P076

# Endosomal Escape Vehicle–Oligonucleotide Conjugates for the Targeted Upregulation and Downregulation of Gene Expression

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## INTRODUCTION

- Intracellular delivery of oligonucleotides is challenging because of poor cell entry and limited escape from the endosome in the target cell.[1,2]
- To improve cellular uptake and enhance endosomal escape of oligonucleotides and other biologics, we developed the endosomal escape vehicle (EEV)™ delivery platform based on cyclic cell-penetrating peptides (**Figure 1**).
- Here, we assessed the broad applicability of the EEV platform to deliver splice-modulating oligonucleotides (phosphorodiamidate morpholino oligomers [PMOs]) to different target cell types.

## MATERIALS & METHODS

***EEV Library Screening and Optimization***

- EEV constructs with robust target cell uptake and efficacy were screened in vitro, and functional validation was assessed. Well-tolerated EEV constructs with desired tissue functional delivery were then assessed in wild-type and disease animal models. Finally, a candidate EEV construct with desired therapeutic effect was selected.

***DMD Studies***

- DMD is caused by mutations in the *DMD* gene, resulting in the production of nonfunctional dystrophin protein (**Figure 2**).[3,4] Currently approved unconjugated PMO therapies were designed to restore the reading frame and produce dystrophin by exon skipping but have shown modest improvements.[5,6]
- EEV-PMO-23, a DMD exon 23 skipping PMO conjugated to the EEV platform, was administered intravenously to assess exon skipping and dystrophin production in D2-*mdx*[7] mice (**Figure 3**). These mice contain a nonsense mutation in exon 23.
- ENTR-601-44, a DMD exon 44 skipping PMO conjugated to the EEV platform, was administered to non-human primates (NHP) to assess exon skipping in cardiac and skeletal muscles (**Figure 4**).
- ENTR-601-45, a DMD exon 45 skipping PMO conjugated to the EEV platform, was evaluated for exon skipping and dystrophin production in patient-derived skeletal and cardiac muscle cells (**Figure 5**).
- Exon skipping efficiency was analyzed by RT-PCR and LabChip. Dystrophin protein restoration in cells was evaluated by Simple Western Jess and immunofluorescence.

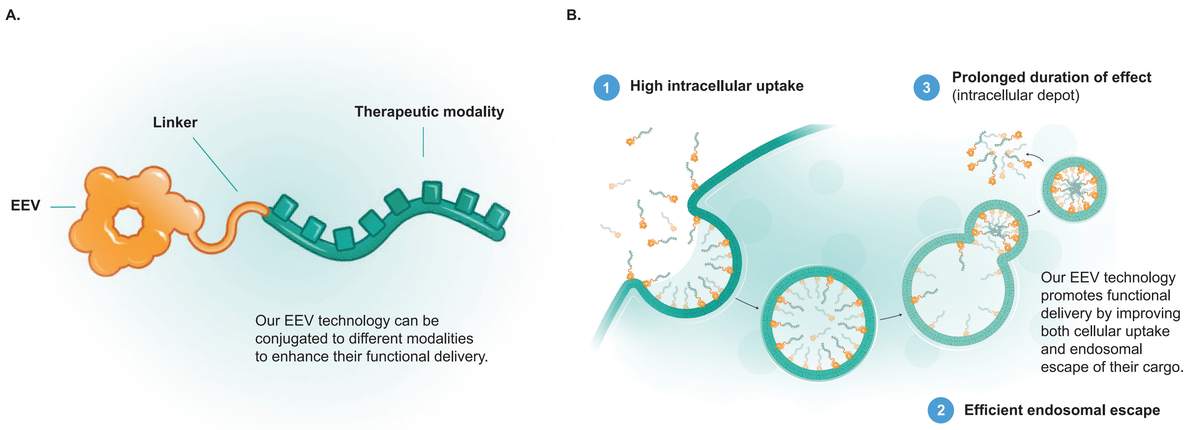
***IRF5 Studies***

- Interferon regulatory factor 5 (IRF5) is a transcription factor that promotes production of several proinflammatory cytokines in macrophages, and overexpression of IRF5 has been implicated in several autoimmune and inflammatory diseases.[8-11]
- IRF5 experiments used several EEV constructs conjugated to an exon skipping PMO.[12] The PMO in this conjugate facilitates exon 4 skipping of IRF5 mRNA that produces a premature stop codon, thereby reducing translation of IRF5 mRNA (**Figure 6A**).[12]

