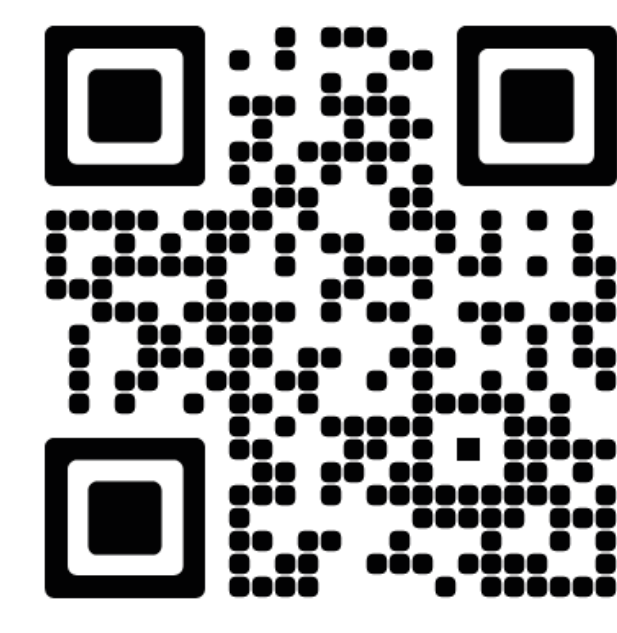




INTRODUCTION

- Intracellular delivery of oligonucleotide therapeutics for the treatment of Duchenne muscular dystrophy (DMD) is challenging because of poor cell entry and limited escape from the endosome in the target cell, necessitating high therapeutic doses.^{1,2}
- To address these limitations, we designed a family of cyclic cell-penetrating peptides that form the core of our Endosomal Escape Vehicle (EEV™) platform, which has been shown to efficiently deliver exon skipping phosphorodiamidate morpholino oligomers (PMOs) to skeletal and cardiac muscle³ (Figure 1).
- EEV peptides have improved delivery of PMOs to skeletal and cardiac muscle in preclinical models of DMD³. EEV-PMOs targeting exon 44 (ENTR-601-44),³ exon 45 (ENTR-601-45)³ and exon 50 (ENTR-601-50)⁴ of *DMD* messenger RNA demonstrated robust exon skipping and increased dystrophin expression in vitro and in vivo.

