Optimization and Application of Endosomal Escape Vehicle (EEV™) Cell-Penetrating Peptides for Enhanced Delivery of Oligonucleotides and Genomic Medicines

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Tides USA, May 16, 2024

entrada THERAPEUTICS

DISCLAIMER

This presentation includes express and implied “forward-looking statements.” Forward looking statements include all statements that are not historical facts, and in some cases, can be identified by terms such as “may,” “might,” “will,” “could,” “would,” “should,” “expect,” “intend,” “plan,” “objective,” “anticipate,” “believe,” “estimate,” “predict,” “potential,” “continue,” “ongoing,” or the negative of these terms, or other comparable terminology intended to identify statements about the future. Forward-looking statements contained in this presentation include, but are not limited to, statements about our product development activities and clinical trials, our regulatory filings and approvals, statements related to our ability to initiate and recruit for a healthy volunteer trial for ENTR-601-44 in the United Kingdom with first subject dosed in September 2023, expectations regarding the timing of data from our Phase 1 trial for ENTR-601-44 in the second half of 2024, the ability to resolve the clinical hold for ENTR-601-44 and subsequent activities, expectations regarding the timing or content of any update regarding our regulatory filings, expectations regarding the safety and therapeutic benefits of ENTR-601-44, our ability to develop and advance our current and future product candidates and discovery programs, our ability to establish and maintain collaborations or strategic relationships, our ability to raise additional funding, the rate and degree of market acceptance and clinical utility of our product candidates, the potential of our EEV product candidates and EEV platform, the ability and willingness of our third-party collaborators to continue research and development activities relating to our product candidates, including our Vertex partnership for ENTR-701, our collaborators’ ability to protect our intellectual property for our products, and the sufficiency of our cash resources through 2025. By their nature, these statements are subject to numerous risks and uncertainties, including factors beyond our control, that could cause actual results, performance or achievement to differ materially and adversely from those anticipated or implied in the statements. You should not rely upon forward-looking statements as predictions of future events. Although our management believes that the expectations reflected in our statements are reasonable, we cannot guarantee that the future results, performance or events and circumstances described in the forward-looking statements will be achieved or occur. Recipients are cautioned not to place undue reliance on these forward-looking statements, which speak only as of the date such statements are made and should not be construed as statements of fact.

Certain information contained in this presentation and statements made orally during this presentation relate to or are based on studies, publications, surveys and other data obtained from third-party sources and our own internal estimates and research. While we believe these third-party studies, publications, surveys and other data to be reliable as of the date of this presentation, it has not independently verified, and makes no representations as to the adequacy, fairness, accuracy or completeness of, any information obtained from third-party sources. In addition, no independent source has evaluated the reasonableness or accuracy of our internal estimates or research and no reliance should be made on any information or statements made in this presentation relating to or based on such internal estimates and research.

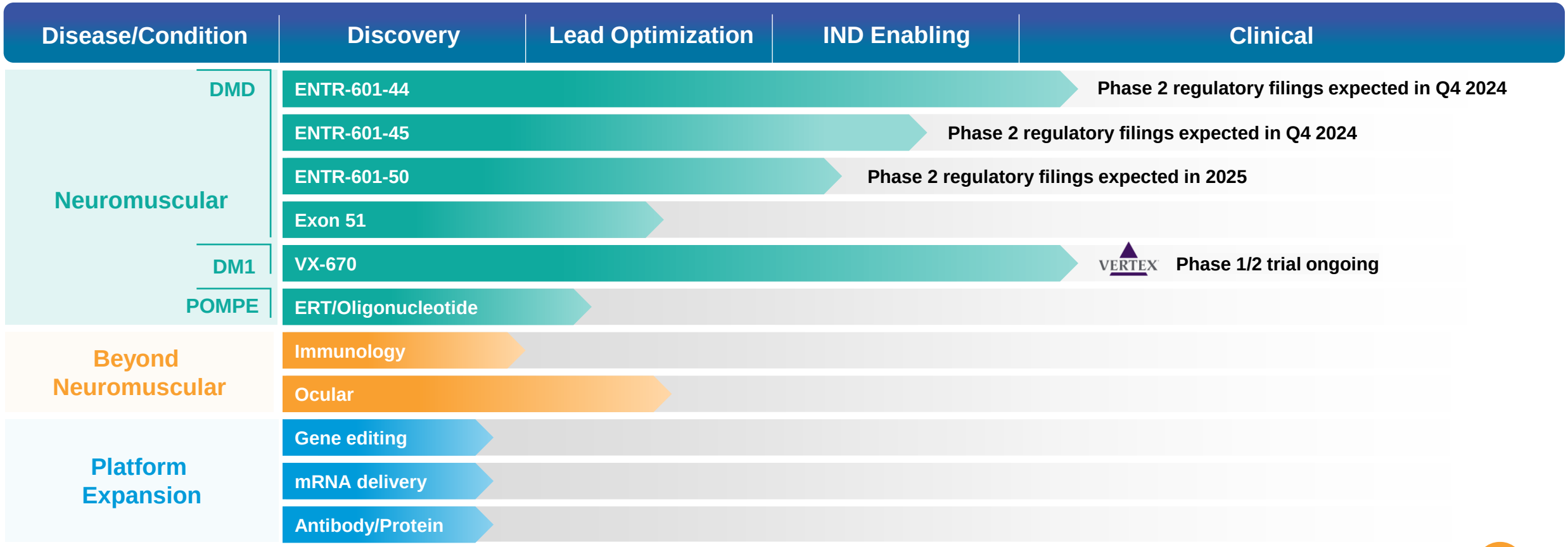
OUR MISSION

To Treat Devastating Diseases with Intracellular Therapeutics

entrada THERAPEUTICS

A Differentiated and Expanding Pipeline

Entrada’s pipeline includes a diverse array of high potential and high value assets;
Each disease has a substantial patient population with a significant unmet medical need



EEV™ PLATFORM DEVELOPMENT AND OPTIMIZATION

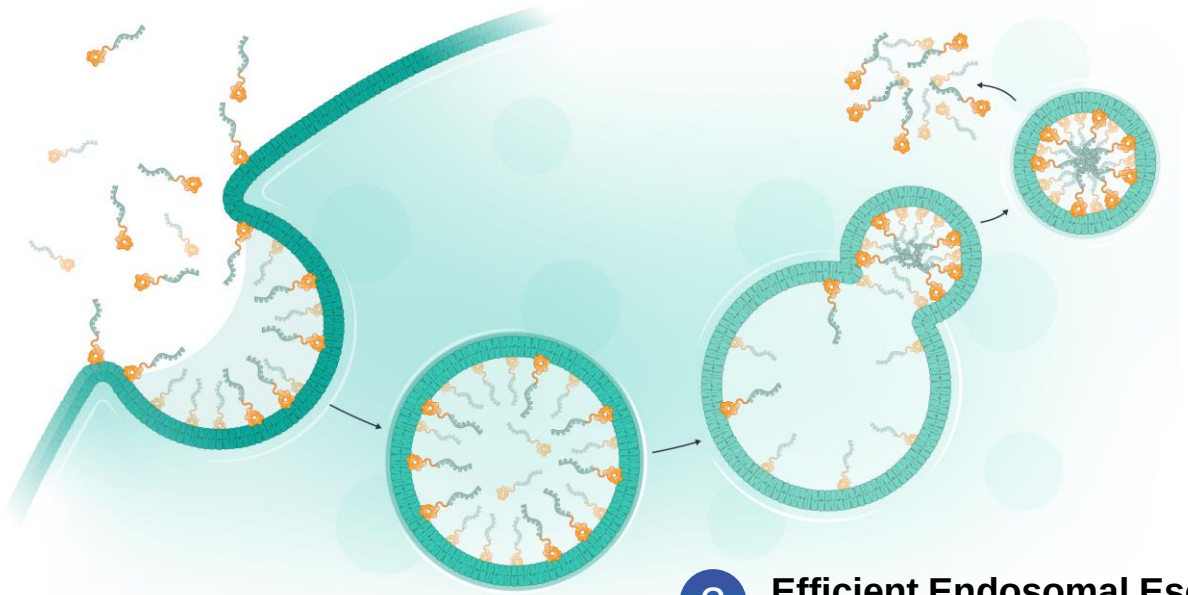
Endosomal Escape Vehicle (EEV™) Therapeutics

- Unique chemistry results in **improved uptake and endosomal escape**
- Cyclic structure designed to **extend half life and increase stability**
- Phospholipid binding potentially **enables broad biodistribution to all cells**
- Mechanism of **internalization conserved across species**

Entrada seeks to solve a fundamental problem: a lack of efficient cellular uptake and escape from the endosome; Both are critical to intracellular target engagement and therapeutic benefit

1 **High Intracellular Uptake**
(~90% Uptake)

3 **Prolonged Duration Of Effect**
(Intracellular Depot)



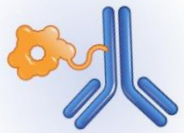
2 **Efficient Endosomal Escape**
(~50% Escape v. 2% Standard)

Qian, Z. et al. *ACS Chem. Biol.* 2013; Qian, Z. et al. *Biochemistry* 2014; Qian, Z. et al. *Biochemistry* 2016; Sahni, A. et al. *ACS Chem. Biol.* 2020; Pei, D. *Acc. Chem. Res.* 2022.

