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Direct Reprogramming of Human DMD Fibroblasts into Myotubes for In Vitro Evaluation of Antisense-Mediated Exon Skipping and Exons 45–55 Skipping Accompanied by Rescue of Dystrophin Expression

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Abstract

Antisense oligonucleotide-mediated exon skipping is a promising therapeutic approach for the treatment of various genetic diseases and a therapy which has gained significant traction in recent years following FDA approval of new antisense-based drugs. Exon skipping for Duchenne muscular dystrophy (DMD) works by modulating dystrophin pre-mRNA splicing, preventing incorporation of frame-disrupting exons into the final mRNA product while maintaining the open reading frame, to produce a shortened-yet-functional protein as seen in milder Becker muscular dystrophy (BMD) patients. Exons 45–55 skipping in *dystrophin* is potentially applicable to approximately 47% of DMD patients because many mutations occur within this "mutation hotspot." In addition, patients naturally harboring a dystrophin exons 45–55 in-frame deletion mutation have an asymptomatic or exceptionally mild phenotype compared to shorter in-frame deletion mutations in this region. As such, exons 45–55 skipping could transform the DMD phenotype into an asymptomatic or very mild BMD phenotype and rescue nearly a half of DMD patients. In addition, this strategy is potentially applicable to some BMD patients as well, who have in-frame deletion mutations in this region. As the degree of exon skipping correlates with therapeutic outcomes, reliable measurements of exon skipping efficiencies are essential to the development of novel antisense-mediated exon skipping therapeutics. In the case of DMD, researchers have often relied upon human muscle fibers obtained from muscle biopsies for testing; however, this method is highly invasive and patient myofibers can display limited proliferative ability. To overcome these challenges, researchers can generate myofibers from patient fibroblast cells by transducing the cells with a viral vector containing MyoD, a myogenic regulatory factor. Here, we describe a methodology for assessing *dystrophin* exons 45–55 multiple skipping efficiency using antisense oligonucleotides in human muscle cells derived from DMD patient fibroblast cells.

Keywords

Phosphorodiamidate morpholino oligomer (PMO); Duchenne/Becker muscular dystrophy (DMD/BMD); Dystrophin; Exon skipping; Fibroblast; Myotube; NS-065/NCNP-01; Golodirsen; Direct reprogramming; Eteplirsen

1 Introduction

Antisense therapeutics have come a long way since their advent approximately 30 years ago. Continuous advancements in anti-sense oligo chemistries have led to the recent FDA approval of the first-ever marketable drugs for treating Duchenne muscular dystrophy (DMD) [1] and spinal muscular atrophy (SMA) [2–4], two of the most lethal genetic diseases among children worldwide. Other antisense drugs have been approved for treating age-related macular degeneration [5], familial hypercholesterolemia [6], and veno-occlusive disease [7].

Antisense oligonucleotides (AOs) function through a number of mechanisms, including exon skipping, exon inclusion, and RNase-H activation. Antisense-mediated exon skipping works by modulating pre-mRNA splicing, preventing incorporation of mutation-carrying exons and/or adjacent exons into the final mRNA product while maintaining the open reading frame, producing a shortened-yet-functional protein [8]. Sarepta Therapeutics' eteplirsen (marketed as Exondys 51) is a 30-nucleotide phosphorodiamidate morpholino oligomer (PMO) AO which facilitates skipping of *dystrophin* exon 51 [9]. Several other PMOs, including golodirsen (Sarepta Therapeutics) and NS-065/NCNP-01 (NS-Pharma) targeting DMD exon 53, are currently in clinical trials [10].

Crucial to the success of most exon skipping strategies is the degree to which functional protein can be restored, such as in the case of DMD where patients lack sufficient levels of dystrophin protein [11]. Suitable methods of quantifying exon skipping and protein rescue are therefore essential to the development of future exon skipping therapies. The recent history of another exon skipping drug candidate, BioMarin's drisapersen, a 2′O-methyl phosphorothioate AO, highlights this principle as it was rejected by the FDA in part due to the inability to restore dystrophin protein to therapeutically beneficial levels [12, 13]. Likewise, controversy still surrounds the FDA's approval of eteplirsen as clinical trial data showed that the drug was only able to rescue dystrophin protein to 0.2–0.3% of normal [14].