## OBJECTIVES

- To evaluate the ability of the EEV platform to deliver PMOs in preclinical models of Duchenne muscular dystrophy (DMD) and murine macrophages.
- To evaluate functional ability of EEV-PMO conjugates to upregulate or downregulate target genes.

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

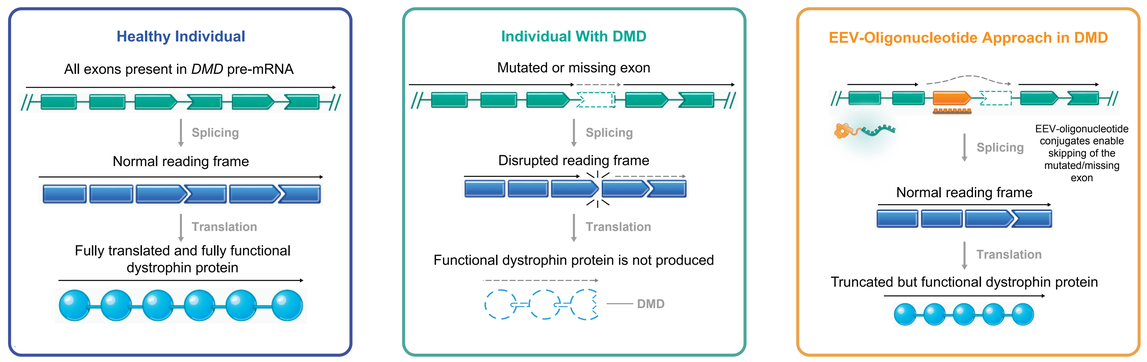



**(A)** Structure of EEV-conjugated therapeutic consisting of cCPP-based EEV construct, linker, and therapeutic modality. **(B)** Mechanism of EEV-mediated delivery of therapeutics. EEV, endosomal escape vehicle; cCPP, cyclic cell-penetrating peptide.

## RESULTS

### Upregulation of Target Genes

### Figure 2. Mechanism of EEV-PMO Conjugates in DMD Models.

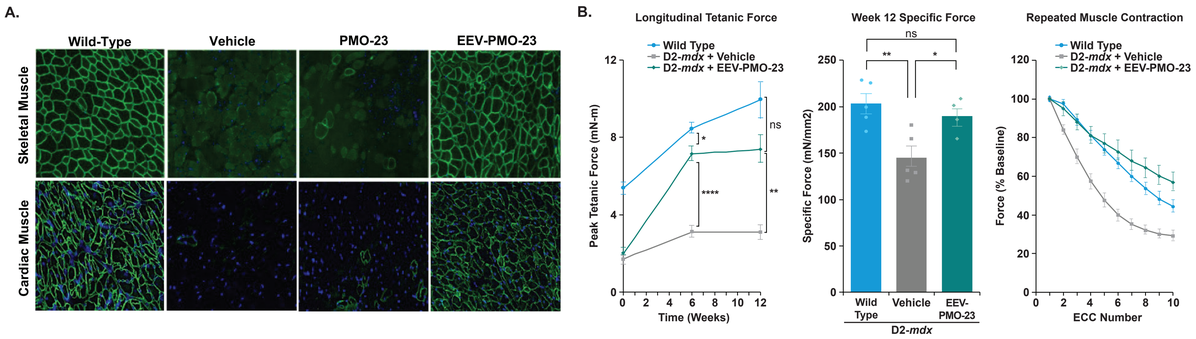



DMD, Duchenne muscular dystrophy; EEV, endosomal escape vehicle.

