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CRISPR/Cas9 Technology in Restoring Dystrophin Expression in iPSC-Derived Muscle Progenitors

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Corresponding Author:	Yaoliang Tang Augusta University Augusta, GA UNITED STATES
Corresponding Author's Institution:	Augusta University
Corresponding Author E-Mail:	YAOTANG@augusta.edu
Order of Authors:	Yue Jin
	Yan Shen
	Xuan Su
	Neal Weintraub
	Yaoliang Tang
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Cover letter

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Xiaoyan Cao
Review Editor at JoVE
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MS: “CRISPR/CAS9 TECHNOLOGY IN RESTORING DYSTROPHIN EXPRESSION IN IPSC-DERIVED MUSLCE PROGENITORS”

Dear Editor(s):

On behalf of the co-authors and myself, I am writing to request that you consider our manuscript, “CRISPR/CAS9 TECHNOLOGY IN RESTORING DYSTROPHIN EXPRESSION IN IPSC-DERIVED MUSLCE PROGENITORS," for publication in JOVE. We have revised twice based on reviewers' critiques.

Duchenne muscular dystrophy (DMD) is a progressive disease caused by an X-linked recessive mutation of the dystrophin gene. In this manuscript, we introduce a feasible protocol of using CRISPR/Cas9 deletion strategy to restore dystrophin expression in iPSC-derived MPC, which has significant potential for developing future therapies for the treatment of DMD.

The work presented in this manuscript has not been reported previously, nor is it being considered for publication by any other journal. All authors have read, reviewed and approved the data presented in this manuscript.

Thank you for your generous consideration of our work.

Sincerely,

Yaoliang Tang, MD, PhD, FAHA,
Professor of Medicine
Vascular Biology Center
Medical College of Georgia at Augusta University,
1459 Laney Walker Blvd, Augusta, GA 30912, USA.
Email: yaotang@augusta.edu

TITLE:

CRISPR/Cas9 Technology in Restoring Dystrophin Expression in iPSC-Derived Muscle Progenitors

AUTHORS AND AFFILIATIONS:

Yue Jin[1], Yan Shen[1], Xuan Su[1], Neal Weintraub[1], Yaoliang Tang[1]

[1]Medical College of Georgia, Augusta University, Augusta, GA, USA

Email addresses of co-authors:

Yue Jin (YUJIN@augusta.edu)
Yan Shen (YASHEN@augusta.edu)
Xuan Su (XSU@augusta.edu)
Neal Weintraub (nweintraub@augusta.edu)

Corresponding author:

Yaoliang Tang (yaotang@augusta.edu)

KEYWORDS:

induced pluripotent stem cells, iPSC, CRISPR/Cas9, DMD, dystrophin, myogenic progenitor, differentiation

SUMMARY:

Here, we present a Cas9-based exon23 deletion protocol to restore dystrophin expression in iPSC from Dmd^{mdx} mouse-derived skin fibroblasts and directly differentiate iPSCs into myogenic progenitor cells (MPC) using the Tet-on MyoD activation system.

ABSTRACT:

Duchenne muscular dystrophy (DMD) is a severe progressive muscle disease caused by mutations in the dystrophin gene, which ultimately leads to the exhaustion of muscle progenitor cells. Clustered regularly interspaced short palindromic repeats/CRISPR-associated 9 (CRISPR/Cas9) gene editing has the potential to restore the expression of the dystrophin gene. Autologous induced pluripotent stem cells (iPSCs)-derived muscle progenitor cells (MPC) can replenish the stem/progenitor cell pool, repair damage, and prevent further complications in DMD without causing an immune response. In this study, we introduce a combination of CRISPR/Cas9 and non-integrated iPSC technologies to obtain muscle progenitors with recovered dystrophin protein expression. Briefly, we use a non-integrating Sendai vector to establish an iPSC line from dermal fibroblasts of Dmd^{mdx} mice. We then use the CRISPR/Cas9 deletion strategy to restore dystrophin expression through a non-homologous end joining of the reframed dystrophin gene. After PCR validation of exon23 depletion in three colonies from 94 picked iPSC colonies, we differentiate iPSC into MPC by doxycycline (Dox)-induced expression of MyoD, a key transcription factor playing a significant role in regulating muscle differentiation. Our results show the feasibility of using CRISPR/Cas9 deletion strategy to restore dystrophin expression in iPSC-derived MPC, which has significant

potential for developing future therapies for the treatment of DMD.

INTRODUCTION:

Duchenne muscular dystrophy (DMD) is one of the most common muscular dystrophies and is characterized by the absence of dystrophin, affecting 1 of approximately 5,000 newborn boys worldwide[1]. Loss of dystrophin gene function results in structural muscle defects leading to progressive myofibers degeneration[1,2]. Recombinant adeno-associated virus (rAAV)-mediated gene therapy system has been tested to restore the dystrophin expression and improve muscle function, such as gene replacement using micro-dystrophins (μ-Dys). However, the rAAV approach requires repeated injections to sustain expression of the functional protein[3,4]. Therefore, we need a strategy that can provide effectively and permanently recover dystrophin gene expression in patients with DMD. The Dmd^{mdx} mouse, a mouse model for DMD, has a point mutation in exon 23 of the dystrophin gene that introduces a premature termination codon and results in a non-functional truncated protein lacking the C-terminal dystroglycan binding domain. Recent studies demonstrated the use of CRISPR/Cas9 technology to restore dystrophin gene expression by accurate gene correction or mutant exon deletion in small and large animal[5-7]. Long et al.[8] reported the method for correcting the dystrophin gene mutation in Dmd^{mdx} mouse germline by homology-directed repair (HDR) based CRISPR/Cas9 genome editing. El Refaey et al.[9] reported that rAAV could efficiently excise the mutant exon 23 in dystrophic mice. In these studies, gRNAs were designed in the introns 20 and 23 to cause double-stranded DNA breaks, which partially restored the dystrophin expression after DNA repair via non-homologous end joining (NHEJ). Even more exciting, Amoasii et al.[10] recently reported the efficacy and feasibility of rAAV-mediated CRISPR gene editing in restoring dystrophin expression in canine models, an essential step in future clinical application.

DMD also causes stem cell disorders[11]. For muscle damage, residential muscle stem cells replenish dying muscle cells after muscle differentiation. However, the consecutive cycles of injury and repair lead to shortening of telomeres in muscle stem cells[12], and premature depletion of stem cell pools[13,14]. Therefore, a combination of autologous stem cell therapy with genome editing to restore dystrophin expression can be a practical approach for treating DMD. The CRISPR/Cas9 technology provides the possibility of generating autologous genetically corrected induced pluripotent stem cells (iPSC) for functional muscle regeneration and prevent further complications of DMD without causing immune rejection. However, iPSCs have a risk of tumor formation, which could be alleviated by the differentiation of iPSC into myogenic progenitor cells.

In this protocol, we describe the use of non-integrating Sendai virus to reprogramming dermal fibroblasts of Dmd^{mdx} mice into iPSCs and then recover dystrophin expression by CRISPR/Cas9 genome deletion. After validation of Exon23 deletion in iPSC by genotyping, we differentiated genome-corrected iPSC into myogenic progenitors (MPC) via MyoD-induced myogenic differentiation.

PROTOCOL:

All animal handling and surgical procedures were performed by a protocol approved by the Augusta University Institutional Animal Care and Use Committee (IACUC). Mice were fed standard diet and water ad libitum.

