Utility of a 3D–Engineered Skeletal Muscle Organoid System to Assess Exon Skipping, Dystrophin Protein Restoration, and Functional Improvement in a Human DMD Cell Model

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INTRODUCTION

- Robust in vitro models of skeletal muscle tissue are important for understanding disease mechanisms and identifying novel therapeutic targets, particularly in Duchenne muscular dystrophy (DMD).
- Culturing muscle cells is challenging because mature myotubes typically detach from their substrate within 1 week of initiating the differentiation regimen when cultured in a 2-dimensional (2D) matrix.
- A 3-dimensional (3D)–engineered skeletal muscle tissue system (ie, engineered muscle tissue [EMT]) was developed to circumvent these challenges.[1] This model system has been shown to accurately depict defects in skeletal muscle cells derived from patients with DMD compared with cells from individuals without DMD; thus, this model can be useful for elucidating the efficacy of therapeutics for DMD.
- One class of therapeutic approved for treating patients with DMD is exon-skipping phosphorodiamidate morpholino oligomers (PMO), which produce dystrophin by restoring the messenger RNA reading frame by exon skipping.
- EMTs derived from DMD patient muscle cells may be a suitable human-relevant model system for evaluating the efficacy of next-generation therapeutics for the treatment of DMD.

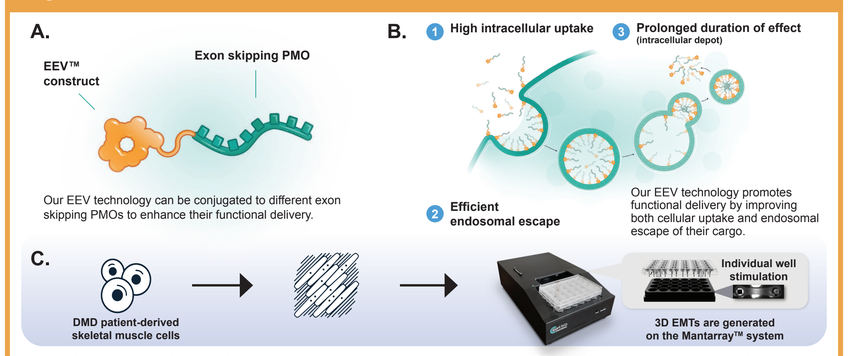
MATERIALS AND METHODS

- EEV-PMO-44 and EEV-PMO-45 are *DMD* exon 44 and exon 45 skipping PMOs, respectively, conjugated to an EEV construct. The EEV platform consists of a cyclic cell-penetrating peptide that was designed to improve cellular uptake and enhance endosomal escape of therapeutic cargo[2] (**Figures 1A** and **1B**).
- 3D EMTs were generated as described by Smith et al.[1] Briefly, immortalized patient-derived skeletal myoblasts harboring exon 44 (DMDΔ45) (Institute of Myologie, Paris, France) or exon 45 (DMDΔ46-48) (Curi Bio, Seattle, WA, USA) skip-amenable deletions in the *DMD* gene were split from 2D surfaces and engineered into EMTs with the Mantarray™ system (Curi Bio, Seattle, WA) (**Figure 1C**). EMTs were exposed to varying doses of EEV-PMO-44 or EEV-PMO-45 for 24 hours, followed by compound washout. EMTs were assessed for *DMD* exon 44 or exon 45 skipping and dystrophin protein restoration 1, 4, and 6 weeks following compound removal.
  - In addition, tetanic force and muscle contraction kinetics were assessed in EMTs treated with EEV-PMO-45 4 weeks after washout using Mantarray's integrated software.
- Dystrophin protein restoration was evaluated by simple Western Jess (Bio-Techne, Minneapolis, MN) and immunofluorescence. *DMD* exon-skipping efficiency was determined by reverse-transcriptase polymerase chain reaction (PCR) and LabChip analysis (Revvity, Santa Clara, CA) for DMDΔ45 EMTs, and by droplet digital PCR for DMDΔ46-48 EMTs.

OBJECTIVE

- To assess the utility of EMTs as an in vitro model system for examining the efficacy of *DMD* exon 44 skipping and exon 45 skipping Endosomal Escape Vehicle (EEV™)-PMO constructs.

Figure 1. EEV-PMO Construct and Generation of 3D EMTs.