#### Exon Skipping and Dystrophin Restoration in D2-*mdx* Model of DMD

Broad dystrophin expression and restoration of skeletal and cardiac muscle integrity were observed with monthly EEV-PMO-23 administration compared with PMO-23 alone (**Figure 3A**). Bi-weekly treatment with EEV-PMO-23 improved skeletal muscle contractile force in D2-*mdx* mice which was not significantly different than wild-type mice at Week 12 (**Figure 3B**).

### Figure 3. Repeat EEV-PMO Treatment and Improved Muscle Contractility in D2-*mdx* Mice.

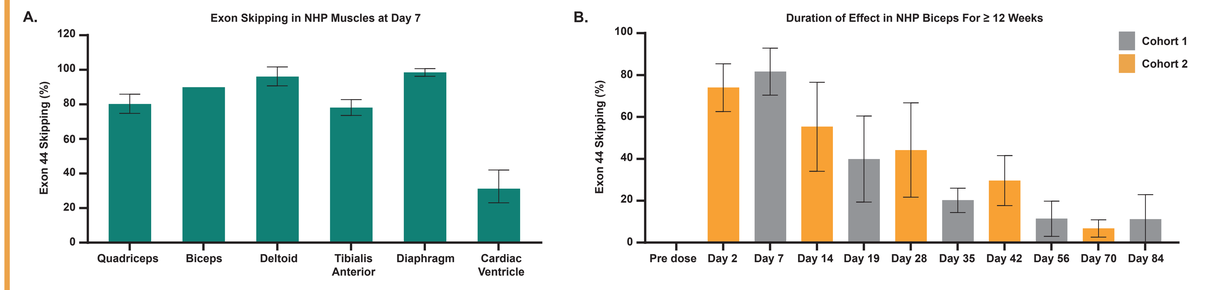



**(A)** D2-*mdx* mice (male, n=6-7) were treated with 4 monthly doses of either vehicle, 20 mg/kg PMO or 20 mg/kg PMO equivalent of EEV-PMO, and the data were collected ~4 weeks after the last dose. Dystrophin in green and DAPI in blue. **(B)** D2-*mdx* mice were treated with vehicle or 80 mg/kg (Q2W) EEV-PMO-23. Muscle contractility was assessed at Weeks 6 and 12 via isometric force generated by tetanic contraction of plantar flexor muscle group and ECC generated by repeated tetanic contraction of the tibialis anterior muscle. *p<0.05, **p<0.01, ****p<0.0001; ns, not significant; shown as mean ± SEM. DAPI, 4′,6-diamidino-2-phenylindole; ECC, eccentric force; EEV, endosomal escape vehicle; PMO, phosphorodiamidate morpholino oligomer; Q2W, once every 2 weeks.

#### Durable Efficacy of ENTR-601-44 in NHP Models of DMD

A single dose of ENTR-601-44 resulted in robust exon skipping in both skeletal and cardiac muscles in NHPs, as well as prolonged duration of effect for at least 12 weeks (**Figure 4**).