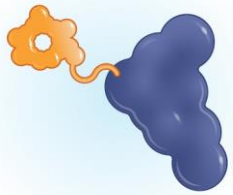
A BROADLY APPLICABLE PLATFORM

Entrada has demonstrated intracellular uptake of unique moieties ranging from 1 kDa to 600 kDa

Antibodies

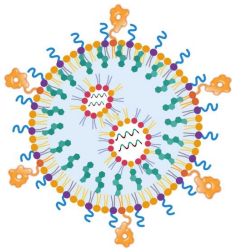


Enzymes

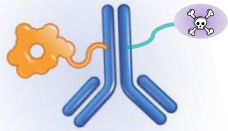


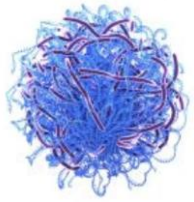
Oligonucleotides

Genomic Medicine



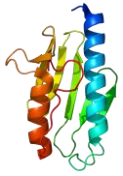
Antibody Drug Conjugates





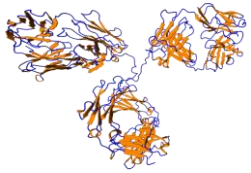
300-3000 KDa

Genomic Medicine Formulation



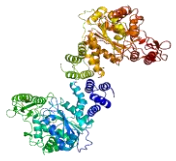
550-600 KDa

Hybrid frataxin



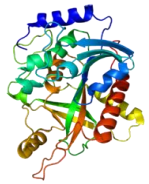
150 KDa

Antibody



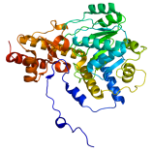
98 KDa

Thymidine phosphorylase



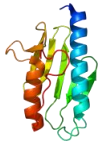
96 KDa

Purine nucleoside phosphorylase



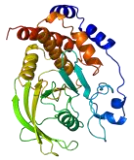
86 KDa

Alanine-glyoxylate aminotransferase



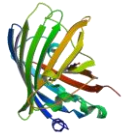
46 KDa

Human frataxin



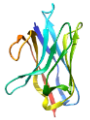
37 KDa

PTP1B catalytic domain



32 KDa

EGFP



16 KDa

Nanobody

6 KDa

Oligonucleotide

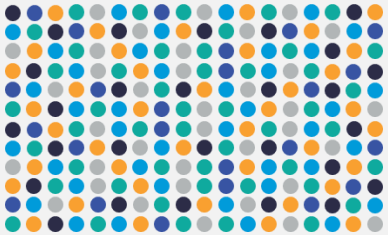
1-3 KDa

Various peptide cargos

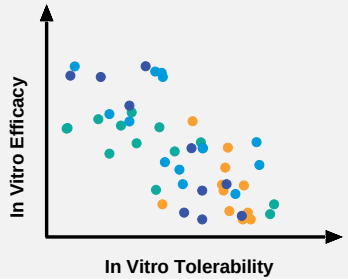
DISCOVERY ENGINE FOR EEV THERAPEUTICS
EEV-OLIGO EXAMPLE

Fit-for-purpose EEVs can be designed for target indications and modalities via iterative optimizations of EEV peptides through medicinal chemistry, *in vitro* and *in vivo* screenings

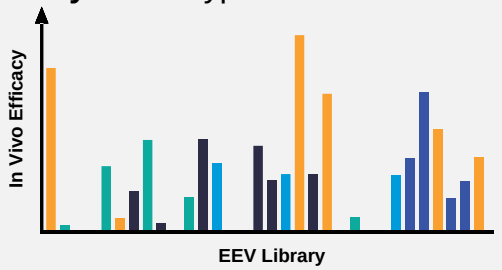
Chemically diverse macrocyclic **EEV library** generated through medicinal and combinatorial chemistry



In vitro **delivery and counter-screening** in relevant cell types with therapeutic payload



In vivo screening to assess **functional delivery and pharmacodynamic activity** in wild-type and disease models

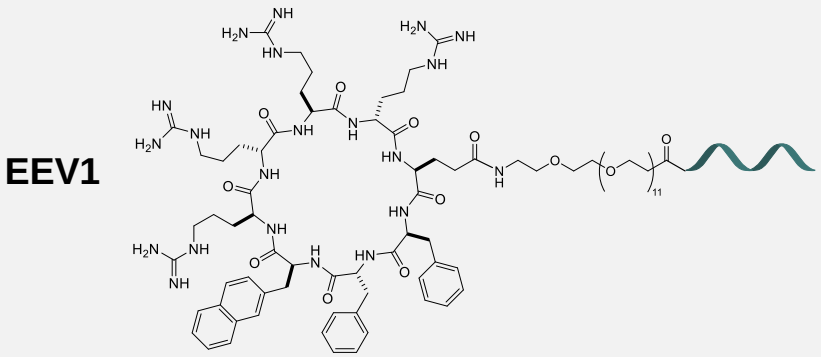


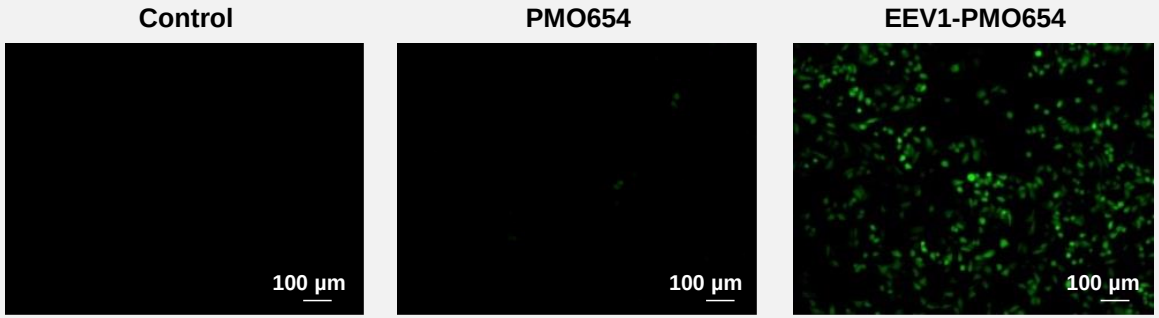
EEV, endosomal escape vehicle.

OLIGO DELIVERY WITH FIRST GENERATION EEV
EEV1 EXAMPLE

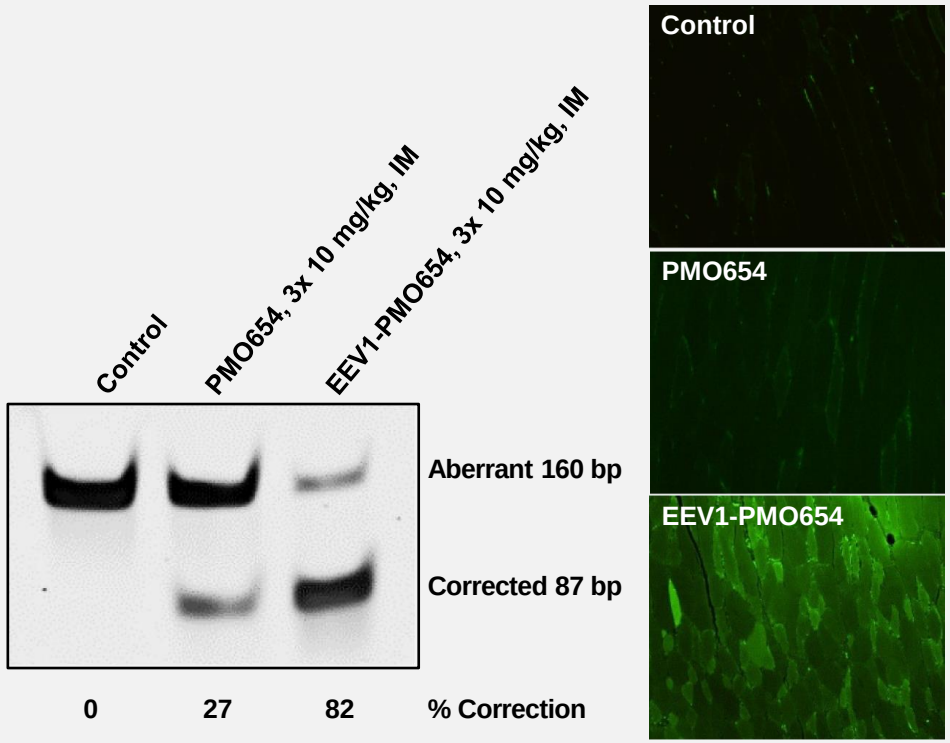
A first-generation EEV1 peptide-PMO construct enhanced splice correction *in vitro* and after local injection, demonstrated functional delivery of oligonucleotides

Splicing Correction in HeLa EGFP-654 Cells





Three Daily IM Doses of EEV1-PMO654



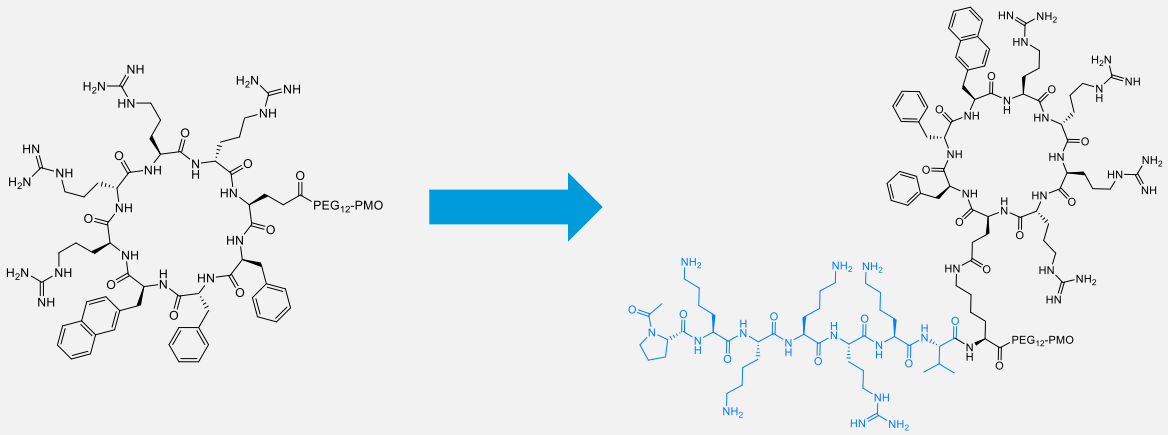
[a]Treatment of HeLa EGFP-654 cells with antisense oligonucleotides, such as PMOs, could switch the splicing and restore expression of EGFP; **EEV**, endosomal escape vehicle; **PMO**, phosphorodiamidate morpholino oligomer; **IM**, intramuscular; Qian, Z. et al. *Biochemistry* 2014, 2016; Li, X. et al. *Mol. Ther. Nucleic Acids* 2023.