1. Isolation of primary mouse fibroblasts from adult Dmd^{mdx} mice

1.1. Euthanize adult Dmd^{mdx} mice (male, 2 months old) by CO_2 asphyxiation and thoracotomy according to IACUC approved by Medical College of Georgia, Augusta University. Cut the tail with a sterile scalpel in a sterile condition under the laminar hood. Rinse the tail with 70% ethanol for 5 min, and then wash with sterile phosphate-buffered saline (PBS) in a 6 cm dish.

1.2. Peel the tail skin off by a sharp incision from the base to the tail tip along the tail skin, and then gently peel off the tail skin with tweezers. Mince the skin to a size of 1 mm^3 using a sterile scalpel and move the minced skin tissue to a 6 cm dish in Dulbecco's minimal essential medium/Ham's F12 (DMEM/F12) containing 0.1% collagenase IV and 1 U/mL of dispase.

1.3. Digest the skin tissue in a culture dish for 2 h at 37 °C in a 5% CO_2 incubator.

1.4. Coat a 6-well plate with fibronectin and 0.2% gelatin (1 mL of fibronectin in 199 mL of 0.2% gelatin; **Table of Materials**) and incubate at 37 °C for 1 h.

1.5. Dissociate the digested skin tissue with a 1 mL pipet tip and transfer the tissue and supernatant to a sterile 15 mL conical centrifuge tube. Centrifuge at 217 x *g* for 3 min at room temperature, discard the supernatant and resuspend the pellet in 1.5 mL of fibroblast medium (**Table of Materials**).

1.6. Culture the pellets including incomplete digested skin tissue from step 1.5 on the 6-well plate coated with fibronectin and 0.2% gelatin from step 1.4 in a 37 °C, 5% CO_2 incubator. Replace the medium 24 h after the initial plating to remove unattached cells, and change the medium every 48 h.

2. Reprogramming mouse skin fibroblasts into iPSCs

2.1. Two days before transduction, digest mouse dermal fibroblasts from step 1.6 with the cell detachment solution (**Table of Materials**) in a 37 °C incubator with a humidified atmosphere of 5% CO_2 for 5 min.

2.2. Count cells using a hemacytometer and centrifuge at 217 x *g* for 3 min.

2.3. Seed cells at a density of 1−2 x 10^5 cells per well onto a 6-well plate and culture with fibroblast medium (**Table of Materials**) in a 37 °C incubator with a humidified atmosphere of 5% CO_2.

2.4. On the day of transduction (day 0), estimate the cells and calculate the volume of each virus required to reach the target multiplicity of infection (MOI) of 5, 5, and 3 (i.e., KOS MOI = 5, hc-Myc MOI = 5, hKlf4 MOI = 3) according to the commercial manual.

$$\text{Volume of virus } (\mu L) = \frac{\text{MOI } \left(\frac{\text{CIU}}{\text{cell}}\right) \times \text{ number of cells}}{\text{titer of virus } \left(\frac{\text{CIU}}{\text{mL}}\right) \times 10^{-3} \left(\frac{\text{mL}}{\mu L}\right)}$$

2.5. Thaw three Sendai tubes in a 37 °C water bath for 5−10 s and add the calculated volumes of each of the three Sendai tubes to 1 mL of fibroblast medium (**Table of Materials**).

2.6. Remove the fibroblast medium from step 2.3 and add the reprogramming virus mixture to the wells containing the cells. Incubate the cells overnight in a 37 °C incubator with a humidified atmosphere of 5% CO_2.

2.7. Replace the medium with fresh fibroblast medium 24 h after transduction. Culture the cells for one week with medium exchange every other day.

2.8. Harvest infected mouse fibroblasts on day 7 after transduction with 0.05% trypsin/EDTA and place on dishes that are previously coated with fibronectin and 0.2% gelatin.

2.9. Culture the infected mouse fibroblasts from step 2.6 with complete mouse embryonic stem cell (ES) growth medium (**Table of Materials**) in a 37 °C incubator with a humidified atmosphere of 5% CO_2 and change medium daily.

2.10. From the 8th day, observe the plate under an inverted microscope every other day to identify the appearance of cell clumps with the morphology of mouse ES.

3. Using alkaline phosphatase live stain and flow cytometry to quantify reprogramming efficiency

3.1. Remove the culture media from each well and rinse with DMEM/F-12 for 2−3 min.

3.2. Apply 2 mL of 1x alkaline phosphatase (AP) live stain working solution (1:500 dilution in DMEM/F-12) to the adherent cells, and incubate in a 37 °C incubator with a humidified atmosphere of 5% CO_2 for 30 min.

3.3. Aspirate the AP live stain and wash twice with PBS for 5 min each.

3.4. Digest the cells with the cell detachment solution (**Table of Materials**) in a 37 °C incubator with a humidified atmosphere of 5% CO_2 for 5 min. Perform flow cytometry to determine the reprogramming efficiency.

4. Selecting and harvesting ES-like cells

4.1. Examine the colonies from step 2.8 under an inverted microscope.

4.2. Mark the colonies at the bottom of the dish with a self-inking object marker.

4.3. Apply greased cloning rings to cover the marked cell colonies. Add 100 µL of 0.05%

trypsin/EDTA to each cloning ring at 37 °C for 5 min, and then transfer the digested cells with 100 µL pipette tips to 48-well culture plates containing mES growth medium.

4.4. Incubate the cells in 48-well culture plates in a 37 °C incubator with a humidified atmosphere of 5% CO_2. Passage the cells to 6 cm dish when they reach 70% confluence.

4.5. Repeat steps 4.3 and 4.4 for several times until uniform dorm-shaped clones are obtained.

5. Freezing iPSCs for cryopreservation

5.1. Dissociate the selected iPSCs from step 4.5 with trypsin, versene, and chick plasma (TVP; **Table of Materials**) solution at 37 °C in a 5% CO_2 incubator for 30 min.

5.2. Collect the cells in a sterile 15 mL conical tube and centrifuge at 217 x *g* for 3 min at room temperature.

5.3. Aspirate the supernatant and re-suspend the cell pellet in 2 mL of mouse ES frozen medium (**Table of Materials**) to obtain 1 mL per cryovial.

5.4. Add cells to cryovials and freeze using a freezer container that provides the critical, repeated -1 °C/min cooling rate required for cryopreservation at -80 °C overnight.

5.5. Transfer frozen vials into a liquid nitrogen tank.

6. Immunofluorescence staining for stem cell markers in iPSCs

6.1. Seed iPSCs from step 5.1 cultured with mES medium from step 2.1 cultured with fibroblast medium into an 8-well chamber slide coated with poly-D-lysine/laminin (**Table of Materials**) at the appropriate density to achieve between 1−2 x 10^4 cells per well and incubate in a 37 °C incubator with a humidified atmosphere of 5% CO_2 for 48 h.

6.2. Immerse the slides in 4% formaldehyde for 15 min at room temperature, and then immerse the slides in PBS twice for 5 min each time.

6.3. Incubate sections with a mouse IgG-blocking reagent (**Table of Materials**), and 5% goat serum for 1 h at room temperature.

6.4. Dilute the primary antibodies in protein diluent (mouse-anti-SSEA1, 1:100, rabbit-anti-Nanog, 1:500; rabbit-anti-POU class 5 homeobox 1 [OCT4], 1:500; rabbit-anti-SRY-box 2 [SOX2], 1:500; rabbit-anti-Lin-28 homolog A [Lin28A], 1:400) (**Table of Materials**). Apply antibodies to the cells and incubate at 4 °C overnight in a humidified chamber.

