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

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3rd Annual Next Generation LNP Delivery Summit

entrada THERAPEUTICS

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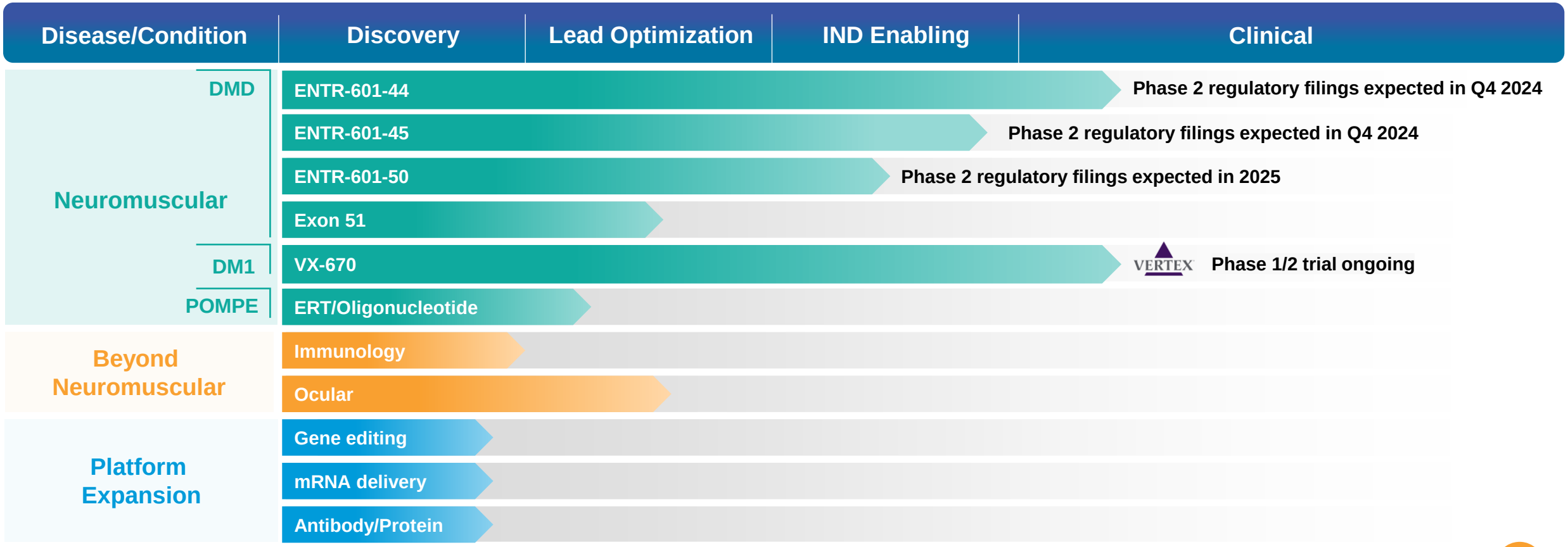
OUR MISSION

To Treat Devastating Diseases with Intracellular Therapeutics

entrada THERAPEUTICS

ENTRADA'S PIPELINE

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



EEV™ PLATFORM DEVELOPMENT AND OPTIMIZATION

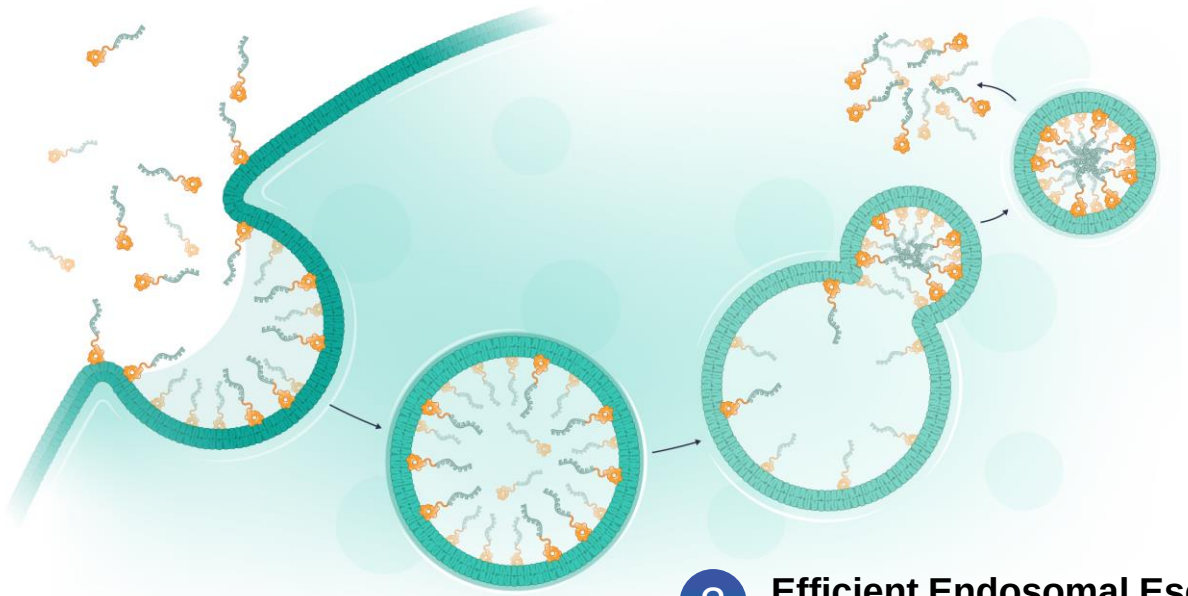
Endosomal Escape Vehicle (EEV™) Therapeutics

- 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)



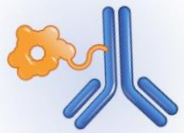
2 **Efficient Endosomal Escape**
(~50% Escape v. 2% Standard)

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.