ENHANCED OLIGONUCLEOTIDE DELIVERY
EEV2 EXAMPLE

The addition of an exocyclic peptide sequence to a first-generation EEV1 peptide improved exon skipping in skeletal and cardiac muscle of *mdx* mice after intravenous injection

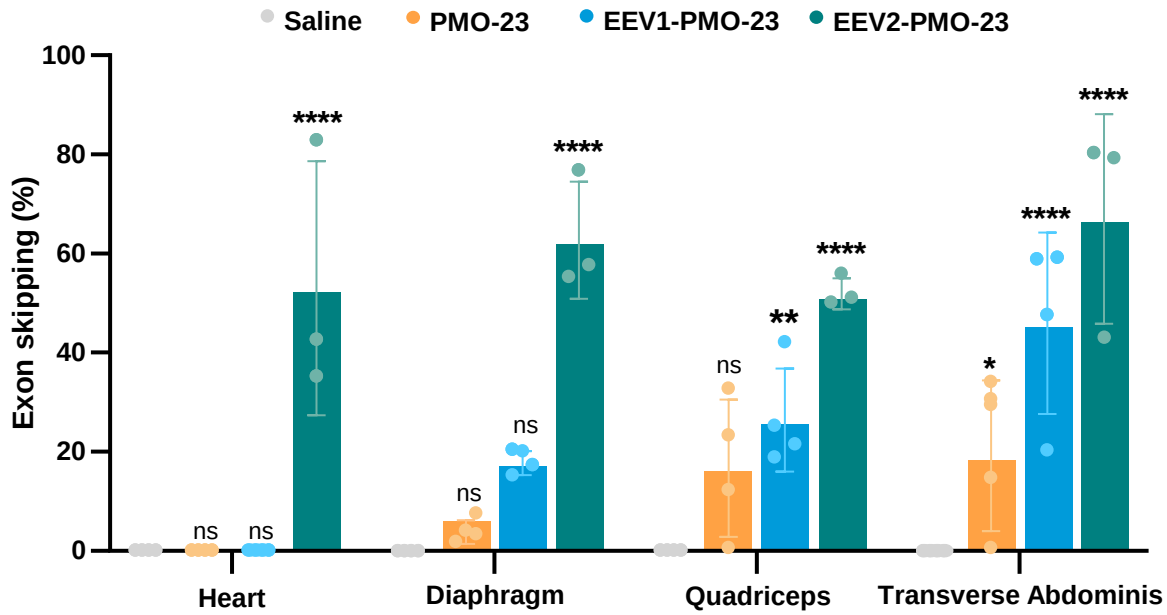
Structure of EEV2 Construct

EEV1 + exocyclic peptide sequence = EEV2



- To create the EEV2 construct, EEV1 was modified to include an exocyclic peptide sequence to improve delivery to the nucleus

Higher In Vivo Exon Skipping with EEV2 vs. EEV1



- *mdx* mice were evaluated for exon skipping (via RT-PCR) 7 days following a single 20-mg/kg IV injection of saline, PMO-23, EEV1-PMO-23, or EEV2-PMO-23

p<0.01, ****p<0.0001 vs. Vehicle. Values are shown as mean ± standard deviation. *mdx* is a DMD mouse model with a nonsense mutation in DMD exon 23; **EEV, endosomal escape vehicle; **IV,** intravenous; **ns**, not significant; **PMO**, phosphorodiamidate morpholino oligomer; **RT-PCR,** reverse transcription polymerase chain reaction; Li, X. et al. *Mol. Ther. Nucleic Acids* 2023.

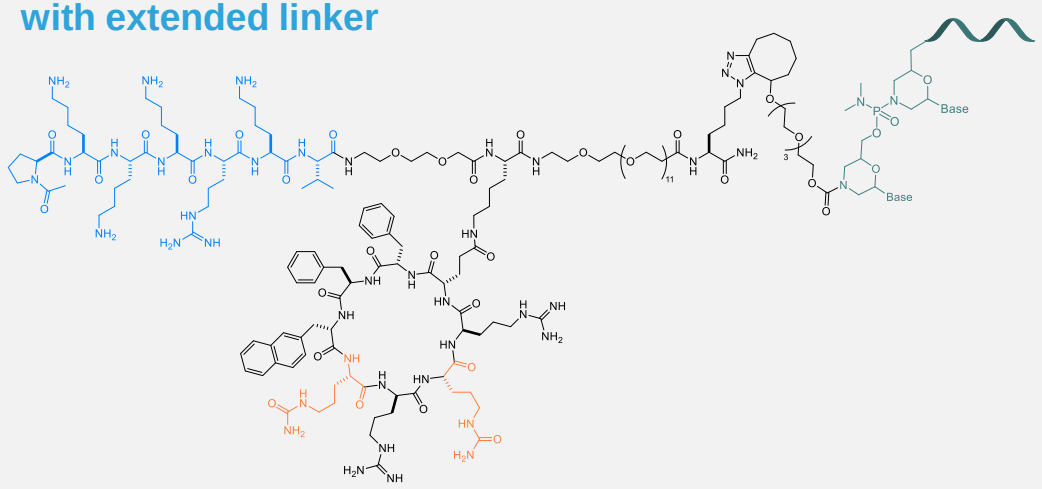
OPTIMIZATION OF EEV FOR MUSCLE DELIVERY
EEV3 Example

Rational substitution of cationic residues with a surrogate results in robust functional delivery to skeletal and cardiac muscle

EEV3-PMO654 Structure and Medicinal Chemistry

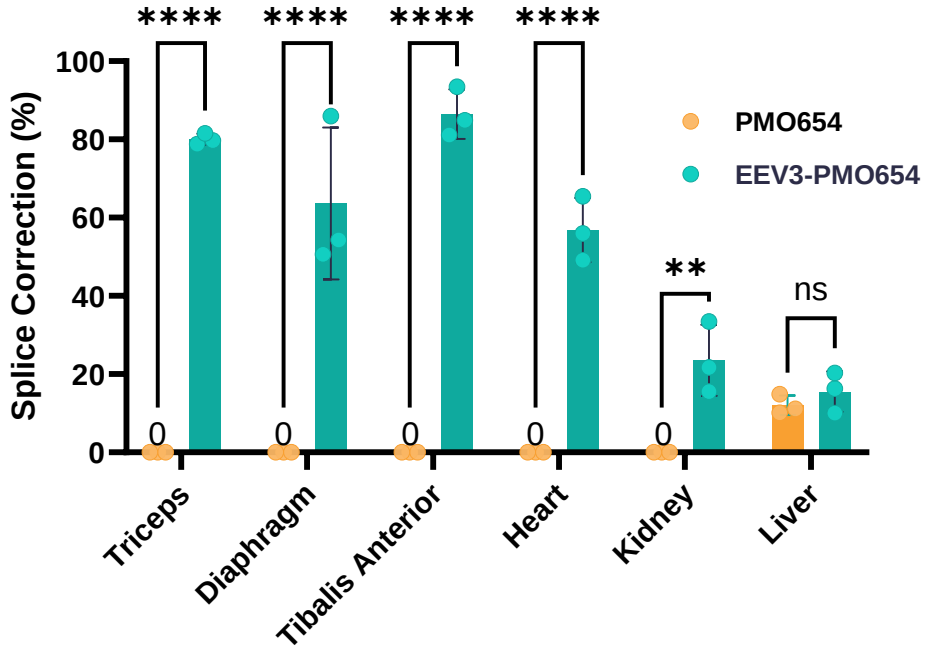
Exocyclic peptide sequence with extended linker

Conjugation with PMO



Substitution of positively charged arginine residues with neutral charged citrullines

Enhanced Functional Delivery to Muscle



- *EGFP654* mice were evaluated for splice correction 7 days following three weekly 10 mg/kg IV injections of PMO654 or EEV3-PMO654

p<0.01, ****p<0.0001; values are shown as mean ± standard deviation. **EEV, endosomal escape vehicle; **IV,** intravenous; **ns**, not significant; **PMO**, phosphorodiamidate morpholino oligomer.

OPTIMIZATION OF EEV FOR MUSCLE DELIVERY

Uptake and Outcomes in a Murine Model

EEV-therapeutic candidates have demonstrated favorable pharmacological properties: efficient intracellular delivery, significant uptake in target tissues and potent pharmacodynamic outcomes

Tissue Uptake in Muscle

- ✓ Skeletal muscle
- ✓ Cardiac muscle

+

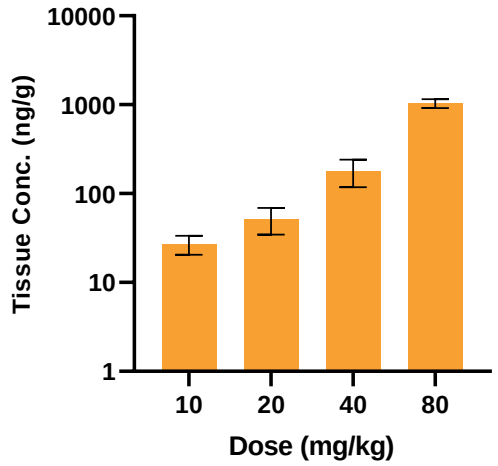
Intracellular Delivery

- ✓ Endosomal escape
- ✓ Nuclear localization

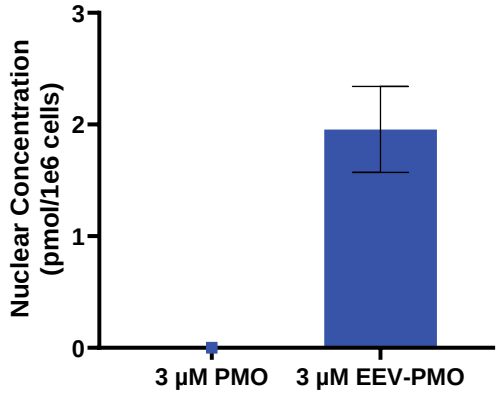
=

Pharmacodynamic Outcome

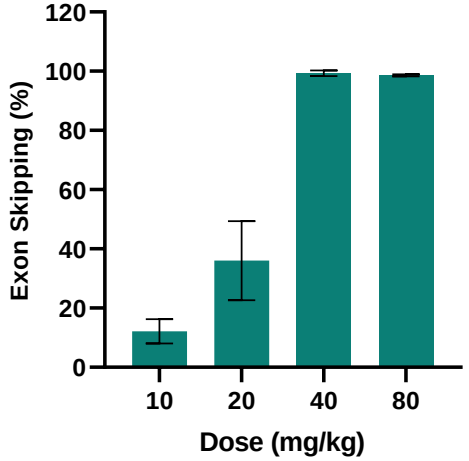
- ✓ Rapid, dose-dependent response
- ✓ Duration of at least 12 weeks