As the clinical severity of DMD and Becker muscular dystrophy (BMD) varies depending on mutation pattern [15], it is important to consider which exons to target for exon skipping. For example, patients harboring *dystrophin* exons 45–55 deletion mutations have an exceptionally mild phenotype [16, 17], even when compared to other in-frame exon deletion patterns [15], suggesting that multiple exon skipping of *dystrophin* exons 45–55 could be a particularly beneficial therapeutic approach [18]. Exon skipping and protein rescue quantification in DMD preclinical and clinical studies generally involves the use of muscle tissue obtained via patient muscle biopsy [19–21]; this method produces several challenges. First, muscle biopsies can be invasive and discomforting for patients. The process of obtaining a muscle biopsy can also be complicated, as it requires coordination between several health specialists (i.e., surgeon, pathologist, lab personnel) as well as the use of specialized cryopreservation techniques, as opposed to preservation in formalin which is routinely done with other tissue biopsies [22]. Additionally, the amount of primary muscle cells obtained via biopsy is limited and these cells display poor proliferative ability [23], resulting in low reproducibility of experimental analysis due to lack of available sample. Finally, with the advancement of highly sensitive molecular diagnostic techniques, such as

multiplex ligation-dependent probe amplification (MLPA) [24], muscle biopsy is now less frequently used for diagnosis [25, 26], further curbing access to patient muscle samples for drug discovery.

It is possible to transdifferentiate fibroblasts into myoblasts in vitro via ectopic expression of the myogenic regulatory factor, MyoD [27–29]. By utilizing muscle cells obtained via direct reprogramming of patient fibroblast cells, one can less invasively obtain a suitable cell model for assessing the exon skipping potential of novel antisense drugs, circumventing many of the challenges associated with a patient muscle biopsy. In this chapter, we discuss a methodology for direct reprogramming of DMD patient fibroblast cells into myoblasts, their differentiation into myotubes, and assessment of exon skipping efficiency using phosphorodiamidate morpholino oligomer (PMO) antisense drugs which catalyze *dystrophin* exons 45–55 skipping [30, 32].

2 Materials

2.1 Cell Culture

1. Growth Media: DMEM/F-12 (Invitrogen), 10% fetal bovine serum (FBS), 1% penicillin/streptomycin. For a T225 cell culture flask, add 36 mL DMEM/F12, 4 mL FBS, and 400 μL pen/strep. Store at 4 °C.
2. Differentiation Media: DMEM/F-12 (Invitrogen), 2% horse serum, 1× ITS Liquid Media Supplement (Sigma-Aldrich), 1% penicillin/streptomycin. Store at 4 °C.
3. DMD fibroblast cells from patients harboring out-of-frame deletion mutations of *dystrophin* exons 45–50 (GM05017) and exons 46–50 (GM05162) can be obtained from Coriell Cell Repository (Camden, NJ, USA).

2.2 MyoD Transduction and FACS

1. Vectors: pRetroX-IRES-ZsGreen1 expression vector (Clontech) containing a subcloned pUC57 vector harboring the human MYOD1 coding sequence, pVSV-G envelope vector, and gap-pol expression vector.
2. Calcium Chloride: 2.5 M solution in water.
3. Polybrene (Sigma-Aldrich): 1:1000 in media, or for a T225 flask, 40 μL in 40 mL.
4. Hank’s Balanced Salt Solution (HBS).

2.3 Antisense PMO Transfection

1. Morpholinos (PMOs) designed according to the guideline as previously published [31]. Purchase from GeneTools LLC, Philomath, Oregon, USA. Exon 45 PMO: GACAACAGTTTGCCGCTGCCCAATGCCATC; Exon 51 PMO: AGGTTGTGTCACCAGAGTAACAGTCTGAGT; Exon 52 PMO; GGTAATGAGTTCTTCCAACTGGGGACGCCT; Exon 53 PMO: GTTCTTGTACTTCATCCCACTGATTCTGAA; Exon 54 PMO:

GAGAAGTTTCAGGGCCAAGTCATTTGCCAC, Exon 55 PMO: TCTTCCAAAGCAGCCTCTCGCTCACTCACC.

2. Endo-Porter (GeneTools LLC, Philomath, Oregon, USA).
3. Opti-MEM (Invitrogen, Carlsbad, California, USA): Add 1% pen/strep. Store at 4 °C.

2.4 RT-PCR and Sequencing of Exon-Skipped Transcripts

1. TRIzol (Invitrogen, Carlsbad, California, USA).
2. SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, California, USA).
3. *DMD* forward primer: GACAAGGGCGATTTGACAG; *DMD* reverse primer: TCCGAAGTTCACTCCACTTG; *GAPDH* forward primer: TCCCTGAGCTGAACGGGAAG; *GAPDH* reverse primer: GGAGGAGTGGGTGTCGCTGT.

