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

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INTRODUCTION

- Robust in vitro models of skeletal muscle tissue are critical to understanding disease mechanism 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 organoid 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 in comparison with cells from individuals without DMD and thus can be useful for elucidating the efficacy of therapeutics for DMD.
- One class of therapeutic approved for the treatment of DMD is exon skipping phosphorodiamidate morpholino oligomers (PMO), which produce dystrophin by restoring the reading frame and by exon skipping.
  - EMTs derived from patients with DMD may be a suitable model system for evaluating the efficacy of next-generation PMOs in development for the treatment of DMD.

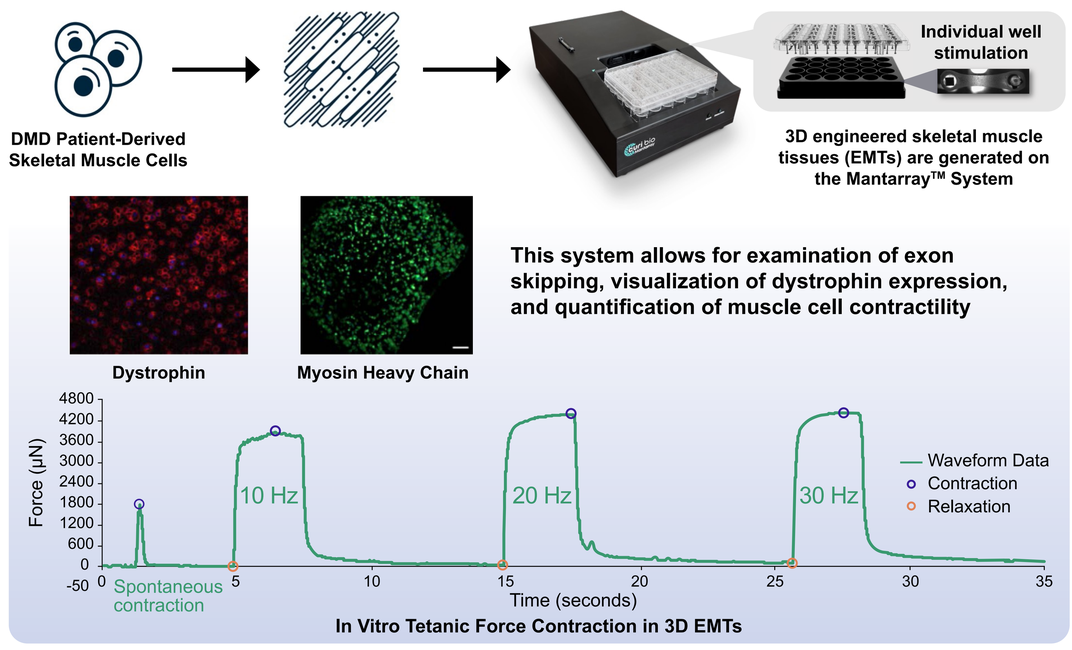
OBJECTIVE

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

MATERIALS & METHODS

- EEV-PMO44 is a *DMD* exon 44 skipping PMO 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]
- 3D EMTs were generated as described by Smith et al (2022).[1] Briefly, immortalized patient-derived skeletal myoblasts harboring an exon 45 deletion in the *DMD* gene (Institute of Myologie, Paris, France) were split from 2D surfaces and engineered into EMTs with the Mantarray™ system (Curi Bio, Seattle, WA) (**Figure 1**). EMTs were exposed to varying doses of EEV-PMO44 (low, medium, high) for 24 hours, followed by compound washout. EMTs were assessed for *DMD* exon 44 skipping and dystrophin protein restoration 1, 4, and 6 weeks following compound removal.
- Dystrophin restoration was evaluated by Simple Western Jess (Bio-Techne, Minneapolis, MN) and immunofluorescence. Exon-skipping efficiency was determined by reverse-transcriptase polymerase chain reaction and LabChip analysis (Revvity, Santa Clara, CA).

Figure 1. Generation of 3D EMTs.