IV, hDMD mice, 5-day post injection



24-hour incubation

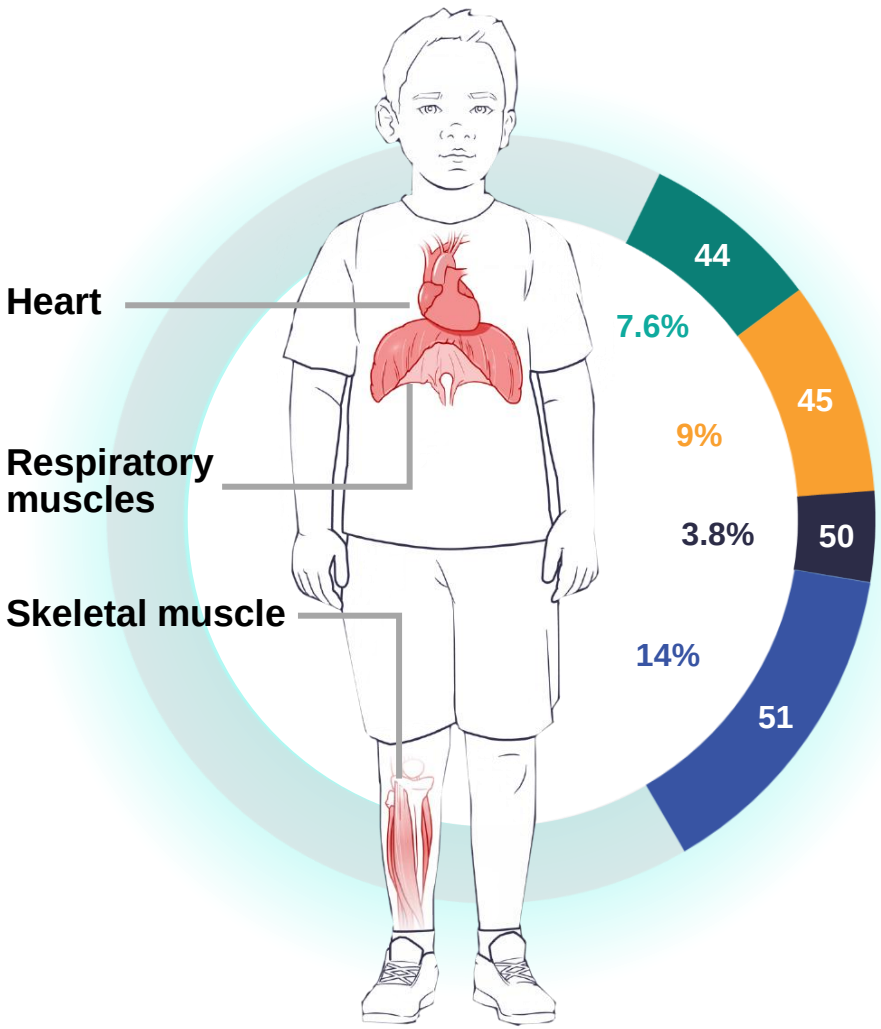


IV, hDMD mice, 5-day post injection

hDMD mice express full-length human dystrophin gene; **IV,** intravenous; **PMO**, phosphorodiamidate morpholino oligomer.

DUCHENNE MUSCULAR DYSTROPHY:
DELIVERY OF OLIGONUCLEOTIDES

DMD: Significant Unmet Need



Duchenne is caused by **mutations in the DMD gene, which lead to a lack of functional dystrophin**, causing progressive loss of muscle function throughout the body

~40,000

people in the **US and Europe** have Duchenne[1]

Duchenne Franchise

ENTR-601-44 Phase 1

Phase 1 data expected October 2024
Phase 2 regulatory filings expected Q4 2024

ENTR-601-45 IND Enabling

Phase 2 regulatory filings expected Q4 2024

ENTR-601-50 IND Enabling

Phase 2 regulatory filings expected 2025

Exon 51 Lead Optimization

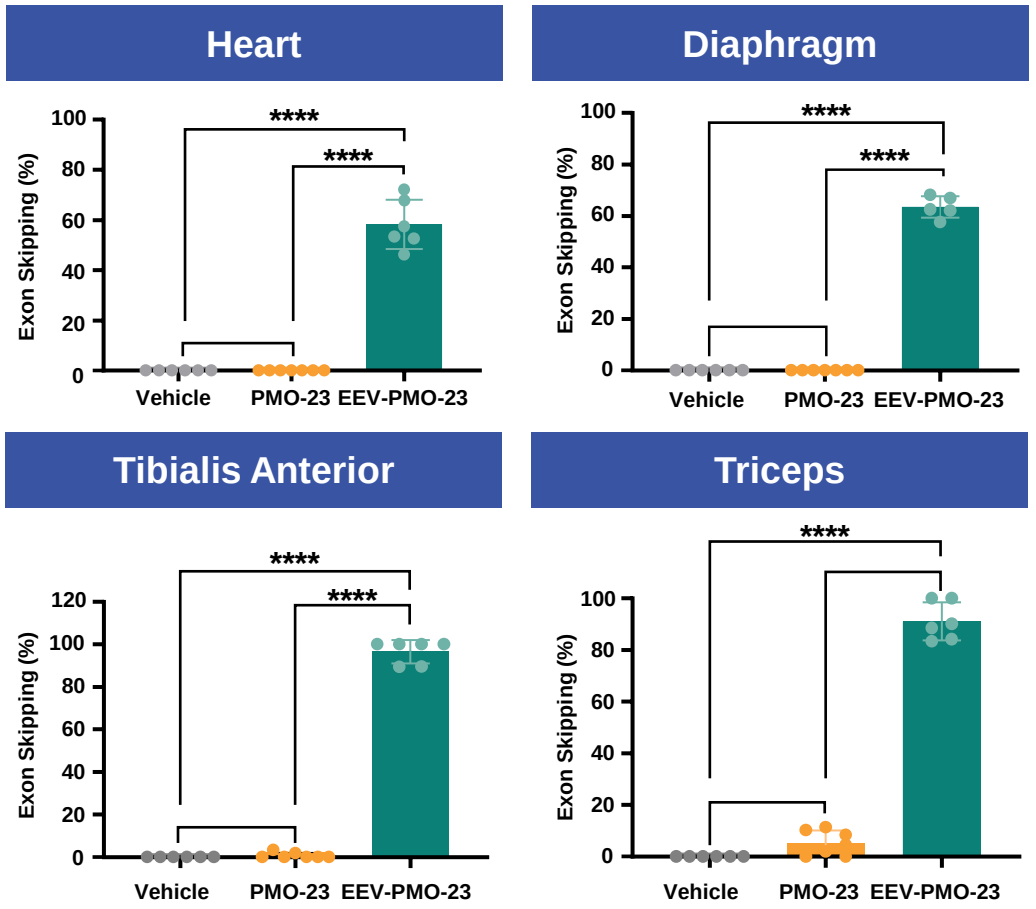
Candidate selection expected in 2024

[1]Parent Project Muscular Dystrophy: About Duchenne. [2]Europeans Medicines Agency: Orphan designation for the treatment of Duchenne muscular dystrophy. [3]Bladen, C.L. et al *HUMAN MUTATION*, 2015.

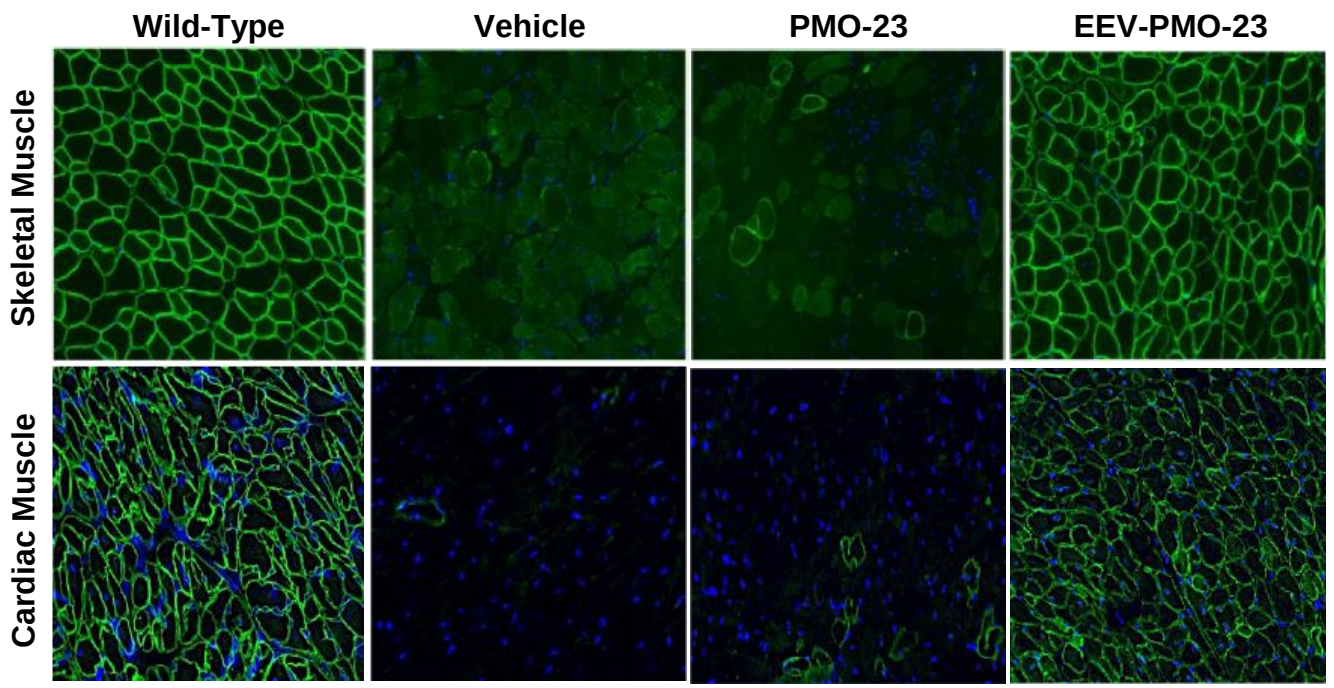
EEV-PMO RESTORES MUSCLE INTEGRITY

D2-*mdx* Mice

Robust exon 23 skipping after 4 monthly IV doses of EEV-PMO-23 in D2-*mdx* mice



Broad dystrophin expression and restoration of muscle integrity after four monthly IV doses of EEV-PMO-23 in D2-*mdx* mice