2.5 Immuno-cytochemistry

1. 4% paraformaldehyde in PBS (VWR): store at 2–8 °C.
2. Permeabilizing/Blocking solution: PBS containing 0.5% Triton X-100 and 10% goat serum.
3. Anti-dystrophin primary antibody: NCL-DYS1 (Novocastra).
4. Anti-desmin primary antibody: NCL-DES-DERII (Novocastra).
5. Anti-myosin heavy chain primary antibody: NCL-MHCf (Novocastra).
6. Anti-mouse IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor 546.
7. ProLong Gold Antifade Mountant (Invitrogen, Carlsbad, California, USA).

3 Methods

3.1 Retrovirus Preparation

1. Seed HEK293T cells in a T225 flask and culture in growth media until 70–80% confluent. Incubate cells in a CO_2 incubator at 37 °C.
2. Wash 70–80% confluent HEK cells with PBS twice and add 40 mL of fresh GM 1–2 h before adding plasmids (*see* Note 1).
3. Combine plasmids in a 50-mL tube at a ratio of 1:1:2 (pVSVG: 18.75 μg; pGP: 18.75 μg; pMyoD: 37.5 μg).
4. Add 400 μL of 2.5 M $CaCl_2$ solution per flask and fill to 4 mL with distilled water (DW).
5. Add plasmid solution dropwise into 4 mL of 2× HBS.

6. Gently aerate the solution using an autopipette, then incubate at room temperature for 20 min.
7. Remove 20 mL of growth media from the flask containing HEK cells and place it in a 50-mL tube. Add the plasmid solution to the tube containing growth media and mix, then add the transfection solution to the flask opposite the cell layer side. Incubate overnight.
8. After overnight incubation, replace the transfection solution with fresh growth media and culture an additional 2–3 days.
9. Collect viral supernatant in fresh 50 mL tubes and centrifuge at 1000 rpm (215 × *g*) for 3 min at room temp to remove debris.
10. Under a flame, filter viral solution using a Corning bottle-top vacuum filter, then prepare aliquots and store at −80 °C.

3.2 MyoD Transduction and FACS

1. Seed patient fibroblast cells in a T225 flask and culture in growth media until 70–80% confluent. Incubate cells in a CO_2 incubator at 37 °C.
2. Add virus solution to 70–80% confluent fibroblasts. If using 40 mL of media in T225 flasks, add 40 μL of polybrene to the virus solution. Incubate for 24 h.
3. Replace virus solution with fresh growth media and incubate an additional 48 h, or until many GFP+ cells (>50%) are observed.
4. To prepare cells for FACS sorting, rinse cells twice with PBS and detach cells with trypsin–EDTA (*see* Note 2); for a T225 flask, use 5 mL of trypsin–EDTA.
5. Stop the trypsin reaction by adding 40 mL of fresh growth media and centrifuge cells at 1000 rpm for 5 min. Discard the supernatant and resuspend the pellet in 1 mL of PBS.
6. Filter cells using a BD Falcon Tube with cell strainer cap, then add 20 μL of propidium iodide solution (*see* Note 3).
7. Sort cells using flow cytometry.
8. Adjust sorted GFP+ cells to 2×10^5/mL with growth media and seed cells at 1×10^5 cells in 500 μL of growth media into each well of a collagen-coated 12-well plate. Incubate cells for 24 h.
9. Replace growth media with differentiation media and incubate until myotubes form. Change media every 2–3 days.

3.3 Antisense PMO Transfection

1. Prepare morpholinos (PMOs) for transfection by warming at 70 °C for 15 min. Check to ensure no aggregates remain in the tube; if necessary, warm for an additional 10–15 min to remove any aggregates.

2. Vortex PMO tubes briefly and then spin down. Cool to room temperature before transfecting.
3. Prepare PMOs and Endo-Porter transfection reagent in differentiation media according to desired final concentration and the number of PMOs used. For single exon skipping using one PMO at a final concentration of 10 μM in 500 μL of differentiation media, combine 10 μL of PMO, 3 μL of Endo-Porter, and 492 μL of differentiation media in a tube.
4. Incubate cells with PMO media for 48 h at 37 °C in a CO_2 incubator. After 48 h, change to fresh differentiation media and incubate an additional 72 h at 37 °C in a CO_2 incubator, then collect cells for analysis.