6.5. Discard the primary antibody solution and wash the slides 3x in PBS.

6.6. Apply second antibodies (Alexa488-conjugated goat-anti-mouse antibody and Alexa555-conjugated goat-anti-rabbit, 1:400 each) in M.O.M. protein diluent on slides and incubate for 45 min at room temperature.

6.7. Wash the slides 3x in PBS and mount sections with mounting medium containing 4'6-diamidino-2-phenylindole (DAPI).

6.8. Take pictures with a confocal microscope.

7. Investigating the pluripotency of iPSCs in vivo

7.1. Dissociate the iPSCs from step 5.1 into single cells using TVP solution in a 37 °C incubator with a humidified atmosphere of 5% CO_2 for 30 min.

7.2. Count cells using a hemacytometer and centrifuge at 217 x *g* for 3 min.

7.3. Aspirate the supernatant and resuspend the pellet with mES medium in a 1.5 mL sterile centrifuge tube at a concentration of 5 x 10^5 cells/30 µL for cell transplantation.

7.4. Remove hair from both hind limbs of immunodeficient mice using hair clippers.

7.5. Anesthetize mice with ketamine (100 mg/kg) and clean the injection site with 75% alcohol.

7.6. Inject 30 µL of iPSC suspension from step 7.3 intramuscularly into the gastrocnemii, using a 31 G needle.

7.7. Two weeks after the injection, harvest the mouse gastrocnemii and embed in optimal cutting temperature (OCT) compound, snap freeze and cut into 5-µm sections[15,16].

7.8. Fix sections in 4% formaldehyde for 15 min at room temperature, and then wash the slides twice in PBS for 5 min each time.

7.9. Block the cells with 5% goat serum protein diluent for 1 h at room temperature.

7.10. Add diluted primary antibody (rabbit anti-AFP, 1:50; rabbit anti-SMA, 1:50; rabbit anti-TH, 1:50) to the slides and incubate at 4 °C overnight in a humidified chamber.

7.11. Discard the primary antibody solution, wash the cells 3x (5 min/wash) in PBS, add a 1:400 diluted Alexa555-conjugated goat-anti-rabbit antibody to slides, and incubate for 45 min at room temperature.

7.12. Wash slides 3x (5 min/wash) in PBS, and mount sections with mounting medium containing DAPI.

8. Construction of CRISPR/Cas9 lentiviral vector targeting introns flanking dystrophin exon 23

8.1. Design two pairs of gRNA oligos targeting intron-flanked dystrophin exon 23 via http://crispor.tefor.net/crispor.py.

NOTE: The designed pairs are as follows:
i22sense: 5'-CACCGTTAAGCTTAGGTAAAATCAA- 3'
i22anti-sense: 5'-AAACTTGATTTTACCTAAGCTTAAC-3'
i23sense: 5'-CACCGAGTAATGTGTCATACCTTCT- 3'
i23anti-sense: 5'-AAACAGAAGGTATGACACATTACTC-3').

8.2. Digest and dephosphorylate 5 µg of lentiviral CRISPR plasmid (lenti-CRISPRv2-blast [a gift from Mohan Babu] and lenti-Guide-Hygro-iRFP670 [a gift from Kristen Brennand]) (**Table of Materials**) with BsmB1/Esp3I for 30 min at 37 °C. For 5 µg of plasmids, add 3 µL of BsmB1 restrict enzyme, 3 µL of fast alkaline phosphatase, 6 µL of 10x enzyme digest buffer and 0.6 µL of 100 mM DTT in 60 µL reaction).

8.3. Load the reactions onto a 0.8% agarose gel. Run the gel at 100−150 V for 30 min.

8.4. Purify digested plasmid in-gel using a gel extraction kit (**Table of Materials**) and elute in 20 µL of H_2O.

8.5. Phosphorylate and anneal each pair of the gRNA oligonucleotides containing 1 µL of each oligonucleotide at 100 µM, 1 µL of 10x T4 ligation buffer, 0.5 µL of T4 polynucleotide kinase (PNK), 6.5 µL of ddH_2O at 37 °C for 30 min, and then 95 °C for 5 min and then ramp down to 25 °C at 5 °C/min.

8.6. Ligate a 1:200 dilution of the annealed gRNA oligonucleotides into the plentiCRISPR V2-Blast or plentiGuide-Hygro-iRFP670. Mix 50 ng of BsmB1/Esp3I digested vectors with 1 µL of diluted oligo duplex and 5 µL of 2x ligase buffer plus 1 µL of ligase in a 11 µL reaction system and incubate for 10 min at room temperature.

8.7. Perform transformation with 3 µL of ligation product into 50 µL competent cells (**Table of Materials**) according to the manufacturer's instructions.

8.8. Spread the transformed competent cells in an agar plate with 100 µg/mL carbenicillin and incubate at 31.5 °C for 18 h.

8.9. Pick colonies with 10 µL sterile pipette tips and culture in 5 mL of terrific broth (**Table of Materials**) containing 100 µg/mL carbenicillin at 31.5 °C, 185 rpm in a shaker incubator for 21 h.

8.10. Purify the plasmid DNA using the mini-prep and midi-prep kits (**Table of Materials**).

8.11. Verify the mini-prep plasmids by restriction digestion. For the 20 µL reaction system, add 1 µg of plasmid DNA, 2 µL of digest reaction buffer (**Table of Materials**) and 1 µL of restriction enzyme mixture (0.5 µL of KpnI-HF and 0.5 µL of AgeI-HF for plentiCRISPR V2-Blast-i22; 0.5 µL of NotI-HF and 0.5 µL of EcoRI-HF for plentiGuide-Hygro- iRFP670- i23). Incubate the reaction system for 1 h at 37 °C.

8.12. Load the reactions onto a 0.8% agarose gel. Run the gel at 100−150 V for 30 min.

NOTE: The correct bands for plentiCRISPR V2-Blast-i22 should be 622 bp and 12.2 kb, and the correct bands for plentiGuide-Hygro- iRFP670- i23 should be 2.6 kb and 7.1 kb.

9. Lentiviral vector packaging

9.1. Culture 7 x 10^5 293FT cells in 5 mL of DMEM media containing 10% fetal bovine serum in a 6 cm dish overnight at 37 °C, 5% CO_2.

9.2. Prepare a cocktail (1 µg of plentiCRISPR V2-Blast-i22 or plentiGuide-Hygro-iRFP670-i23, 750 ng of psPAX2 packaging plasmid, 250 ng of pMD2.G envelope plasmid, and 5 µL of transfection reagent A [**Table of Materials**] in 100 µL of reduced serum MEM media).

9.3. Prepare a mixture of 5 µL of transfection reagent B (**Table of Materials**) in 100 µL of reduced serum MEM media.

9.4. Add 100 µL of transfection reagent B mixture to plasmid mixture from step 9.2 and incubate for 5 min at room temperature.

9.5. Add the DNA-lipid complex (from step 9.4) dropwise to the 293FT cells. Incubate overnight at 37 °C with 5% CO_2.

9.6. Add virus production enhancer (500x) (**Table of Materials**) to each dish the next day and incubate for 24 h at 37 °C, 5% CO_2.

