In Vitro Efficacy of an Endosomal Escape Vehicle (EEV™) – *DMD* Exon 45 Skipping Oligonucleotide Conjugate in DMD Patient-Derived Skeletal and Cardiac Muscle Cells

Christopher M. Brennan, Holly McKee, Zekun Liu, Cindy Wang, Matthew Streeter, Mahboubeh Kheirabadi, Ziqing Leo Qian, Mahasweta Girgenrath, Nelsa L. Estrella

Entrada Therapeutics, Boston, MA

INTRODUCTION

- Duchenne muscular dystrophy (DMD) is an X-linked, progressive disease caused by mutations in the *DMD* gene, resulting in the production of nonfunctional dystrophin protein.[1,2]
- Currently approved exon-skipping phosphorodiamidate morpholino oligomer (PMO) therapies for DMD have shown modest improvements in dystrophin production.[3-5]
- To enhance the delivery of PMOs to target tissues, we designed a family of proprietary cyclic cell-penetrating peptides that form the core of the Endosomal Escape Vehicle (EEV™) platform[6] (**Figure 1**).
  - Previous studies have demonstrated robust *DMD* exon 44 and 45 skipping and dystrophin restoration in vitro and in vivo with EEV-PMO conjugates ENTR-601-44 and ENTR-601-45, respectively.[7,8]

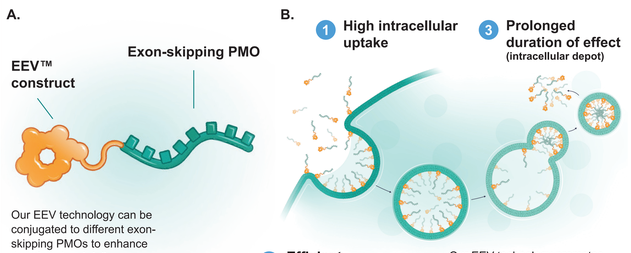
OBJECTIVE

- To further examine the *DMD* exon 45 skipping efficacy of EEV-PMO constructs in vitro using skeletal and cardiac muscle cell lines.

MATERIALS AND METHODS

- EEV-PMO-45 is a *DMD* exon 45 skipping PMO conjugated to the EEV platform.
- Primary skeletal muscle cells were derived from a patient with exon 45 skip-amenable DMD harboring a *DMD* exon 44 deletion (DMDΔ44).
- Induced pluripotent stem cell (iPSC)-derived skeletal muscle cells and cardiomyocytes were derived from a patient with exon 45 skip-amenable DMD harboring a deletion of *DMD* exons 46, 47, and 48 (DMDΔ46-48).
- Dystrophin protein levels were evaluated by simple Western Jess (Bio-Techne, Minneapolis, MN) and immunofluorescence. Exon-skipping efficiency was analyzed by reverse-transcriptase polymerase chain reaction (RT-PCR) and LabChip (Perkin Elmer, Santa Clara, CA).

Figure 1. EEV-PMO Construct Structure and Mechanism of Action.



(A) Structure of EEV-PMO construct consisting of a cyclic cell-penetrating peptide-based EEV construct and an exon-skipping PMO. **(B)** Mechanism of EEV construct-mediated delivery of PMOs. EEV, Endosomal Escape Vehicle; PMO, phosphorodiamidate morpholino oligomer.

CONCLUSIONS

- *DMD* exon 45 skipping EEV-PMO constructs produced robust exon skipping and dystrophin production in three distinct DMD patient-derived muscle cell models amenable to exon 45 skipping.
  - The significant exon skipping and dystrophin production observed in both skeletal and cardiac muscle cells underscore the therapeutic potential of the EEV-PMO approach for the treatment of DMD.
- These findings are consistent with earlier studies showing the preclinical efficacy of ENTR-601-45 and support further study in patients with exon 45 skip-amenable DMD.