3.4 RNA Isolation

1. Remove media from the dish and add 1 mL of TRIzol (Invitrogen) reagent.
2. Using a micropipette, rinse the cells on the dish repeatedly with the 1 mL of TRIzol until they become detached and transfer the TRIzol–cell mixture to a fresh 1.5 mL microcentrifuge tube (*see* Note 4). Incubate cells in TRIzol at room temperature for 5 min.
3. Add 200 μL of chloroform per 1 mL of TRIzol used.
4. Cap tubes and shake vigorously for 15 s. Incubate at room temperature for 3 min.
5. Centrifuge at 12,000 × *g* (rcf) for 15 min at 4 °C.
6. Transfer clear aqueous phase into fresh tubes (*see* Note 5).
7. Add 500 μL of isopropyl alcohol per 1 mL of TRIzol used.
8. Add 2 μL of glycogen and vortex. Incubate at room temperature for 10 min.
9. Centrifuge at 12,000 × *g* for 10 min at 4 °C.
10. Remove supernatant and wash with 1 mL of 75% ethanol and vortex. Centrifuge at 7500 × *g* for 5 min at 4 °C.
11. Remove supernatant and air-dry pellet for 10–15 min (*see* Note 6).
12. Dissolve pellet in 35 μL of RNase-free water and incubate at 60 °C for 10 min. Store in −80 °C freezer.

3.5 RT-PCR and Sequencing of Exon-Skipped Transcripts

1. Use 200 ng of total RNA and SuperScript III Reverse Transcriptase to generate cDNA.
2. Perform RT-PCR reaction using the following settings: 94 °C for 15 s, 60 °C for 30 s, 68 °C for 75 s, for 35 cycles.
3. Run RT-PCR products on a 1.5% agarose gel—135 V for 5 min, then 100 V for 35 min.

4. Excise bands of interest from the gel and use a gel and PCR cleanup kit to retrieve RT-PCR products.

5. Prepare RT-PCR products for Sanger Sequencing according to sequencing facility instructions.

3.6 Immuno-cytochemistry

1. Discard old media by pipetting and fix cells with 500 μL/well of 4% paraformaldehyde (PFA) for 5 min at room temp.

2. Wash cells with 1 mL phosphate buffered saline (PBS) containing 0.1% Triton X-100 (0.1% PBST) for 5 min under agitation. Repeat three times.

3. Permeabilize and block with 500 μL/well of PBS containing 0.5% Triton X-100 (0.5% PBST) and 10% goat serum for 20 min.

4. Incubate each well with 300 μL of 0.5% PBST containing 10% goat serum and primary antibody for 1 h at room temperature. Use the following ratios for antibodies: anti-desmin (NCL-DES-DERII, Novocastra) 1:30, anti-dystrophin (NCL-DYS1, Novocastra) 1:30, anti-myosin heavy chain (NCL-MHCf, Novocastra) 1:30.

5. After incubation, wash cells with 1 mL 0.1% PBST for 5 min under agitation. Repeat three times.

6. Incubate cells with anti-mouse IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor 546 (1:500) in 0.1% PBST for 1 h at room temp.

7. Wash cells with 1 mL of 0.1% PBST for 5 min under agitation. Repeat three times.

8. Add 300 μL of aqueous antifade reagent containing DAPI to each well.

9. Incubate for at least 30 min and observe using fluorescence microscope. Store plates at 4 °C.

4 Notes

1. When washing cells with PBS, always add PBS to the side of the flask opposite the cell layer to avoid sloughing off cells. Using PBS prewarmed in a 37 °C water bath is also helpful to reduce cell stress.

2. When performing a trypsin digest to detach cells, placing the flask in a 37 °C CO_2 incubator can help speed the detachment. Gently tapping the side of the flask against a lab bench or table edge can help loosen cell attachment, although this can also cause cell clumping and should not be done until the latter part of the cell-detachment process.

3. When preparing cells for FACS sorting and using the cell strainer cap, place the pipette tip firmly against the straining mesh of the cap, but not so firm as to puncture through the mesh. Pipette slowly and smoothly.

4. When collecting total RNA from cells using TRIzol, gentle scraping of the bottom of the dish with the pipette tip can help detach cells. Pipetting should be done gently, as vigorous pipetting can cause the liquid streaming from the pipette tip to strike the bottom of the dish with such force that splashing can occur; this can result in contamination if adjacent wells are being used for other treatment groups.

5. When transferring the clear aqueous phase, it is important to pipette very slowly and smoothly to avoid pipetting the red phenol phase. If phenol phase contamination occurs, another round of centrifugation at 12,000 × *g* can separate out the different phases again.

6. Excess ethanol can be removed via careful pipetting; we do not recommend using a micropipette tip larger than 2–20 μL, to avoid aspirating the pellet. Alternatively, use a micropipette tip (0.2–2 μL) and capillary action to remove excess ethanol.

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