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Exon Skipping in Directly Reprogrammed Myotubes Obtained from Human Urine-Derived Cells

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Cover Letter

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Review Editor

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Re: JoVE60840R2

"Exon skipping in directly reprogrammed myotubes obtained from human urine-derived cells"

Dear Dr Xiaoyan Cao,

Thank you very much for your consideration of our manuscript and request for a revised version. We also much appreciate the reviewer for their valuable comments and suggestions. We addressed and responded to the reviewer's comments and recommendations. We hope that our responses and revisions to the manuscript satisfactorily address the reviewer's comments and that the manuscript is now suitable for publication in Journal of Visualized Experiments.

By submitting the manuscript, the authors assert that the materials presented in this manuscript have not been published before in another scientific journal or is being considered for publication elsewhere. The authors declare no competing financial interests. All authors have read and approved all versions of the manuscript, its content, and its submission.

Thank you for your consideration in advance,

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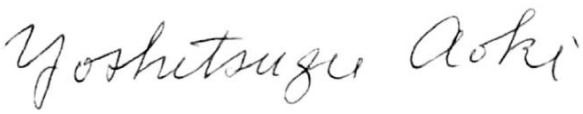

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TITLE:

Exon Skipping in Directly Reprogrammed Myotubes Obtained from Human Urine-Derived Cells

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KEYWORDS:

Duchenne muscular dystrophy (DMD), urine-derived cells, exon skipping, precision medicine, skeletal muscle, clinical trial

SUMMARY:

In this article, we describe a detailed protocol for efficient modelling of Duchenne muscular dystrophy muscle using *MYOD1*-converted urine-derived cells to evaluate the restoration of dystrophin mRNA and protein levels after exon skipping.

ABSTRACT:

Duchenne muscular dystrophy (DMD), a progressive and fatal muscle disease, is caused by mutations in the *DMD* gene that result in the absence of dystrophin protein. To date, we have completed an investigator-initiated first-in-human study at the National Center of Neurology and Psychiatry based on the systemic injection of the morpholino oligonucleotides which are prone to exon-53 skipping. For the effective treatment of DMD, in vitro testing with myoblasts derived from DMD patients to screen drugs and assess patient eligibility before undertaking clinical trials is thought to be essential. Very recently, we reported a new *MYOD1*-converted urine-derived cell (UDC) treated with the histone methyltransferase inhibitor (3-deazaneplanocin A hydrochloride), as a cellular model of DMD. The new autologous UDC might show phenocopy of the disease-specific phenotypes of DMD, leading to the application of precision medicine in a variety of muscle-related diseases. In this article, we describe a detailed protocol for efficient modelling of DMD muscle cells using *MYOD1*-converted UDCs along with reverse transcriptase polymerase chain reaction (RT-PCR), Western blotting, and immunocytochemistry to evaluate the restoration of dystrophin mRNA and protein levels after exon skipping.

INTRODUCTION:

Duchenne muscular dystrophy (DMD), a progressive, fatal muscle disease, is caused by frame-shift mutations in the *DMD* gene that result in the absence of dystrophin protein[1]. Antisense oligonucleotide-based exon skipping therapy is thought to be promising for DMD. This therapy

is based on the conversion of the severer DMD phenotype to the milder Becker muscular dystrophy-like phenotype by altering pre-mRNA splicing to restore the *DMD* reading frame[2]. We have recently completed a first-in-human study based on repeated intravenous administration of the phosphorodiamidate morpholino oligomer (PMO) viltolarsen, which can induce exon 53 skipping in DMD, and demonstrated an excellent safety profile, promising efficacy, and acceptable pharmacokinetic parameters (registered as UMIN: 000010964 and ClinicalTrials.gov: NCT02081625)[3].

However, to develop cost-effective and efficient treatments for the disease, in vitro tests using primary muscle cells obtained from DMD patients are essential for drug screening and patient eligibility verification before undertaking clinical trials, as well as biomarkers that reflect the efficacy of exon skipping therapies during human trials[4]. Very recently, we reported a novel technology to develop patient-specific *MYOD1*-converted urine-derived cells (UDCs)[5,6] as a primary myoblast model of DMD[7]. Thus, to generate the myoblasts, only the collection of urine from patients is required and no invasive procedure is needed. In this article, we describe a detailed protocol for efficient modelling of DMD muscle using *MYOD1*-converted UDCs treated with 3-deazaneplanocin A hydrochloride to evaluate the restored dystrophin mRNA and protein after exon skipping.

PROTOCOL:

The Ethics Committee of the National Center of Neurology and Psychiatry approved this study (approval ID: A2017-018, A2018-029). All individuals gave informed consent before providing urine. All experiments were performed under the relevant guidelines and regulations.

1. Isolation and primary culture of UDCs

NOTE: UDCs were isolated according to a previously published protocol[8,9,10] with some modifications.

1.1. Collect urine samples during spontaneous micturition in sterilized plastic bottles.

NOTE: Sterilization of the external urethral orifice is not needed. Midstream urine is desirable to reduce the risk of viral contamination. If the stock time before the next procedure is >1 h, urine samples should be transferred to 4 °C to preserve the cell viability. However, a temperature of <4 °C should be avoided because insoluble precipitates may appear.

1.2. Centrifuge the entire urine sample at 400 x *g* for 10 min at room temperature.

1.3. Aspirate the supernatant, leaving 1 mL in the tube.

1.4. Resuspend the pellets individually in the remaining 1 mL of urine and then collect those in a single 50 mL tube.

1.5. Add 10 mL of washing buffer consisting of 99 mL of PBS without calcium and magnesium, 1% penicillin/streptomycin (P/S), 0.5 µg/mL amphotericin B, and centrifuge the samples at 200 x *g* for 10 min at room temperature.

