Title

Validation and Detection of Exon Skipping Boosters in DMD Patient Cell Models and mdx Mouse

Authors

Barthelemy, Florian
Wang, Dereck
Nelson, Stanley F
et al.

Publication Date

2018

DOI

10.1007/978-1-4939-8651-4_19

Chapter 19

Validation and Detection of Exon Skipping Boosters in DMD Patient Cell Models and mdx Mouse

Florian Barthelemy, Dereck Wang, Stanley F. Nelson, and M. Carrie Miceli

Abstract

Duchenne muscular dystrophy (DMD) is caused by mutations in the *DMD* gene. Most deletions, duplications, or indels lead to shift of mRNA reading frame, which prevent the production of dystrophin protein. DMD is the leading fatal genetic disorder in childhood. One therapeutic strategy aims to skip one or more exons to restore reading frame to enable the production of internally truncated proteins with partial functionality. However, to date the efficiency of this strategy is suboptimal. Here we present methods for assessing exon skipping using AON alone or in combination with skip booster in the context of human DMD patient fibroblast derived myotubes and in the mdx mouse model of DMD.

Key words Dystrophin, Exon skipping, Dantrolene, Muscular dystrophies, Therapies

1 Introduction

DMD is the most common childhood lethal genetic disease, affecting 1 in 3500 male births [1]. Mutations in *DMD* result in the loss of expression of dystrophin protein, critical to muscle and cardiac health. Antisense oligonucleotide (AON)-mediated exon skipping is personalized genetic medicine aimed at restoring DMD mRNA reading frame to rescue expression of a partially functional dystrophin protein [2]. Initial DMD exon skipping trials using Eterplirsen/Exondys51, an exon51 skipping AON, demonstrated dystrophin rescue in a small cohort treated for 2 years, leading to its recent FDA approval [3, 4]. Boys receiving the drug walked further in 6 min walked test than matched historical controls. Levels of rescued dystrophin remain suboptimal and even modest increases in dystrophin expression predict increased functional efficacy [5, 6]. Here we describe a methodology for identification and validation of agents that boost exon skipping and assessing their efficacy alone or in combination with AON in human mutation specific DMD culture models and the mdx DMD mouse model.

Toshifumi Yokota and Rika Maruyama (eds.), *Exon Skipping and Inclusion Therapies: Methods and Protocols*, Methods in Molecular Biology, vol. 1828, https://doi.org/10.1007/978-1-4939-8651-4_19,

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing of waste materials (*see* **Note 1**). We do not add sodium azide to reagents.

2.1 Establishment of Induced Directly Reprogrammed Myotubes

1. Collagenase Type AFA (*see* **Note 2**).
2. Dulbecco's Phosphate Buffered Saline (D-PBS).
3. Hank's Balanced Salt Solution (HBSS).
4. Clorox Bleach (*see* **Note 3**).
5. 1% Gelatin: Weigh 5 g of gelatin (from pork skin) powder. Combine with 500 mL endotoxin-free (e.g., MilliQ) water in a sterile bottle. Swirl the bottle to mix (At this stage, the gelatin is not soluble). Autoclave for 30 min (per SOP-SP-003, Sterilization of Solutions) within 2 h after mixing. Cool the 1% gelatin solution to room temperature. Store the 1% gelatin solution bottle at 4–8 °C in a refrigerator until use (*see* **Note 2**).
6. Feather Disposable Scalpel Handle #10.
7. Protamine Sulfate (1000×): Weigh 10 mg of protamine sulfate powder. Make up to 1 mL with of endotoxin-free (e.g., MilliQ) water. Swirl to mix and filter the solution using a 22 μM filter. Aliquot and store the solution at 4–8 °C in a refrigerator until use (*see* **Note 2**).
8. 4-OH Tamoxifen: Weigh 0.97 mg of 4-OH tamoxifen and make up to 1 mL with 200 proof ethanol. Swirl to mix and filter the solution using a 22 μM filter. Aliquot and store the solution at 4–8 °C in a refrigerator until use (*see* **Notes 2** and **4**).
9. Human fibroblast growth media (GM)**:** 15% FBS, 1% nonessential amino acids, 1% pen/strep, DMEM (*see* **Note 5**).
10. Human fibroblast differentiation media (DM): 2% horse serum, 1% pen/strep, DMEM/Ham's F-10 (V/V), 2% insulin–transferrin–selenium mix (*see* **Note 5**).
11. Lentiviruses (LV) RRL-SIN-cPPT-hCMV-hTERT and LV-MyoD-ERT-IRES-PURO (*see* **Note 6**).

2.2 Immunofluorescence

1. 4% and 2% PFA solutions: dilute 16% paraformaldehyde aqueous solution in PBS to achieve a 4% final solution and a 2% solution (*see* **Note 2**).
2. 1.5% BSA solution: Weigh 1.5 g of BSA. Make up to 100 mL with PBS (*see* **Notes 2** and 7).