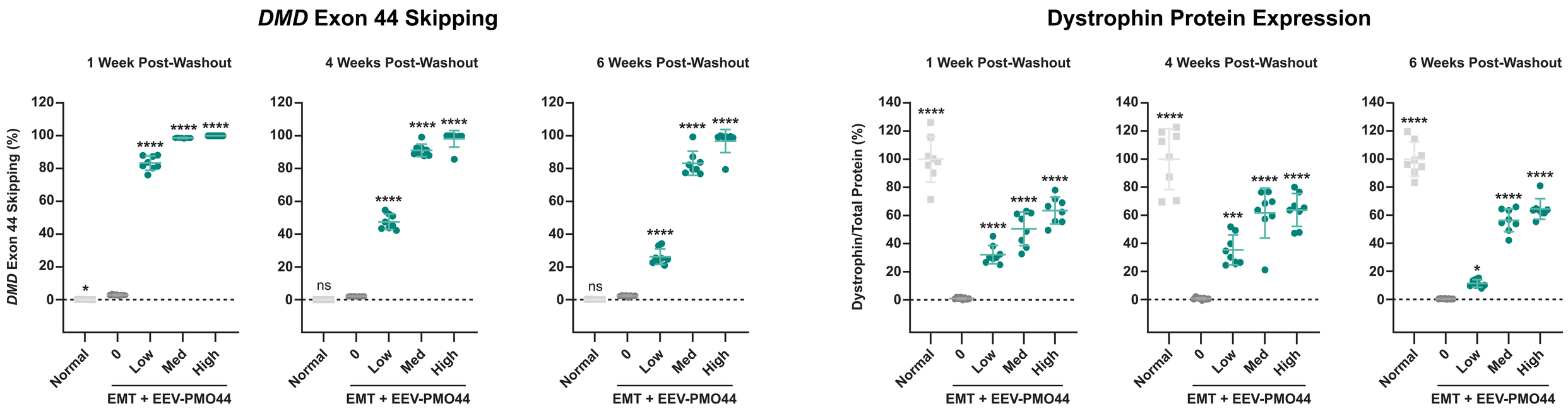


Immortalized patient-derived skeletal myoblasts harboring an exon 45 deletion in the *DMD* gene were split from 2D surfaces and engineered into EMTs with the Mantarray™ Use system (Curi Bio, Seattle, WA). 2D, 2-dimensional; 3D, 3-dimensional; DMD, Duchenne muscular dystrophy; EMT, engineered muscle tissue.

RESULTS

- Significant dose-dependent *DMD* exon 44 skipping and dystrophin expression were observed in EMTs for up to 6 weeks post-washout following treatment with EEV-PMO44 (**Figure 2**).

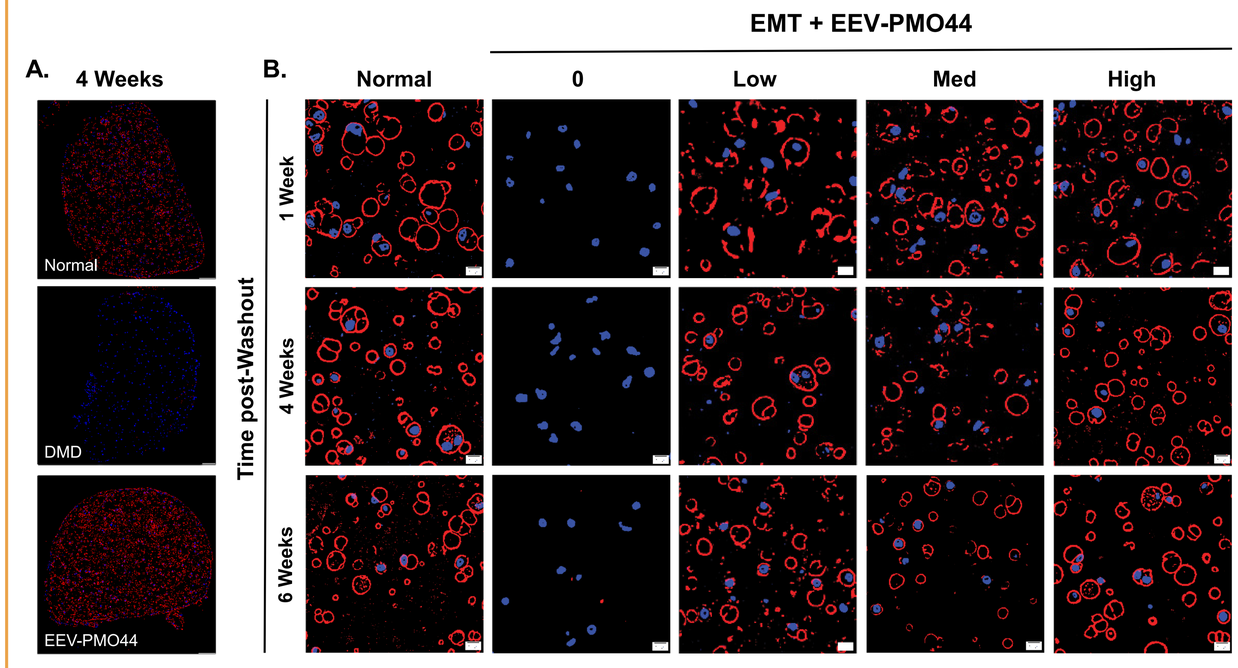
Figure 2. Exon 44 Skipping and Dystrophin Expression with EEV-PMO44 in EMTs.



EMTs (N=8) were treated with EEV-PMO44 for 24 hours and analyzed at 1-, 4-, and 6-weeks post-washout. Normal cells from individuals without DMD were used as controls. Data are shown as mean ± SD; Ordinary 1-way ANOVA and Dunnett's multiple comparison test; **p*<0.05, ****p*<0.001, *****p*<0.0001, in relation to untreated EMTs. ANOVA, analysis of variance; DMD, Duchenne muscular dystrophy; EEV, endosomal escape vehicle construct; EMT, engineered muscle tissue; Med, medium; ns, not significant; PMO, phosphorodiamidate morpholino oligomer.