MATERIALS AND METHODS

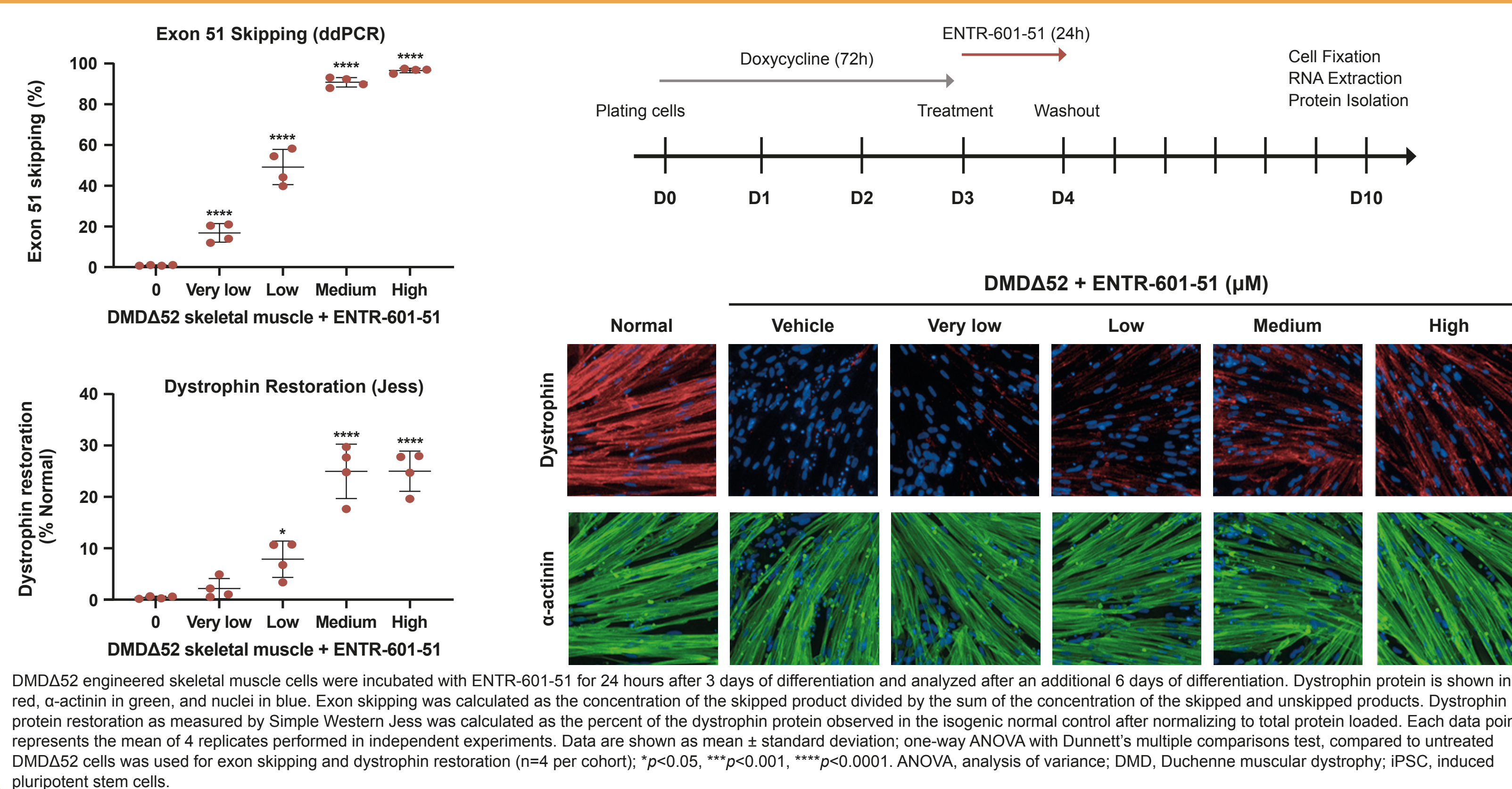
- ENTR-601-51 is a *DMD* exon 51 skipping PMO conjugated to an EEV cyclic peptide and is in development for the treatment of patients with exon 51 skip–amenable DMD.
- DMDΔ52 induced pluripotent stem cells (iPSC) were genetically engineered from a normal control iPSC line to include a deletion of *DMD* exon 52. Additional myogenic cells were derived from primary myogenic cells isolated from patients with exon 51 skip–amenable DMD harboring a deletion of *DMD* exons 48-50 (DMDΔ48-50), exons 45-50 (DMDΔ45-50), or exon 50 (DMDΔ50).
- Del52hDMD *mdx* are human dystrophin (hDMD)–expressing mice engineered with a deletion in the hDMD exon 52 on the *mdx* background, resulting in an exon 51 skip–amenable mouse line.⁵ hDMD *mdx* mice carrying the full-length hDMD were used as healthy controls for dystrophin quantification and muscle function; PMO localization in satellite cells was also assessed.
- Exon skipping efficiency was analyzed by digital droplet polymerase chain reaction (ddPCR) (Bio-Rad, Hercules, CA). Dystrophin restoration was evaluated by Simple Western Jess (Bio-Techne, Minneapolis, MN).
- Muscle strength (specific tetanic force) and resistance to repeated eccentric (ECC)–induced muscle damage were assessed in the gastrocnemius muscle. These measurements were performed using a 3-in-1 Whole Animal Muscle Physiology system (Aurora Scientific, Canada).
- Gastrocnemius muscle sections from mice were assessed by immunohistochemistry to detect PMO localization in centrally nucleated fibers and PAX7-positive satellite cells.