3. 0.25% Triton X-100 solution: prepare 0.25 mL of Triton X-100 in a bottle and make up to 100 mL with PBS.
4. 0.1% Triton X-100: prepare 0.1 mL of Triton X-100 in a bottle and make up to 100 mL with PBS.
5. MHC antibody (Developmental Studies Hybridoma Bank at the University of Iowa, cat#MF 20).
6. Monoclonal Mouse Anti-MyoD1 (Dako, cat# M3512).
7. igG1 antibody (BD Pharmigen, cat# 554121).
8. Purified Mouse IgG2b (Thermo Fisher Scientific, cat# 02–6300.
9. DAPI stain mounting media.

2.3 Exon Skipping and Drug Treatment

1. Oligofectamine.
2. Opti-MEM.
3. Human exon 45 AON (2′OMe-PS): CCA AUG CCA UCC UGG AGU UCC UGU AA.
4. Human exon 44 AON (2′OMe-PS): UGU UCA GCU UCU GUU AGC CAC UGA.
5. 100 μM 2′OMe-PS solution: suspend the oligonucleotide in a sterile solvent (Water for short term usage or 1× TE pH 7.0) to achieve the desired concentration. Vortex well and allow the solution to equilibrate. After 5 min, vortex again to complete the resuspension. Aliquot and place at −20 °C.
6. Revonto solution: Weight 151 mg of revonto. Make up to 1 mL with of endotoxin-free (e.g., MilliQ) water. Swirl to mix and filter the solution using a 22 μM filter. Aliquot and store the solution at 4–8 °C in a refrigerator until use (*see* **Note 8**).
7. Mannitol solution: Weight 150 mg of Mannitol. Make up to 1 mL with of endotoxin-free (e.g., MilliQ) water. Swirl to mix and filter the solution using a 22 μM filter. Aliquot and store the solution at 4–8 °C in a refrigerator until use.
8. Primers.
9. Superscript IV.
10. Platinium taq.
11. Random primers.
12. 2100 Bioanalyzer.
13. DNA 1000 chips.

2.4 Exon Skipping and Drug Treatment (In Vivo)

1. Dulbecco's Phosphate Buffered Saline (D-PBS).
2. Glucose/fructose solution: weight 250 mg of glucose and 250 mg of fructose and make up to 10 mL with PBS. Filter using a 22 μM filter.

3. Morpholino mouse M23: GGC CAA ACC TCG GCT TAC CTG AAA T.
4. Morpholino solution: Solubilize PMO at the desired concentration in Sterile-Filtered Glucose–Fructose Solution or in PBS.
5. Dantrolene chow: Formulate dantrolene sodium from capsules (NDC 0115-4433-1) into 58YP chow at 0, 1121, and 1328 parts per million (ppm) and irradiate.

2.5 RNA Extraction (from Tissue)

1. RNA Isolation Kit: RNeasy Mini Kit.
2. TissueRuptor II.
3. TissueRuptor Disposable Probes.
4. Inactivate potential RNase enzymes on disposable probes by soaking in DEPC water overnight. Then wrap in aluminum foil and autoclave for 30 min.

2.6 Reverse Transcription and ddPCR for Mouse Exon Skipping

1. RT-ddPCR Supermix for Probes.
2. Droplet Generation Oil for Probes.
3. DG8 Cartridge and Gasket.
4. *Dmd* splice junctions of exon 22–24 (exon skip) primers and probe are:
   (a) Forward: TCGGGAAATTACAGAATCACATAAAA, FAM.
   (b) Probe: CCTTACAGAAATGGATGGCTGAAGTTGA TGTTT.
   (c) Reverse: GCAGGCCATTCCTCTTTCAG.
5. *Dmd* unskipped junctions of exon 22–23 (full-length) primers and probe are:
   (a) Forward: GTTACTGAATATGAAATAATGGAGGAG AGA, FAM.
   (b) Probe: TCGGGAAATTACAGGCTCTGCAAA,
   (c) Reverse: CCATTTTGTTGCTCTTTCAAAGAA.

2.7 Immunohistochemistry

1. Tissue-Tek O.C.T. Compound.
2. Mouse on mouse kit.
3. Primary antibodies: MANDYS8 (dystrophin rod domain), Ab15277 (dystrophin C terminus; Abcam), and Manex1A (dystrophin N terminus; Developmental Studies Hybridoma Bank). a-Sarcoglycan (NCL-a-SARC (Novocastra)), b-dystroglycan (NCLb-DG (Novocastra)) and DNA (4,6-diamidino-2-phenylindole).
4. Secondary antibodies: fluorescein isothiocyanate (FITC) anti-mouse or FITC anti-rabbit (Vector Labs).