1.6. Aspirate the supernatant, leaving 0.2 mL in the tube.

1.7. Resuspend the cell pellets in 4.5 mL of primary medium composed of a 1:1 mixture of high glucose Dulbecco's modified Eagle medium (DMEM) without sodium pyruvate and Ham's F-12 nutrient mix supplemented with recombinant human epidermal growth factor (EGF), insulin, hydrocortisone, epinephrine, T3, transferrin, 10% tetracycline-free fetal bovine serum (FBS), 1% P/S, and 0.5 µg/mL amphotericin B.

1.8. Seed the cells in three wells of gelatine-coated six well plates (total volume of each well, 1.5 mL). Culture humidified at 37 °C and 5% CO_2 for 24 h.

1.9. Add 1.5 mL of the primary medium daily for the next 3 days.

1.10. On day 4, replace the medium with 1.5 mL of growth medium supplemented with recombinant human EGF, insulin, hydrocortisone, epinephrine, T3, transferrin, 15% tetracycline-free FBS, 0.5% L-alanine-L-glutamine, 0.5% nonessential amino acids, and 2.5 ng/mL fibroblast growth factor-basic (bFGF), recombinant human platelet-derived growth factor (PDGF), EGF, and 1% P/S.

1.11. Change the growth medium every other day.

NOTE: UDC colonies appear within a week.

1.12. When the UDC culture becomes 80–90% confluent, remove the medium and wash cells with PBS, split all the cells using 0.25% trypsin-EDTA and seed at 3,000–5,000 cells/cm^2 onto a new gelatine-coated 60 mm dish (passage 1).

NOTE: The UDCs can be stored in liquid nitrogen. UDCs are usually divided at 60−70% confluency in 60 mm culture dish into three stock tubes.

2. Retroviral construct

2.1. Amplify the coding region of *MYOD1* (NM_002478.4) plasmid by polymerase chain reaction (PCR).

NOTE: The mixture for *MYOD1* amplification and conditions for the thermal cycler are shown in **Table 1** and **Table 2**, respectively.

2.2. Detect a single band of about 1,000 bp size by 0.7% agarose gel electrophoresis using 1 µL of the amplified PCR product to confirm that the *MYOD1* sequence is amplified successfully.

2.3. Clean the PCR product using the clean-up kit and determine its concentration with a spectrophotometer.

2.4. Incubate the mixture as shown in **Table 3** at 37 °C overnight to digest retroviral vector with a Tet-on system and puromycin resistant gene at restriction enzyme-targeted regions in the multiple cloning site.

2.5. Detect a single band by 0.7% agarose gel electrophoresis using 1 µL of the digested product to confirm that the retroviral vector was digested successfully.

2.6. Clean the digested product using clean-up kit and determine its concentration by spectrophotometer.

2.7. To clone the amplified *MYOD1* fragment (produced by steps 2.1−2.3) into the digested retroviral vector (produced by steps 2.4−2.6), perform an in-fusion cloning reaction. Set up the reaction as shown in **Table 4**, incubate the reaction for 15 min at 50 °C, and then place on ice.

2.8. Perform transformation using *E. coli* competent cells according to the manufacturer's instructions (**Table of Materials**).

2.9. Select the transformed competent cells by culturing on an LB culture plate consisting of 10 g/L bacto tryptone, 5 g/L bacto yeast extract, 5 g/L NaCl, 15 g/L bacto agar, and 50 mg/L ampicillin.

2.10. Pick up the selected colony and culture in LB culture medium without bacto agar at 200 rpm at 37 °C overnight.

2.11. Purify the *MYOD1*-inserted retroviral vectors using a plasmid purification kit (**Table of Materials**) and quantify using a spectrophotometer.

2.12. Confirm that *MYOD1* is correctly inserted into the retroviral vector by direct sequencing of the PCR product amplified by the forward and reverse primers targeted respectively on both sides across the inserted *MYOD1* sequence.

NOTE: The *MYOD1* sequence can be detected sandwiched between retroviral vector sequences when in-fusion cloning is successful.

2.13. For retroviral production, seed the packaging cells at 50,000 cells/cm^2 on 10 cm collagen-coated plates and culture in DMEM with 10% FBS humidified at 37 °C and 5% CO_2 for 24 h.

2.14. When the packaging cells proliferate to 80% confluency, mix 30 µg of *MYOD1*-inserted retroviral vectors, 30 µg of packaging vectors, and transfection reagent containing cell-penetrating peptide (**Table of Materials**) by vortexing, and incubate them for 10 min.

2.15. Add the incubated mixture to the medium of packaging cells and culture humidified at 37 °C and 5% CO_2.

NOTE: Collagen coating is necessary. Transfection may be better at 24 h or more after seeding because packaging cells easily detach from the culture plate.

2.16. After 4 h or overnight, change the medium to fresh growth medium.

2.17. Collect the viral supernatant and replace with fresh medium at 24 and 48 h after cotransfection and combine the supernatant.

2.18. To concentrate the viral supernatant, mix it with the concentrator reagent and incubate at 37 °C overnight, then centrifuge at 1,500 x *g* for 45 min at 4 °C.

2.19. Filter the retrovirus supernatant through a PVDF filter with 0.45 µm pores.

2.20. Check the titer of the retroviral vector using a quantitative PCR kit and thermal cycler system according to the manufacturer's instructions.

2.21. Divide the viral supernatant into small aliquots and stock them at -80 °C.

3. Infection with *MYOD1*-retroviral vector in UDCs

3.1. Seed the UDCs at 3,000–5,000 cells/cm^2 on a gelatine-coated 60 mm dish.

3.2. After 24 h of seeding, infect the thawed retrovirus (step 2.21) at a multiplicity of infection of 200 by adding hexadimethrine bromide at a concentration of 8 µg/mL.