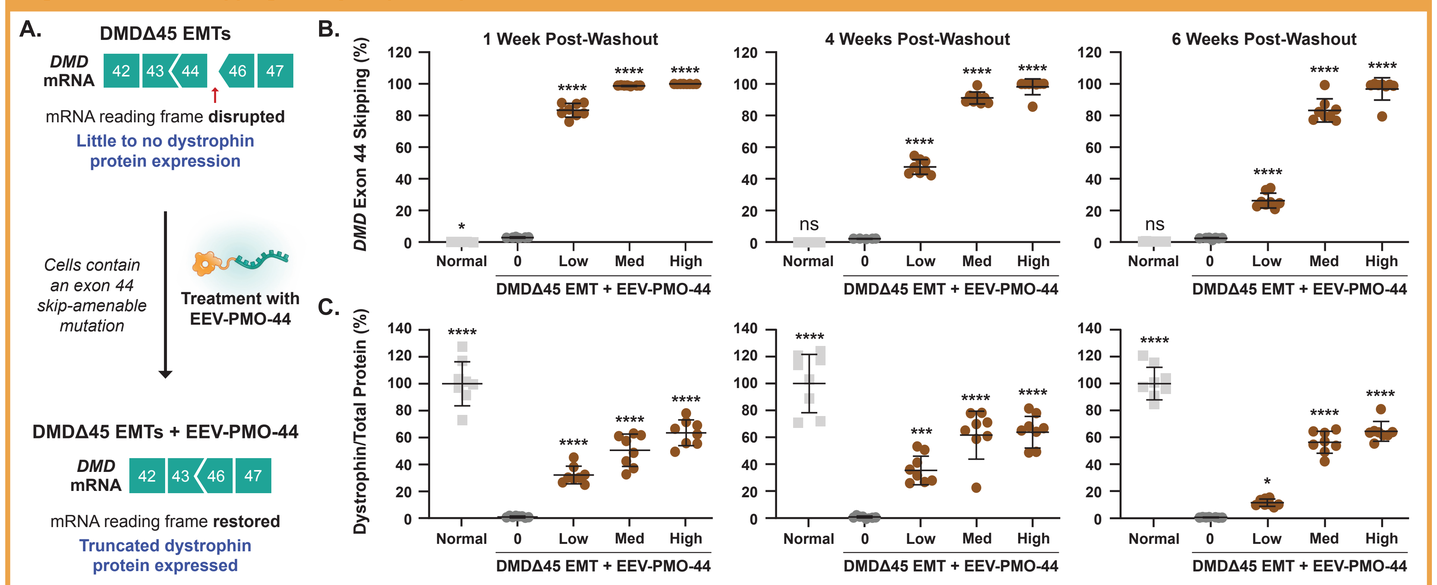
(A) Structure of EEV-PMO construct consisting of a cyclic cell-penetrating peptide-based EEV construct and exon-skipping PMO; **(B)** Mechanism of EEV construct-mediated delivery of PMOs. **(C)** Patient-derived skeletal myoblasts harboring an exon 45 or exon 46-48 deletion in the *DMD* gene were split from 2D surfaces and engineered into EMTs with the Mantarray system (Curi Bio, Seattle, WA). 2D, 2-dimensional; 3D, 3-dimensional; DMD, Duchenne muscular dystrophy; *DMD*, dystrophin gene; EEV, Endosomal Escape Vehicle; EMT, engineered muscle tissue; PMO, phosphorodiamidate morpholino oligomer.