5. Vectashield Mounting Medium.
6. Ariol SL-50.

2.8 Immunoblot

1. MitoBuffer: 0.2 mM EDTA, 0.25 mM sucrose, 10 mM tris–HCl (pH 7.4) with protease/phosphatase inhibitor cocktail.
2. Extraction buffer: 50 mM tris–HCl (pH 7.4), 7 M urea, 2 M thiourea, 4% CHAPS, 2% SDS, 50 mM b-mercaptoethanol.
3. 2-D Quant Kit.
4. 6% polyacrylamide gel.
5. Nitrocellulose membrane.
6. Tris-buffered saline (TBS).
7. TBS containing 5% Tween 20 (TBST).
8. Diluent solution: 5% milk in TBST. Store at 4–8 °C.
9. Anti- dystrophin (MANDYS8 (Sigma)).
10. Anti-vinculin (hVin-1 (Sigma)).
11. ImageLab 5.1 gel documentation system.

3 Methods

Carry out all procedures at room temperature, unless otherwise specified.

3.1 Derivation of Fibroblasts From Human Skin Punch Biopsies

1. Aspirate the transportation medium from the tissue (*see* **Notes 9** and **10**) and rinse the tissue twice with HBSS.
2. Add 3 mL of HBSS to the tissue and transfer HBSS with tissue into a 6 cm dish.
3. Aspirate the HBSS gently and add 300ul of freshly prepared 2% AOF collagenase (*see* **Notes 2**, **3**, and **11**).
4. Chop the tissue into 1 mm pieces with sterile individual use scalpels (*see* **Note 12**).
5. Add 3,7 mL collagenase to the well and incubate with chopped tissue for 90 min at 37 °C. Gently shake every 20 min.
6. Add 5 mL of DMEM to the collagenase and pipette into 15 mL tube. Add another 5 mL of DMEM to 6 cm dish for final rinse and add to the same 15 mL tube. Centrifuge 300 × *g* for 5 min.
7. Aspirate the supernatant and without disturbing the pellet rinse with 5 mL of DMEM (*see* **Note 13**).
8. Centrifuge at 300 × *g* 5 min.
9. Aspirate the supernatant.

10. Solubilize cell pellet in 3 mL of GM and add to gelatin coated well (aspirate off gelatin beforehand). Shake slightly to separate.
11. Incubate the plate at 37 °C with 5% CO_2 and 95% humidity for 3 days without disturbing the plate.
12. After 72 h, observed for attached cells and clumps by gently transferring the plate to a microscope.
13. If you observe attached single cells and clumps, transfer the medium containing the floating clumps to the next well and add fresh medium to the well containing the attached cells.
14. Allow the cells and clumps to settle in the new well for 2 min and partially aspirate the medium gently using a pipette and replace with fresh DMEM
15. Incubate the plate at 37 °C with 5% CO_2 and 95% humidity for 3 days without disturbing the plate.
16. Change GM every 3 days by gently aspirating with a pipette.
17. After 2–3 weeks transfer cells into a tT75 flask, this is P1.
18. After 3–4 days (or until subconfluent) transfer cells into a tT175. This is p2.
19. When Confluent split into three T225. When subconfluent split and freeze ten vials at 1×10^6 cells/vial frozen in 10% DMSO–FBS.
20. Seed remainder into appropriate flask. Expand until ready to immortalized with hTERT.

3.2 Immortalization of Human Primary Fibroblasts

1. Prior to handling the virus, make sure to wear appropriate personal protective equipment and to have prepared all the reagents for proper handling of biohazard waste (*see* **Notes 14–20**).
2. On day 0, Plate 2×10^5 cells/well on a 6-well plate in 2 mL of Fibroblast Growth Media.
3. On day 1:

   Aspirate off medium from the 6-well plates containing cells.

   Rinse once with 1× PBS.

   Add sequentially (for $n = 1$):

   (a) Fibroblast GM: up to 1 mL.

   (b) Protamine sulfate: 4 μL.

   (c) LV-hTERT: 10 ng (*see* **Note 6**).

   On day 2 (24H postinfection):

   (a) Wash 2× with fibroblast GM.

   (b) Add 2 mL of fresh GM.

Day 3 or 4 (48 or 72H postinfection) depending when your cells are confluent:

(a) Put one well of each in a T75 for expansion.

(b) When the cells in the T75 are confluent, split into three T225. When the cells in the T225 are confluent, freeze two T225 and proceed to the Reprogramming of hTERT immortalized DMD patient fibroblasts with LV-MyoD-ERT-IRES-PURO Selectable Vector with the last T225.

3.3 Reprogramming of hTERT Immortalized DMD Patient Fibroblasts with MyoD

1. Prior to handling the virus, make sure to wear appropriate personal protective equipment and to have prepared all the reagents for proper handling of biohazard waste (*see* **Notes 14–20**).
2. On day 0, Plate 2×10^5 cells/well on a 6-well plate in 2 mL of Fibroblast Growth Media.
3. On day 1:

Aspirate off medium from the 6-well plates containing cells.

Rinse 1× with PBS.

Add sequentially (for $n = 1$):

(a) Fibroblast GM: up to 1 mL.

(b) Protamine sulfate 4 μL.

(c) LV-MyoD-ERT-IRES-PURO: 100 ng (*see* **Note 6**).

Day 2:

(a) Wash 2× with fibroblast GM.

(b) Put one well of each in a T75 for expansion.