3.3. After a 24 h incubation humidified at 37 °C and 5% CO_2, replace the culture medium with fresh growth medium containing 1 µg/mL puromycin to select the *MYOD1*-transduced cells. Change the medium every other day.

NOTE: When selecting for *MYOD1*-positive cells, 1 µg/mL puromycin is usually used for selection. The appropriate dose should be determined. Use a plate containing untransfected cells and choose the dose that kills all the cells in 3−5 days. *MYOD1*-positive cells should be selected within 7−10 days after adding puromycin. The *MYOD1*-transduced UDCs can be stored in liquid nitrogen.

4. Myogenic differentiation of *MYOD1*-transduced UDCs treated with 3-deazaneplanocin A hydrochloride (DZNep)

NOTE: Recently, it has been reported that DZNep, a histone methyltransferase inhibitor, could significantly promote the expression of *MYOGENIN*, one of the late muscle regulatory factors, and also lead to myotube differentiation[7].

4.1. Plate *MYOD1*-transduced UDCs in the collagen-coated wells at a density of 3.5 x 10^4 cells/cm^2. Culture humidified at 37 °C and 5% CO_2.

4.2. After 24 h, change the growth medium to differentiation medium composed of high glucose DMEM with L-alanine-L-glutamine, 5% horse serum, ITS supplement, 1 µg/mL doxycycline, and 5 µM DZNep.

NOTE: The 10 mM DZNep solution can be stored at -80 °C for 3 months. Use a defrost freezer and avoid repeated freeze-thaw cycles. Both *MYOD1* activated by doxycycline and DZNep suppress proliferation and promote the myogenic differentiation of UDCs. Therefore, it is recommended that doxycycline and DZNep are added after the induction of myogenic differentiation. DZNep promotes myogenic differentiation of the *MYOD1*-UDCs in a dose-dependent manner. On the other hand, it shows cytotoxicity at a high concentration. Therefore, determine the appropriate concentration of DZNep, ranging from 1−10 µM depending on its effects on myogenic differentiation and cellular bioavailability.

4.3. After 3 days, change the differentiation medium to fresh differentiation medium without DZNep. Then, change the medium every 3 days.

NOTE: UDCs fuse to each other and form myotubes within 1−2 weeks after differentiation.

5. Exon skipping in *MYOD1*-converted UDCs

NOTE: Here, three protocols are described to evaluate exon skipping in patient-derived cells: 1) reverse transcriptase polymerase chain reaction (RT-PCR) of dystrophin mRNA; 2) semiquantification of restored dystrophin protein signal by Western blot; and 3) semiquantification of restored dystrophin fluorescence signal by immunocytochemistry. All the methods can detect exon skipping in a dose-dependent manner.

5.1. Evaluation of exon skipping efficiency by RT-PCR

5.1.1. To transfect antisense oligonucleotide (ASO) into *MYOD1*-converted UDCs obtained from DMD patients on the day 7 after differentiation, mix ASO, transfection reagent (**Table of Materials**), and differentiation medium to a final concentration of 1−10 µM. Culture humidified at 37 °C and 5% CO_2.

5.1.2. After 72 h of incubation with ASO, change the medium to fresh differentiation medium without ASO.

5.1.3. From 3−7 days after ASO transfection, remove differentiation medium and wash 1x with PBS. Add cell lysis buffer, lyse the UDCs and harvest the total RNA using an RNA extraction kit.

5.1.4. Measure the RNA concentration with a spectrophotometer.

5.1.5. Combine the required reagents for one-step RT-PCR reaction in PCR tubes according to **Table 5**.

5.1.6. Place the PCR tubes with the mixture in a thermocycler. Run the thermocycler, according to **Table 6**.

5.1.7. Perform microchip electrophoresis and calculate the exon skipping efficiency using the molar concentration as below.

Exon skipping efficiency (%) = skipped band / (skipped band + non-skipped band) x 100

NOTE: Store the PCR product in a refrigerator at 4 °C for short-term storage or -20 °C for long-term storage.

5.2. Detection of dystrophin after exon skipping by Western blotting

5.2.1. Transfect ASO and culture *MYOD1*-UDCs according to steps 5.1.1 and 5.1.2.

5.2.2. Change the medium every 3 days.

5.2.3. After 2 weeks of differentiation, extract the total protein from the cultured cells using radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors.

5.2.4. Sonicate the lysates on ice and centrifuge at 14,000 x *g* for 15 min at 4 °C.

5.2.5. Collect the supernatant and determine protein concentrations using a BCA protein assay kit.

5.2.6. Add 15 µg of total protein in a 0.5 mL tube and dilute by adding the RIPA buffer containing protease inhibitors to a total volume of 10 µL. Run 15 µg of total protein per lane.

5.2.7. Add sample buffer, reducing agent, and deionized water as shown in **Table 7**. Denature the cell lysates at 70 °C for 10 min.

5.2.8. Prepare the tris-acetate running buffer containing 8.95 g/L tricine, 6.06 g/L tris base, 1.0 g/L sodium dodecyl sulfate (SDS).

5.2.9. Load the sample (20 µL) onto tris-acetate 3−8% gel and perform electrophoresis at 150 V for 75 min.

5.2.10. Prepare the blotting buffer without methanol.

5.2.11. Soak the PVDF membrane for 20 s in methanol and then in the blotting buffer until use (at least 10 min). Cut the PVDF membrane to a size of 6 x 8 cm using mini gels and 8 x 12 cm using midi gels.

5.2.12. Cut the blotting papers to the same size as that of the PVDF membrane and soak those in the blotting buffer until use.

5.2.13. After electrophoresis, cut the gel to the same size as that of the PVDF membrane and soak the gel in distilled water.

5.2.14. Place the blotting papers, PVDF membrane, and gel on the semidry transfer apparatus (**Figure 1**). Transfer at 4 mA/cm^2 for 30 min.