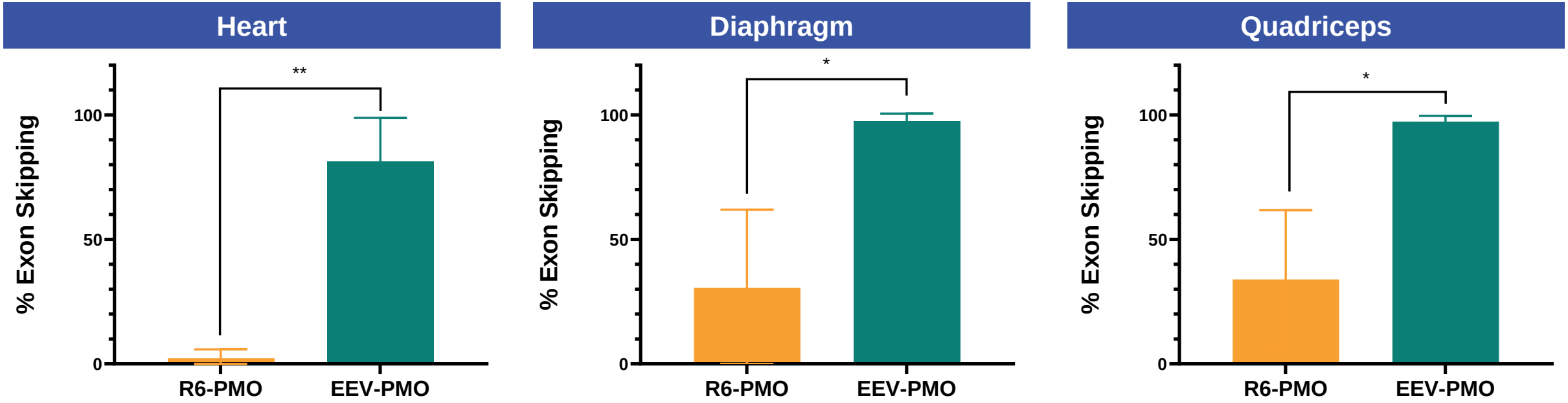


- D2-*mdx* mice (male, n=6-7) were treated with 4 monthly doses of either vehicle, 20 mg/kg unconjugated PMO-23 or 20 mg/kg PMO-23 equivalent of EEV-PMO-23, and the data were collected ~4 weeks after the last dose.

****p<0.0001. Values are shown as mean ± standard deviation. **D2-*mdx*** is a DMD mouse model with a nonsense mutation in *DMD* exon 23 (Coley, W.D. et al. *Hum. Mol. Genet*. 2016). Li, X. et al. *Mol. Ther. Nucleic Acids* 2023. **EEV**, endosomal escape vehicle; **DMD**, Duchenne muscular dystrophy; **IV,** intravenous; **ns**, not significant; **PMO**, phosphorodiamidate morpholino oligomer; **PMO-23**, mouse *DMD* exon 23 skipping phosphorodiamidate morpholino oligomer.

SUPERIOR TO ALTERNATIVE PEPTIDES
R6-PMO Example

EEV-PMO significantly improved exon 23 skipping after 3 days in *mdx* mice as compared to competitive R6-PMO



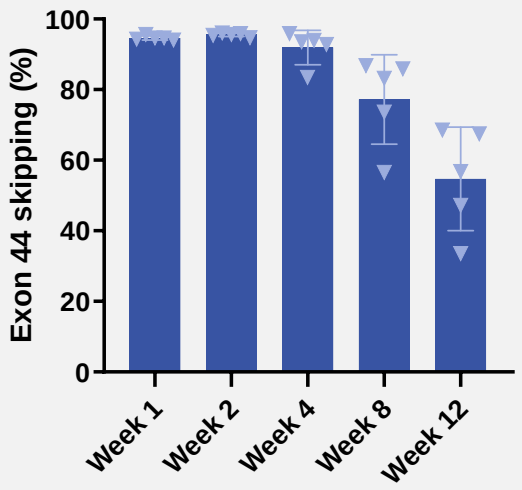
- EEV-PMO-23 demonstrates significantly improved PD effects after single 40 mg/kg IV dose in *mdx* mice

Values are shown as mean ± standard deviation; *p<0.05, **p<0.01; ***mdx*** is a DMD mouse model with a nonsense mutation in *DMD* exon 23; **PMO-23**, mouse *DMD* exon 23 skipping phosphorodiamidate morpholino oligomer; **IV,** intravenous.

CONSISTENT AND DURABLE EFFICACY OF EEV-PMO WAS DEMONSTRATED ACROSS SPECIES

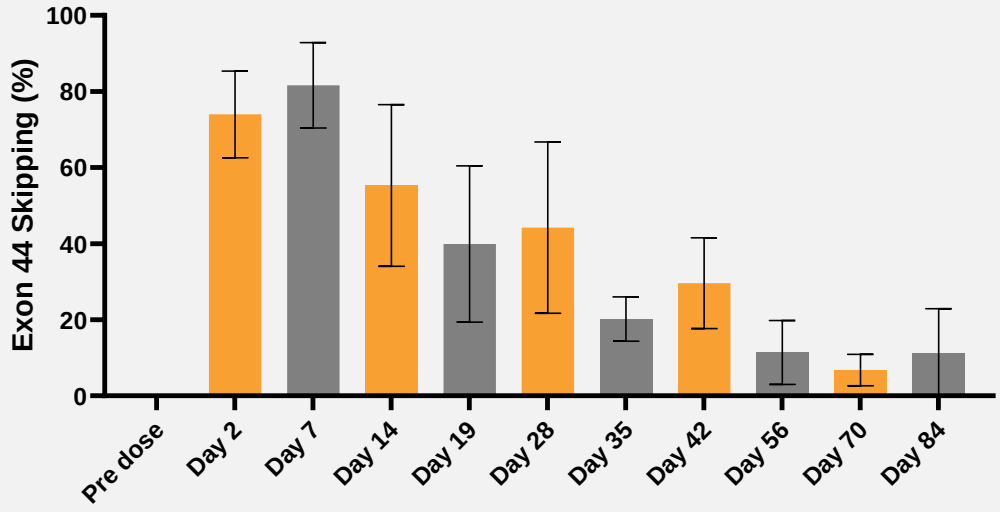
Significant patient benefit is implied by data in the mouse and the monkey at clinically relevant levels; *in vitro* data suggests much higher target engagement in patient cells

Exon 44 Skipping in hDMD Mouse



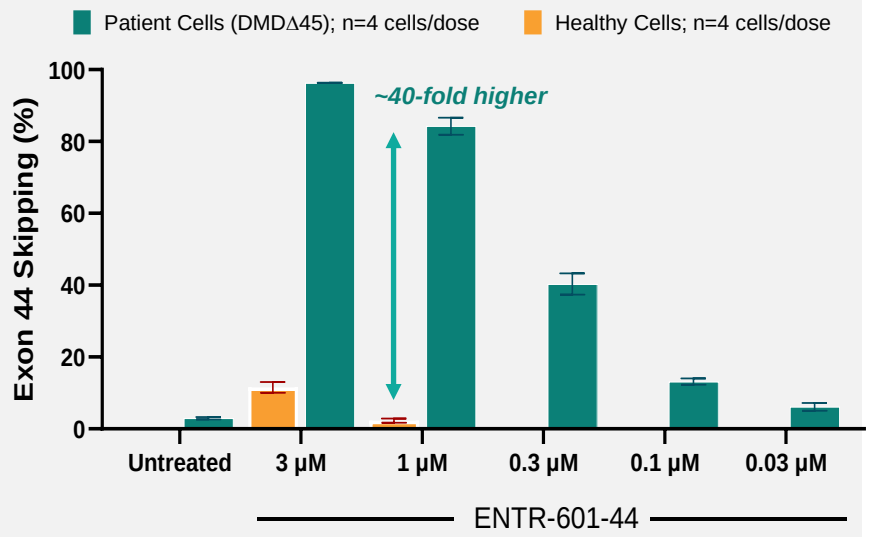
- Single IV 60 mg/kg dose of ENTR-601-44
- Tibialis Anterior

Exon 44 Skipping in Monkey



- Post IV infusion of single 35 mg/kg dose of ENTR-601-44**,** robust exon 44 skipping observed in biceps of treated monkeys (n=3 per cohort) for at least 12 weeks

Exon 44 Skipping in Healthy and Patient-Derived Muscle Cells



- Robust dose-dependent exon 44 skipping was observed in DMD patient-derived muscle cells harboring an exon 44 skip-amenable mutation

hDMD transgenic mice (left) express full-length human dystrophin gene ('t Hoen, A.C. et al. *J. Biol. Chem.* 2008). **DMDΔ45** (right) are immortalized myoblasts from DMD patients harboring an out-of-frame exon 45 deletion and further differentiated into myotubes. Values are shown as mean ± standard deviation. **ENTR-601-44** is a DMD exon 44 skipping EEV-oligonucleotide construct. **DMD**, Duchenne muscular dystrophy; **hDMD,** human Duchenne muscular dystrophy; **IV,** intravenous.