Day 3 or 4:

As soon as the first T75 reach confluence, split in two T75 for selection. Keep one out of two under selective media (as described below) until there is enough to validate for MyoD and MyHC staining, and to freeze for future use.

(a) One T75 (LV-MyoD-ERT-IRES-PURO): 0 ug/ml puromycin.

(b) One T75 (LV-MyoD-ERT-IRES-PURO): 1 μg/mL puromycin.

(c) One T75 (mock): 1 μg/mL puromycin.

(d) One T75 (mock): 0 ug/ml puromycin.

3.4 IHC Validation of Reprogramming with MyoD

1. On day 0 plate 75,000 cells on a coverslip previously added into a well of 24-well plates in GM (*see* **Note 21**).
2. After 24 h or when the cells reach 80% of confluence, add 5 μM of tamoxifen (*see* **Note 19**) to the GM.

3. After 2 days with tamoxifen, aspirate media.
4. Wash with 1× PBS.
5. Add 250 μL of 2% PFA per well to fix for 15 min.
6. Rinse 1× with PBS (aspirate slow, pipet slow).
7. Wash 3× with PBS.
8. Permeabilize cells with 0.25% Triton–PBS for 5 min.
9. Wash 3× with PBS.
10. Block in 1.5% BSA–PBS for 2 h.
11. Add primary antibody anti MyoD or isotype control IgG1 overnight at 4 °C or 2 h at RT (1:100 in 0.1% Triton–PBS).
12. Wash 3× with 0.1% Triton–PBS.
13. Add 350 μL secondary antibody (alexa 488/FITC) for 1 h at 1:400 in 0.1% Triton–PBS (goat anti mouse, FITC-conjugated).
14. Wash 1× with 0.1% Triton–PBS.
15. Wash 1× with PBS for 5 min.
16. Dry, and mount slides with DAPI stain mounting media (Prolong Gold antifade reagent with DAPI). Seal with clear nail polish. Store at 4 °C until the pictures are taken (better if it is done the next day).

3.5 IHC Validation of Myotubes Formation

1. On day 0 plate 75,000 cells on a coverslip previously added into a well of 24-well plate in GM.
2. After 24 h or when the cells reach 80% of confluence, add 5 μM of tamoxifen to the GM.
3. After 2 days with tamoxifen, aspirate media.
4. Wash: PBS 1× Wash 1× with PBS for 5 min.
5. Add 2 mL of the Human myoblast differentiation media with tamoxifen.
6. Change the media after 2 days.
7. After 4 days in Human myoblast differentiation media + tamoxifen, rinse with PBS.
8. Replace media with Human fibroblast growth media.
9. After 72 h change the media for Human fibroblast growth media.
10. After 5 days in Human fibroblast growth media (10 days after initiation of differentiation), rinse the cells with PBS 1×.
11. Fix with 4% paraformaldehyde for 10 min.
12. Wash once in PBS.
13. Add 200 μL of PBS with 0.5% Triton 10 min.

14. Replace by PBS + 1% BSA 30 min.
15. Add Primary Antibody MF20 or isotype control IgG2B for 2 h at room temperature or overnight in the fridge.
16. Wash Once in PBS.
17. Add secondary antibody for 1 h at room temperature.
18. Wash Once in PBS.
19. Mounting: Dry, and mount slides with DAPI stain mounting media (Prolong Gold antifade reagent with DAPI), Seal with clear nail polish. Store at 4 °C until the pictures are taken (better if it is done the next day.

3.6 Drug Boosting of Antisense-Mediated Exon Skipping

Transfect your cells with the AON according to the manufacturer protocol after 4 days of differentiation in corresponding media. Let the AON on contact with the cells for 24 h in growth media. For the first time, you may try different concentration of AON (25, 50, 100, 250, and 500 nM) and different concentrations of dantrolene (25 or 50 μM) as follow:

1. For each transfection sample, prepare complexes as follows (for each Well of a 6-Well Plate):
   (a) Dilute 5 μL of a 100 μM stock AON in 175 μL of Opti-MEM® I Reduced Serum Medium to a final volume of 175 μL. Mix gently.
   (b) Mix Oligofectamine™ Reagent gently before use, then dilute 4 μL in Opti-MEM® I Medium to a final volume of 20 μL. Mix gently and incubate for 5–10 min.
   (c) Combine the diluted oligonucleotide with diluted Oligofectamine™ Reagent (total volume = 190 μL). Mix gently and incubate for 15–20 min.
2. While complexes are forming, remove the differentiation medium from the cells and wash once with PBS. Add 800 μL of Opti-MEM® I Reduced Serum Medium to each well containing cells.
3. Add 200 μL of the mix oligonucleotides/AON to the cells.
4. Incubate the cells at 37 °C in a CO_2 incubator for 4 h.
5. Add 500 μL of growth medium containing 3× the normal concentration of serum without removing the transfection mixture.
6. After 24 h replace the growth media for media containing the dantrolene at the desired concentration. Prepare 33.2 μL of Revonto (stock solution at 1 mg/mL) (*see* **Notes 20** and **21**) + 1966.8 μL of GM to achieve a 50 μM final. Add 2 mL of solution per well.
7. After 48 h, harvest the cells for RNA analysis directly in TRIzol. Extract RNA or keep in the −80 °C until extraction.

