-in cells were selected by G418, and the monoclonal cell population was isolated. The loxP-Neo-loxP cassette was removed by Cre recombinase. (B) Genotyping using RT-PCR with Taqman probe hybridizing at exon1 in the *PAX3* gene. The results show #142 is heterozygous. (C) Fluorescence micrographs of hiPSC-derived EBs that expressed PAX3-GFP. Scale bar, 200 μm. (D) Representative flow cytometry plots showing GFP+ and GFP- populations. (E) Gene expression of *PAX3* in the GFP+ and GFP- populations was analyzed by RT-PCR. (F) Immunocytochemistry shows PAX3 expression in GFP+ and GFP- cells after single cell plating.

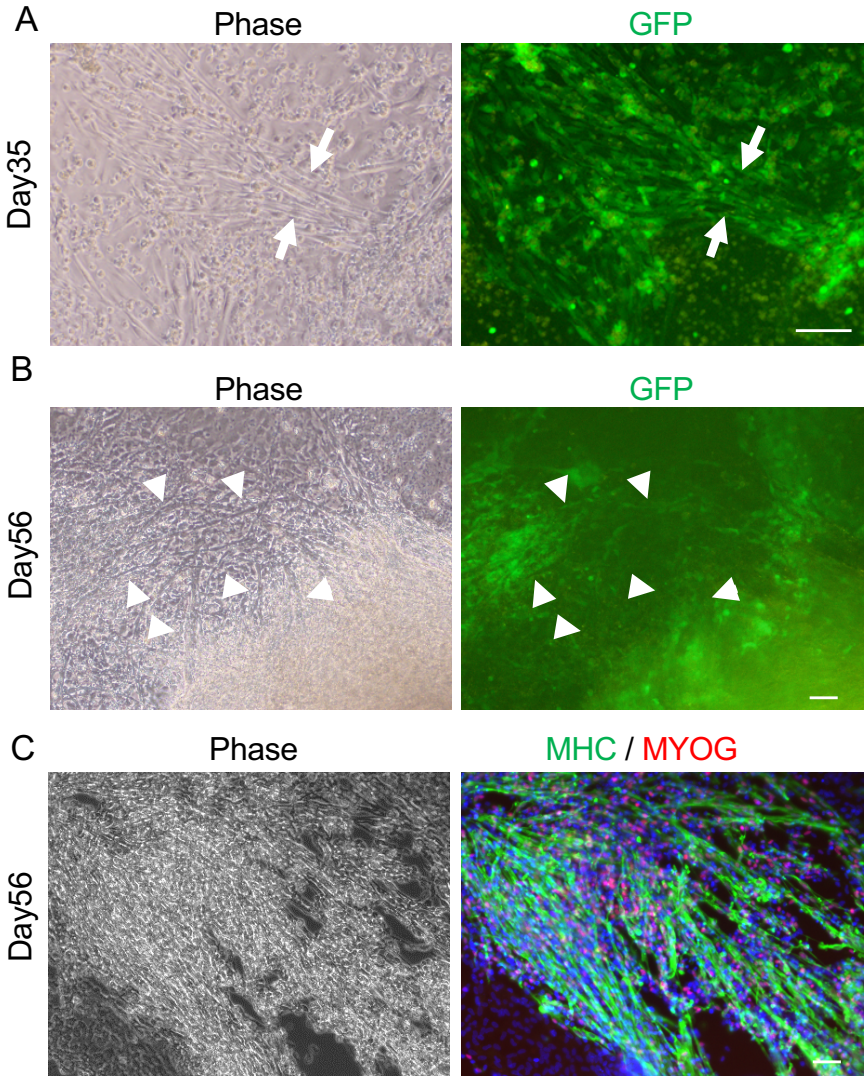
Figure. S2



Supplemental Fig S2. Detecting neural crest marker expression in PAX3+/CD271- and PAX3+/CD271+ populations. Related to Figure 2.