ENTR-601-44 DATA SUMMARY

Significant patient benefit is implied by data in the mouse and the monkey at clinically relevant levels; *in vitro* data suggests much higher target engagement in patient cells

- ✓ High levels of exon skipping across *mdx*, D2-*mdx,* human dystrophin mouse and NHP studies
- ✓ Exon skipping translates to promising dystrophin production in heart and skeletal muscles
- ✓ Dystrophin production observed results in functional improvement in D2-*mdx* mouse
- ✓ Extended circulating half-life and durable exon skipping over 12+ weeks from a single injection of ENTR-601-44 was observed in the NHP

ENTR-601-44-101:
Phase 1 clinical trial ongoing

- First participant dosed in September 2023
- Initiated dosing of the fourth and final cohort
- Data readout anticipated in October 2024
- Phase 1 clinical data will support the global clinical trial in patients*

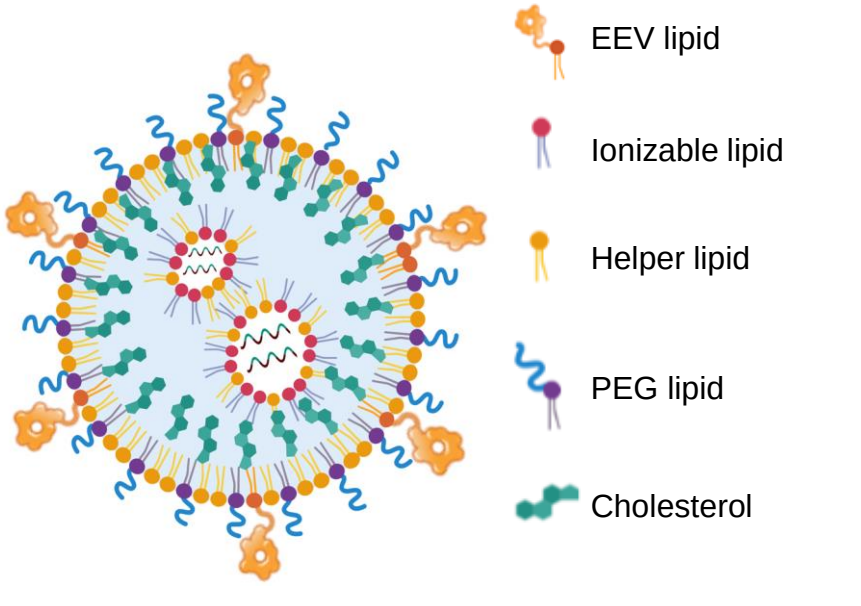
*MAD/Phase 2b study is subject to regulatory feedback and the outcome of the SAD study.

PLATFORM EXPANSION:
DELIVERY OF GENOMIC MEDICINES

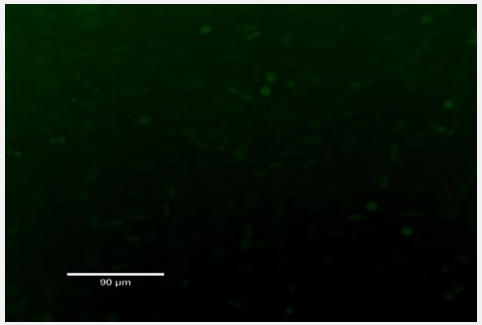
EEV PLATFORM FOR GENOMIC MEDICINE DELIVERY

EEV-incorporated Lipid Nanoparticle (EEV-LNP) showed ~9-fold mRNA delivery improvement over LNP in HeLa cells, allowing the expansion of genomic medicine delivery

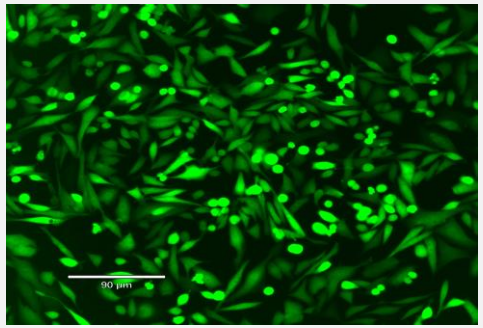
EEV-LNP Construct



EGFP mRNA Delivery

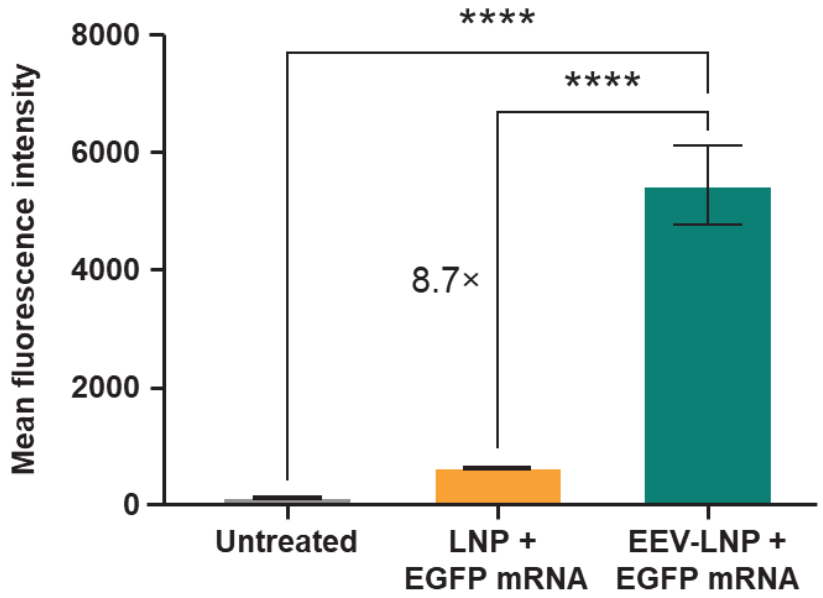


LNP + EGFP mRNA



EEV-LNP + EGFP mRNA

FACS Quantification of mRNA Delivery



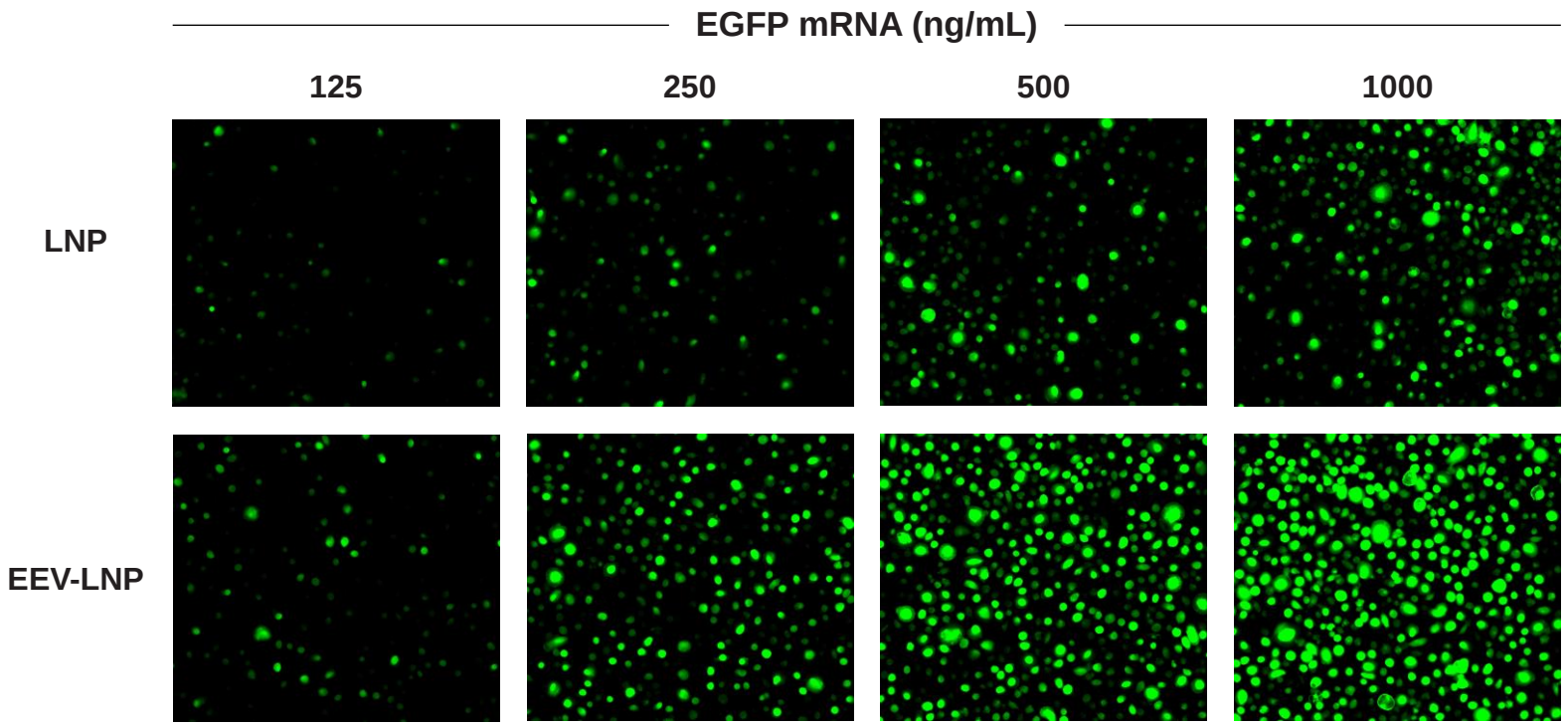
- EEV-LNP improved EGFP mRNA delivery by approximately 9-fold compared to LNP

Hela cells were treated with 250 ng/mL EEV-LNP or LNP, both containing EGFP mRNA. Cells were harvested after 24 hours and analyzed for GFP expression by FACS analysis. One-way ANOVA was used for statistical comparison; ****p<0.0001. **ANOVA**, analysis of variance; **EEV**, Endosomal Escape Vehicle; **EGFP**, enhanced green fluorescent protein; **FACS**, fluorescence-activated cell sorting; **EGFP**, enhanced green fluorescent protein; **LNP**, lipid nanoparticle; **mRNA**, messenger RNA.