RESULTS

DMD Exon 44 Skipping and Dystrophin Production in DMDΔ45 EMTs

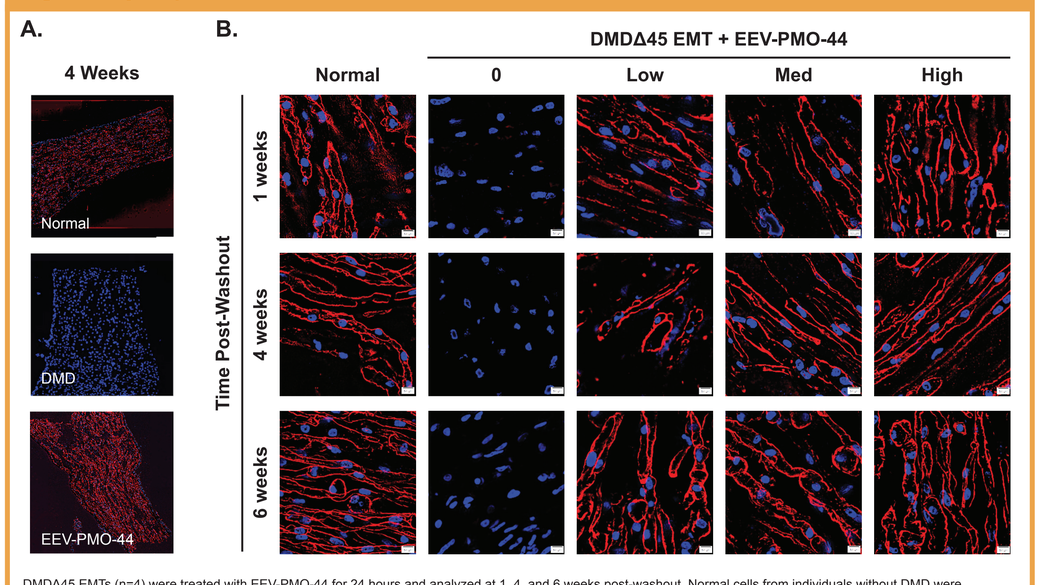
- Significant dose-dependent *DMD* exon 44 skipping and dystrophin protein expression/restoration were observed in DMDΔ45 EMTs for up to 6 weeks post-washout following treatment with EEV-PMO-44 (**Figure 2**).
- Longitudinal sections of DMDΔ45 EMTs also showed broad dystrophin expression across the entire tissue sample following EEV-PMO-44 treatment (**Figure 3A**) and proper localization to the sarcolemma (**Figure 3B**).

Figure 2. Exon 44 Skipping and Dystrophin Expression With EEV-PMO-44 in DMDΔ45 EMTs.



DMDΔ45 EMTs (n=8) were treated with EEV-PMO-44 **(A)** for 24 hours and analyzed at 1, 4, and 6 weeks post-washout for **(B)** *DMD* exon 44 skipping and **(C)** dystrophin protein expression. Normal cells from individuals without DMD were used as controls. Data are shown as mean ± SD; statistical significance was determined by ordinary one-way ANOVA and Dunnett's multiple comparison post-hoc test. Reported *p* values are relative to the untreated DMDΔ45 group; *$p<0.05$, ***$p<0.001$, ****$p<0.0001$. ANOVA, analysis of variance; DMD, Duchenne muscular dystrophy; *DMD*, dystrophin gene; EEV, Endosomal Escape Vehicle; EMT, engineered muscle tissue; Med, medium; ns, not significant; PMO, phosphorodiamidate morpholino oligomer.

Figure 3. Dystrophin Restoration With EEV-PMO-44 in DMDΔ45 EMTs.