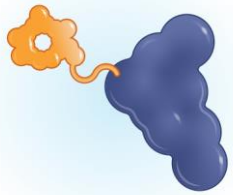
A BROADLY APPLICABLE PLATFORM

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

Antibodies

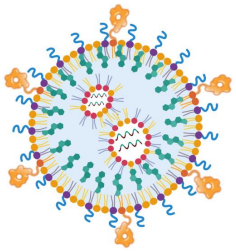


Enzymes

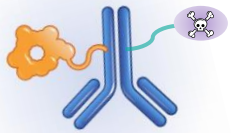


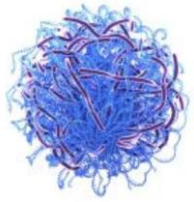
Oligonucleotides

Genomic Medicine



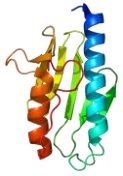
Antibody Drug Conjugates





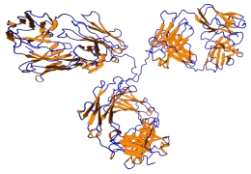
300-3000 KDa

Genomic Medicine Formulation



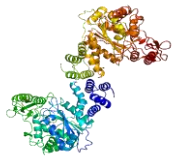
550-600 KDa

Hybrid frataxin



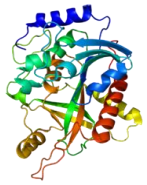
150 KDa

Antibody



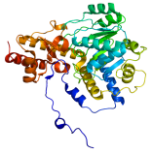
98 KDa

Thymidine phosphorylase



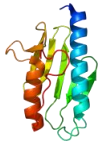
96 KDa

Purine nucleoside phosphorylase



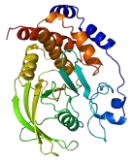
86 KDa

Alanine-glyoxylate aminotransferase



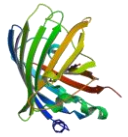
46 KDa

Human frataxin



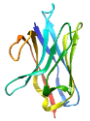
37 KDa

PTP1B catalytic domain



32 KDa

EGFP



16 KDa

Nanobody

6 KDa

Oligonucleotide

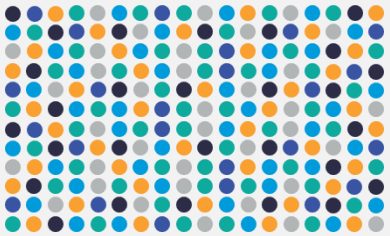
1-3 KDa

Various peptide cargos

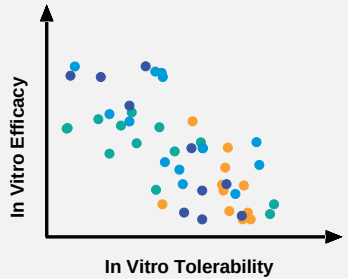
DISCOVERY ENGINE FOR EEV THERAPEUTICS
EEV-OLIGO EXAMPLE

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

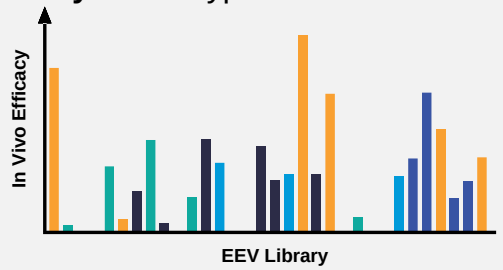
Chemically diverse macrocyclic **EEV library** generated through medicinal and combinatorial chemistry