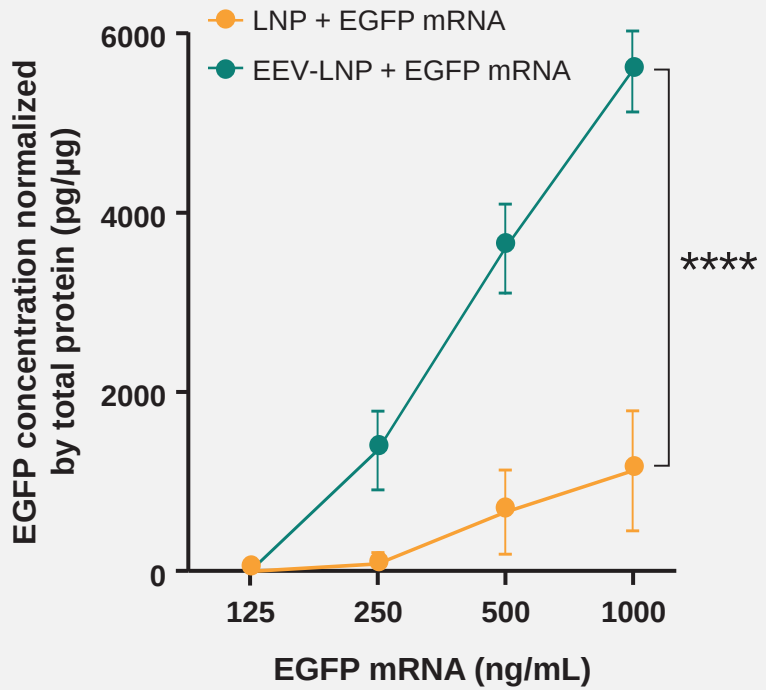
IMPROVED mRNA DELIVERY: PRIMARY MACROPHAGE

EEV-LNP demonstrated dose-dependent and up to ~ 6-fold improvement in EGFP transfection efficacy in primary human macrophages *ex vivo* when compared to LNP control

EEV-LNP EGFP mRNA Uptake in Primary Human Macrophages



ELISA Quantification

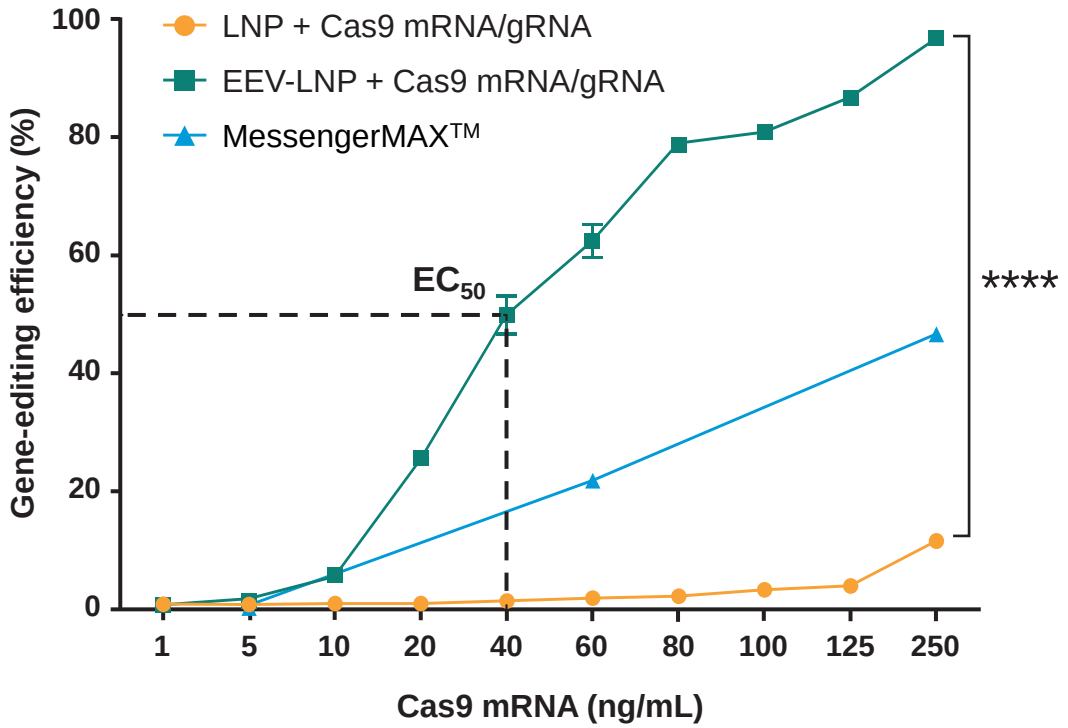


Primary CD14+ human monocytes were treated with granulocyte-macrophage colony-stimulating factor for 7 days to drive differentiation into macrophages. Cells were treated with EEV-LNP or LNP (both containing EGFP mRNA) and assessed EGFP expression by (A) fluorescence microscopy or (B) ELISA 24 hours post-treatment. Two-way ANOVA was used for statistical comparison; ****p<0.0001. **ANOVA**, analysis of variance; **EEV**, Endosomal Escape Vehicle; **EGFP**, enhanced green fluorescent protein; **ELISA**, enzyme-linked immunosorbent assay; **LNP**, lipid nanoparticle; **mRNA**, messenger RNA.

GENE EDITING EFFICIENCY WITH EEV-LNP

EEV-LNP demonstrated dose-dependent gene editing, with editing efficiency consistently higher than LNP or transfection across a wide concentration range of Cas9 mRNA

Gene Editing Efficiency in HEK293-uGFP Cells[1]



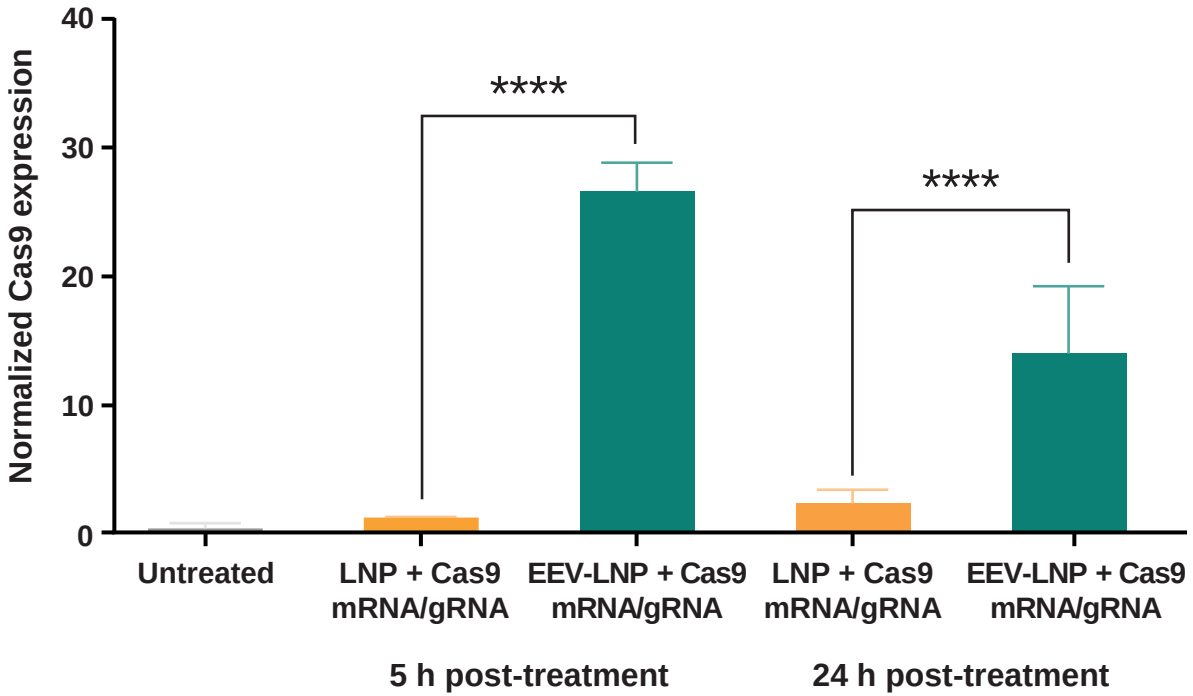
- **EEV-LNP based co-delivery of Cas9 mRNA/gRNA showed dose-dependent gene editing *in vitro***
  - Gene editing efficiency is quantified by FACS as percent of GFP-negative population in HEK293-uGFP cell line
- **EEV-LNP resulted in higher gene editing efficiency than LNP alone as well as transfection by MessengerMAX™**
  - Dose dependent delivery of gene editing with EEV-LNP with an EC50 around 40 ng/mL
  - ~33-fold improvement at a dose as low as 40 ng/mL Cas9 compared to LNP

1. Li X, et al. *J Biol Chem*. 1998. After 48 hours of treatment, HEK293-uGFP6 cells were analyzed by flow cytometry to quantify the percentage of GFP-knockout cells. Two-way ANOVA test was used for statistical comparison; ****p<0.0001. **ANOVA**, analysis of variance; **EC50**, half maximal effective concentration; **EEV**, Endosomal Escape Vehicle; **GFP**, green fluorescent protein; **gRNA**, guide RNA; **LNP**, lipid nanoparticle; **mRNA**, messenger RNA; **uGFP**, unstable green fluorescence protein.