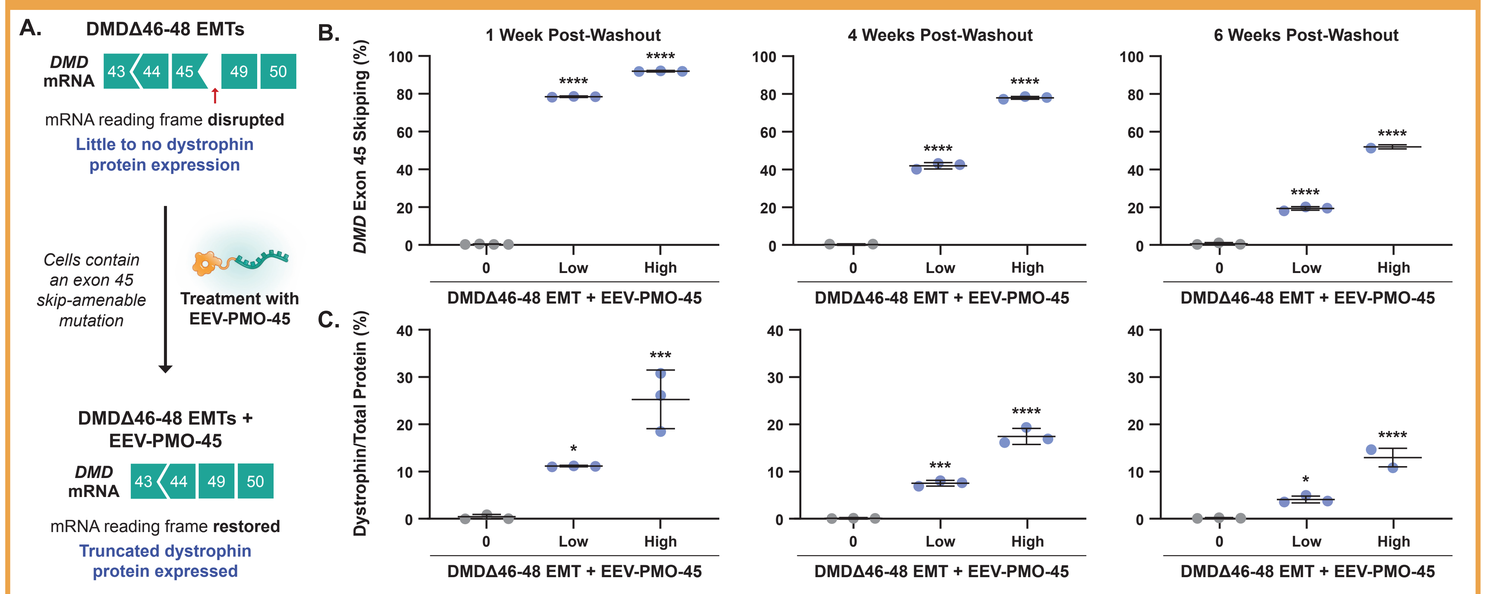


DMDΔ45 EMTs (n=4) were treated with EEV-PMO-44 for 24 hours and analyzed at 1, 4, and 6 weeks post-washout. Normal cells from individuals without DMD were used as controls. Red, dystrophin; blue, DAPI. **(A)** Longitudinal images (40x stitched) of whole tissue treated with high-dose EEV-PMO-44 were examined 4 weeks post-washout, demonstrating even dystrophin distribution. **(B)** Longitudinal images (40x cropped) of EMTs, demonstrating proper dystrophin expression on sarcolemma. DAPI, 4′,6-diamidino-2-phenylindole; DMD, Duchenne muscular dystrophy; EEV, Endosomal Escape Vehicle; EMT, engineered muscle tissue; Med, medium; PMO, phosphorodiamidate morpholino oligomer.

DMD Exon 45 Skipping, Dystrophin Production, and Muscle Cell Function in DMDΔ46-48 EMTs

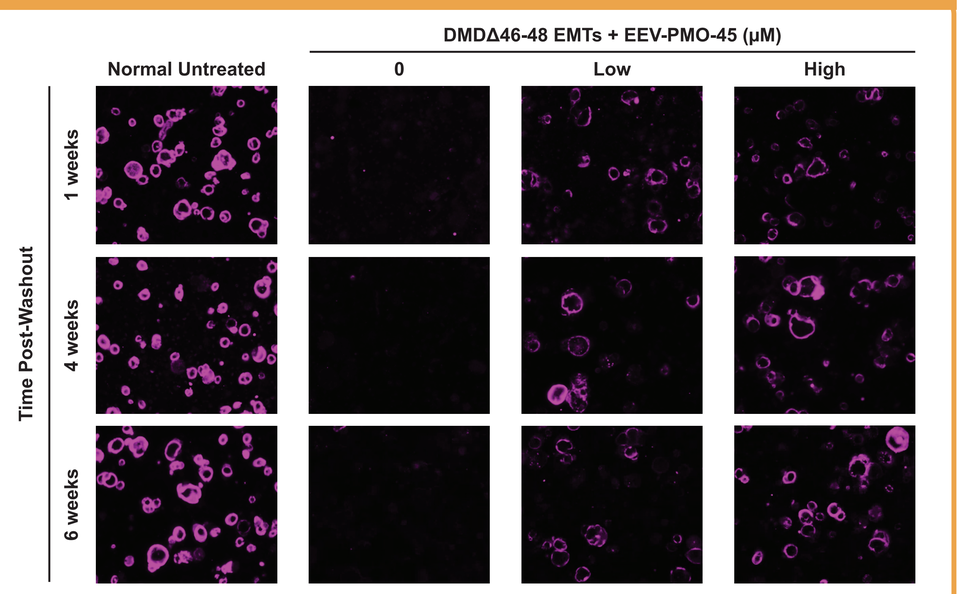
- Significant dose-dependent *DMD* exon 45 skipping and dystrophin protein expression/restoration were observed in DMDΔ46-48 EMTs for up to 6 weeks post-washout following treatment with EEV-PMO-45 (**Figure 4**).
- Cross-sections of DMDΔ46-48 EMTs showed dystrophin expression in the sarcolemma following treatment with EEV-PMO-45 (**Figure 5**).

Figure 4. Exon 44 Skipping and Dystrophin Expression With EEV-PMO-45 in DMDΔ46-48 EMTs.