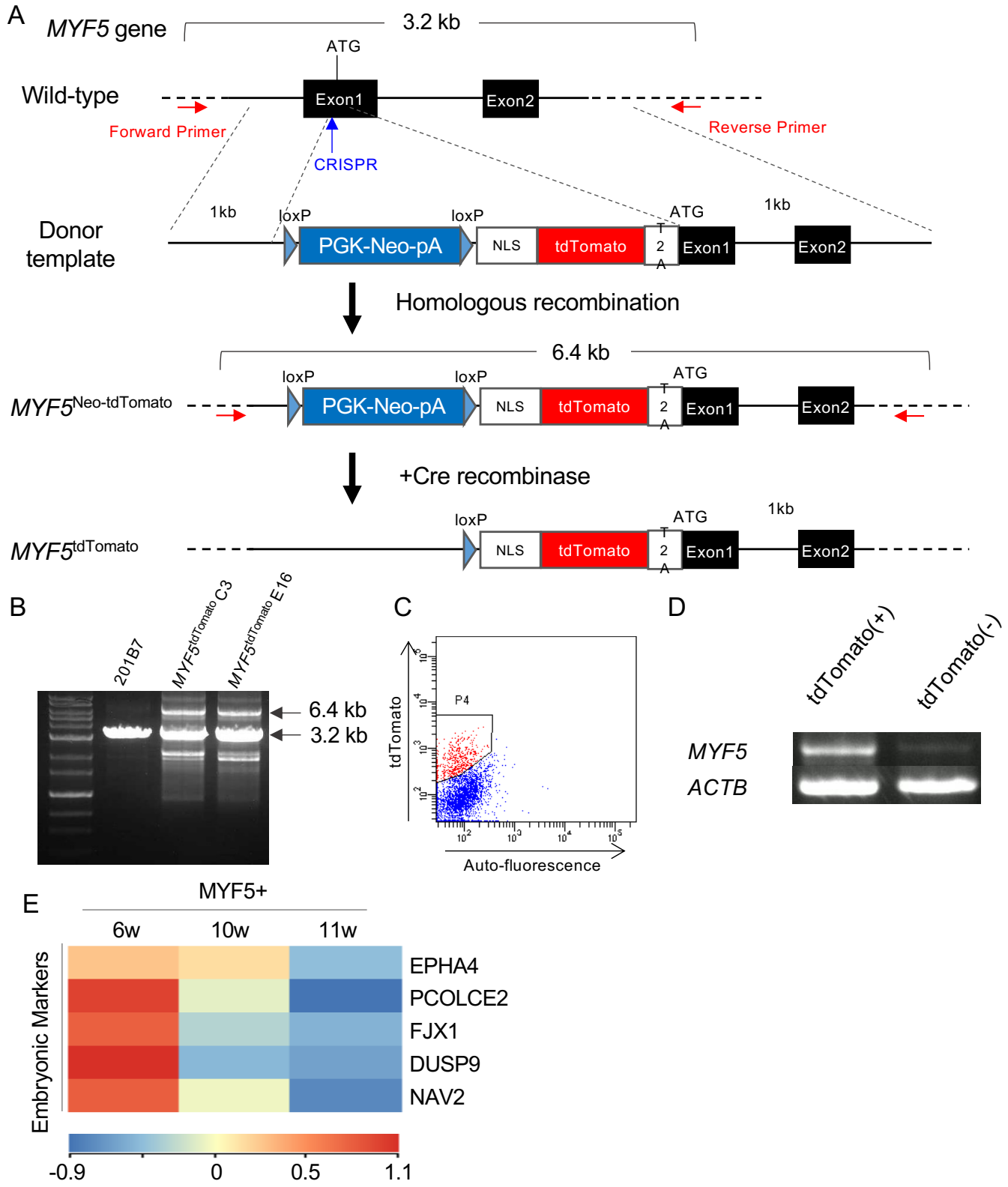
(A) Timeline of hiPSCs to neural crest lineage using a neural crest differentiation protocol (Fukuta et al., 2014) and timeline of hiPSCs to dermomyotome differentiation using method Z (Zhao et al. this work). (B) FACS sorting of PAX3+/CD271+ at day 10 (D10) differentiation in the neural crest differentiation protocol. FACS sorting of PAX3+/CD271- and PAX3+/CD271+ population at day 12 (D12) of differentiation in method Z. (C) The neural crest marker gene expression in PAX3+/CD271- and PAX3+/CD271+ cells in method Z. The PAX3+/CD271+ population in the neural crest differentiation protocol was a positive control (Neural crest POS). Data of three independent experiment are shown as means ± S.D., *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (D) After 12 days of differentiation in method Z, method S, or method C, the cells were passaged on Matrigel-coated plates at the same cell density and stimulated with myogenic differentiation medium. (E) The myogenesis marker gene expression in method Z, method S and method C. Data of three independent experiment are shown as means ± S.D., *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure. S3



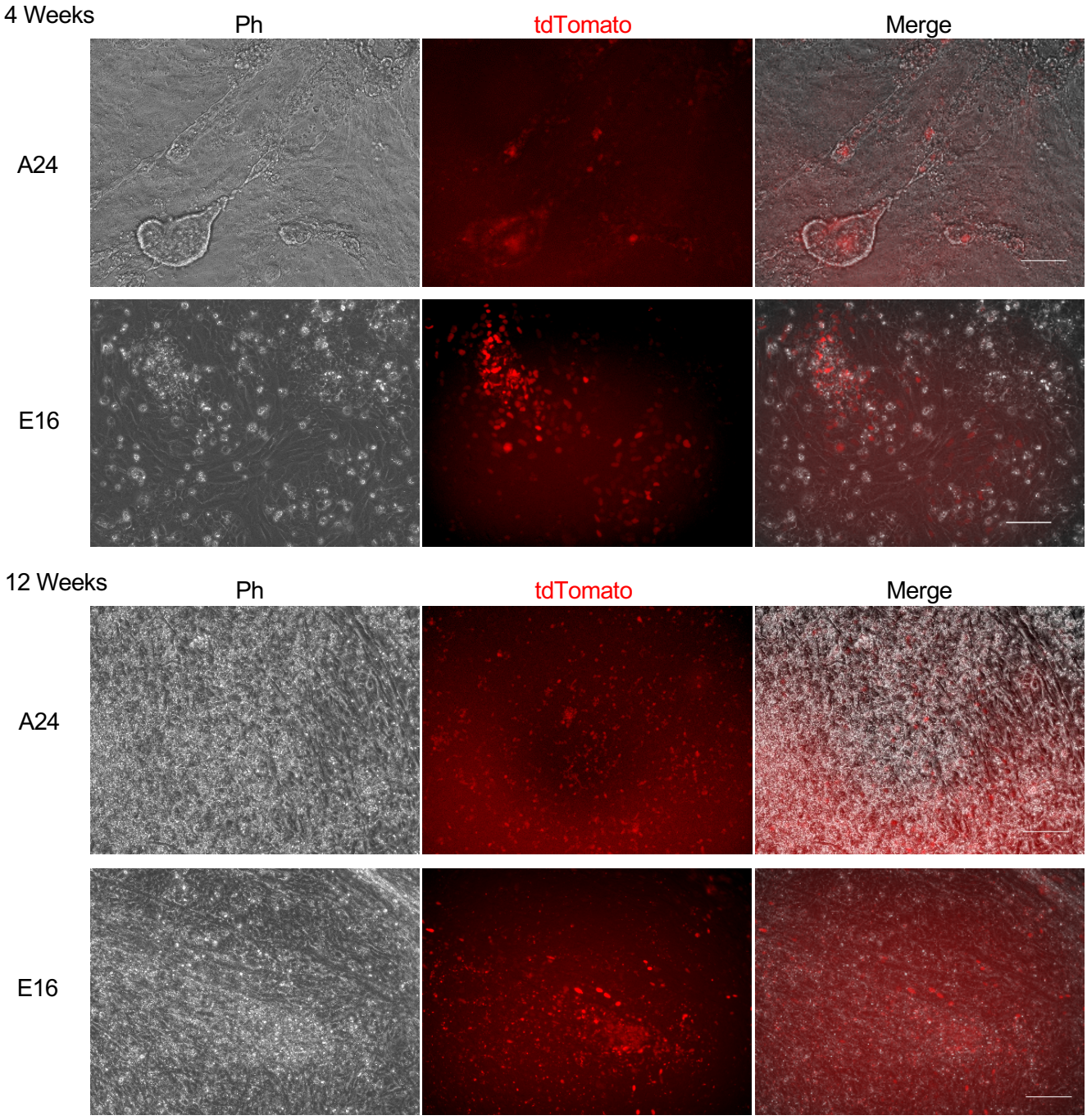
Supplemental Fig S3. Myogenic differentiation of the PAX3-GFP KI cell line. Related to Figure 1.
(A) Phase and fluorescence micrographs of hiPSCs differentiated for 35 days. Arrows, differentiated myofibers with GFP+ cells. Scale bar, 100 μm. (B) Phase and fluorescence micrographs of hiPSCs differentiated for 56 days. Arrowheads, differentiated myofibers without GFP+ cells. Scale bar, 100 μm. (C) Phase and fluorescence micrographs of hiPSCs differentiated for 56 days. Immunohistochemistry with myosin heavy chain (MHC) (Green) and MYOGENIN (MYOG) (Red) antibodies. Scale bar, 100 μm.