5.2.15. Rinse the membrane 2x with distilled water.

5.2.16. Prepare anti-dystrophin (1:500) and anti-α-tubulin (1:1,200) antibody as the primary antibodies.

5.2.17. Prepare HRP-conjugated anti-mouse antibody (1:100) as a secondary antibody.

5.2.18. Incubate the membranes with the primary antibodies, wash with wash buffer, then incubate with the secondary antibody using an automated Western-processing device (**Table of Materials**) at room temperature.

NOTE: Anti-α-tubulin antibody is usually used as a loading control. The mixed primary antibody solutions, including 1:500 anti-dystrophin and 1:1,200 anti-α-tubulin antibodies work well when the antibody reaction is performed simultaneously.

5.2.19. Rinse the membrane in distilled water.

5.2.20. Detect the proteins using chemiluminescent detection reagent and a charge-coupled device (CCD) camera-based imager.

5.2.21. Analyse the data using appropriate software.

5.3. Detection of dystrophin after exon skipping by immunocytochemistry

5.3.1. Directly reprogram the UDCs into the myotubes in collagen-coated 96 well plate according to steps 4.1−4.3.

5.3.2. Transfect the ASO and culture *MYOD1*-UDCs according to steps 5.1.2 and 5.1.3.

5.3.3. After 2 weeks of differentiation, wash the cells with PBS and fix them in 4% paraformaldehyde for 10 min at 4 °C.

5.3.4. Permeabilize *MYOD1*-UDCs in 0.1% nonionic detergent for 10 min at room temperature and block those with 10% goat serum for 15 min at 37 °C.

5.3.5. Incubate the cells with a primary antibody overnight at 4 °C.

5.3.6. Wash the cells with PBS and incubate those with the secondary antibody for 30 min at room temperature.

NOTE: Here, mouse anti-dystrophin (1:30) is used as the primary antibody, anti-mouse IgG is used as the secondary antibody, and Hoechst (1:10,000) is used for nuclei staining.

5.3.7. Image the plates using a fluorescent microscope and use an analyzer to semiquantify the fluorescence signal automatically in every well under the same condition.

REPRESENTATIVE RESULTS:

We could collect the UDCs easily and non-invasively. UDCs formed colonies within a week after starting primary cell culture we observed a marked proliferative ability. The culture of

UDCs was straightforward, and bacterial or fungal contamination was rare when the procedure was performed correctly.

Figure 2 shows representative phase-contrast images of the UDC colony a week after primary culture (**Figure 2A**) and *MYOD1*-UDCs a week after differentiation (**Figure 2B**). **Figure 3** shows the successful detection of exon skipping in UDCs obtained from DMD patients by RT-PCR. **Figure 3A** shows RT-PCR analysis of dystrophin after antisense oligonucleotide treatment in DZNep-treated *MYOD1*-UDCs derived from a 6-year-old male with an exon 45–54 deletion in the *DMD* gene. The open reading frame was restored by exon 44 skipping. On day 14 following differentiation, we confirmed the induction of exon skipping in a dose-dependent manner (**Figure 3B**). The upper bands denote native products, and the lower bands denote exon 44-skipped products that restored the open reading frame.

Figure 4 shows the successful detection of dystrophin after exon skipping in the UDCs obtained from DMD patients by Western blotting in a dose-dependent manner. We also detected the restored dystrophin expression using immunocytochemistry (**Figure 5**). We measured the intensities of dystrophin with a fluorescent microscope 1 week after the antisense oligonucleotide (ASO) transfection on a 96 well plate (**Figure 5A**). Markedly higher fluorescent signals were observed in *MYOD1*-UDCs treated with ASO than in *MYOD1*-UDCs treated with control ASO (**Figure 5B**).

These results suggest that our new assay can evaluate exon skipping efficiently in *MYOD1*-UDCs obtained from DMD patients at the mRNA and protein level.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic representation of the transfer stack for semidry Western blot. Two papers soaked in the blotting buffer were laid down at the negative terminal, and two papers soaked in the buffer were stacked on top of this. The gel, which has been soaked in the buffer, was laid gently over the PVDF membrane.

Figure 2: Representative images of the UDCs. (**A**) Phase-contrast image of UDCs a week after primary culture. Scale bar = 200 µm. Inset: A magnified image of the area in the white rectangle. (**B**) Phase-contrast image of *MYOD1*-UDCs a week after differentiation. Scale bar = 50 µm. This figure has been modified from Takizawa et al.[7].

Figure 3: Successful evaluation of exon skipping in urine-derived cells (UDCs) obtained from DMD patients by RT-PCR. (**A**) RT-PCR analysis of dystrophin after antisense oligonucleotide treatment in 3-deazaneplanocin A hydrochloride (DZNep)-treated *MYOD1*-UDCs derived from Duchenne muscular dystrophy (DMD) patient with an exon 45–54 deletion. DZNep-treated *MYOD1*-UDCs were also treated with the control antisense at 1–10 µM concentration as controls. The upper bands were unskipped products (Ex 45–54 deletion) that remained out of the reading frame. The lower bands were the exon 44-skipped products (Ex 44–54 deletion and Ex 44 skipped) that restored the open reading frame. (**B**) Skipping efficiency was calculated as (exon 44-skipped transcript molarity)/(native + exon 44-skipped transcript molarity [marked with arrows]) x 100% using a microchip electrophoresis system. One-way ANOVA followed by Bonferroni's post hoc test was used to compare the skipping efficiencies

(n = 3 for each group, ****P < 0.0001). The data are expressed as the mean ± SEM. This figure has been modified from Takizawa et al.[7].