3.7 RNA Extraction

1. If you start with fresh cells, put them on Ice (recover your ice with foil to ensure a proper repartition of the cold) for 5 min (*see* **Note 22**).
2. Wash once with PBS 1×.
3. Add 500 µL of TRIzol per well for a 6-well plate and scrape your cells with a cell scraper and transfer to a new autoclaved eppendorfs. Your eppendorfs should stay on the ice until you have completely finished to take all the conditions
4. Take out of ice your eppendorfs simultaneously and wait 5 min.
5. Add 100 µL of chloroform solution directly in the TRIzol suspension cell, for 3 min and then centrifuge at 12,000 × *g* for 15 min at 4 °C.
6. Transfer the aqueous phase (upper phase) to a clean RNase-free tube (*see* **Note 23**).
7. Add one volume 70% ethanol to each volume of cell homogenate to have a final concentration of 35% ethanol. Then vortex and invert 2–3 times.
8. Transfer up to 700 µL of the sample (including any remaining precipitate) to the Spin Cartridge (with the Collection Tube).
9. Centrifuge at 12,000 × *g* for 15 s. Discard the flow-through, and reinsert the Spin Cartridge into the same Collection Tube (*see* **Note 24**).
10. Repeat **steps 3–4** until the entire sample has been processed.
11. Add 350 µL Wash Buffer I to the Spin Cartridge. Centrifuge at 12,000 × *g* for 15 s. Discard the flow-through and the Collection Tube. Place the Spin Cartridge into a new Collection Tube.
12. Add 80 µL PureLink® DNase mixture (composed of 8 µL of 10× DNase I Reaction Buffer; 10 µL of prepared DNase (~3 U/µL); 62 µL of RNase-free water).
13. Incubate for 15 min.
14. Add 350 µL Wash Buffer I to the Spin Cartridge. Centrifuge at 12000 × *g* for 15 s. Discard flow-through and the Collection Tube and insert the Spin Cartridge into a new Collection Tube.
15. Add 500 µL Wash Buffer II with ethanol (page 11) to the Spin Cartridge. Centrifuge at 12,000 × *g* for 15 s. Discard flow-through and reinsert the Spin Cartridge into the same Collection Tube.
16. Repeat the last step once.

17. Centrifuge the Spin Cartridge at 12,000 × *g* for 1–2 min to dry the membrane with attached the RNA. Discard the Collection Tube and insert the Spin Cartridge into a Recovery Tube.
18. Add 15 μL RNase–Free Water to the center of the Spin Cartridge. Incubate at for 1 min. Centrifuge the Spin Cartridge for 2 min at ≥12,000 × *g* to elute the RNA from the membrane into the Recovery tube.
19. Repeat last step once with the same water.
20. Incubate for 5 min and then dose RNA concentration with nanodrop.

3.8 Reverse Transcription and Nested PCR

1. After RNA extraction, we perform a Reverse transcription using the superscript IV with 300 ng of total RNA for each sample. We use a combination of a specific primer and random hexamers as described below.
2. For one condition:
   (a) 50 μM of random hexamers.
   (b) 2 μM gene-specific reverse primer.
   (c) 1 μL of dNTP (at 10 mM),
   (d) 300 ng of total RNA.
   (e) Complete with water up to 13 μL.

   Incubate at 65° for 5 min and let cool down for at least 1 min at 4°, then add 4 μL of Superscript IV buffer, 1 μL of DTT (100 mM), 1 μL of RNAseOUT and 1 μL of Superscript IV to achieve a total volume of 20 μL. Incubate at 23° for 10 min followed by 10 min at 55° and 10 min at 80°. Keep at −20° if not use right away or proceed to the first PCR.
3. For the First PCR, Use 5 μL of cDNA per Condition in 25 μL Reaction Final.
   Here we use the following concentration for one condition:
   (a) 2.5 μL of 10× PCR buffer.
   (b) 0.5 μL of dNTP (at 10 mM).
   (c) 1 μL of DMSO.
   (d) 1 μL of $MgCl_2$ (at 50 mM),
   (e) 0.5 μL of primers (at 10 mM).
   (f) 0.125 μL of platinum taq.
   (g) Water up to 25 μL.

   The reaction mix is incubated for 5 min at 94° and then for 20 cycles of 40 s at 94°, 40 s at melting temperature and 1 min at 72°, before a final extension of 10 min at 72°. Keep at 4° if not use right away or proceed to the second PCR.

4. For the second PCR, we use 2 μL of the first per condition in 25 μL reaction final. Here we use the following concentration for one condition:
   (a) 2.5 μL of 10× PCR buffer.
   (b) 0.5 μL of dNTP (at 10 mM).
   (c) 1 μL of DMSO.
   (d) 0.5 μL of $MgCl_2$ (at 50 mM).
   (e) 0.5 μL of primers (at 10 mM).
   (f) 0.125 μL of platinum taq.
   (g) Water up to 25 μL.