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



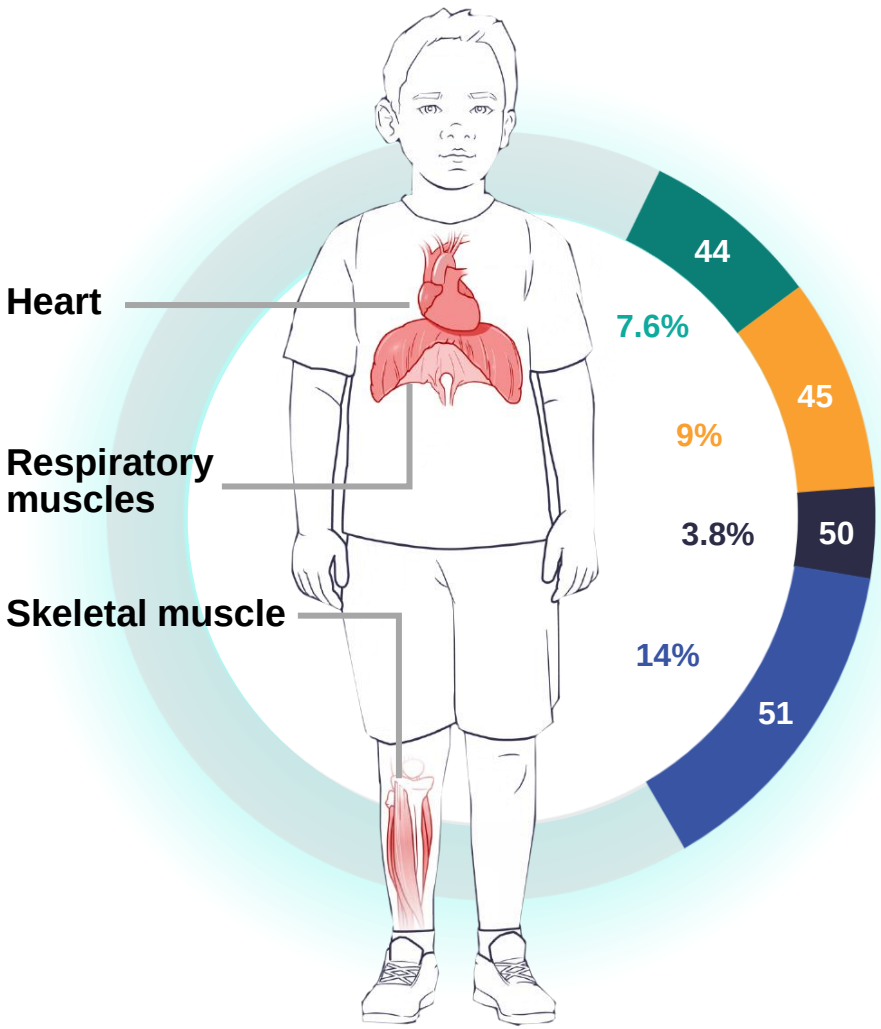
In vivo screening to assess **functional delivery and pharmacodynamic activity** in wild-type and disease models



EEV, endosomal escape vehicle

DUCHENNE MUSCULAR DYSTROPHY:
DELIVERY OF OLIGONUCLEOTIDES

DUCHENNE: 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: Positive preliminary data reported; Data to be presented October 2024

Phase 2: Regulatory filings expected Q4 2024

ENTR-601-45

Phase 2: Regulatory filings expected Q4 2024

ENTR-601-50

Phase 2: Regulatory filings expected 2025

Exon 51

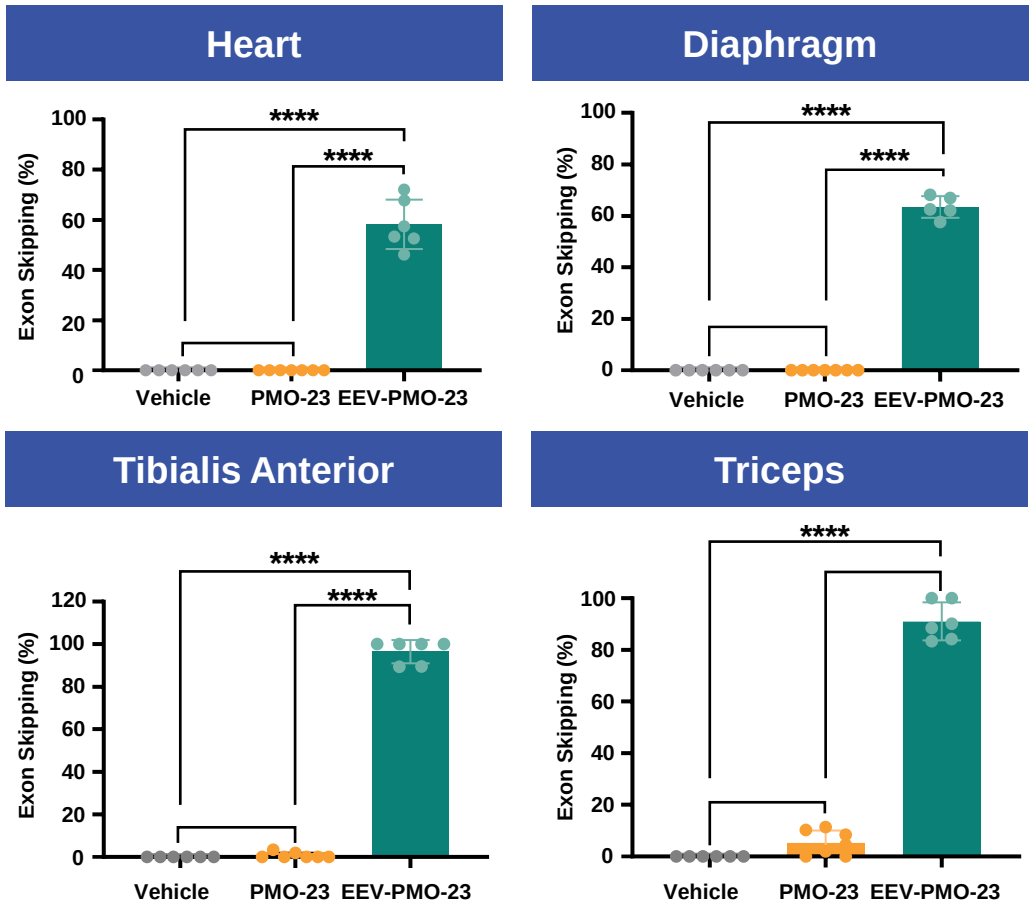
Candidate selection expected in 2024

[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.