Figure. S4



Supplemental Fig S4. Generation of MYF5-tdTomato KI hiPSC line (201B7). Related to Figure 3 & 4.
(A) Schematic overview depicting the MYF5-tdTomato cell line establishment strategy. Top line shows the *MYF5* gene structure. The blue arrow indicates the CRISPR cut site, and the red arrows indicate the forward and reverse primers for genotyping. The homologous arms of the donor vector are indicated as the left arm (1 kb) and right arm (1 kb). Knock-in cells were selected by G418, and the monoclonal cell population was isolated. The loxP-Neo-loxP cassette was removed by Cre recombinase. (B) PCR genotyping using the primers in (A) indicates C3 and E16 are heterozygous. PCR revealed the wild type band (3.2 kb) and Neo-tdTomato KI band (6.4 kb). (C) Representative flow cytometric plots showing the MYF5-tdTomato^{+} and MYF5-tdTomato^{-} populations. (D) RT-PCR analysis of the *MYF5* gene expression in the tdTomato+ and tdTomato- populations. (E) Heat map of embryonic marker gene expression in MYF5+ cells differentiated for 6, 10 or 11 weeks (green is low expression and red is high).

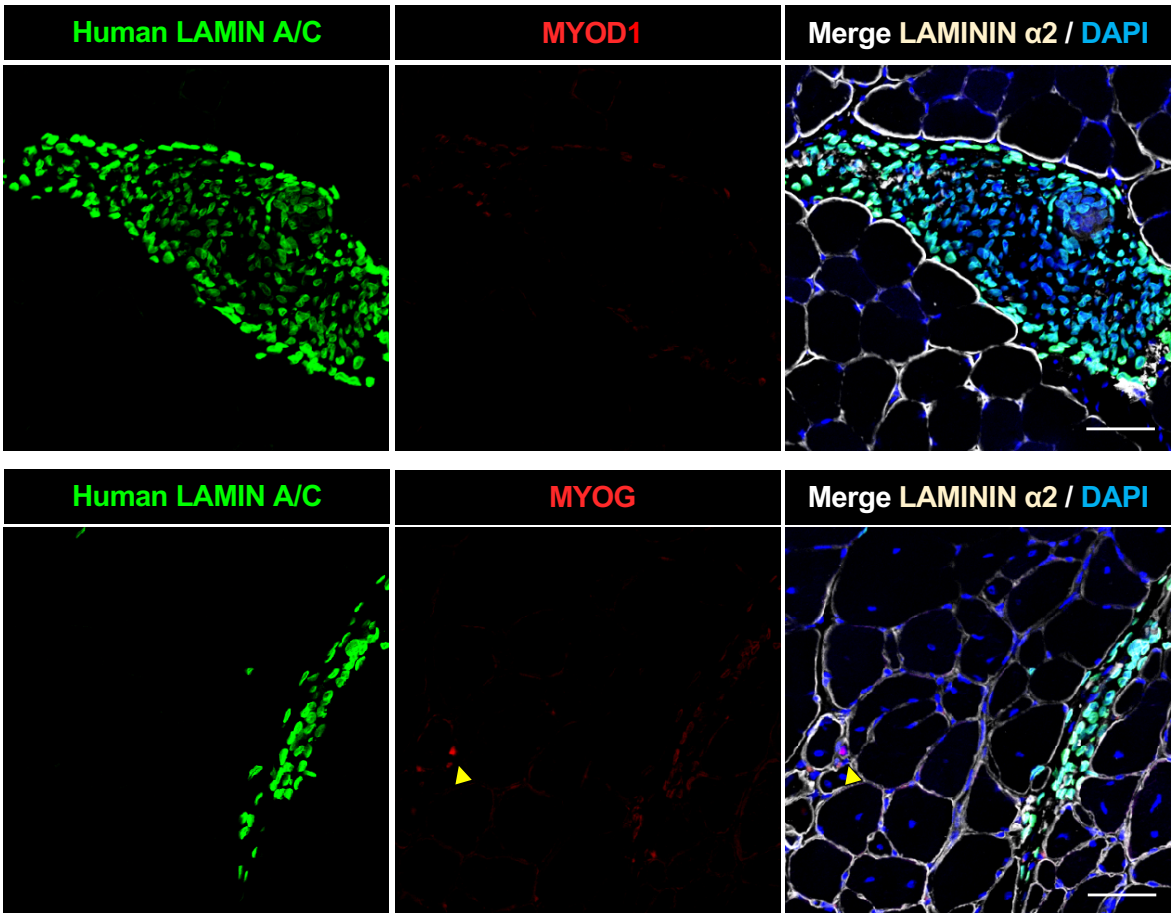
Figure. S5



Supplemental Fig S5. Phase and fluorescence images of MYF5-tdTomato reporter cells. Related to Figure 3. Representative bright field (Phase, Ph), tdTomato and merged images of live cultures of MYF5-tdTomato cells at 4 weeks and 12 weeks in A24 and E16 cell lines. Scale bars = 50 µm.

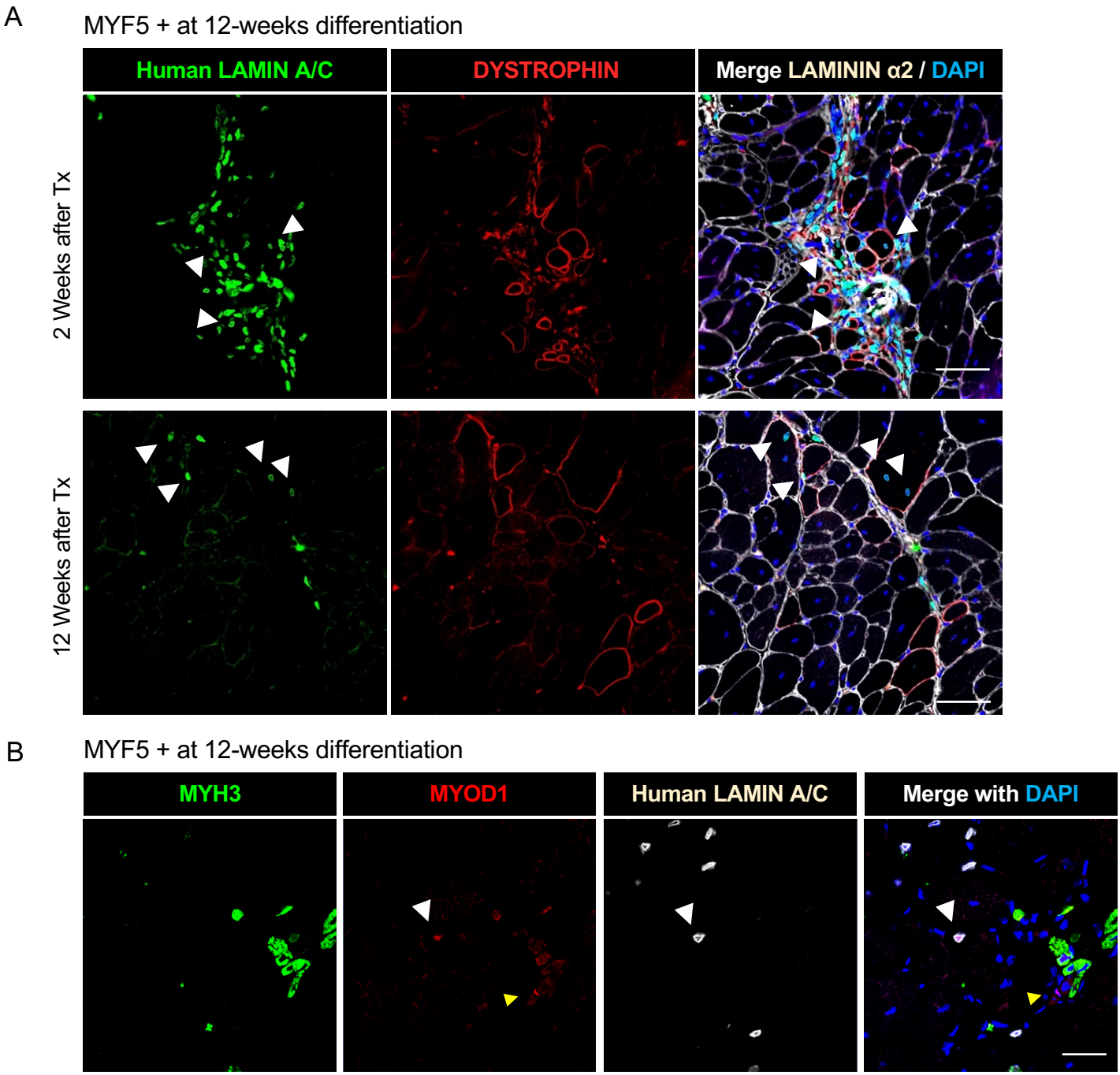
Figure. S6

MYF5- at 12-weeks of differentiation



Supplemental Fig S6. Characterization of the engrafted cells derived from MYF5- cells. Related to Figure 5
12-weeks differentiated MYF5- cells were sorted and transplanted into the tibialis anterior muscles of Ctx treated NSG mice. The engrafted cells were evaluated by immunohistochemistry. Immunohistochemistry of human LAMIN A/C (green), MYOD1 (upper, red), MYOGENIN (MYOG) (lower, red), LAMININ α2 (white) and DAPI (blue) at 4 weeks after transplantation. Scale bars, 50 μm. Yellow arrowheads indicate murine myogenic cells. MYF5- cell-derived engrafted cells mainly consisted of non-myogenic cells.