RESULTS

Exon Skipping and Dystrophin Restoration After Treatment With ENTR-601-51 in iPSC-Derived Muscle Cells

- Treatment of iPSC-derived muscle cells with ENTR-601-51 resulted in dose-dependent *DMD* exon 51 skipping and dystrophin protein expression (Figure 2).

Figure 2. Efficacy of ENTR-601-51 in Engineered DMDΔ52 iPSC-Derived Myotubes



ENTR-601-51-Induced Exon Skipping and Dystrophin Protein Restoration Across Multiple Exon 51 Skip–Amenable Genotypes

- ENTR-601-51 drives exon 51 skipping and promotes dystrophin restoration in DMDΔ45-50, DMDΔ48-50, DMDΔ50, and DMDΔ52 cell lines (Table 1).

Table 1. ENTR-601-51 in Multiple Exon 51 Skip–Amenable Myotubes

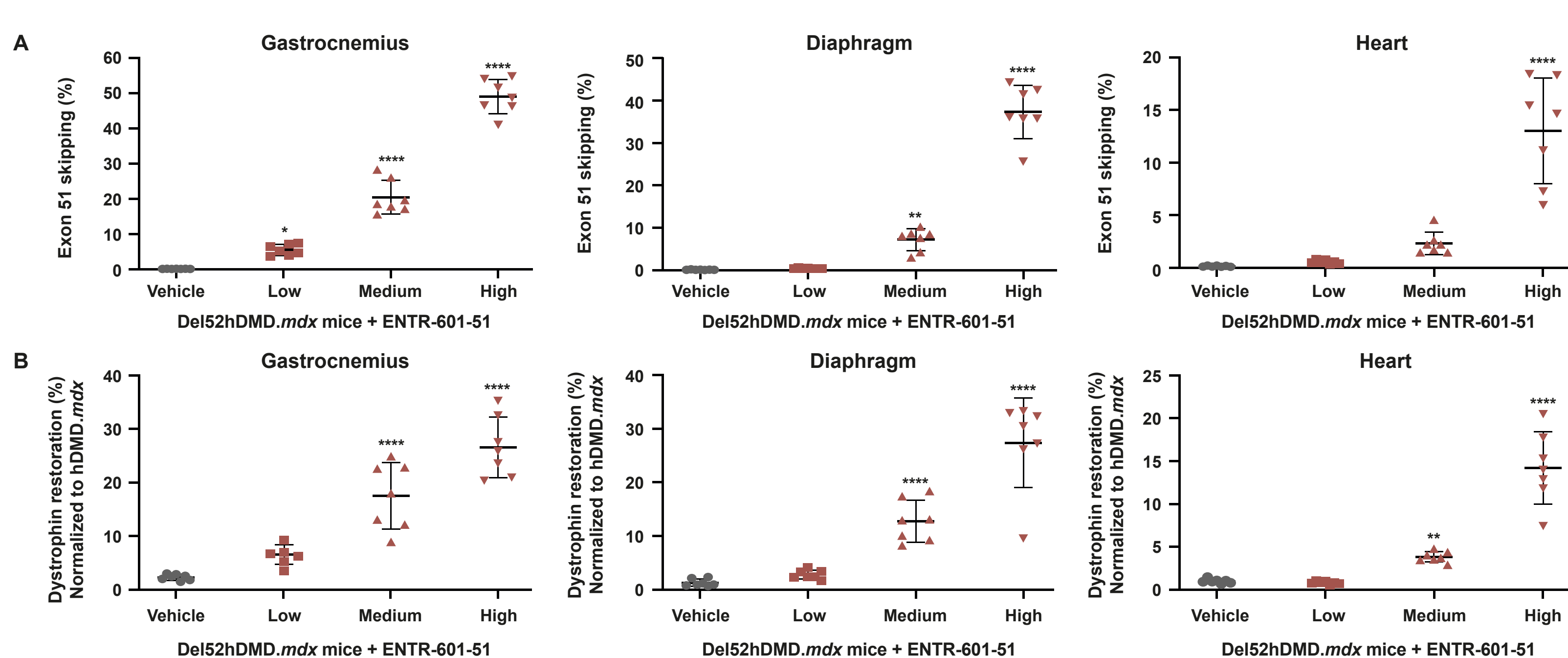
Dose response	Myotube genotype			
	DMDΔ45-50	DMDΔ48-50	DMDΔ50	iPSC-engineered DMDΔ52
Exon 51 skipping	✓	✓	✓	✓
Dystrophin protein restoration	✓	✓	✓	✓

DMD, Duchenne muscular dystrophy; iPSC, induced pluripotent stem cells.