ACKNOWLEDGMENTS

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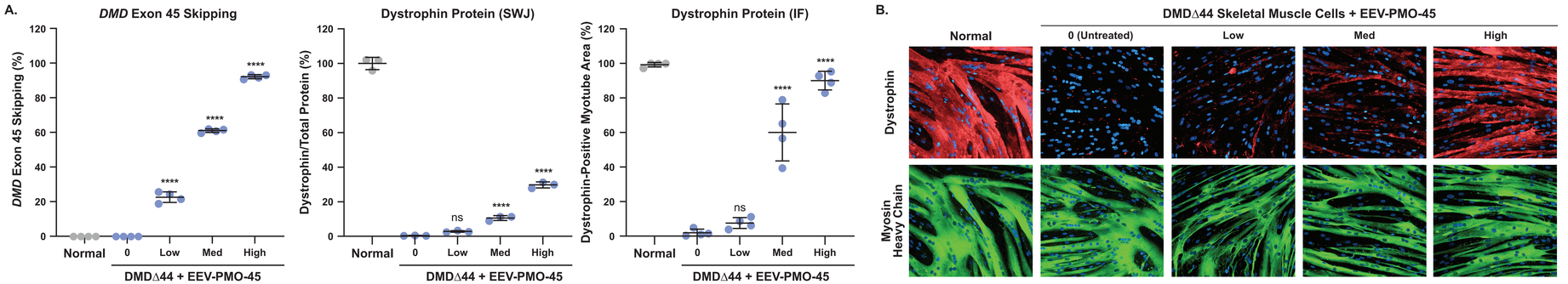
RESULTS

Exon 45 Skipping and Dystrophin Production in Patient-Derived Primary Muscle Cells

- Treatment of DMDΔ44 primary skeletal muscle cells with EEV-PMO-45 resulted in dose-dependent *DMD* exon 45 skipping and dystrophin protein restoration (**Figure 2**).

Figure 2. *DMD* Exon 45 Skipping and Dystrophin Restoration in Primary DMDΔ44 Skeletal Muscle Cells.

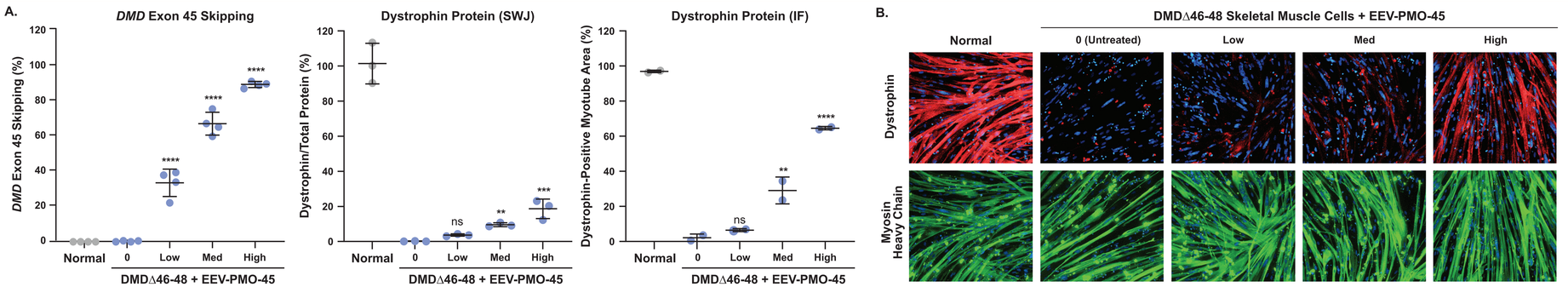


DMDΔ44 patient-derived primary skeletal muscle cells were treated with three different doses of EEV-PMO-45 for 24 hours and analyzed after 5 days of differentiation. **(A)** Exon skipping (RT-PCR) and dystrophin protein (SWJ, IF). **(B)** Visualization of dystrophin (red), myosin heavy chain (green), and Hoechst (blue) by immunofluorescence. Data are shown as mean ± SD. Ordinary one-way ANOVA and Dunnett's multiple comparison test. **p<0.01, ***p<0.001, ****p<0.0001 compared with untreated DMDΔ44 cells. ANOVA, analysis of variance; *DMD*, dystrophin gene; EEV, Endosomal Escape Vehicle; ns, not significant; IF, immunofluorescence; PMO, phosphorodiamidate morpholino oligomer; SD, standard deviation; SWJ, Simple Western Jess.