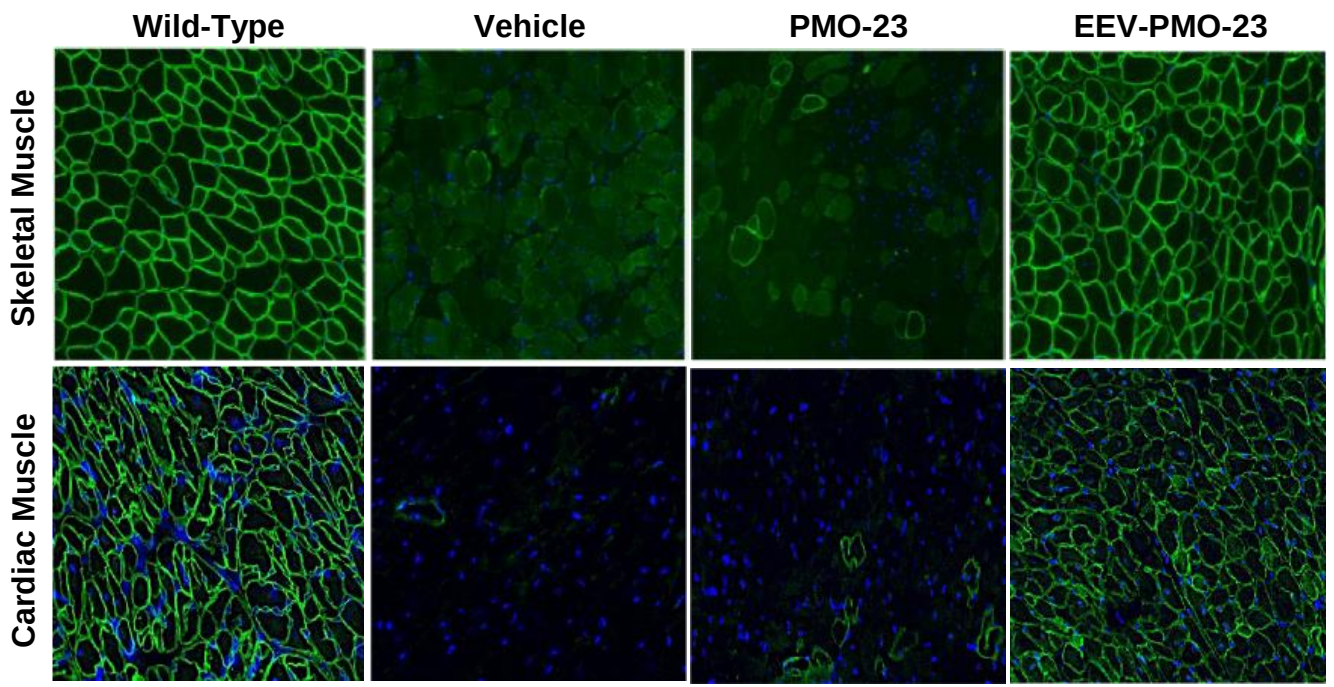
EEV-PMO RESTORES MUSCLE INTEGRITY

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

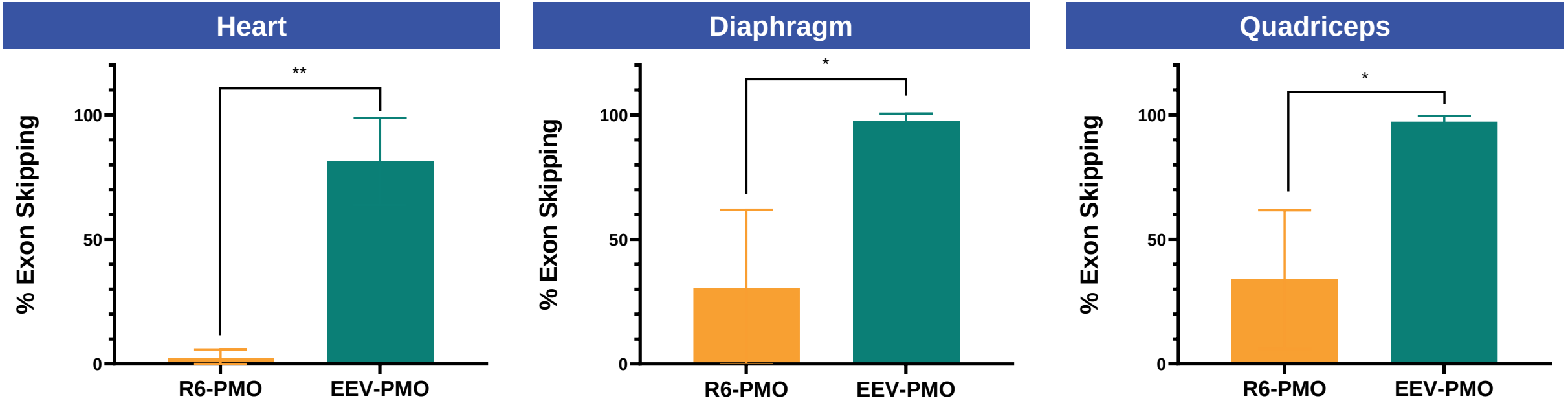


- D2-*mdx* mice (male, n=6-7) were treated with 4 monthly doses of either vehicle, 20 mg/kg unconjugated PMO-23 or 20 mg/kg PMO-23 equivalent of EEV-PMO-23, and the data were collected ~4 weeks after the last dose.