### Figure 4. ENTR-601-44 in NHPs.

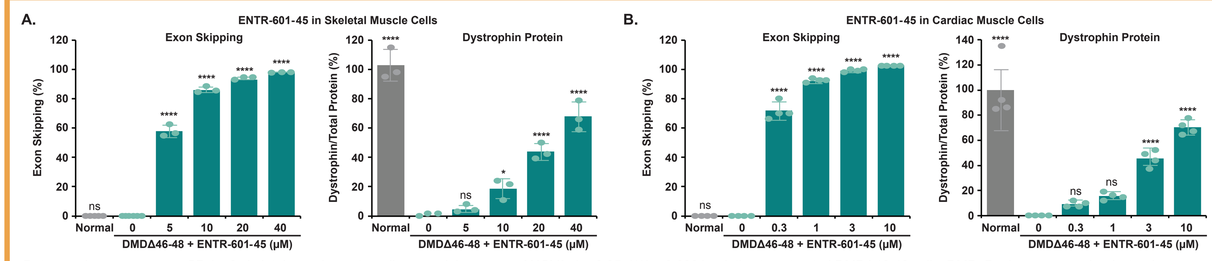



**(A)** At 7 days post-IV infusion of 30 mg/kg (PMO equivalent), robust exon 44 skipping across different muscle groups isolated from the ENTR-601-44–treated NHP. **(B)** Post-intravenous infusion of 35 mg/kg (PMO equivalent), robust exon 44 skipping observed in biceps in the ENTR-601-44–treated NHP (n=3 per cohort) for ≥12 weeks. NHP, non-human primate; PMO, phosphorodiamidate morpholino oligomer.

#### In Vitro Efficacy of ENTR-601-45 in Patient-Derived Skeletal and Cardiac Muscle Cells

DMD patient-derived skeletal and cardiac muscle cells (DMDΔ46-48) were treated with ENTR-601-45 for 24 hours. ENTR-601-45 showed robust exon skipping and dystrophin production in patient-derived skeletal (**Figure 5A**) and cardiac muscle cells (**Figure 5B**).

### Figure 5. ENTR-601-45 In Vitro Efficacy.



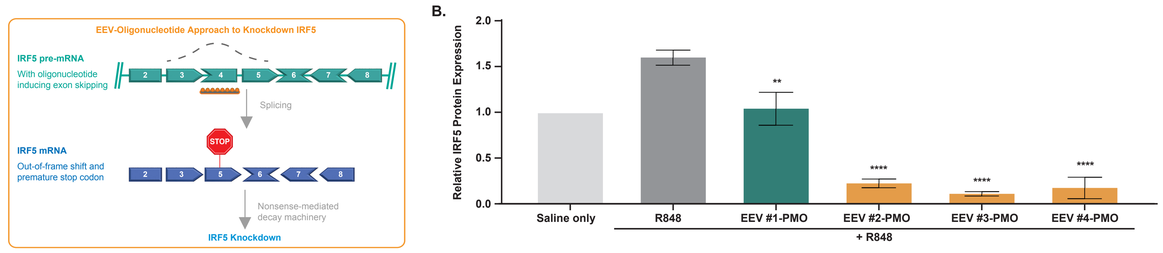

Data are shown as mean ± SD (n=3 skeletal muscle; n=4 cardiac muscle); one-way ANOVA; *p<0.05, ****p<0.0001; relative to untreated DMDΔ46-48 cells. DMD, Duchenne muscular dystrophy; SD, standard deviation.

### Downregulation of Target Genes

#### EEV-PMO Treatment Reduced IRF5 Expression In Vitro and In Vivo

Four different EEV-PMO conjugates reduced IRF5 expression in mouse macrophages stimulated to produce IRF5 (**Figure 6B**). EEV#1-PMO also induced downregulation of IRF5 in liver, small intestine, and skeletal muscle of wild-type mice.

### Figure 6. EEV-PMO Knockdown of IRF5 Expression.




**(A)** Mechanism of EEV#1-PMO for IRF5 knockdown. **(B)** Mouse macrophage cells were pre-treated with different EEV (#1-4) conjugated to PMO at 2 micromolar for 4 hours, followed by stimulation with R848 to stimulate IRF5 production overnight. At 24 hours post treatment, cells were harvested and evaluated by Western Blot. R848 is a TLR7/8 agonist that upregulates IRF5. **p<0.01, ****p<0.0001 versus R848-treated cells. EEV, endosomal escape vehicle; IRF5, interferon regulatory factor 5; PMO, phosphorodiamidate morpholino oligomer; TLR, toll-like receptor.

## CONCLUSIONS

- The EEV™ platform consists of a library of proprietary cyclic peptides with unique chemistry that enable improved cellular uptake, endosomal escape, and consistent translation across species.
- The results presented here demonstrate that the EEV platform successfully and efficiently delivers oligonucleotides to specific cell and tissue types.
- The EEV-PMO approach has broad applicability to upregulate or downregulate target genes through distinct mechanisms of action.

## ACKNOWLEDGEMENTS

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