Figure. S7



Supplemental Fig S7. Characteristics of the engrafted MYF5+ cells in DMD-null/NSG model mice. Related to Figure 6

(A) 12-weeks differentiated MYF5+ cells were sorted and transplanted into the tibialis anterior muscles of DMD-null/NSG model mice. The engrafted cells were evaluated by immunohistochemistry. Immunohistochemistry of human LAMIN A/C (green), DYSTROPHIN (red), LAMININ α2 (white) and DAPI (blue) at 2 (upper) or 12 (lower) weeks after transplantation. White arrows indicate DYSTROPHIN+ myofibers. White arrowheads indicate engrafted human cells. Scale bars, 50 μm. The size of the DYSTROPHIN+ myofibers at 2 weeks after treatment was smaller than normal mouse myofibers, but the same size at 12 weeks. (B) 12-weeks differentiated MYF+ cells were sorted and transplanted into the tibialis anterior muscles of DMD-null/NSG model mice. The engrafted cells were evaluated by immunohistochemistry. Immunohistochemistry of embryonic myosin heavy chain (MYH3, green), MYOD1 (red), human LAMIN A/C (white) and DAPI (blue) at 4 weeks after transplantation. White arrows indicate DYSTROPHIN+ myofibers. White arrowheads indicate human cell-derived MYOD1 positive cell. Yellow arrowheads indicate murine MYOD1 positive cell. Scale bars, 50 μm.

References

Fukuta, M., Nakai, Y., Kirino, K., Nakagawa, M., Sekiguchi, K., Nagata, S., Matsumoto, Y., Yamamoto, T., Umeda, K., Heike, T.*, et al.* (2014). Derivation of mesenchymal stromal cells from pluripotent stem cells through a neural crest lineage using small molecule compounds with defined media. PLoS One *9*, e112291.

Supplemental Table 1. qRT-PCR Primer Sets

Gene	Forward Sequence	Reverse Sequence
T	ACCCAGTTCATAGCGGTGAC	CATTGGGAGTACCCAGGTTG
TBX6	AGCCTGTGTCTTTCCATCGT	AGGCTGTCACGGAGATGAAT
PAX3	AGGAAGGAGGCAGAGGAAAG	CAGCTGTTCTGCTGTGAAGG
MEOX1	GAGATTGCGGTAAACCTGGA	GAACTTGGAGAGGCTGTGGA
SIX1	AGTTCTCGCCTCACAACCAC	ACACCCCTCGACTTCTCCTT
MYF5	TCACCTCCTCAGAGCAACCT	GGAACTAGAAGCCCCTGGAG
PAX7	AGGGCCTCCTGCTTGTTTAT	GGTTTTGCCCAACTCAGTGT
MYOD1	ACGTGAGGACGAGCATGTG	GTGCAGCGCTTGAGTGTCT
MYOGENIN(MYOG)	TGGGCGTGTAAGGTGTGTAA	CGATGTACTGGATGGCACTG
NFIX	CTGGAGTCAGACCAGGGAGC	GGGCTCGACTGCTGGATGAT
COL15A1	CGTGTTAGAGATGGCTGGA	GTTTGGTGGAGGCAGAAG
CD44	CCAGAAGGAACAGTGGTTTGGC	ACTGTCCTCTGGGCTTGGTGTT
CRYAB	CTTTGACCAGTTCTTCGGAG	CCTCAATCACATCTCCCAAC
DLK1	AAGGACTGCCAGAAAAAGGAC	GCAGAAATTGCCTGAGAAGC
RPLP0	AAACGAGTCCTGGCCTTGTCT	GCAGATGGATCAGCCAAGAAG
NOGGIN	CCTCATCGAACACCCAGACC	CATGAAGCCTGGGTCGTAGTG
PARAXIS	TCCTGGAGAGCTGTGAGGAT	CACACCCTGTCACCAACAGT
DMRT2	GAACCACCAAGCAAGGACTTC	CCCAGACCCTGAATACTGCAT
ALX4	TCCACTGCATATGAGCTGC	GTTGTTGCCGAGCCAGGA
EN1	GACTCGGACAGGTGCTATCG	AGTTCGCAGTTTCGTCCCTT
MYH3	GCAGATTGAGCTGGAAAAGG	TCAGCTGCTCGATCTCTTCA
SOX10	CGGACCAGTACCCGCACCT	GGCGCTTGTCACTTTCGTTCA
TFAP2A	AAGAGTTCACCGACCTGCTG	AGGGCCTCGGTGAGATAGTT
NGFR	CAGGCTTTGCAGCACTCAC	CTGCTGCTGTTGCTGCTTCT
ITGA4	GCTTCTCAGATCTGCTCGTG	GTCACTTCCAACGAGGTTTG

Supplemental Table 2. Primers Lists and Template Genes for Construction of Plasmids