****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



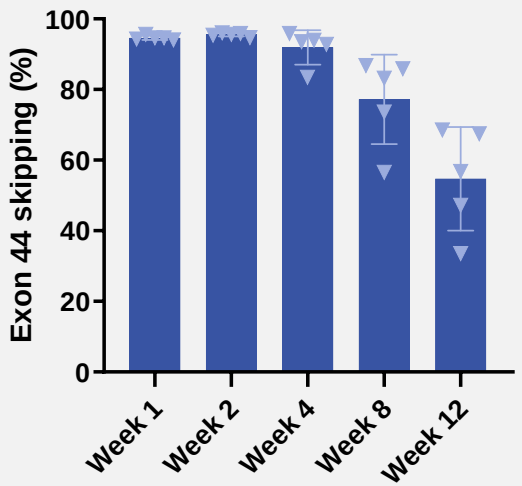
- EEV-PMO-23 demonstrates significantly improved PD effects after single 40 mg/kg IV dose in *mdx* mice

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

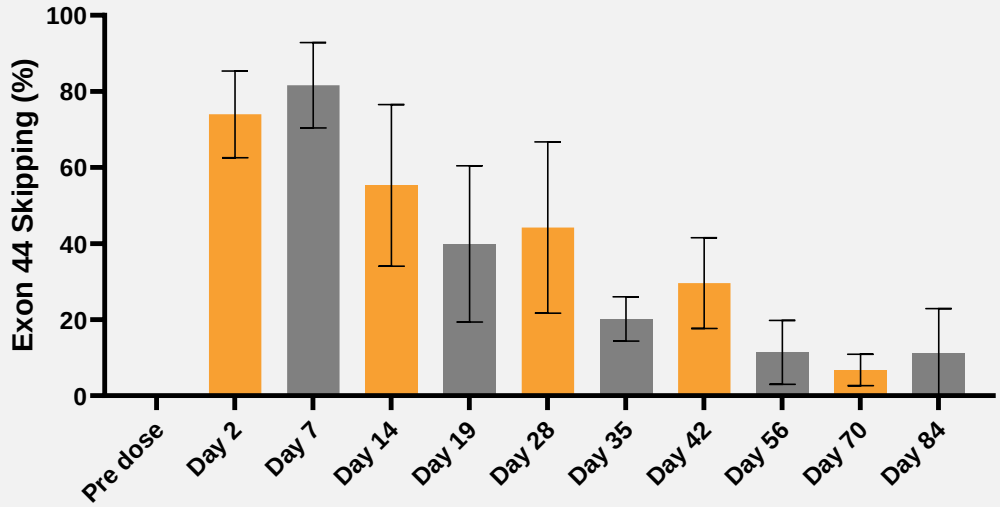
Significant patient benefit is implied by data in the mouse and the monkey at clinically relevant levels; *in vitro* data suggests much higher target engagement in patient cells

Exon 44 Skipping in hDMD Mouse



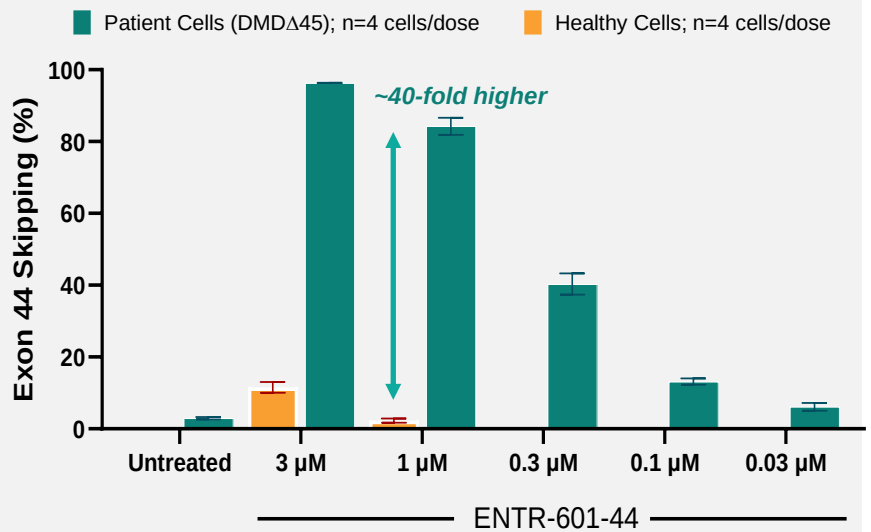
- Single IV 60 mg/kg dose of ENTR-601-44
- Tibialis Anterior

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 in healthy volunteers has completed dosing

- Cohorts 1, 2, and 3 have completed dosing and follow-up
- Fourth and final cohort has completed PK, PD, and safety assessments
- Preliminary results demonstrated exon 44 skipping with no treatment-related adverse events. Final results available by October 2024.
- Phase 1 clinical data will support the global clinical trial in patients*