The reaction mix is incubated for 5 min at 94° and then for 35 cycles of 40 s at 94°, 40 s at melting temperature and 1 min at 72°, before a final extension of 10 min at 72°. Keep at 4° until visualization on a 2% agarose gel or for analysis using the Bioanalyzer.

3.9 Bioanalyzer Quantification of Exon Skipping Efficiency

1. After the verification on agarose gel, samples are loaded on a DNA1000 Bioanalyzer chip and run on the 2100 Bioanalyzer.
2. Results are analyzed as followed: the molarity of each peak is used to calculate the skipping ratio (skipped peak versus unskipped + skipped peaks).

3.10 Drug Boosting of Antisense Mediated Exon Skipping In Vivo

1. Inject the morpholino in mice intravenously retro-orbitally in 100 μL of saline weekly.
2. Mice were given ad libitum access to chow. The amount of chow consumed was weighed weekly.
3. At the end of the experiments, the quadriceps, gastrocnemius, TA, and triceps were harvested.
4. The right muscle was frozen in tissue-tek o.c.t. compound for sectioning and analysis by immunohistochemistry, whereas the left muscle was cut in half and snap-frozen for analysis by Western Blot and ddPCR.
5. For the diaphragm and heart, one half of the muscle was designated for immunohistochemistry and one-fourth for Western blot and RT-PCR.

3.11 RNA Extraction from Mouse Tissue

1. Take frozen muscle out of −180 °C freezer and place into 2 mL round-bottom Eppendorf tubes.
2. Keep each individual sample on dry ice until it is ready to process.
3. Move one sample/tube to a container with ice.
4. Add 300 μL RLT Buffer to tube and immediately homogenize using TissueRuptor®.

5. Homogenize tissue at high speed in bursts of 5 s. Keep sample touching ice to avoid heating due to friction.
6. Once sample is fully homogenized, place on ice. Repeat **steps 3–5** for each sample individually.
7. Once all samples are homogenized, add 295 μL RNase-free water and 5 μL Proteinase K solution. Mix thoroughly by inversion.
8. Incubate at 55 °C for 10 min.
9. Centrifuge for 3 min at 10,000 rpm 9000 × *g* at room temperature.
10. Pipet supernatant into a new tube.
11. Add 150 μL 100% ethanol. Mix by inversion.
12. Transfer sample to RNeasy MinElute spin column placed in a 2 mL collection tube. Close the lid and centrifuge for 15 s at 10,000 rpm 9000 x g. Discard flow-through.
13. Add 350 μL Buffer RW1 to RNeasy MinElute spin column. Close lid and centrifuge for 15 s at 10,000 rpm 9000 × *g*. Discard flow-through.
14. In a separate tube, add 10 μL DNase I solution to 70 μL Buffer RDD (for each sample). Mix by inversion.
15. Add DNase I incubation mix (80 μL) directly to RNeasy MinElute spin column membrane, and incubate on desktop for 15 min.
16. Add 350 μL Buffer RW1 to RNeasy MinElute spin column. Close lid and centrifuge for 15 s at 10,000 rpm 9000 × *g*. Discard flow-through.
17. Place RNeasy MinElute spin column in a new 2 mL collection tube. Add 500 μL Buffer RPE to the spin column. Close lid and centrifuge for 15 s at 10,000 rpm 9000 × *g*. Discard flow-through.
18. Add 500 μL of 80% ethanol to the RNeasy MinElute spin column. Close lid and centrifuge for 2 min at 10,000 rpm 9000 × *g*. Discard flow-through and collection tube.
19. Place RNeasy MinElute spin column in a new 2 mL collection tube. Open lid of spin column, and centrifuge for 5 min at 10,000 rpm 9000 × *g*. Discard flow-through.
20. Place RNeasy MinElute spin column in a new 1.5 mL Eppendorf tube. Add 50 μL RNase-free water directly to center of spin membrane. Let sit at room temperature for 1 min. Close lid and centrifuge for 1 min at 10,000 rpm 9000 × *g*.
21. Determine RNA concentration by nanodrop.
22. Aliquot RNA as desired and immediately use or freeze at −80 °C.