Figure 4: Successful evaluation of exon skipping in urine-derived cells (UDCs) from DMD patients by Western blot. (**A**) Representative Western blot for dystrophin in DZNep-treated *MYOD1*-UDCs from DMD patient with an exon 45–54 deletion after exon 44 skipping. For dystrophin detection, anti-dystrophin (against C-terminal) was used. (**B**) The relative intensities of the bands normalized to α-tubulin expression were compared in patient-derived cells with and without antisense oligonucleotide treatment by performing one-way ANOVA followed by Bonferroni's post hoc test (n = 3 for each group, **P < 0.01, ***P < 0.001, HI = healthy individual). This figure has been modified from Takizawa et al.[7].

Figure 5: Heatmaps of immunocytochemistry for dystrophin after antisense oligonucleotide treatment in DZNep-treated *MYOD1*-UDCs obtained from DMD patient with exon 45–54 deletion. (**A**) Deletion of exon 45–54 restored the open reading frame based on the exon skipping of exon 44. (**B**) The signal intensity was quantified using a fluorescent microscope after 1 week of antisense oligonucleotide transfection on a 96 well plate. One-way ANOVA followed by Bonferroni's post hoc test was used for the comparison (n = 3–4 for each group, ****P < 0.0001). This figure has been modified from Takizawa et al.[7].

Table 1: Mixture for *MYOD1* amplification by RT-PCR.

Table 2: Conditions for the thermal cycler for *MYOD1* amplification.

Table 3: Mixture for a tube to digest retroviral vector.

Table 4: Mixture for the in-fusion cloning reaction.

Table 5: Necessary compounds for one reaction of the one-step RT-PCR.

Table 6: Thermal cycler condition for one-step RT-PCR.

Table 7: Preparation of samples for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

DISCUSSION:

Here, we describe a detailed protocol of exon skipping in *MYOD1*-converted UDCs obtained from DMD patients. Using the assay system, we screened optimal antisense sequences efficiently. We assume that *MYOD1*-converted UDCs can be useful for the investigation of the pathophysiology of the disease.

Evaluation of exon skipping using patient-derived cells at the mRNA level is indispensable for screening new drugs and assessing patient eligibility before undertaking clinical trials. Calculation of exon skipping efficiency can be evaluated only at an mRNA level.

Evaluation of exon skipping at the protein level is also important because dystrophin restoration is important as a surrogate biomarker to predict the benefits of exon skipping. To

date, screening of antisense oligonucleotide sequences is often performed using primary muscle cell lines or immortalized myoblast cell lines including human rhabdomyosarcoma (RD) cells, but we cannot measure the recovery of dystrophin levels using muscle cell lines or RD cell lines because they express this protein endogenously. We can clearly detect the restoration of dystrophin in DMD patient-derived *MYOD1*-UDCs in a dose-dependent manner. In our new assay, we consider that the evaluation of restored protein by Western blotting is superior in quantifiability. On the other hand, evaluation by immunocytochemistry using 96 well plates is ideal for screening many candidate compounds simultaneously.

In this article, we describe a detailed protocol for an efficient modelling DMD muscle using *MYOD1*-converted UDCs along with RT-PCR, Western blotting, and immunocytochemistry to evaluate the restored dystrophin at the mRNA and protein levels after exon skipping. UDCs can be collected noninvasively and easily. Therefore, we assume that the brand-new in vitro assay can be applied to a wide range of basic and translational studies regardless of the type of muscular disorders.

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DISCLOSURES:

The National Center of Neurology and Psychiatry is now developing NS-065/NCNP-01, an exon 53 skipping drug for DMD, with Nippon Shinyaku Co., Ltd.