9.7. Collect medium from cells using pipettes on the next two days and filter the medium through a 0.45 µm filter to remove the cells.

10. Concentration and purification of lentiviral vectors

10.1. Precipitate lentiviral vector in the medium of step 9.7 overnight at 4 °C with 5x polyethylene glycol 4000 (PEG4000, 8.5% final concentration) and 4 M NaCl (0.4 M final concentration).

10.2. Centrifuge the viral media containing the PEG4000 solution at 2,095 x *g* and 4 °C for 30 min, remove and discard the supernatant.

10.3. Resuspend the pellets with 500 µL of serum reduced MEM media (lentivirus titer: lenti-CRISPR V2-gRNAi22: 1.56 x 10^8, lenti-iRFP670-gRNAi23: 1.3 x 10^8, lenti-CRISPR V2-control: 3.13 x 10^7, lenti-iRFP670-control: 5.9 x 10^7). Store at -80 °C until use.

11. Deletion of exon 23 in mouse iPSCs with two guide RNAs (gRNAs) coupled with Cas9

11.1. Plate the mouse iPSCs from step 4.5 in a 24-well plate coated with fibronectin and gelatin.

11.2. After the cells reach 50% confluence, switch to the fresh culture medium (complete mouse embryonic stem cell growth medium) containing 8 µg/mL polybrene).

11.3. Add 100 µL of the lentiviral particle solution from step 10.3 including lenti-CRISPR V2-gRNAi22, lenti-iRFP670-gRNAi23 and control (empty vector: lenti-CRISPR V2, lenti-iRFP670) to mouse iPSCs. Incubate cells for 3 days at 37 °C with 5% CO_2.

11.4. Select stably infected cells with media containing 2.5 µg/mL blasticidin and 100 µg/mL hygromycin B by determining the minimum concentration of blasticidin and hygromycin B required to kill the un-infected cell.

NOTE: Un-infected cells would be killed by blasticidin and hygromycin B.

11.5. Digest the selected mouse iPSCs with 0.5 mL of TVP solution each well (24-well plate) and incubate cells for 30 min at 37 °C with 5% CO_2.

11.6. Dissociate the iPSCs into single cells by pipetting, count cells with a cell counting chamber and then dilute about 150 digested single cells with mES medium into 10 cm dish for culture at 37 °C with 5% CO_2.

11.7. After about 10 days, pick single colonies under an inverted microscope using 10 µL sterile pipette tips (96 colonies need to be picked).

11.8. Transfer the picked colonies into 50 µL of TVP solution each well (96-well plate, one colony each well), digest at 37 °C for 30 min, and then seed the digested cells into two 96-well culture plates to keep culture (one for genotyping).

11.9. Incubate in a CO_2 incubator at 37 °C until 70% confluent.

12. Identification of iPSC colonies with exon23 deletion

12.1. Remove the medium in 96-well plate when the cell colonies reach 70% confluence.

12.2. Add 25 µL of lysis reagent (**Table of Materials**) containing proteinase K solution (1 mL of proteinase K in 100 mL of lysis reagent) to each well, and transfer the lysate to a 96-well PCR plate.

12.3. Seal the PCR plate and incubate the plates at 55 °C for 30 min, and then at 95 °C for 45 min to lyse the cells and denature the proteinase K.

12.4. Carry out PCR reaction with 2 µL of lysate from step 12.3. For the 20 µL PCR reaction, add 2 µL of lysate, 10 µL of 2x DNA polymerase premix (**Table of Materials**), 7 µL of DNase-free water, and 1 µL of DMD exon 23 primers (**Table 1**).

12.5. Use the following parameters for PCR reaction: 98 °C for 1 min, 35 cycles of 98 °C for 10 s, 60 °C for 15 s, 72 °C for 30 s, and a final extension at 72 °C for 1 min.

12.6. Load the PCR reaction onto a 2% agarose gel. Run the gel at 100–150 V for 30 min.

12.7. Inspect the gel under UV light (the knockout efficiency is 3/94).

13. Using the Tet-on MyoD activation system to directly differentiate iPSC into myogenic progenitor cells (MPC)

13.1. Package the LV-TRE-VP64-mouse MyoD-T2A-dsRedExpress2 and LV-TRE-VP16 mouse MyoD-T2A-dsRedExpress2 (a gift from Charles Gersbach) (**Table of Materials**) as previously described for lenti-CRISPRv2-blast and lenti-gRNA-iRFP670 vectors in sections 9 and 10.

13.2. Infect mouse iPSC with lentivirus-TRE-VP64-MyoD-T2A-dsRed-Express2 or lentivirus-TRE-VP16-MyoD-T2A-dsRedExpress2 as previously described for lenti-CRISPRv2-blast and lenti-gRNA-iRFP670 vectors in steps 11.1−11.3.

13.3. Select cells with 1 µg/mL puromycin after three days of infection to obtain a pure transduced cell population.

13.4. Add 3 µg/mL doxycycline into culture media (10% FBS DMEM) to MPC differentiation. Replace fresh medium supplemented with doxycycline every two days.

14. Quantitative reverse transcription PCR for evaluating dynamic muscle differentiation and DMD exon 22-24 expression

14.1. Extract cellular RNA after 0, 3, 6, and 10 days after doxycycline treatment using RNA isolation reagent, reversely transcribe RNA into cDNA by using first strand cDNA synthesis kit (**Table of Materials**).

14.2. For the 20 µL qPCR reaction system, add 1 µL of cDNA, 10 µL of PCR reaction buffer (**Table of Materials**), 8 µL of DNA H_2O, and 1 µL of mixture of forward and reverse primers (glyceraldehyde-3-phosphate dehydrogenase [GAPDH], skeletal muscle [ACTA1], OCT4 and DMD exon22, DMD exon23, and DMD exon24, see **Table 1**).

14.3. Use the following parameters for PCR reaction: 50 °C for 2 min, 95 °C for 2 min, 40 cycles of 95 °C for 15 s, 60 °C for 1 min, 90 °C for 5 s, melt curve 65.0 °C to 95.0 °C, increment 0.5 °C.

15. Immunofluorescence staining of myosin heavy chain 2 (MYH2) and dystrophin protein expression

15.1. Plate doxycycline-induced, lenti-TRE-MyoD modified cells from step 13.4 onto 8-well culture slides.

15.2. Fix cells in 4% formaldehyde for 15 min at room temperature, and then wash the slides twice in PBS for 5 min each time.

15.3. Block the cells with 5% goat serum protein diluent for 1 h at room temperature.

15.4. Add rabbit-anti-dystrophin antibody (1:300) and mouse-anti-MYH2 antibody (1:100) to the slides, incubate at 4 °C overnight in a humidified chamber.

15.5. Discard the primary antibody solution, wash the cells 3x (5 min/wash) in PBS, add 1:400 diluted Alexa488-conjugated goat-anti-rabbit antibody and Alexa555-conjugated goat-anti-mouse antibody to slides, and incubate for 45 min at room temperature.