3.12 Reverse Transcription and ddPCR for Mouse Exon Skipping

1. After RNA extraction, we prepare our RT-ddPCR reaction mix with 250 ng of total RNA for each sample.
2. For one condition:
   (a) 12.5 μL of 2× One-step RT-ddPCR supermix.
   (b) 1 μL of 25 mM Manganese Acetate solution.
   (c) 1.25 μL of 20× Taqman RPLP0.
   (d) 0.45 μL of primers (at 50 μm).
   (e) 0.5 μL of Probe-FAM (at 10 μM).
   (f) 8 μL of template RNA (250 ng total).
   (g) 0.85 μL of water.
3. Generate oil droplets by transferring 20 μL of sample mix to middle row of DG8 Cartridge.
4. Add 70 μL of Droplet Generation Oil for Probes to top row of DG8 cartridge.
5. Place DG8 Gasket over DG8 Cartridge.
6. Insert DG8 cartridge into droplet generation machine and run.
7. Following droplet generation, transfer 70 μL droplets to 96-well ddPCR plate.
8. Repeat **steps 3**–7 for all samples.
9. Cover 96-well ddPCR plate with ddPCR foil and run samples in thermocycler.
10. For PCR reaction, use a heated lid set to 105 °C. The reaction mix is incubated for 30 min at 60 °C (for reverse transcription. Then 5 min at 95 °C (for enzyme activation). Then 40 cycles of the following: 1) 30 s at 94 °C (for denaturation) and 1 min at 60 °C (for annealing). Then 10 min at 98 °C (for enzyme heat kill). Then hold at 4°.
11. Following PCR, insert plate into ddPCR machine to analyze results.

3.13 Immunohistochemistry

1. Immunohistochemistry (IHC) is performed on unfixed 10-mm tissue sections.
2. Sections were mounted in Vectashield Mounting Medium.
3. Whole-muscle cross-sections were scanned with a 5×—objective and were analyzed with Ariol SL-50.
4. For quantifying dystrophin immunofluorescence, the minimum threshold was placed above background level for all sections. Each sample's dystrophin immunofluorescent levels are expressed as a percentage of C57 (100%).

5. For quantifying dystrophin positive fibers, the minimum threshold was placed above background level for all sections. For each sample, fibers were counted across the entire section. Dystrophin positive fibers were defined as fibers that had sarcolemmal dystrophin expression above threshold and surrounding >75–80% of each fiber (*see* **Note 25**).
6. For quantifying necrotic area, diaphragms from the three mice per group that had total dystrophin levels by western blot near the median were selected. The total area of IgG staining was determined using ImageJ analysis. A standard protocol was used for hematoxylin and eosin (H&E) staining. The average percentage of fibers with centrally located nuclei in the diaphragm per group was determined by counting 750–1000 fibers from the three mice per group which had total dystrophin levels by western blot near the median.

3.14 Immunoblot

1. To isolate total protein from flash-frozen quadriceps and diaphragms from the treated mdx and control C57 mice, tissues were homogenized in Mito buffer and subjected to low-speed (3000 × *g*) centrifugation for 10 min at 4 °C.
2. The supernatant was centrifuged at 100,000 × *g* (high-speed centrifugation) for 1 h for isolation of membrane fraction.
3. Isolated membranes and pellet after low-speed centrifugation were combined and prepared in 300 mL of extraction buffer.
4. 2-D Quant Kit determined protein concentration.
5. Forty micrograms of total protein were run on a 6% polyacrylamide gel and transferred onto a nitrocellulose membrane for 2 h at 4 °C.
6. The membrane was blocked for 1 h in 5% milk.
7. Incubate with MANDYS8 (Sigma), 1:400 in TBS-T and anti-vinculin hVin-1 (Sigma), 1:5000 in TBS-T.
8. Densitometry analysis is performed with ImageLab 5.1 gel documentation system.
9. Dystrophin protein levels are normalized to vinculin.
10. After normalization, results are pooled across treatment groups to determine the average dystrophin rescue (*see* **Note 26**).

4 Notes

1. Biohazard trash bins should contain only biohazardous red bags. Always put double bags on this bins. Wait for 30 min before disposing of all the Biohazardous waste according to institutional hazardous waste protocols.

2. Prepare all the requirements in the list. *Prior to use of any chemical, be sure to read the related MSDS.* All chemical agents should be weighted using proper personal protective equipment (labcoat, nitrile gloves, eye protection, surgical mask (if volatile or powder)) and should be changed just after the preparation of the stock solutions.
3. Prepare biohazard vacuum flask in PSMII with 400 mL of pure bleach (to have a final concentration of 10% bleach when the flask is full).
4. 4-OH Tamoxifen could be hard to solubilize. In this case heat at 55 °C. When solubilized, filter through a 22 μM filter and aliquots at −20 °C within a secondary container. When filtering, be extremely careful since the solution may be leaking from the filter.
5. All the cell media should be filtered using a 500 mL Filter System (Pore size: 0.22 μM; PES Membrane).
6. Lentiviruses were obtained from the UCLA vector core. The MyoD-ER(T) construct has been previously described [7]. The puromycin selectable vector was generated by amplifying the MyoD-ER(T) portion of the plasmid using the following primers: FWD 5′-GTATCGACTAGTCCTCGACAGGACAGGAC-3′ and REV 5′-ATAGCCGAATTCTCGCCCGCTTGA-3′ (Integrated DNA Technologies). The resulting product was gel purified (Qiagen) and ligated into the pRRLsin.cPPT.CMV.MCS.IRES.Puro vector backbone obtained from the UCLA Vector Core, and validated by Sanger sequencing.
7. BSA solution should be kept at 4 °C and PFA solution should be kept under the chemical hood or in the fridge in a secondary container.
8. Dantrolene (Revonto) should be protected from light and used within 6 h after preparation.
9. Skin punches should be taken and stored in 10 mL DMEM (high glucose, −Phenol Red, −L-glutamine) + 1× Penicillin/streptomycin and stored on ice. Tissue should be processed as soon as possible but within the hour is preferable.
10. Follow all aseptic precautions throughout the process.
11. 2% AOF collagenase is prepared fresh every time and resuspended in HBSS (+calcium, +$MgCl_2$, no serum).
12. Be careful that the blades do not touch anything besides the tissue. All the sharp containers should be closed and dispose in Biohazardous bags when full.
13. Using a pipette, remove as much as possible to get rid of the collagenase only with a pipette and very carefully. Do not use a vacuum aspiration.