Part of Fragment		Sequence
For Reporter Plasmids		
MYF5-5' Arm	Fw	CGGTATCGATAAGCTCTCCCCAACCCAAATAACC
	Rv	GCTATACGAAGTTATATAAAGAACCCCAACCCCAG
	Template	Human Genome
LoxP-Neo-pA-LoxP	Fw	TGGAGCCATGGTGGCATAACTTCGTATAATGTATGCTATACGAAGTTATCAATTGTCTAGACAATTGGC
	Rv	GCTATACGAAGTTATATAAAGAACCCCAACCCCAG
	Template	pBS-FloxedNeo
NLS-tdTomato T2A	Fw	GCCACCATGGCTCCAAAA
	Rv	TGGGCCGGGATTCTCCTCCACGTCGCCGCATGTCAGCAGGCTTCCTCTGCCCTCCTTGTACAGCTCGTCCATGC
	Template	pCMV-NLS-tdTomato
MYF5-ATG-Exon1	Fw	GAGAATCCCGGCCCAATGGACGTGATGGATGGC
	Rv	AGCTGGAGCTCCACCCTGACCAAAGGTGAGGCTGT
	Template	Human Genome
For sgRNA Plasmids		
MYF5 sgRNA	Fw	GAGACCACTTGGATCCGATCCCTCTCGCTGCCGTCCGTTTTAGAGCTAGAAATAGCA
	Rv	GCCCGGGTTTGAATTCAAAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTC
	Template	-

Supplemental Table 3. Primers Lists for Validation of Reporter Line.

Use		Sequence
For Integration Check	Fw1	TGAACTGGGTTACCCTAGATTGAGTTGAG
	Rv1	AGGCCAGAGGCCACTTGTGTAGC
	Fw2	ATGCGGCGACGTGGAGGAGAATC
	Rv2	ACCCAGAGGTCAGGTGGTCACAG
For Copy Number Check	Fw	CCTGAATGAACTGCAGGACG
	Rv	CTTCCCGCTTCAGTGACAAC
For Sequencing	Fw1	TGAACTGGGTTACCCTAGATTGAG
	Fw2	AGGACAACAACATGGCCGTCATC
	Fw3	ATGCGGCGACGTGGAGGAGAATC
	Fw4	CCTGAATGAACTGCAGGACG
	Rv1	ACCCAGAGGTCAGGTGGTCACAG
	Rv2	AGGCCAGAGGCCACTTGTGTAGC
	Rv3	CCTAAACTGGGTACATGAGAATGG
	Rv4	CTTCCCGCTTCAGTGACAAC

Supplemental Table 4. Antibody list and dilution ratio

1st Antibody	Source	Clonarity	Dilution	Company
CD271-AF647	mouse mono	IgG1	1:20	BD Pharmingen
Myoshin Heavy chain (MHC) MF20	mouse mono	IgG2b	1:800	eBioscience
MYOGENIN (MYOG) F5D	mouse mono	IgG1	1:500	Santacruz
PAX7	mouse mono	IgG1	1:100	DSHB
MYOD1	rabbit mono	IgG	1:500	Abcam
Human SPECTRIN	mouse mono	IgG2b	1:200	Leica
Human Nuclei	mouse mono	IgG1	1:200	Millipore
LAMININ α2	rat mono	IgG	1:150	ALEXIS
DYSTROPHIN	rabbit poly	IgG	1:1000	Abcam
Human LAMIN A/C	mouse mono	IgG2b	1:200	Leica
eMyHC (MYH3)	rabbit poly	IgG	1:400	Sigma
PAX3	rabbit poly	IgG	1:100	Gene Tex
2nd Antibody			Dilution	Company
Alexa Fluor 568 conjugated goat-anti-mouse IgG2b			1:500	Invitrogen
Alexa Fluor 488 conjugated goat-anti-mouse IgG1			1:500	Invitrogen
Alexa Fluor 568 conjugated goat-anti-mouse IgG1			1:500	Invitrogen
Alexa Fluor 488 conjugated goat-anti-rabbit IgG			1:500	Invitrogen
Alexa Fluor 488 conjugated goat-anti-mouse IgG2b			1:500	Invitrogen
Alexa Fluor 647 conjugated goat-anti-rat IgG			1:500	Invitrogen
Alexa Fluor 568 conjugated goat-anti-rabbit IgG			1:500	Invitrogen

Supplemental Experimental Procedures

Human iPSC lines and maintenance culture

HiPSC clone 201B7(Takahashi et al., 2007) was used as the parental cell line for generating the PAX3-GFP and MYF5-tdTomato reporter lines. All hiPSCs were cultured and maintained in feeder-free culture on iMatrix-511 (Nippi) in StemFit AK02N medium (Ajinomoto) as previously described(Nakagawa et al., 2014).

RNA extraction and quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted with the ReliaPrep RNA Cell Miniprep system (Promega, Z6012). cDNA synthesis was carried out with the ReverTra Ace qPCR RT kit (TOYOBO, FSQ-101). qRT-PCR was carried out with the SYBR Green system (Applied Biosystems) and a One Step thermal cycler (Applied Biosystems) and was performed in triplicate for each sample. *Beta-actin* or *RPLP0* was used as the internal control. Primer sets used in this study are listed in Supplemental Table S1.