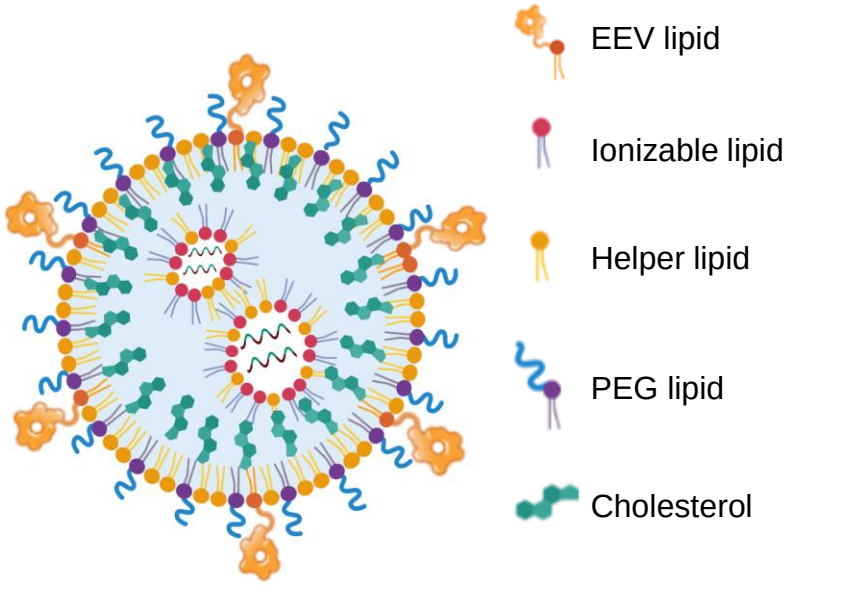
*MAD/Phase 2b study is subject to regulatory feedback and the outcome of the SAD study.

PLATFORM EXPANSION:
DELIVERY OF GENOMIC MEDICINES

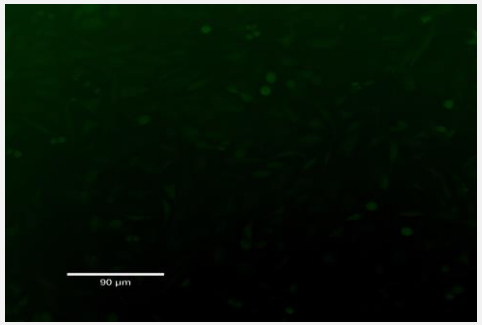
EEV PLATFORM FOR GENOMIC MEDICINE DELIVERY