Exon Skipping and Dystrophin Production After Treatment With ENTR-601-51 in Deletion 52 Mice

- Three doses every 6 weeks (Q6W) of ENTR-601-51 lead to a significant, dose-dependent increase in exon 51 skipping (A) and dystrophin protein restoration (B) in skeletal and cardiac muscles in del52hDMD *mdx* mice (Figure 3).

Figure 3. Efficacy of ENTR-601-51 in Del52hDMD *mdx* Mice

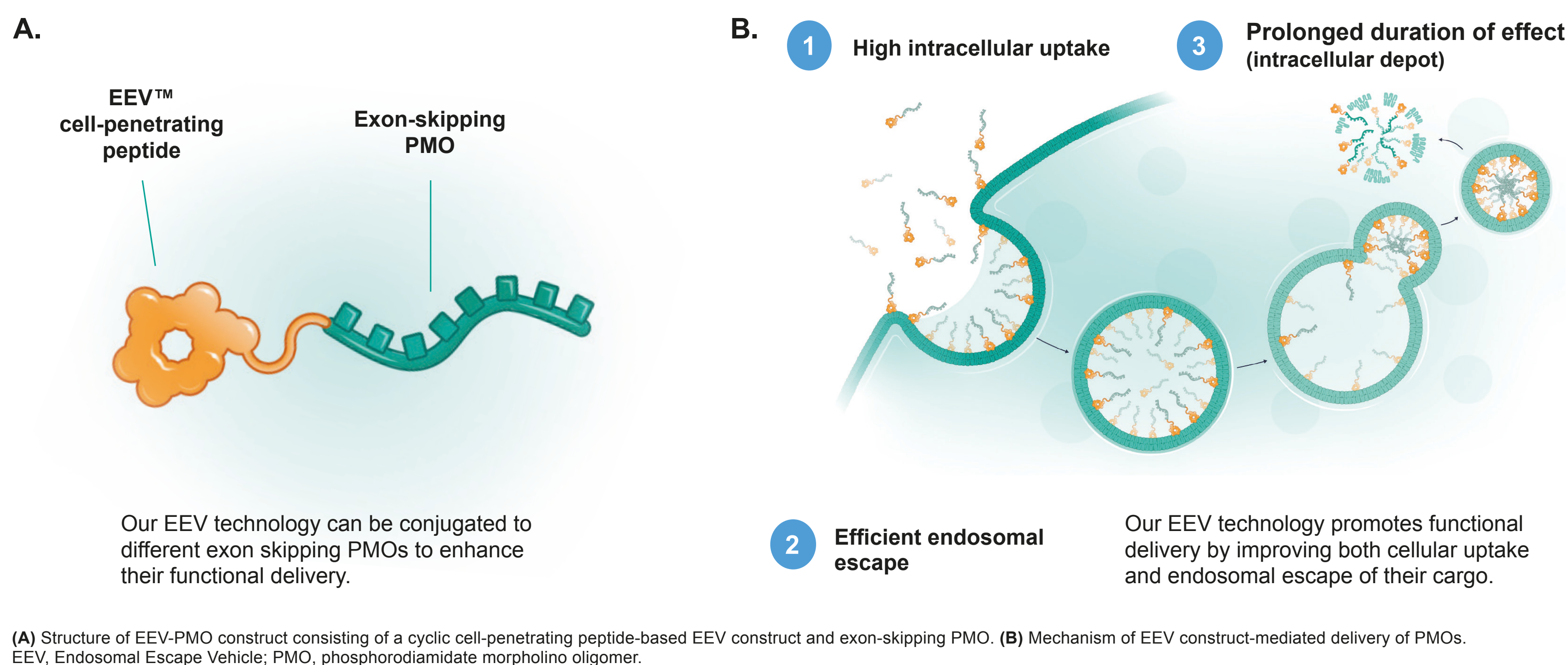


del52hDMD *mdx* mice were treated with 3 IV injections of ENTR-601-51 or vehicle 6 weeks apart. Human DMD exon 51 skipping (A) and dystrophin protein expression (B) were analyzed in the gastrocnemius, diaphragm, and heart 6 weeks