15.6. Wash the slides 3x (5 min/wash) in PBS, and mount sections with mounting medium containing DAPI.

REPRESENTATIVE RESULTS:

Establishment of Dmd^{mdx} skin fibroblasts derived iPSC. We demonstrated the efficiency of generating mouse iPSCs from Dmd^{mdx} mice derived skin fibroblast using the integration-free reprogramming vectors. **Figure 1A** demonstrated that the appearance of embryonic stem cell (ESC)-like colonies at three weeks after infection. We evaluate the efficiency of iPSC induction by live alkaline phosphatase (AP) stain; **Figure 1B** shows that the percentage of AP-positive cells was around 1.8% by FACS analysis. SSEA1, Lin28, Nanog, OCT4 and SOX2, pluripotency markers for mouse embryonic stem cells, were positive for iPSC colonies by immunofluorescent staining, (**Figure 1C**). To investigate the three germline differentiation of iPSCs in vivo, we intramuscularly injected iPSCs into the mouse gastrocnemii. We observed that the injected iPSCs differentiated into liver cells (endoderm), smooth muscle cells (mesoderm), and adrenergic neuron cells (ectoderm) (**Figure 1D**), indicting the pluripotency of iPSCs.

CRISPR/Cas9-mediated exon23 deletion*.* We designed two guide RNAs that flank the mutant exon 23. After Cas9-mediated double-stranded breaks (DSB) and non-homologous end joining (NHEJ), mutant exon 23 was deleted, allowing for truncated but functional dystrophin production (**Figure 2A**). To identify exon 23 deleted mouse iPSC, cells were sparsely seeded, and individual colonies were picked and propagated. Genomic DNAs extracted from these colonies were subjected to PCR genotyping. **Figure 2B** demonstrated that colony #1 and #2 have exon 23 deletions indicating a successful deletion of the exon 23.

Differentiating mouse iPSCs into a myogenic lineage and restoring dystrophin expression*.* We use a tetracycline-inducible MyoD expression system to induce myogenic differentiation of iPSCs. Doxycycline was used to induce MyoD expression in iPSCs. **Figure 3A** shows the time course of muscle differentiation in Dox-treated iPSCs. qRT-PCR showed that the mRNA level of OCT4, a pluripotent marker, gradually decreased, while the expression of ACTA1, a skeletal muscle marker, increased after Dox induction. Also, we observed the myotubes formation at two weeks after Dox treatment (**Figure 3B**). Importantly, the qRT-PCR assay showed the recovery of DMD exon 24 mRNA expression in Dox-induced, Cas9-mediated Exon23 deleted line in comparison to Cas9-control line (**Figure 3C**). Inconsistent with qRT-PCR, immunofluorescent staining shows the dystrophin protein expression in Cas9-mediated exon 23 deleted cells, whereas the dystrophin expression was absent in control cells (**Figure 3D**).

FIGURE AND TABLE LEGENDS:

Figure 1: Reprogramming skin fibroblasts from Dmd^{mdx} mice into iPSCs. (**A**) Representative image of ES-like colonies (scale bar = 200 μm). (**B**) FACS analysis of the reprogramming

efficiency of mouse skin fibroblasts into iPSCs after 8 days of Sendai virus transduction by live AP staining. (**C**) Immunofluorescent staining of SSEA1, Lin28, Nanog, Oct4, and SOX2 in iPSCs (scale bar = 50 μm). (**D**) Immunofluorescent staining for AFP (endoderm), SMA (mesoderm), and tyrosine hydrolase (TH) (ectoderm) of teratoma 2 weeks after iPSC injection into gastrocnemii (scale bar = 20 μm).

Figure 2: CRISPR/Cas9-mediated exon23 deletion. (**A**) Schematic diagram of CRISPR/Cas9-mediated exon 23 deletions. The Cas9 nuclease targets intron 22 and intron 23 by two gRNAs. Double-stranded breaks (DSBs) by Cas9 results in the excision of the mutant exon 23. The distal ends are repaired by non-homologous end joining (NHEJ), resulting in the restoration of the reading frame of the dystrophin gene. (**B**) PCR genotyping analysis of exon 23. The arrow indicates the PCR product of exon 23. GAPDH serves as a reference.

Figure 3: Differentiating mouse iPSCs into the myogenic lineage and restoring dystrophin expression. (**A**) qRT-PCR showed the time course of mRNA level of Oct4 and ACTA1 in Dox-treated exon 23-deleted Dmd^{mdx} iPSC (*P < 0.05 vs D0, D6, D10, #P < 0.05 vs D0, D3, D10, $P < 0.05 vs D0, D3, D6, n = 4 for Oct4) (*P < 0.05 vs D6 and D10, #P < 0.05 vs D0, D3, and D10, $P < 0.05 vs D0, D3, and D6, n = 3 for ACTA1). (**B**) Left: Representative image of myotube formation from Dox-induced mouse iPSCs (scale bar = 200 μm). Right: Immunofluorescent analysis of MYH2 in myotube formation from Dox-induced mouse iPSCs (scale bar = 20 μm). (**C**) Upper: the PCR primer positions for DMD Exon22, Exon23 and Exon24; Bottom: qRT-PCR analysis of the mRNA level of DMD Exon22, Exon23, and Exon24 expression in MPC (****P < 0.0001, n = 3). (**D**) Immunofluorescent analysis of dystrophin expression in Dox-induced MPC from iPSC $^{Cas9\text{-}Ctrl}$ and iPSC $^{Cas9\text{-}gRNA}$ (scale bar = 50 μm).

Table 1: Primer sequence.

DISCUSSION:

Duchenne Muscular Dystrophy (DMD) is a destructive and ultimately fatal hereditary disease characterized by a lack of dystrophin, leading to progressive muscle atrophy[1,2]. Our results demonstrate the restored dystrophin gene expression in Dmd^{mdx} iPSC-derived myogenic progenitor cells by the approach of CRISPR/Cas9-mediated exon23 deletion. This approach has three advantages.

First, we generated iPSCs from Dmd^{mdx} mouse-derived dermal fibroblasts using a non-integrated RNA vector. A variety of methods have been developed to generate iPSCs, such as lentiviral and retroviral vectors, which will integrate into host chromosomes to express reprogramming genes, thus bearing safety concerns. DNA-based vectors such as plasmid vectors, adeno-associated viruses and adenoviruses exist in a non-integrated manner; however, they may still integrate into the host chromosome at a low frequency. In this study, we used a modified, non-transmissible Sendai virus, a non-integrated RNA vector, to safely and effectively deliver stem cell transcription factors to fibroblasts for reprogramming.

Next, we use CRISPR-mediated genome deletion, rather than CRISPR/Cas9-mediated

precision gene correction, to restore dystrophin expression in iPSCs. This method is feasible and efficient; it is easy to design multiple gRNAs to delete multiple mutant exons, which occur in many human DMD patients[17]. Exon deletion utilizes a relatively efficient non-homologous end joining pathway, and the method also avoids the need to deliver a DNA repair template. Therefore, in comparison to Cas9-mediated precision correction, Cas9–mediated exon deletion is suitable for DMD patients with multiple gene mutations.

Finally, we induced undifferentiated iPSCs into myogenic progenitor cells, which may reduce the risk of tumorigenesis caused by iPSCs. In this protocol, we induced MyoD expression via an inducible tetracycline-regulated (Tet-On) vector system to differentiate iPSCs into skeletal muscle progenitors[18,19].

In conclusion, the combination of CRISPR/Cas9 genome editing with Tet-on MyoD activation system may provide a safe, feasible, and efficient strategy for mutant DMD-Exon23 deletion in stem cells for cell transplantation in DMD patients.