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

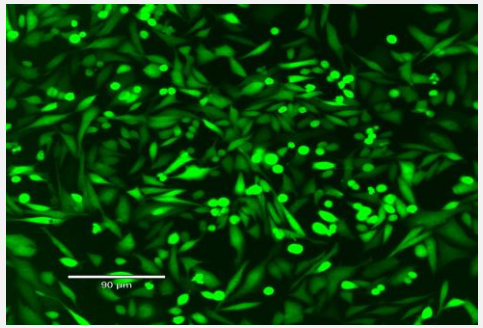
EEV-LNP Construct



EGFP mRNA Delivery

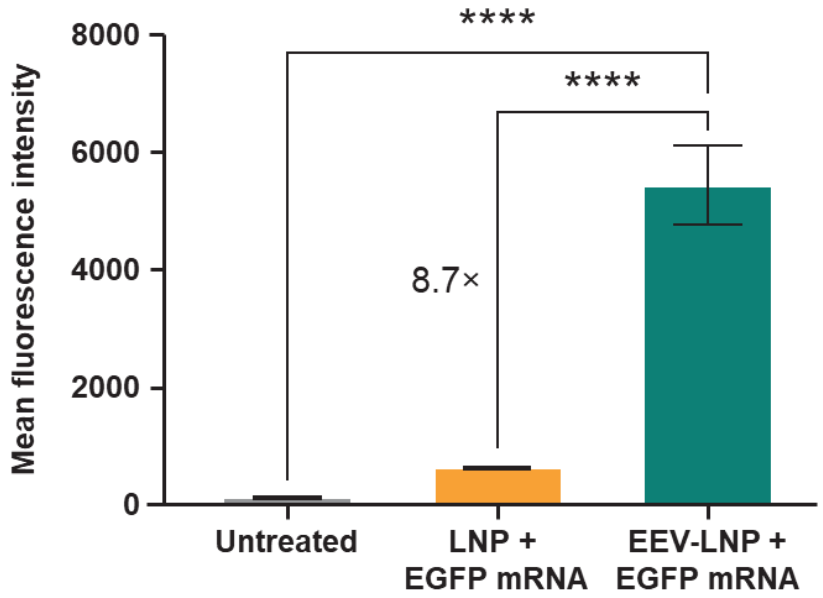


LNP + EGFP mRNA



EEV-LNP + EGFP mRNA

FACS Quantification of mRNA Delivery



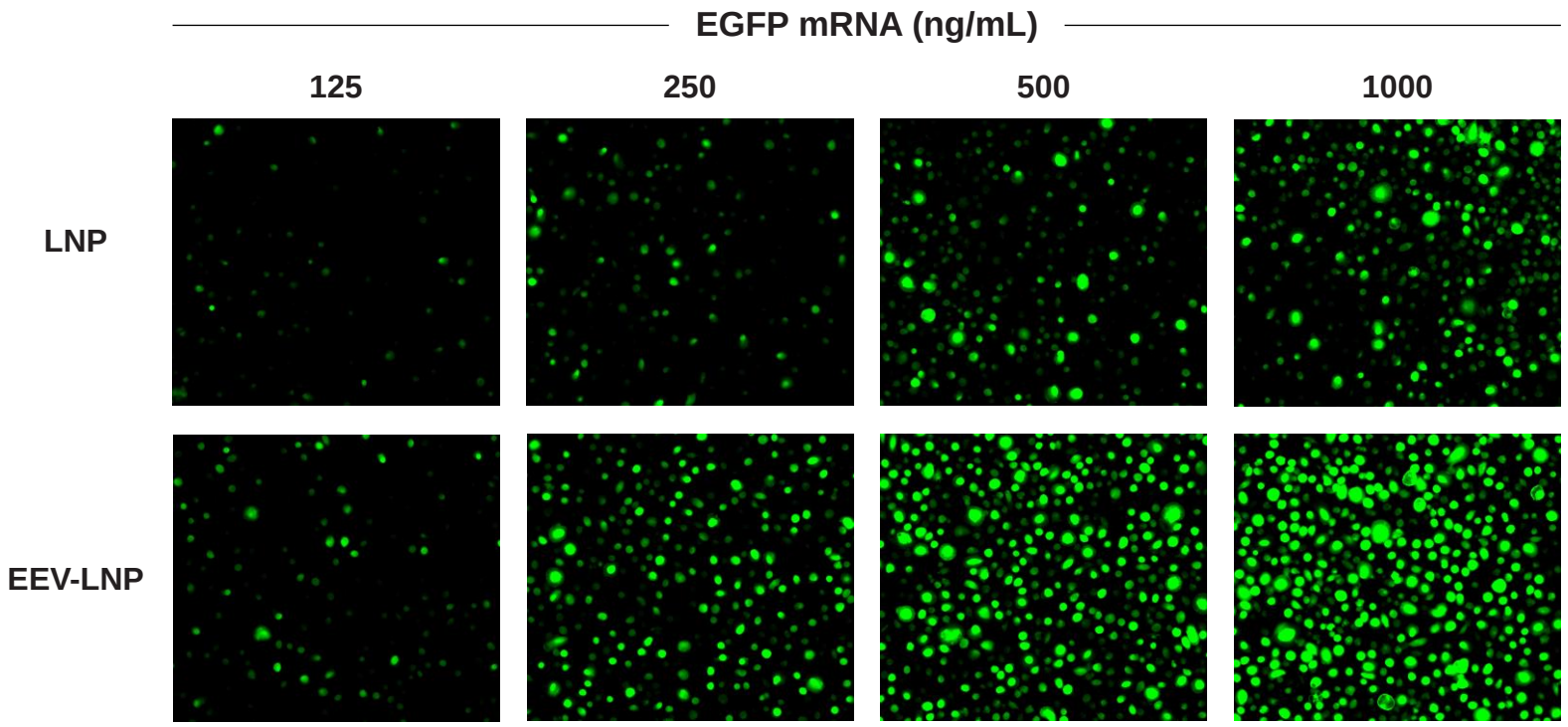
- EEV-LNP improved EGFP mRNA delivery by approximately 9-fold compared to LNP

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.

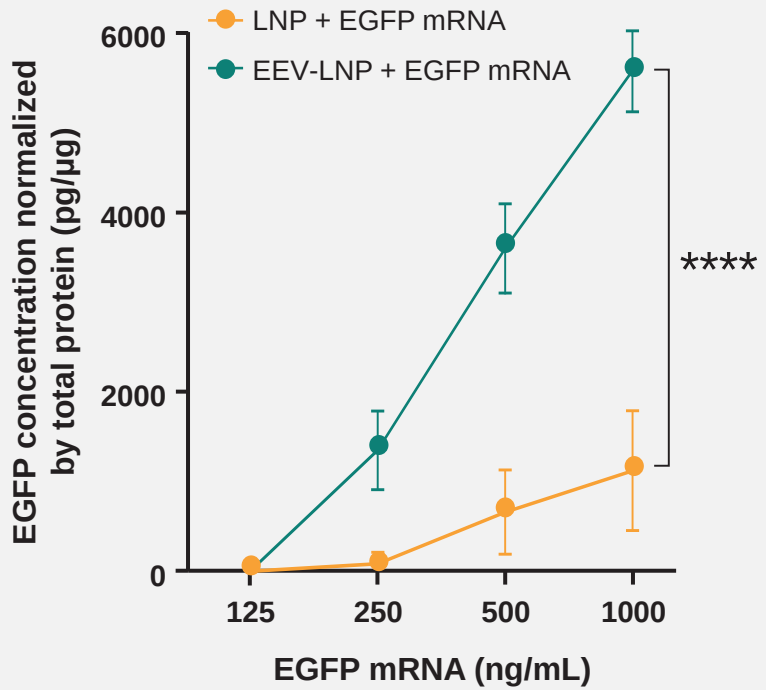
IMPROVED mRNA DELIVERY: PRIMARY MACROPHAGES