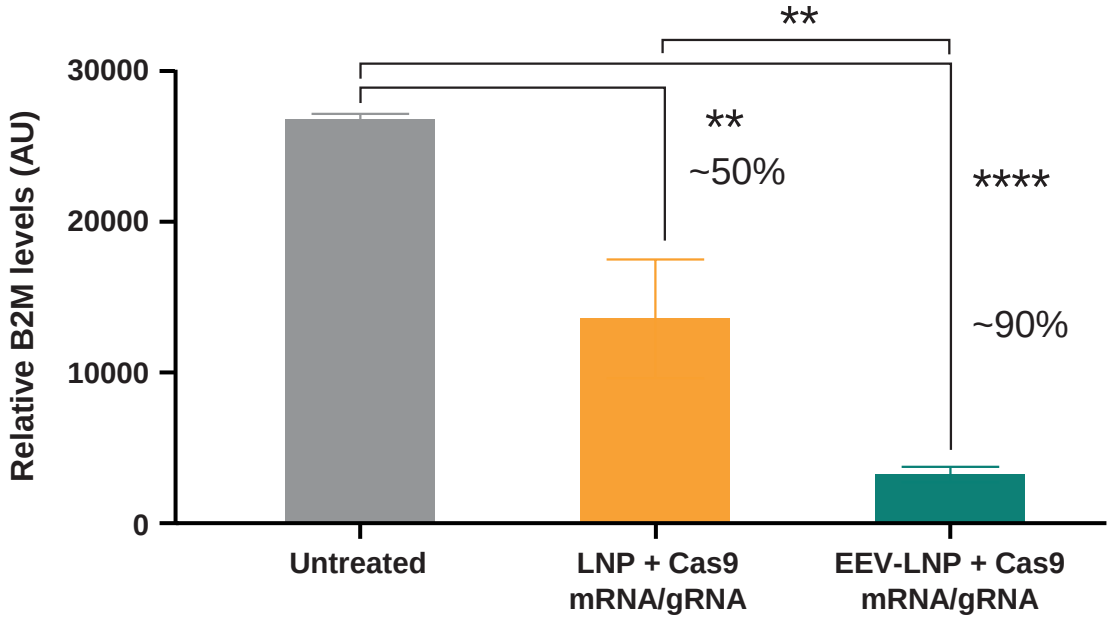
GENE KNOCKDOWN WITH EEV-LNP COMPARED TO LNP ALONE

EEV-LNP showed 26-fold higher Cas9 expression at 5 hours post-transfection compared to LNP, demonstrating enhanced gene editing efficiency in primary macrophages

Cas9 Expression in Primary Macrophages



Endogenous B2M Knockdown (7 days post-treatment)



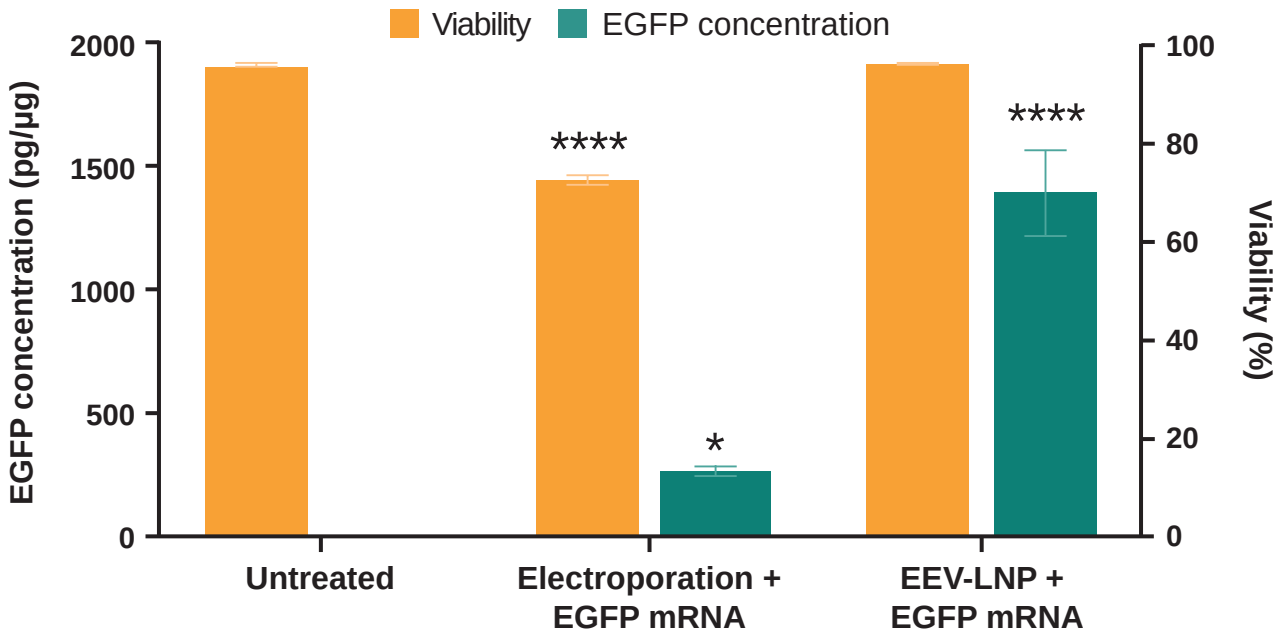
- The beta-2-microglobulin (B2M) gene is a component of the class I major histocompatibility complex involved in the presentation of peptide antigens to the immune system and expressed in human macrophages

Human primary macrophages were treated for 5 hours with EEV-LNP or LNP containing Cas9 mRNA and gRNA targeting B2M gene. (Left) Cas9 protein expression was quantified by Western blot at 5 and 24 hours post-treatment. (Right) B2M knockdown was assessed at 7 days post-treatment by FACS analysis. One-way ANOVA was used for statistical comparison; ****$p<0.0001$, **$p<0.01$. **ANOVA**, analysis of variance; **AU**, arbitrary unit; **EEV**, Endosomal Escape Vehicle; **FACS**, fluorescence-activated cell sorting; **gRNA**, guide RNA; **LNP**, lipid nanoparticle; **mRNA**, messenger RNA.

EEV-LNP mRNA DELIVERY COMPARED TO ELECTROPORATION

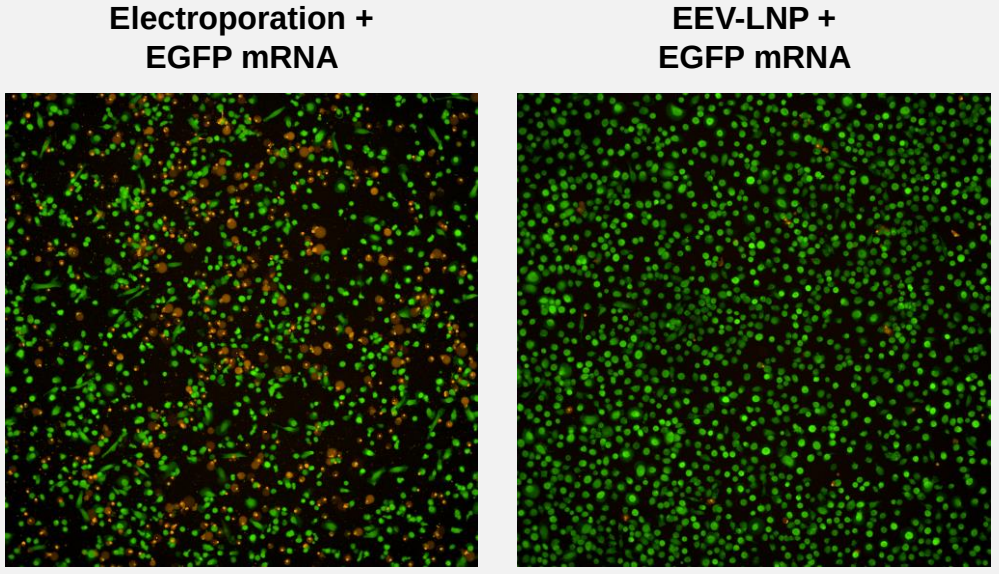
EEV-LNP demonstrated 5-fold improvement in mRNA delivery compared to electroporation, with reduced impact on cell viability

EEV-LNP vs. Electroporation (24 hours post-treatment)



EGFP: *p<0.05 vs. untreated; ****p<0.0001 vs. untreated and electroporation
Viability: ****p<0.0001 vs. untreated and EEV-LNP

Viability of Primary Macrophages



Green: Live cells (EGFP)

Red: Necrotic/Dead Cells (Propidium Iodide)

Human primary macrophages were differentiated from monocytes isolated from PBMCs and treated with EEV-LNP or electroporated (EGFP mRNA dose 2500 ng/10^6 cells) using Lonza nucleofection method. EGFP expression was measured by ELISA after 24 hours (left). Cell viability quantified by LIVE/DEAD™ Cell Imaging Kit (right). One-way ANOVA was used for statistical comparison; **ANOVA**, analysis of variance; **EEV**, Endosomal Escape Vehicle; **EGFP**, enhanced green fluorescent protein; **ELISA**, enzyme-linked immunosorbent assay; **LNP**, lipid nanoparticle; **PBMC**, peripheral blood mononuclear cell.

PLATFORM OPPORTUNITIES

Entrada continues to invest in and build upon our EEV platform to extend our efforts in developing novel EEV-therapeutic candidates

TARGET	APPROACH	GOAL
DNA	Gene Editing	Deliver CRISPR enzyme and repair gene function with guide RNA
RNA	RNA Editing	Deliver oligonucleotide therapeutics for RNA editing
RNA	RNA Splicing	Modify RNA via exon/intron splicing to activate protein expression
RNA	RNA Blocking	Block trinucleotide repeats in RNA to inhibit adverse binding
RNA	RNA Silencing	Silence or knockdown RNA to prevent protein expression
PROTEIN	Protein Replacement	Replace proteins and enzymes
PROTEIN	Protein Inhibition	Inhibit protein signaling pathways
PROTEIN	Protein Degradation	Degrade disease-causing proteins

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