Generation of PAX3-GFP reporter cell line

The human BAC clone RP11-109L24 was purchased from Invitrogen. The construction and recombination of the targeting vector were performed as described(Mae et al., 2013). The primers used for the construction were as follows: hPAX3-EGFP-S, 5'-TAATTTCCGAGCGAAGCTGCCCCCAGGATGACCACGCTGGCCGGCGCTGTGCCCAGGATGATGGTGAGCAAGGGCGAGG-3'; and hPAX3-PN-AS, 5'-GGGTGAGGCAGCCGGTCCCAGGCCCTGGGATCCAGGCGGCGCGCTGAGGCCCTCCCTTACGTCGACGGCGAGCTCAGACG-3'. Electroporation of the BAC construct into 201B7 was carried out as previously described(Mae et al., 2013). The gene targeted clone was selected by the Taqman PCR assay. RT-PCR reactions were carried out with 100 ng of genomic DNA, 250 nM of Taqman probes and 500 nM of primers. The sequences of the primers were as follows: PAX3 Fw, 5'-GGATATATTTCCGAGCGAAGCT-3'; PAX3 Rv, 5'-GGCCCTCCCTTACCTTCCA-3'; and PAX3 probe, 5'-(FAM)-ACGCGGGTAGTTCT-3'. We selected clone #142, which showed approximately half the expression level of the targeted site, as a heterozygous targeted PAX3-GFP knocked-in clone. To remove the PGK-Neo cassette, 2 μg Cre expression vector pcDNA3-nVenus-Cre was transfected into the targeted 201B7 cells by the FuGENE HD lipofection system (Roche). After the isolation of 24 clones, removal of the PGK-Neo cassette was confirmed by adding G418 (Nacalai tesque) in a separately prepared culture well.

Generation of MYF5-tdTomato reporter cell line

To construct the donor template vector for td-Tomato knock-in of the *MYF5* gene, the following four fragments, *MYF5*-5'-UTR, LoxP-Neo-pA-LoxP, tdTomato-P2A and *MYF5*-Exon1, were integrated into the HindⅢ-Sac Ⅰ site of the pBS Ⅱ SK(+) vector by the In-Fusion HD Cloning kit (Clontech). Each fragment was cloned by PCR (KOD Plus Neo, TOYOBO). All primers used in the PCR are listed in Supplemental Table 2. Before the transfection experiments, plasmid DNAs were purified by the NucleoBond Xtra Maxi Plasmid DNA Purification kit (Macherey-Nagel).

To construct the sgRNA expression vector, two oligos containing the sgRNA target site and a universal reverse primer were PCR amplified and cloned into the BamHI-EcoRI site of the pHL-H1-ccdB-mEF1α-RiH vector. All primers are listed in Supplemental Table 2.

Together with the Cas9 expression vector, the donor template vector and the sgRNA expression vector were electroporated into 201B7 iPSCs as previously described(Li et al., 2015). Briefly, transfection was performed with a NEPA 21 Electroporator (Nepagene) as follows. The poring pulse was set to pulse voltage, 125 V; pulse width, 5 ms; pulse number, 2. HiPSCs were pretreated with a ROCK inhibitor (10 μM Y-27632; Sigma) for at least 1 hr before electroporation and dissociated into single cells with 0.25% Trypsin solution treatment for 5 min at 37℃. 1.6 μg of Cas9 plasmid, 0.5 μg of sgRNA plasmid and 2.5 μg of reporter plasmid were electroporated into $1x10^6$ cells. After electroporation, the cells were plated onto an iMatrix-511-coated 10 cm dish in the presence of 10 μM Y-27632 for 2 days.

48 hr after the electroporation, the hiPSCs were treated with 100 μg/ml G418 for the selection of transfected cells. After subcloning the 24 colonies, genome DNA was extracted from each clone for genotyping. To establish reporter lines with heterozygous td-Tomato knocked-in, genomic PCR was done with Fw1-Rv1 primer pairs (Supplemental Table 3), and copy numbers of the knocked-in construct were checked by qRT-PCR with Fw-Rv primer pairs (Supplemental Table 3). The sequences of these clones were checked with the primers listed in Supplemental Table 3. After confirmation, two clones were selected and then transfected with 5 μg CRE expression vector pCMV-Cre-puro to remove the LoxP-Neo-pA-LoxP cassette. After transfection, we picked up several clones and sequenced each genome in order to confirm the construction was correctly knocked-in. Two heterozygous clones (C3 and E16) and one homozygous clone (A24) were selected for the subsequent experiments.

Myogenic differentiation

HiPSCs were dissociated with Accutase (Nacalai) and plated as single cells on a Matrigel-coated 6-well plate (10,000 cells/well) in Stemfit (AK02N, Ajinomoto) supplemented with ROCK inhibitor (10 μM, Y-

27632, Sigma) for 2 days. The medium was changed to fresh Stemfit, in which the cells were kept for one day. Afterwards, the medium was changed to differentiation medium, which contained CDMi supplemented with CHIR99021 (CHIR, Axon MedChem, Tocris) and SB431542 (SB, Sigma). CDMi contains IMDM (+) L-Glutamine (+) 25 mM HEPES (Invitrogen, 12440053) and F12 (1X) Nutrient Mixture (Ham) (+) L-Glutamine (Invitrogen, 11765054) at the ratio 1:1 supplemented with 1% BSA (Sigma), 1% Penicillin-Streptomycin Mixed Solution (Nacalai), 1% CD Lipid Concentrate (Invitrogen), 1% Insulin-Transferrin-Selenium (Invitrogen) and 450 µM 1-Thioglycerol (Sigma). After 7 days of differentiation, the cells were dissociated with Accutase (Nacalai) and plated as single cells on a Matrigel-coated 6-well plate (400,000 cells/well) in CDM supplemented with CHIR, SB and a ROCK inhibitor (10 µM Y-27632, Sigma). At differentiation day 14, the cells were dissociated with Accutase (Nacalai) and plated as single cells on a Matrigel-coated 6-well plate (400,000 cells/well) in CDM supplemented with Y-27632. The medium was changed to serum-free culture medium (SF-O3; Sanko Junyaku) supplemented with 0.2% bovine serum albumin (BSA), 0.1 mM 2-mercaptoethanol (2-ME), 10 ng/ml recombinant human IGF-1 (PeproTech), 10 ng/ml recombinant human bFGF (Oriental Yeast CO., LTD, NIB 47079000) and 10 ng/ml recombinant human HGF (PeproTech). The medium was then changed every 2 or 3 days from day 17 to day 38. Finally, the medium was changed to DMEM (Invitrogen, 11960069) supplemented with 0.5% Penicillin-Streptomycin (Nacalai, 26253-84), 2 mM L-glutamine (Nacalai, 16948-04), 0.1 mM 2-ME, 2% Horse Serum (HS, Sigma), 5 µM SB and 10 ng/ml IGF-1 (Peprotech) until week 12. In figure 2, the cells were differentiated to dermomyotome-like cells following the protocol of Method S (Shelton et al., 2014) or Method C (Chal et al., 2015).