after the final dose. Percent dystrophin protein restoration is normalized to total protein and normalized to hDMD *mdx* controls. Data shown as mean ± standard deviation. One-way ANOVA with Dunnett's post-test was used for statistical comparison; **p*≤0.01, ***p*≤0.01, ****p*≤0.0001 vs. vehicle. ANOVA, analysis of variance; hDMD, human dystrophin transgene; IV, intravenous.

OBJECTIVE

- To assess the efficacy and therapeutic potential of ENTR-601-51 using in vitro human cell lines and in vivo mouse models of DMD amenable to exon 51 skipping.

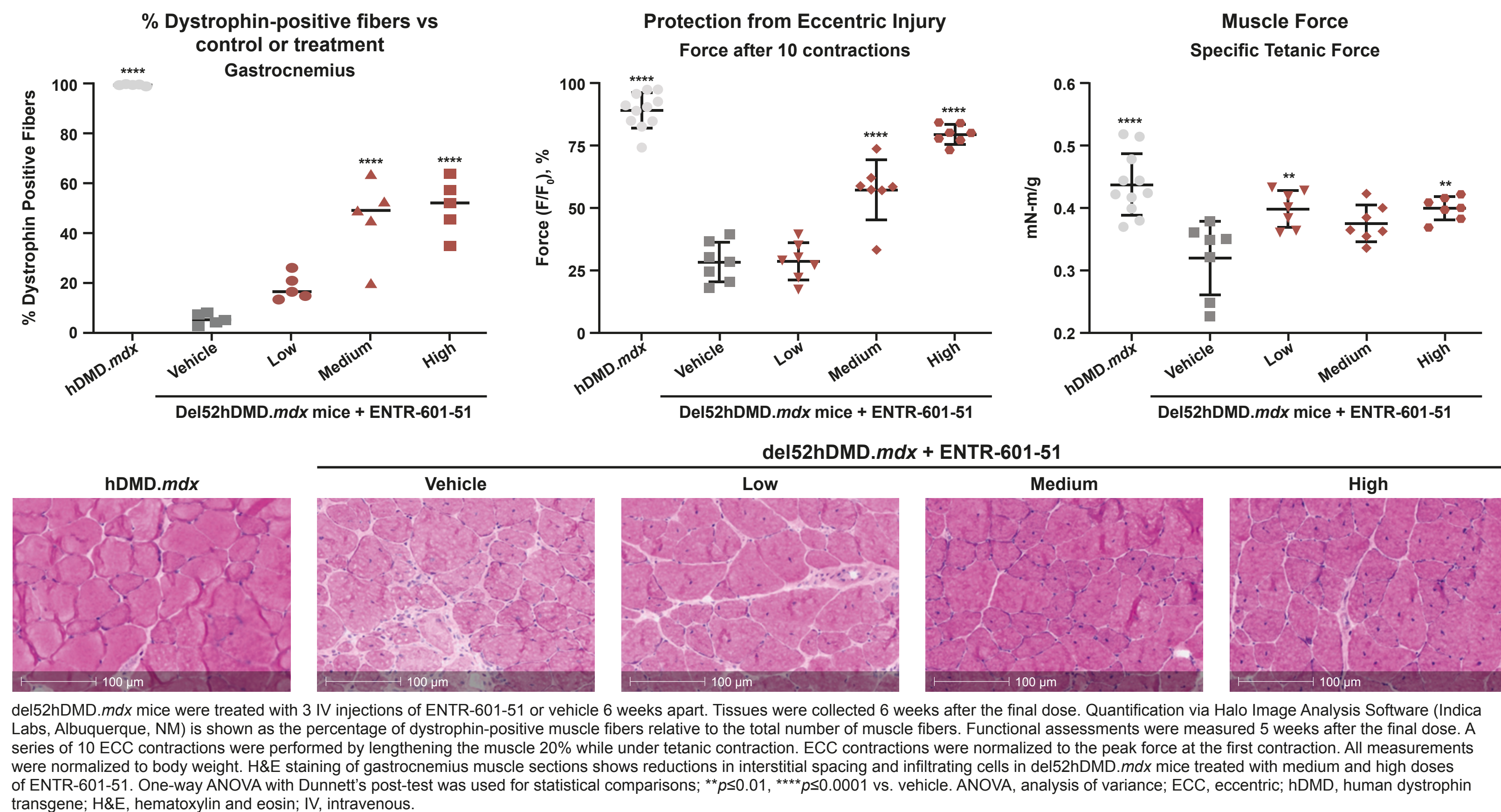
Figure 1. EEV-PMO Construct Structure and Mechanism of Action.