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Figure 1

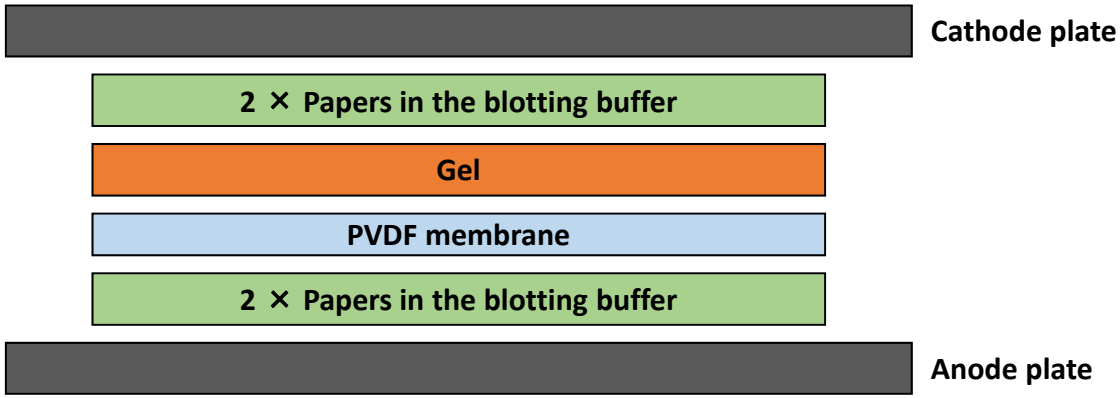

Figure 2

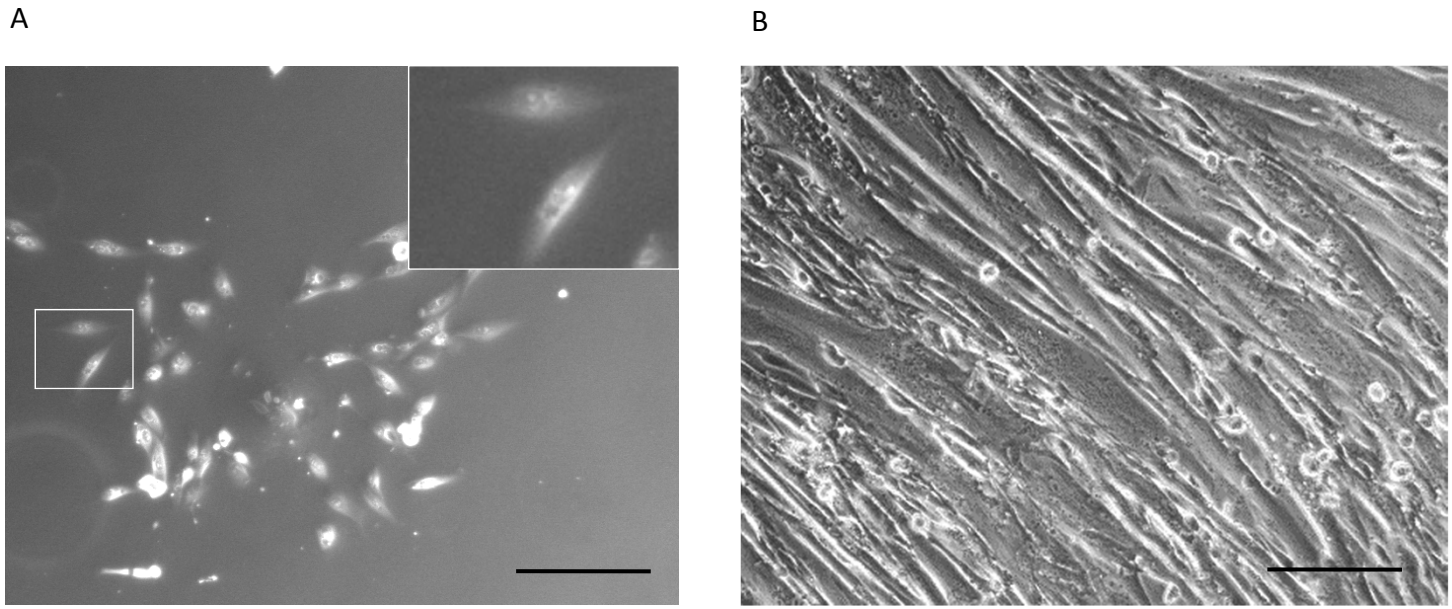

Figure 3

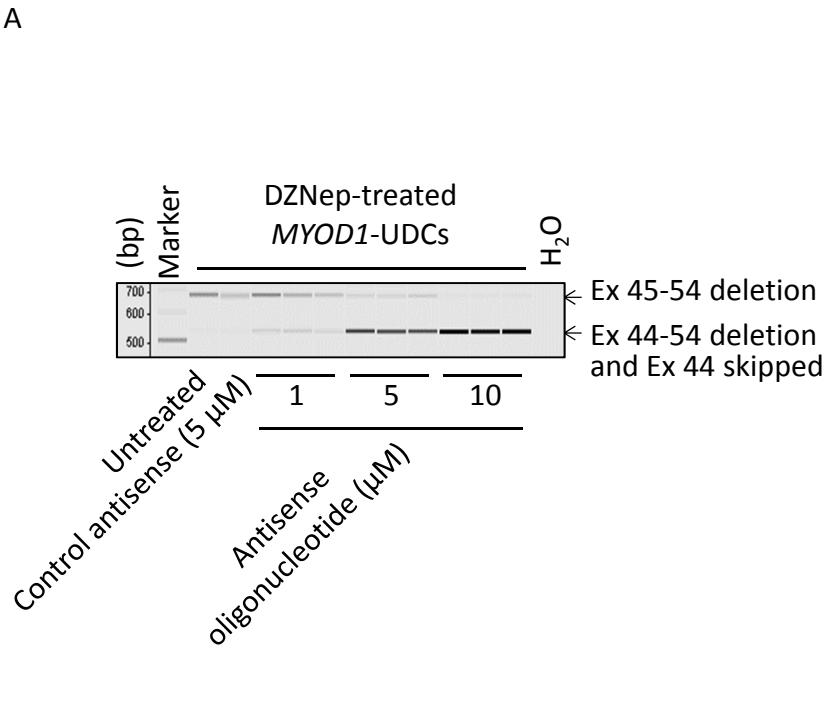

Figure 4

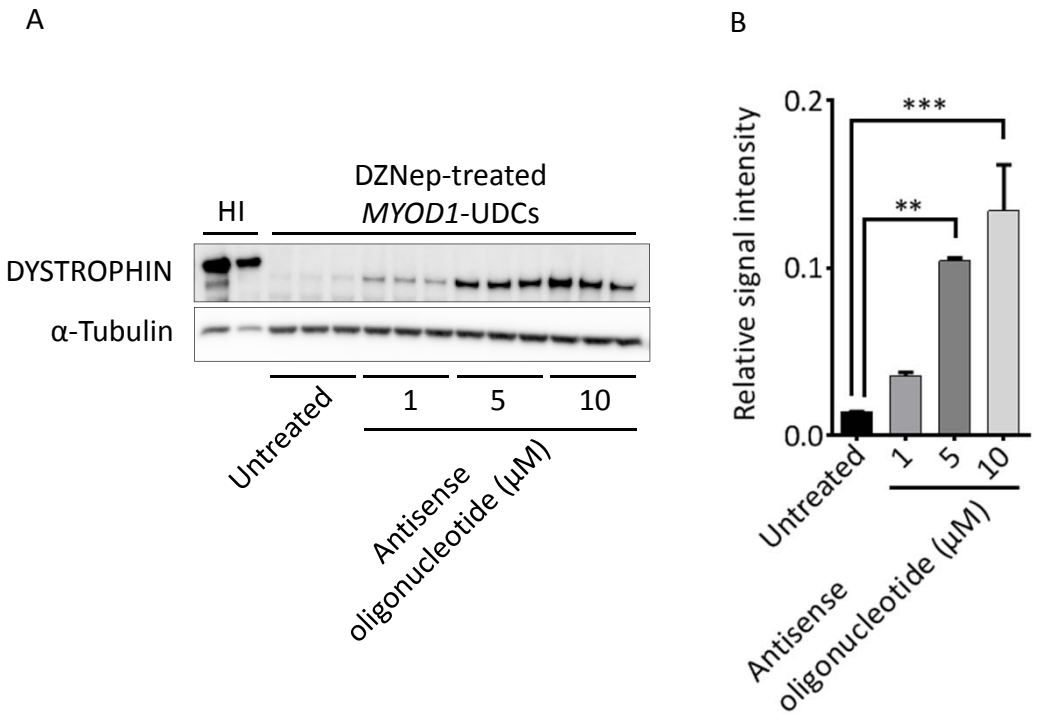

Figure 5

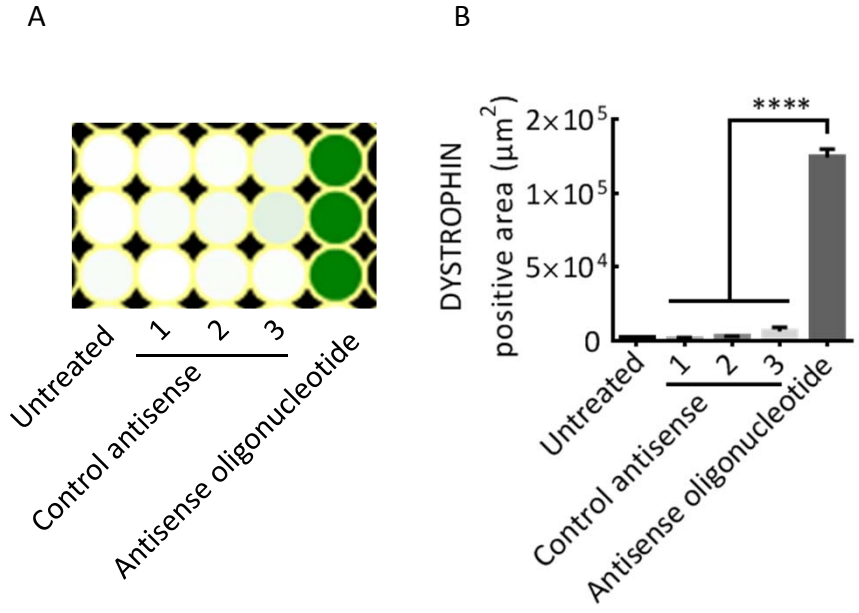

Table 1

Reagent	**Volume**	**Final concentration**
2x PCR premix	12.5 µL	1x
Forward primer	5 pmol	0.2 µM
Reverse primer	5 pmol	0.2 µM
Template	80 ng	
Sterilized distilled water	up to 25 µL	
Total volume per reaction	**25 µL**	

Table 2

Click here to access/download;Table;JoVE Table 2.xlsx

98 °C	10 s	35 cycles
55 °C	10 s	
72 °C	10 s	

Table 3 Click here to access/download;Table;JoVE Table 3_1105.xlsx

Reagent	**Volume**
10x K buffer	2 µL
Retroviral vector （500 ng/µL）	2 µL
Restriction enzyme 1 (2−15 U)	1 µL
Restriction enzyme 2 (2−15 U)	1 µL
Sterilized distilled water	14 µL
Total volume	**20 µL**

Table 4

Reagent	**Volume**