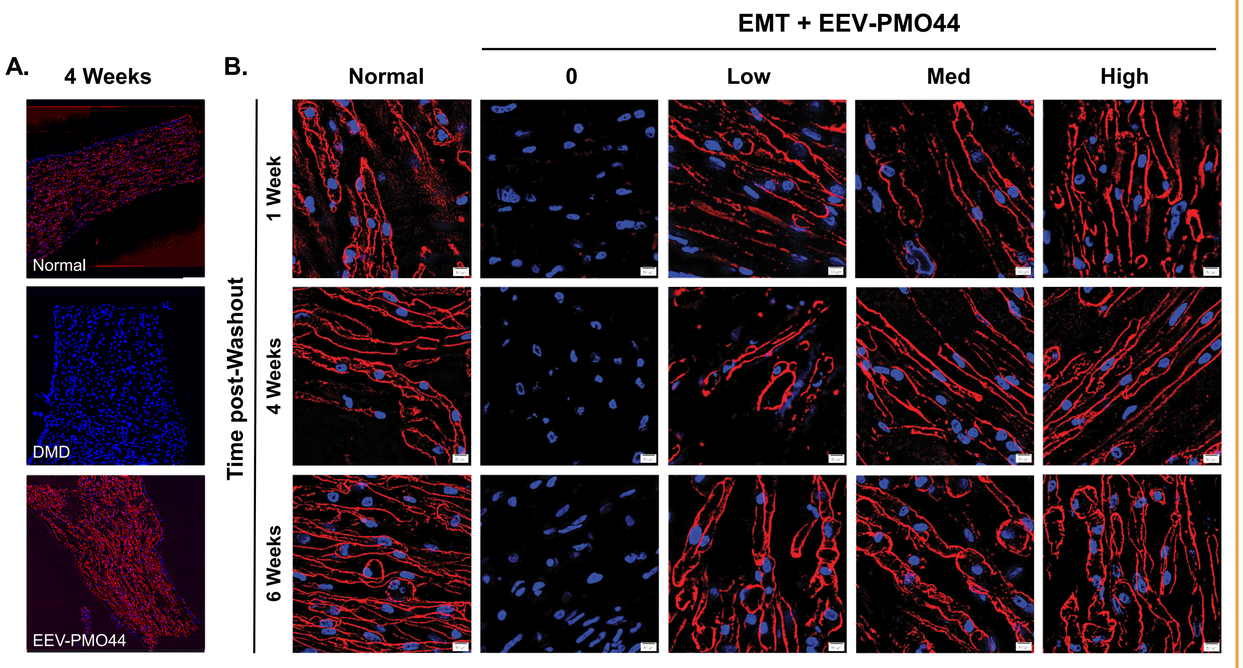
- EMT cross-sections showed even expression of dystrophin across the entire tissue sample following treatment with EEV-PMO44 (**Figure 3A**). In addition, dystrophin was properly localized to the sarcolemma of EMTs (**Figure 3B**).
- Longitudinal sections of EMT also showed broad dystrophin expression across the entire tissue sample following EEV-PMO44 treatment (**Figure 4A**) and proper localization to the sarcolemma (**Figure 4B**).

Figure 3. Dystrophin Restoration with EEV-PMO44 in EMTs (Cross-sectional).



EMTs (N=4) were treated with EEV-PMO44 for 24 hours and analyzed at 1-, 4-, and 6-weeks post-washout. Normal cells from non-DMD individuals were used as controls. Red, dystrophin; blue, DAPI. **(A)** Cross-sectional images (40× stitched) of whole tissue treated with high dose EEV-PMO44 were examined 4 weeks post-washout, demonstrating even dystrophin distribution. **(B)** Cross-sectional images (40× cropped) of EMTs demonstrating proper dystrophin expression on sarcolemma. DAPI, 4′,6-diamidino-2-phenylindole; DMD, Duchenne muscular dystrophy; EEV, endosomal escape vehicle construct; EMT, engineered muscle tissue; Med, medium; PMO, phosphorodiamidate morpholino oligomer.

Figure 4. Dystrophin Restoration with EEV-PMO44 in EMTs (Longitudinal).



EMTs (N=4) were treated with EEV-PMO44 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 (40× stitched) of whole tissue treated with high dose EEV-PMO44 were examined 4 weeks post-washout, demonstrating even dystrophin distribution. **(B)** Longitudinal images (40× cropped) of EMTs, demonstrating proper dystrophin expression on sarcolemma. DAPI, 4′,6-diamidino-2-phenylindole; DMD, Duchenne muscular dystrophy; EEV, endosomal escape vehicle construct; EMT, engineered muscle tissue; Med, medium; PMO, phosphorodiamidate morpholino oligomer.

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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.
- Dystrophin protein was observed in EMTs treated with EEV-PMO44 as early as 7 days post-washout, detectable for at least 6-weeks post-washout, and localized correctly to the sarcolemma.
- This 3D organoid system allowed us to demonstrate EEV-PMO construct efficacy and long-term duration of effect, underscoring the utility of this 3D culture system for the drug development process in Duchenne muscular dystrophy.