To select and harvest ES-like cells efficiently, we should identify the undifferentiated iPSC cells via their dome-like morphology, and an inking object marker can help us label individual clones from the bottom of the culture dish with a 1.8 mm circle around the iPSC clones. To avoid leakage of trypsin solution, we need to apply grease evenly to the bottom of rings. Also, after placing the grease-coated rings on the top of the labeled cell colonies, care should be taken not to touch the rings. Otherwise, the iPSC clones will be detached.

The protocol has its limitations; for example, we chose a non-integrated RNA vector system to generate iPSCs. However, we used a lentiviral CRISPR/Cas9 system to delete DMD exon 23 and a lentiviral-based MyoD activation system to induce iPSC myogenic differentiation; these integrative lentiviral vectors have safety concerns. However, these issues can be solved by the application of a ribonucleoprotein (RNP) complex comprising a recombinant, high-purity S. pyogenes Cas9 nuclease with a crRNA:tracrRNA duplex; we can choose chemically modified MyoD mRNA transfection to directly differentiate iPSCs into myogenic progenitors, although the efficiency may be challenging.

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DISCLOSURES:

The authors have nothing to disclose.

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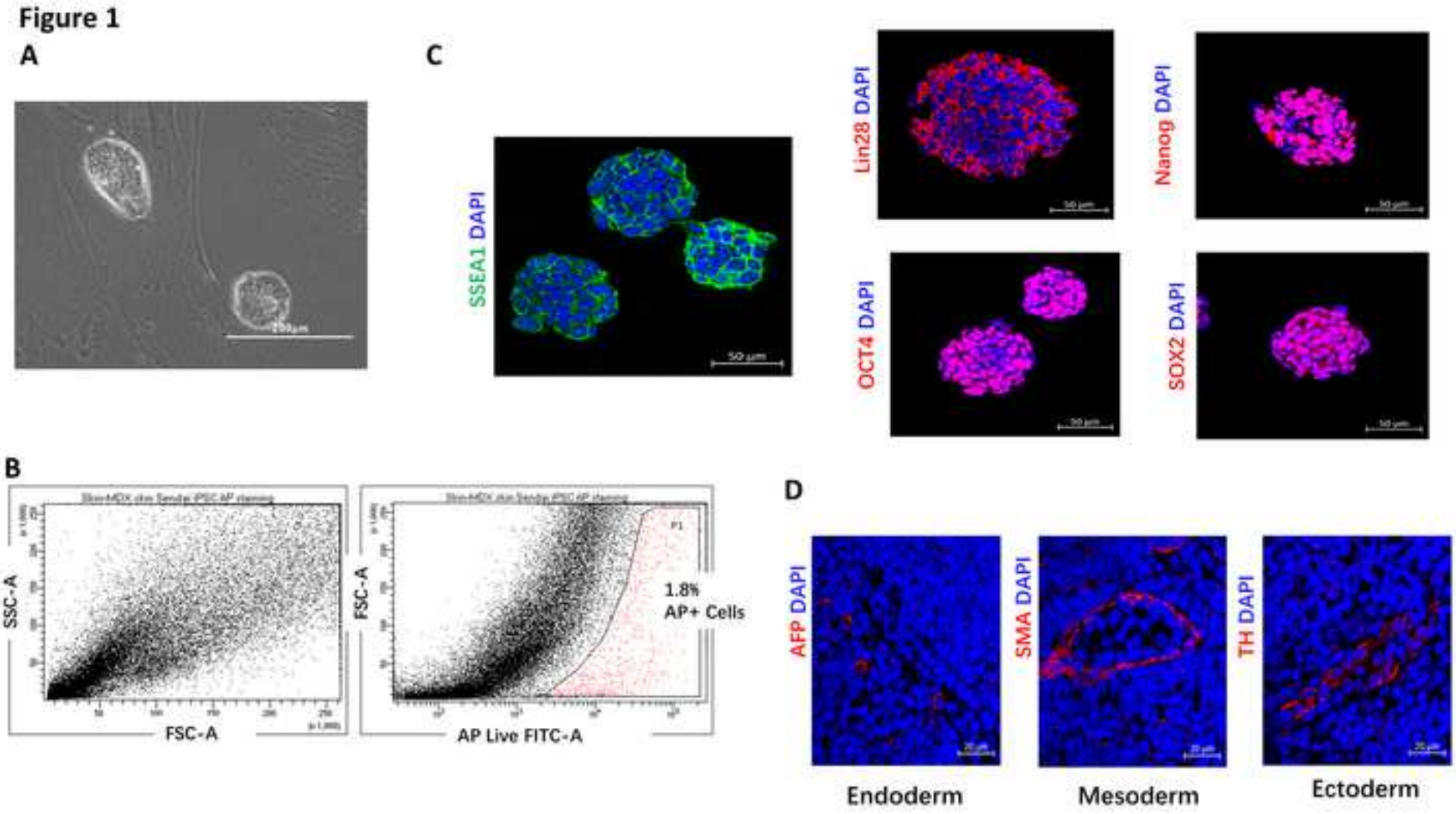