Purified MYOD1 fragment	100 ng
Digested retroviral vector	100 ng
5x Enzyme premix	4 µL
Sterilized distilled water	up to 20 µL
Total volume	**20 µL**

Table 5 Click here to access/download;Table;JoVE Table 5.xlsx

Solution	Volume/Reaction (µL)	Final concentration
RNase-free water	Variable	-
One-step RT-PCR buffer	4	1x
dNTP mix (containing 10 mM of each dNTP)	0.8	400 mM of each dNTP
Forward primer (10 mM)	1.2	0.6 mM
Reverse primer (10 mM)	1.2	0.6 mM
One-step RT-PCR enzyme mix	0.8	-
RNase inhibitor (optional)	Variable	5–10 units/reaction
Template RNA	50–400 ng	
Total volume	**20**	

Table 6

1 cycle	Reverse transcription	30 min	50 °C
1 cycle	Initial PCR activation step	15 min	95 °C
1 cycle	Denaturation	1 min	94 °C
	Annealing	1 min	60 °C
	Extension	1 min	72 °C
1 cycle	Final extension	7 min	72 °C
Hold		∞	4 °C

Table 7

Reagent	**Volume**
Protein (15 µg)	10 µL
Sample buffer (4x)	5 µL
Reducing agent (10x)	2 µL
Deionized water	3 µL
Total volume	**20 µL**