Treatment With ENTR-601-51 Increases Dystrophin-Positive Fibers and Improves Muscle Function in Deletion 52 Mice

- Three Q6W doses of ENTR-601-51 administered to del52hDMD *mdx* mice produced significant, dose-dependent increases in dystrophin-positive muscle fibers in the gastrocnemius.
- ENTR-601-51 also produced significant, dose-dependent increases in specific tetanic force and provides protection from force loss following repeated ECC contractions (Figure 4).
- Histological staining of gastrocnemius muscle shows reduced interstitial spacing, decreased numbers of infiltrating cells, and improved muscle tissue architecture in del52hDMD *mdx* mice with increasing dose levels of ENTR-601-51.

Figure 4. Improved Skeletal Muscle Morphology and Function With ENTR-601-51 in del52hDMD *mdx* Mice



ENTR-601-51 Localizes to the Satellite Cell Compartment

- Six weeks after administration of ENTR-601-51 (3 doses, Q6W) to del52hDMD *mdx* mice, PMO localized in centrally nucleated fibers (CNFs) and satellite cells (Pax7 positive) in gastrocnemius muscle (Figure 5).

Figure 5. PMO Localization in CNFs and Satellite Cells in Gastrocnemius Muscle of del52hDMD *mdx* Mice



del52hDMD *mdx* mice were treated with a high dose of ENTR-601-51 (3 doses, Q6W). Gastrocnemius muscle was collected 6 weeks after the final dose. Distribution of PMO (red), PAX7 (magenta), and laminin (green) was analyzed by IHC. Nuclei are labeled with Hoechst 33342 trihydrochloride (blue). Magenta arrow shows PMO co-localized in a satellite cell; yellow arrow shows PMO localized to a CNF. CNF, centrally nucleated fiber; IHC, immunohistochemistry; PMO, phosphorodiamidate morpholino oligomer; Q6W, every 6 weeks.

CONCLUSIONS

- ENTR-601-51 demonstrated significant dose-dependent exon skipping and dystrophin production in both in vitro (cell-based) and in vivo (mouse) models of DMD amenable to exon 51 skipping.
- All cell lines with different mutations amenable to 51 skipping demonstrated efficient exon 51 skipping and dystrophin protein production upon exposure to ENTR-601-51.
- Treatment with ENTR-601-51 resulted in expression of partially functional dystrophin, as evidenced by enhanced muscle function and restored tissue architecture in the deletion 52 mouse model.
- ENTR-601-51 PMO were co-localized in the satellite cells and in the CNFs.
- These findings demonstrate the therapeutic potential of ENTR-601-51 and support further studies in patients with DMD amenable to exon 51 skipping.

ACKNOWLEDGMENTS

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