Figure 2

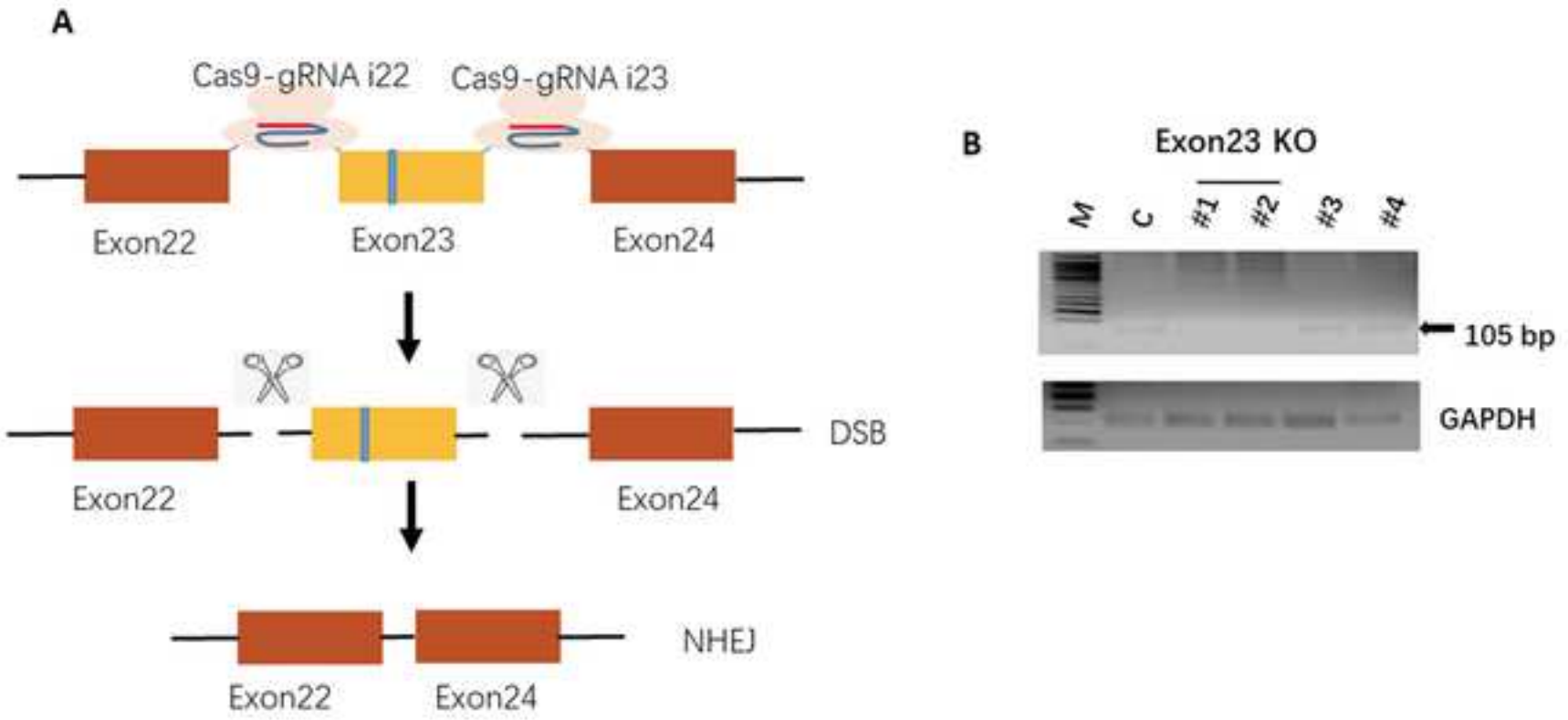
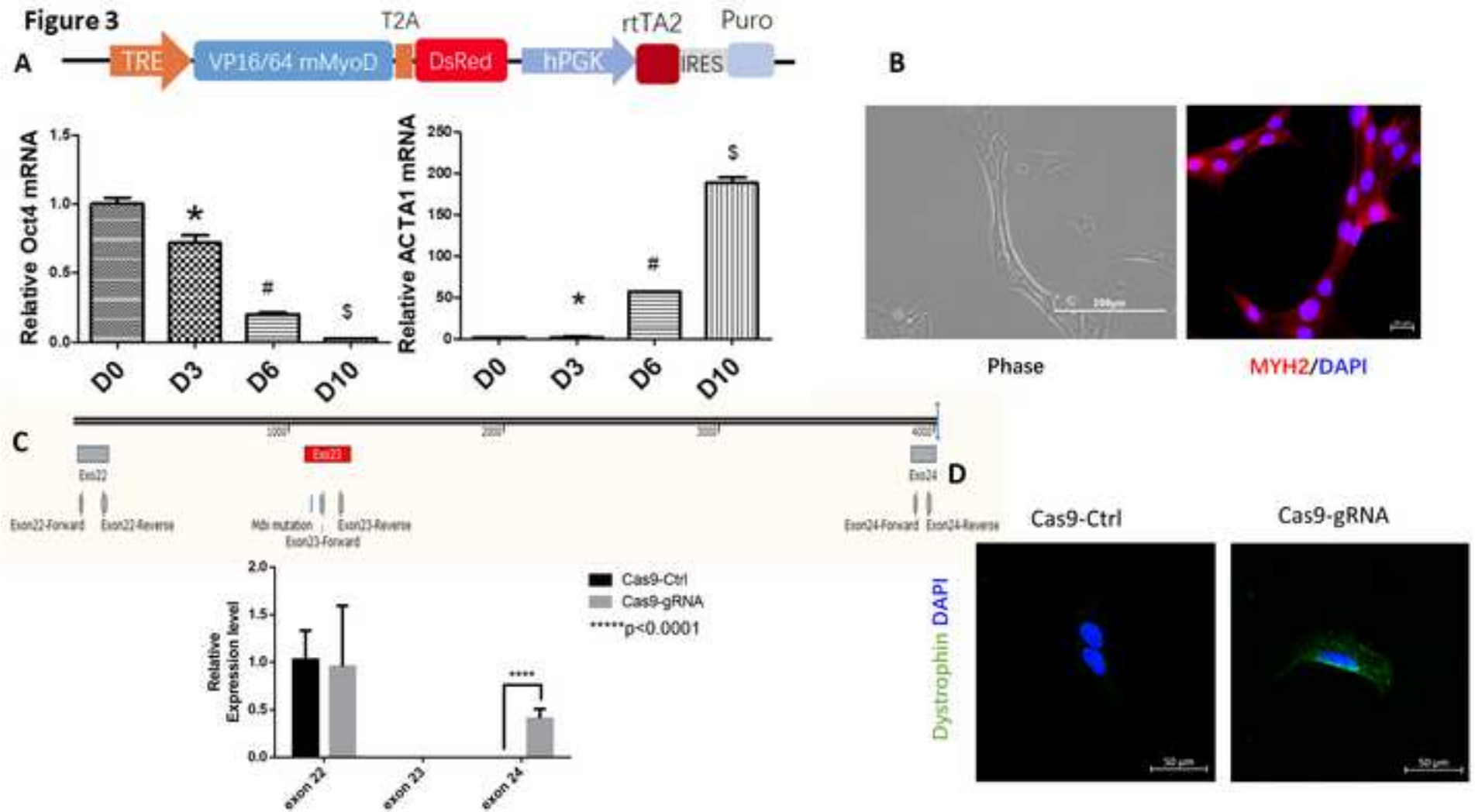


Figure 3

Click here to access/download;Figure;Figure 3.TIF



Table

Guide primers	
i22 sense	5'-CACCGTTAAGCTTAGGTAAAATCAA- 3'
i22 antisense	5’-AAACTTGATTTTACCTAAGCTTAAC-3’
i23 sense	5'-CACCGAGTAATGTGTCATACCTTCT- 3'
I23 antisense	5’-AAACAGAAGGTATGACACATTACTC-3’
PCR primers	
OCT4-Forward	5'-AGCTGCTGAAGCAGAAGAGGATCA-3'
OCT4-Reverse	5'-TCTCATTGTTGTCGGCTTCCTCCA-3'
ACTA1-Forward	5'-GATCCATGAGACCACCTACAAC-3'
ACTA1-Reverse	5'-TCAGCGATACCAGGGTACAT-3'
Exon22-Forward	5'-TTACCACCAATGCGCTATCA-3'
Exon22-Reverse	5'-CCGAGTCTCTCCTCCATTATTTC-3'
Exon23-Forward	5'-CCAACAAAGCACCTTCAGAAATATG-3'
Exon23-Reverse	5'-TTTGGCAGCTTTCCACCA-3'
Exon24-Forward	5'-AAC CTT ACA GAA ATG GAT GGC-3'
Exon24-Reverse	5'-TTTCAGGATTTCAGCATCCC-3'
GAPDH-Forward	5'-TGACAAGCTTCCCATTCTCG-3'
GAPDH-Reverse	5'-CCCTTCATTGACCTCAACTACAT-3'