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Material_Rev_1117.xlsx

Name of Material/Equipment	Company	Catalog Number
1% P/S Solution Stabilized	Thermo Fisher	15070-063
Amphotericin B	Sigma Aldrich	A2942
Anti-dystrophin	Abcam	ab15277
Anti-dystrophin	Leica	NCL-DYS1
Anti-mouse IgG, Dylight 488	Vector Laboratories	DK-2488
Anti-α-tubulin	Sigma	T6199
BZ-X800	KEYENCE	BZ-800
CELLBANKER	ZENOAQ	CB011
ChemiDoc MP Imaging System	Bio-Rad	170-8280J1
CloneAmp HiFi PCR premix	Clontech	639298
cOmplete Protease Inhibitor Cocktail	Roche	4693116001
E.coli DH5 α Competent Cells	TAKARA	9057
ECL Prime Western Blotting Detection Reagent	GE healthcare	RPN2232
EGF	Peprotech	AF-100-15
Endo-Porter	GeneTools	2922498000
Extra Thick Blot Filter Paper	Bio-Rad	1703965
EzFastBlot HMW	Atto	AE-1460
fibroblast growth factor-basic	Sigma-Aldrich	F0291
Glutamax	Thermo Fisher Scientific	35050-061
GP2-293 packaging cells	Clontech	631458
Ham's F-12 Nutrient Mix	Thermo Fisher Scientific	11765-054
High glucose DMEM with GlutaMAX-I	Thermo Fisher Scientific	10569-010
High glucose DMEM without sodium pyruvate	GE Healthcare	SH30022.01
HiSpeed Plasmid Purification Kit	QIAGEN	12643
Histofine Simple Stain MAX PO	NICHIREI BIOSCIENCE INC.	424151
Hoechst 33342	Thermo Fisher Scientific	H3570
Human PDGF-AB	Peprotech	100-00AB-10UG
iBind Flex Solution	Thermo Fisher Scientific	SLF2020
iBind Flex Western Device	Thermo Fisher Scientific	SLF2000
Immobilon-P Transfer Membrane (PVDF)	MERCK	IPVH304F0
In-Fusion HD cloning Kit	Clontech	639648
ITS Liquid Media Supplement	Sigma-Aldrich	I3146

MILTEX HV 0.45 µm filter	MERCK	SLHV033RS
MultiNA	SHIMADZU	MCE-202
MYOD1 (GFP-tagged)	ORIGENE	RG209108
NanoDrop	Thermo Fisher	ND-ONE-W
Nonessential amino acids	Thermo Fisher	11140-050
NucleoSpin Gel and PCR Clean-Up Kit	Clontech	740986.20
NuPAGE 3-8% Tris-Acetate Protein Gels	Invitrogen	EA03785BOX
NuPAGE Antioxidant	Invitrogen	NP0005
NuPAGE LDS Sample Buffer	Invitrogen	NP0007
NuPAGE Sample Reducing Agent	Invitrogen	NP0009
NuPAGE Tris-Acetate SDS Running Buffer	Invitrogen	LA0041
One Step TB Green PrimeScript RT-PCR Kit	TAKARA	RR066A
PBS	Thermo Fisher Scientific	14190-250
Pierce BCA Protein Assay Kit	Thermo Scientific	23227
Polybrene Infection / Transfection Reagent	Sigma-Aldrich	TR-1003
pRetroX-TetOne-Puro Vector	Clontech	634307
Puromycin	Clontech	631305
QIAGEN OneStep RT-PCR Kit	Qiagen	210212
REGM Bullet Kit	Lonza	CC-3190
REGM SingleQuots	Lonza	CC-4127
Retrovirus Titer Set	TAKARA	6166
Retro-X Concentrator	Clontech	631455
RIPA buffer	Thermo Fisher Scientific	89901
RNeasy kit	Qiagen	74104
Tetracycline-free foetal bovine serum	Clontech	631106
Triton-X	MP Biomedicals	9002-93-1
Trypsin-EDTA (0.05%)	Gibco	25300054
XCell SureLock Mini-Cell	Invitrogen	EI0001
Xfect transfection reagent	Clontech	631317

Comments/Description

Cell culture

Western blot (WB)
Immunocytochemistry(ICC)
ICC
Western bot and ICC
Fluorescent microscope
Cell stock in liquid nitrogen
WB
Retroviral production
Protein extraction for WB

WB
Cell culture
ASO transfection
WB
WB
Cell culture
Cell culture
Retroviral production
Cell culture
Cell culture
Cell culture
Retroviral production
WB
ICC
Cell culture
WB
WB (Automated mestern-processing device)
WB
Retroviral production
Cell culture

Retroviral production
Microchip electrophoresis
Retroviral production
Spectrophotometer
Cell culture
PCR clean up
WB
WB
WB
WB
WB
Titer check of retroviral vector
Cell culture
WB
Retroviral infection
Retroviral vecor
Cell culture
PCR
Material for growth medium of UDCs
Material for primary medium of UDCs
Titer check of retroviral vector
Retroviral production
WB
RNA extraction for PCR
Cell culture
ICC
Cell culture
WB
Transfection of plasmids into packaging cells

Updated on 12th of December 2019

OUR RESPONSES TO THE REVIEWER'S COMMENTS ARE SHOWN IN RED.

We truly appreciate all the constructive comments and suggestions from the reviewers. We have adopted all the suggestions in our revised manuscript. The following are our point-to-point responses to the reviewers' comments (the comments are shown with Italic font).

Editorial comments:

The manuscript has been modified and the updated manuscript, 60840_R0.docx, is attached and located in your Editorial Manager account. Please use the updated version to make your revisions.

1. The updated manuscript (60840_R2_RE) is attached and please use this version to incorporate the changes that are requested.

Response: Thank you for your kind assistance.

2. Please revise lines 27-31, 42-43, 45-47, 381-382, 387-390, 456-458, 463-466, 470-474, 475-477, 482-484 to avoid textual overlap with previously published work.

Response: Based on your suggestion, we carefully revised the lines to avoid textual overlap with previously published work.

3. For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Response: We clearly answered how the steps were performed or added references to published material specifying how to perform the protocol action.

4. 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.

Response: We addressed specific comments marked in the attached manuscript and turned on the Word Track Changes to keep track of the changes we made to the manuscript.

5. Figures: Please add panel labels (A, B, etc.) in the figures.

Response: We have added panel labels in the figures based on your comment.

6. Table of Materials: In the comments column, please indicate in which step the commercial kit/reagent is used if possible. Otherwise it is not clear (for instance, two transfection reagents are listed and it is unclear which reagent is used in which step).

Response: We indicated in which step the commercial kit/reagent was used in the comments column.