Exon 45 Skipping and Dystrophin Production in Patient iPSC-Derived Skeletal Muscle Cells

- EEV-PMO-45 produced dose-dependent *DMD* exon 45 skipping and dystrophin protein restoration in DMDΔ46-48 myotubes (**Figure 3**).

Figure 3. *DMD* Exon 45 Skipping and Dystrophin Restoration in DMDΔ46-48 iPSC-Derived Skeletal Muscle Cells.

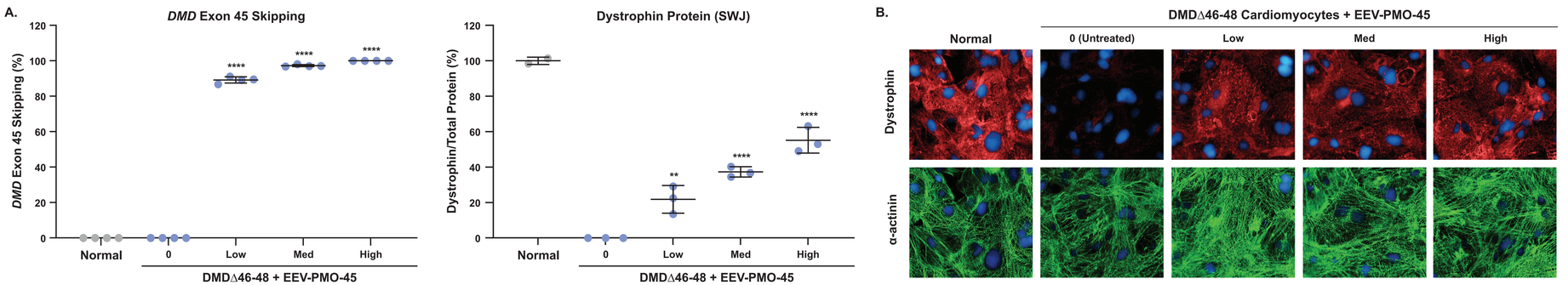


DMDΔ46-48 patient iPSC-derived skeletal muscle cells were treated with three different doses of EEV-PMO-45 for 24 hours and analyzed after 5 days of differentiation. **(A)** Exon skipping (RT-PCR) and dystrophin protein (SWJ, IF). **(B)** Visualization of dystrophin (red), myosin heavy chain (green), and Hoechst (blue) by immunofluorescence. Data are shown as mean ± SD. Ordinary one-way ANOVA and Dunnett's multiple comparison test. **p<0.01, ***p<0.001, ****p<0.0001 compared with untreated DMDΔ46-48 skeletal muscle cells. ANOVA, analysis of variance; *DMD*, dystrophin gene; EEV, Endosomal Escape Vehicle; IF, immunofluorescence; iPSC, induced pluripotent stem cell; ns, not significant; PMO, phosphorodiamidate morpholino oligomer; SD, standard deviation; SWJ, Simple Western Jess.

Exon 45 Skipping and Dystrophin Production in Patient iPSC-Derived Cardiomyocytes

- DMDΔ46-48 cardiomyocytes treated with EEV-PMO-45 showed dose-dependent *DMD* exon 45 skipping and dystrophin protein restoration (**Figure 4**).

Figure 4. Exon 45 Skipping and Dystrophin Production in DMDΔ46-48 iPSC-Derived Cardiomyocytes.



DMDΔ46-48 patient iPSC-derived cardiomyocytes were treated with three different doses of EEV-PMO-45 for 24 hours and analyzed 3 days after treatment washout. **(A)** Exon skipping (RT-PCR) and dystrophin protein (SWJ). **(B)** Visualization of dystrophin (red), α-actinin (green), and Hoechst (blue) by immunofluorescence. Data are shown as mean ± SD. Ordinary one-way ANOVA and Dunnett's multiple comparison test. **p<0.01, ****p<0.0001 compared with untreated DMDΔ46-48 cardiac muscle cells. ANOVA, analysis of variance; *DMD*, dystrophin gene; EEV, Endosomal Escape Vehicle; iPSC, induced pluripotent stem cell; ns, not significant; PMO, phosphorodiamidate morpholino oligomer; SD, standard deviation; SWJ, Simple Western Jess.