Cell preparation for FACS analysis

Cells after 6 weeks of differentiation were treated with collagenase-1 for 30-60 min at 37℃ and neutralized with DMEM +2% HS. Then the cells were centrifuged at 1000 rpm, 4℃ for 10 min. Cells before 6-weeks differentiation did not undergo this collagenase treatment. After removal of the supernatant, the cells were treated with Accutase at 37℃ for 5 min and neutralized with DMEM +2% HS. Then the cells were centrifuged at 1000 rpm, 4℃ for 10 min. After removal of the supernatant, the cells were suspended with HBSS buffer containing 1% Hoechst and filtered with a 40 µm mesh. The prepared cells were stocked on ice before the FACS analysis.

FACS Analysis

Analysis. FACS analysis was performed by using an AriaII (BD) according to the manufacturer's protocol. 1 x 10^6 PAX3-GFP cells in a 100 µl experimental sample were stained with 5 µl CD271-AF647

antibody (BD, 560326) for 20 min on ice. Gating was determined for the PAX3-GFP cell line using hiPSCs and undifferentiated culture as the baseline control.

Sorting. MYF5+ cells were isolated by FACS at 6, 10, 11, or 12 weeks of differentiation. Sorted populations were either processed for qRT-PCR or for the transplantation experiments.

Re-culture Protocol

Sorted cells were centrifuged at 4℃ for 30 min. After removal of the supernatant, the cells were suspended in the maturation medium with 10 μM Y-27632. Then the cells were plated onto a 6-well collagen type 1-coated plate at 1-3x10^5 cells/well.

Immunocytochemistry

Differentiated cell samples were directly fixed with 2% paraformaldehyde (PFA, Nacalai tesque) for 10 min at 4℃ in a culture dish. Alternatively, sorted cells were seeded onto glass slides with Smear Gel (GenoStaff) and fixed with 2% PFA for 10 min at 4℃. Then the cells were washed twice with PBS and blocked by Blocking One (Nacalai) for 30 min at 4°C. The cells were stained with the appropriate primary antibodies diluted in 10% Blocking One in PBS for 16 hr at 4°C. After three washings with 0.2% Triton X-100 (Sigma-Aldrich) in PBS (PBST), the cells were stained with the appropriate secondary antibodies for 1 hr at room temperature. DAPI, a nuclear stain (Sigma), was loaded at 1:5000 dilution for 5 min. All antibodies are listed in Supplemental Table 4. The samples were observed by using a BZ-X700 (Keyence, Osaka, Japan), and the MHC-positive area was measured by using BZ-X analyzer software (Keyence, Osaka, Japan).

Immunohistochemistry

Engrafted muscles were frozen in isopentane (Wako) cooled in liquid nitrogen. Serial 10 μm cryosections were collected. Tissue sections or cells were fixed in 4% PFA for 20 min at room temperature and washed twice with PBS for 5 min at room temperature. After blocking with Blocking One for 1 hr at room temperature, the tissue samples were incubated with primary antibodies diluted in Can Get Signal B solution (TOYOBO) for 16 hr at 4°C. After washing with PBST three times, the samples were stained with the appropriate secondary antibodies diluted in Can Get Signal B solution for 1 hr at room temperature. The immunostained samples were mounted in VECTA SHIELD mounting medium with DAPI (Vector Laboratories). To measure the localization of the engrafted cells, human LAMIN A/C positive nuclei were counted in the entire area of six TA sections per one transplanted tissue co-stained with DYSTROPHIN and

LAMININ α2. A total of four transplanted tissues were analyzed. The samples were observed by using a LSM700 confocal microscope (Carl Zeiss, Oberkochen, Germany). The primary and secondary antibodies are listed in Supplemental Table 4.

Microarrays analysis

Total RNA was isolated using the ReliaPrep^{TM} RNA Miniprep System (Z6012). The sequencing libraries were constructed using the TruSeq Stranded mRNA Library Prep Kit (Illumina) and sequenced in 100 cycle Single-Read mode of HiSeq2500. All sequenced reads were extracted in FASTQ format using BCL2FASTQ Conversion Software 1.8.4 in the CASAVA 1.8.2 pipeline. FASTQ converted reads were mapped to hg19 reference genes using TopHat v2.0.8b and quantified using RPKMforgenes. The analyses were performed using R ver. 3.1.0. Heat maps of embryonic and fetal muscle progenitor marker expression were generated by Genespring GX 13 software.

Muscle contraction assay

1-2 million MYF5-tdTomato positive cells were transplanted into the right gastrocnemius of DMD-null/NSG mice. The left gastrocnemius was used as the shammed control. Living mice were anesthetized by isoflurane (AbbVie). The maximum muscle contraction force of both sides of the gastrocnemius was analyzed at 2, 4 and 6 weeks after the transplantation with the custom-made mouse ankle joint motor function analysis system (Bio Research Center) previously described(Itoh et al., 2017).

Supplemental References

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