DMDΔ46-48 EMTs (n=3) were treated with EEV-PMO-45 **(A)** for 24 hours and analyzed at 1, 4, and 6 weeks post-washout for **(B)** *DMD* exon 45 skipping and **(C)** dystrophin protein expression. Data are shown as mean ± SD; ordinary one-way ANOVA and Dunnett's multiple comparison post-hoc test; *$p<0.05$, ***$p<0.001$, ****$p<0.0001$, versus untreated DMDΔ46-48 EMTs. ANOVA, analysis of variance; DMD, Duchenne muscular dystrophy; EEV, endosomal escape vehicle construct; EMT, engineered muscle tissue; ns, not significant; PMO, phosphorodiamidate morpholino oligomer.

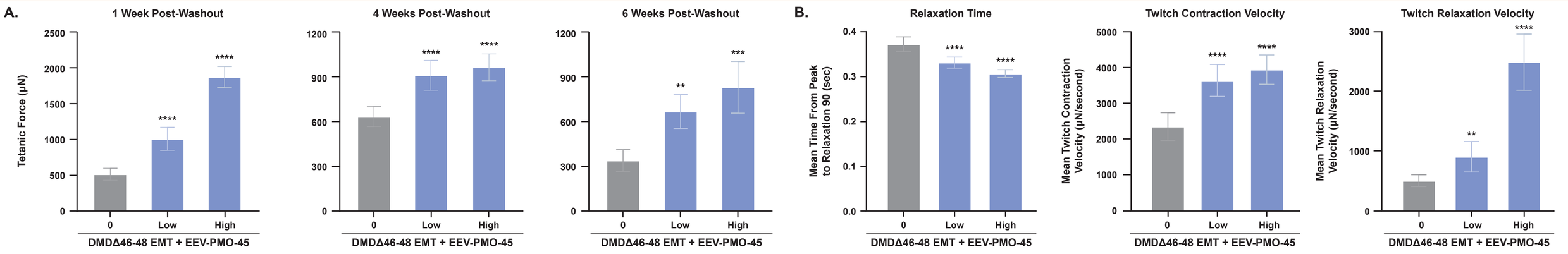
Figure 5. Dystrophin Restoration With EEV-PMO-45 in DMDΔ46-48 EMTs.



DMDΔ46-48 EMTs (n=1) were treated with EEV-PMO-45 for 24 hours and analyzed at 1, 4, and 6 weeks post-washout for dystrophin restoration in the sarcolemma (purple). EEV, endosomal escape vehicle; EMT, engineered muscle tissue; PMO, phosphorodiamidate morpholino oligomer.

- Tetanic force is significantly improved in DMDΔ46-48 EMTs for up to 6 weeks post-washout following treatment with EEV-PMO-45 (**Figure 6A**).
- Relaxation time is reduced, and twitch contraction/relaxation velocities are increased with EEV-PMO-45 treatment 4 weeks after washout, suggesting improved calcium handling and muscle function (**Figure 6B**).

Figure 6. In Vitro Muscle Cell Function in DMDΔ46-48 EMTs With EEV-PMO-45.



DMDΔ46-48 EMTs (n=3) were treated with EEV-PMO-45 for 24 hours. **(A)** EMTs were analyzed at 1, 4, and 6 weeks post-washout for tetanic force. **(B)** EMTs were analyzed for muscle cell contraction kinetics 4 weeks post-washout. Data are shown as mean ± SD; ordinary one-way ANOVA and Dunnett's multiple comparison post-hoc test; **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, versus untreated DMDΔ46-48 EMTs. ANOVA, analysis of variance; DMD, Duchenne muscular dystrophy; EEV, endosomal escape vehicle construct; EMT, engineered muscle tissue; ns, not significant; PMO, phosphorodiamidate morpholino oligomer.

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References: 1. Smith AS, et al. *J Tissue Eng*. 2022. 2. Li X, et al. *Mol Ther Nucleic Acids*. 2023.

CONCLUSIONS

- Consistent with previous studies in 2D cultures and animal models, these findings demonstrate the ability of the EEV™ platform to efficiently deliver exon-skipping oligonucleotides to 3D-engineered skeletal muscle tissues as evident by exon skipping and dystrophin protein production.
- Dystrophin protein was observed in EMTs treated with EEV-PMO-44 or EEV-PMO-45 as early as 7 days post-washout, detectable for at least 6 weeks post-washout, and localized correctly to the sarcolemma.
- Tetanic force increased, and contractile kinetics improved in DMDΔ46-48 EMTs treated with EEV-PMO-45.
- These results underscore the potential utility of this 3D culture system for the drug development process in DMD.