Table of Materials

Click here to access/download;Table of Materials;59432_Materials 6-10-2019.xlsx

Name of Material/Equipment	Company	Catalog Number
Surgical Instruments		
31-gauge needle	Various	
Sharp Incision	Various	
Sterile Scalpels	Various	
Tweezers	Various	
Fibroblast medium (for 100 mL of complete medium)	**Company**	**Catalog Number**
2-Mercaptoethanol (55 mM)	Gibco	21-985-023
Antibiotic Antimycotic Slution 100x	CORNING	MT30004CI
Dulbecco's Modified Eagle's Medium - high glucose	SIGMA	RNBH0544
Fetal Bovine Serum Characterized	HyClone	SH30396.03
L-Glutamine solution	SIGMA	G7513
MEM Non-Essential Amino Acids Solution (100x)	Gibco	11140076
TVP solution (for 500 mL of complete solution)	**Company**	**Catalog Number**
Chicken Serum	Gibco	16110-082
EDTA	Sigma-Aldrich	E6758
Phosphat-buffered saline		
Trypsin (2.5%)	Thermo	15090046
mES growth medium(for 500 mL of complete solution)	**Company**	**Catalog Number**
2-Mercaptoethanol (55 mM)	Gibco	21-985-023
Antibiotic Antimycotic Slution 100x	CORNING	MT30004CI
Dulbecco's Modified Eagle's Medium - high glucose	SIGMA	RNBH0544
Fetal Bovine Serum Characterized	HyClone	SH30396.03
L-Glutamine solution	SIGMA	G7513
Mouse recombinant Leukemia Inhibitory Factor (LIF), 0.5 x 106 U/mL	EMD Millipore Crop	CS210511
MEK/GS3 Inhibitor Supplement	EMD Millipore Crop	CS210510-500UL
MEM Non-Essential Amino Acids Solution (100x)	Gibco	11140076

The ES cell media should not be stored for more than 4 weeks and with inhibitors not more than 2 weeks.

mES frozen medium(for 50 mL of complete solution)	Company	Catalog Number
Dimethyl sulfoxide (DMSO)	SIGMA	D2650
Dulbecco's Modified Eagle's Medium - high glucose	SIGMA	RNBH0544
Fetal Bovine Serum Characterized	HyClone	SH30396.03
Mouse recombinant Leukemia Inhibitory Factor (LIF), 0.5 x 106 U/mL	EMD Millipore Crop	CS210511

Name of Material/ Equipment	Company	Catalog Number
0.05% Trypsin/0.53 mM EDTA	CORNING	25-052-CI
4% Paraformaldehyde	Thermo scientific	J19943-k2
Accutase solution	SIGMA	A6964
AgeI-HF	NEB	R3552L
Alexa488-conjugated goat-anti-mouse antibody	Invitrogen	A32723
Alexa488-conjugated goat-anti-rabbit antibody	Invitrogen	A32731
Alexa555-conjugated goat-anti-rabbit antibody	Invitrogen	A32732
anti-AFP	Thermo scientific	RB-365-A1
anti-α-Smooth Muscle Actin (D4K9N) XP	CST	19245S
anti-Dystrophin	Thermo	PA5-32388
anti-LIN28A（D1A1A）XP	CST	8641S
anti-MYH2	DSHB	mAb2F7
anti-Nanog-XP	CST	8822S
anti-Oct-4A (D6C8T)	CST	83932S
anti-Sox2	abcam	ab97959
anti-SSEA1(MC480)	CST	4744s
anti-TH (H-196)	SANTA CRUZ	sc-14007
Alkaline Phosphatase Live Stain (500x)	Thermo	A14353
Blasticidin S	Sigma-Aldrich	203350
BsmBI/Esp3I	NEB	R0580L/R0734L
Carbenicillin	Millipore	205805-250MG

Collagenase IV	Worthington Biochemical Corporation	LS004189
Competent Cells	TakaRa	636763
CutSmart	NEB	B7204S
CytoTune-iPS 2.0 Sendai Reprogramming Kit	Thermo	A16517
DirectPCR Lysis Reagent (cell)	VIAGEN BIOTECH	302-C
Dispase (1 U/mL)	STEMCELL Technologies	7923
Doxycycline Hydrochloride	Fisher BioReagents	BP26535
EcoRI-HF	NEB	R3101L
Fibronectin bovine plasma	SIGMA	F1141
QIAEX II Gel Extraction Kit (500)	QIAGEN	20051
Gelatin from porcine skin, type A	SIGMA	G1890
HardSet Antifade Mounting Medium with DAPI	Vector	H-1500
Hygromycin B (50 mg/mL)	Invitrogen	10687010
Ketamine HCL Injection	HENRY SCHEIN ANIMAL HEALTH	45822
KpnI-HF	NEB	R3142L
lenti-CRISPRv2-blast	Addgene	83480
lenti-Guide-Hygro-iRFP670	Addgene	99377
Lipofectamin 3000 Transfection Kit	Invitrogen	L3000015
LV-TRE-VP64-mouse MyoD-T2A-dsRedExpress2	Addgene	60625
LV-TRE-VP16 mouse MyoD-T2A-dsRedExpress2	Addgene	60626
Mouse on Mouse (M.O.M.) Basic Kit	Vector	BMK-2202
NotI-HF	NEB	R3189L
Opti-MEM I Reduced Serum Media	ThermoFisher	31985070
Polyethylene glycol 4,000	Alfa Aesar	AAA161510B
Polybrene	SIGMA	TR1003
Corning BioCoat Poly-D-Lysine/Laminin Culture Slide	CORNING	CB354688
PowerUp SYBR Green Master Mix	ThermoFisher	A25742
PrimeSTAR Max Premix	TakaRa	R045
Proteinase K	VIAGEN BIOTECH	507-PKP
Puromycin Dihydrochloride	MP Biomedicals	ICN19453980
qPCR Lentivirus Titration Kit	abm	LV900
Quick ligation kit	NEB	M2200S

QIAprep Spin Miniprep Kit (250)	QIAGEN	27106
QIAGEN Plasmid Plus Midi Kit (100)	QIAGEN	12945
RevertAid RT Reverse Transcription Kit	Thermo scientific	K1691
RNAzol RT	Molecular Research Center, INC	RN 190
T4 DNA Ligase Reaction Buffer	NEB	B0202S
T4 Polynucleotide Kinase	NEB	M0201S
Terrific Broth Modified	Fisher BioReagents	BP9729-600
ViralBoost Reagent (500x)	ALSTEM	VB100

Comments/Description

Volume
0.1 mL
1 mL
87 mL
10 mL
1 mL
1 mL

Volume
5 mL
186 mg
to 500 mL
5 mL

Volume

0.5 mL
5 mL
408.5 mL
75 mL
5 mL

500 µL
500 µL
5 mL

Volume
5 mL
24.9 mL
25 mL

50 µL

RRID

Cell detachment solution

AB_2633275
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AB_2633281
AB_59574
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AB_10997528
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CORRESPONDING AUTHOR:

Name:	Yaoliang Tang
Department:	Vascular Biology Center
Institution:	Medical College of Georgia
Article Title:	CRISPR/CAS9 TECHNOLOGY IN RESTORING DYSTROPHIN EXPRESSION IN IPSC-DERIVED MUSLCE PROGENITORS
Signature:	Yaoliang Tang
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Point to point response to reviewer's critiques

1. Please note that the editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached and please use this version to incorporate the changes that are requested.

   Answer: Thanks for formatting the manuscript.

2. Please address specific comments marked in the attached manuscript. Please turn on Track Changes to keep track of the changes you make to the manuscript.

   Answer: We addressed each comments marked in the attached manuscript.

3. Please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

   Answer: We revised.

4. When reviewing the highlighting length for the protocol, please watch out for repeated steps. Please ensure that the repeated step has been highlighted previously.

   Answer: We highlighted the repeated steps had been highlighted previously.

5. Figure 1: Please submit multipanel figures (A, B, C, etc.) as a single image file that contains the entire figure. Alternatively, split Figure 1 into two figures and update figures numbers correspondingly.

   Answer: We submitted multipanel figures (A, B, C, D.) as a single image file.

6. References: Please do not abbreviate journal titles; use full journal name.

   Answer: Thanks for pointing it out, we revised.