14. Work only in a cell culture hood (PSMII) in a BSL2+ room with proper personal protective equipment (disposable lab-coat, double gloves, eye protection, surgical mask, long pants and closed toe shoes) when handling Human derived-cell lines and lentiviruses.
15. When retrieving virus from −80 freezer place in 50 mL conical as a secondary container. Discard one pair of gloves after placing the virus in the secondary container and put on a second pair when handling the virus.
16. Prepare biohazard vacuum flask in PSMII with 400 mL of pure bleach (to have a final concentration of 10% bleach when the flask is full).
17. Place a beaker with 100% bleach solution in the PSMII for soaking tips and pipettes for at least 30 min before disposal as biohazard.
18. Place all tips and pipettes in the beaker with bleach after pipetting up and down.
19. Wait 30 min before disposing of all the biohazardous waste according to institutional hazardous waste protocols.
20. At the end of your work under the PSMII, wipe the working area with 10% bleach then rinse with ddH_2O and finish with 70% EtOH.
21. Place a sign on the CO_2 incubator door indicating that lentivirus-infected cells are inside.
22. Cover your ice with foil to ensure a proper repartition of the cold.
23. If you are processing the maximum starting amount of sample, you may centrifuge for up to 10 min.
24. Do not try to take too much aqueous phase if the frontier between the aqueous phase and the white fluffy protein band.
25. For both measurements, all of our readings were well below the maximal intensity threshold (pixel saturation was avoided).
26. Serial dilutions of C57 sample into untreated mdx sample were run simultaneously. All samples shown were run through quality control, as defined by linearity of dilution of C57 controls. The average densitometry value for 100% of dystrophin in C57 was calculated as mean of densitometry values of C57 serial dilutions multiplied by dilution factor.

Acknowledgments

F.B. has received a postdoc fellowship grant from the UCLA Center for Duchenne Muscular Dystrophy-NIH NIAMS U54

AR052646 Wellstone Center of Excellence Training Fellowship. This work was funded from grants from the NIH (1T32AR65972A1) and the California Institute of Regenerative Medicine (TRX-05426). Support for the research was also provided by the UCLA Muscular Dystrophy Core Center (NIH grant 5P30AR057230) within the Center for Duchenne Muscular Dystrophy at UCLA. Competing interests: S.F.N. and M.C.M. are inventors on a pending patent on identification of small molecules that enhance exon skipping, filed by UCLA.

References

1. Theadom A, Rodrigues M, Roxburgh R et al (2014) Prevalence of muscular dystrophies: a systematic literature review. Neuroepidemiology 43(3–4):259–268. https://doi.org/10.1159/000369343
2. Foster H, Popplewell L, Dickson G (2012) Genetic therapeutic approaches for Duchenne muscular dystrophy. Hum Gene Ther 23(7):676–687. https://doi.org/10.1089/hum.2012.099
3. Mendell JR, Goemans N, Lowes LP et al (2016) Longitudinal effect of eteplirsen versus historical control on ambulation in Duchenne muscular dystrophy. Ann Neurol 79(2):257–271. https://doi.org/10.1002/ana.24555
4. Aartsma-Rus A, Krieg AM (2017) FDA approves Eteplirsen for Duchenne muscular dystrophy: the next chapter in the Eteplirsen saga. Nucleic Acid Ther 27(1):1–3. https://doi.org/10.1089/nat.2016.0657
5. Cirak S, Arechavala-Gomeza V, Guglieri M et al (2011) Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: an open-label, phase 2, dose-escalation study. Lancet 378(9791):595–605. https://doi.org/10.1016/S0140-6736(11)60756-3
6. Goemans NM, Tulinius M, van den Akker JT et al (2011) Systemic administration of PRO051 in Duchenne's muscular dystrophy. N Engl J Med 364(16):1513–1522. https://doi.org/10.1056/NEJMoa1011367
7. Kimura E, Han JJ, Li S et al (2008) Cell-lineage regulated myogenesis for dystrophin replacement: a novel therapeutic approach for treatment of muscular dystrophy. Hum Mol Genet 17(16):2507–2517. https://doi.org/10.1093/hmg/ddn151