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

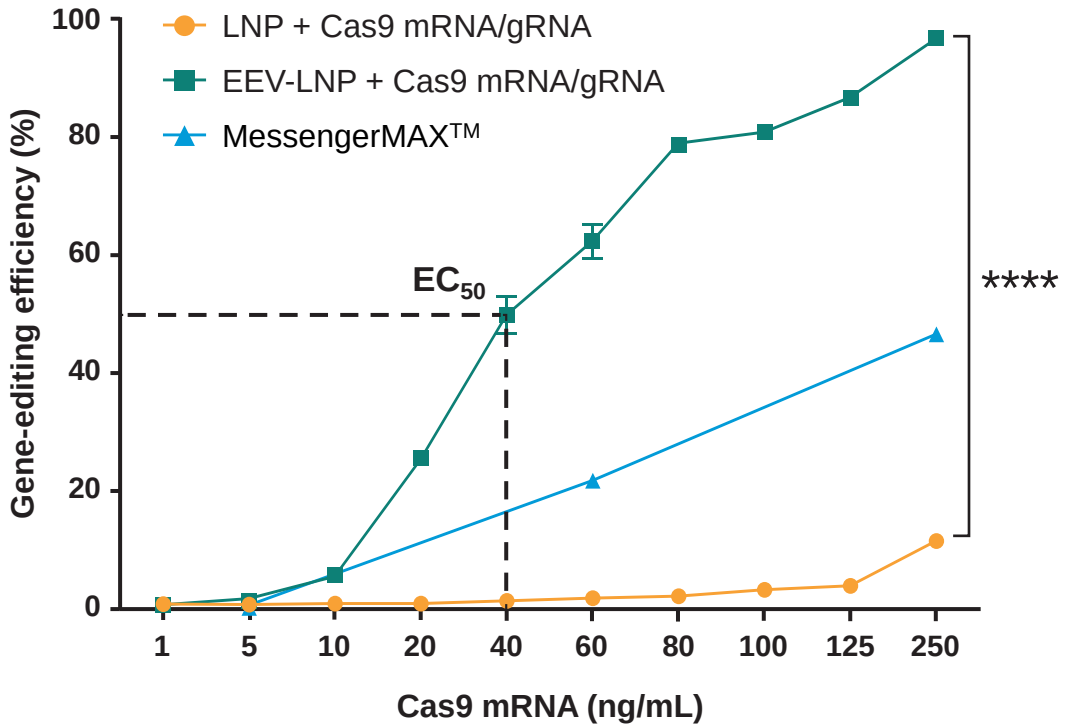


Primary CD14+ human monocytes were treated with granulocyte-macrophage colony-stimulating factor for 7 days to drive differentiation into macrophages. Cells were treated with EEV-LNP or LNP (both containing EGFP mRNA) and assessed EGFP expression by (A) fluorescence microscopy or (B) ELISA 24 hours post-treatment. 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]



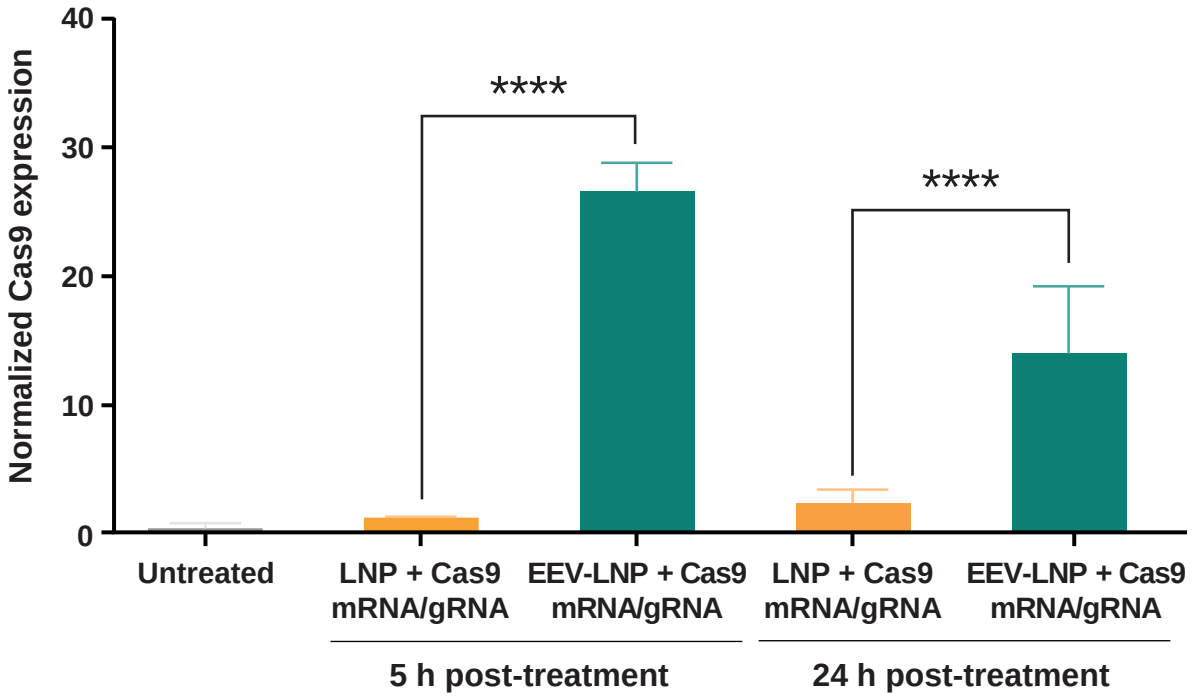
- **EEV-LNP based co-delivery of Cas9 mRNA/gRNA showed dose-dependent gene editing *in vitro***
  - Gene editing efficiency is quantified by FACS as percent of GFP-negative population in HEK293-uGFP cell line
- **EEV-LNP resulted in higher gene editing efficiency than LNP alone as well as transfection 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.

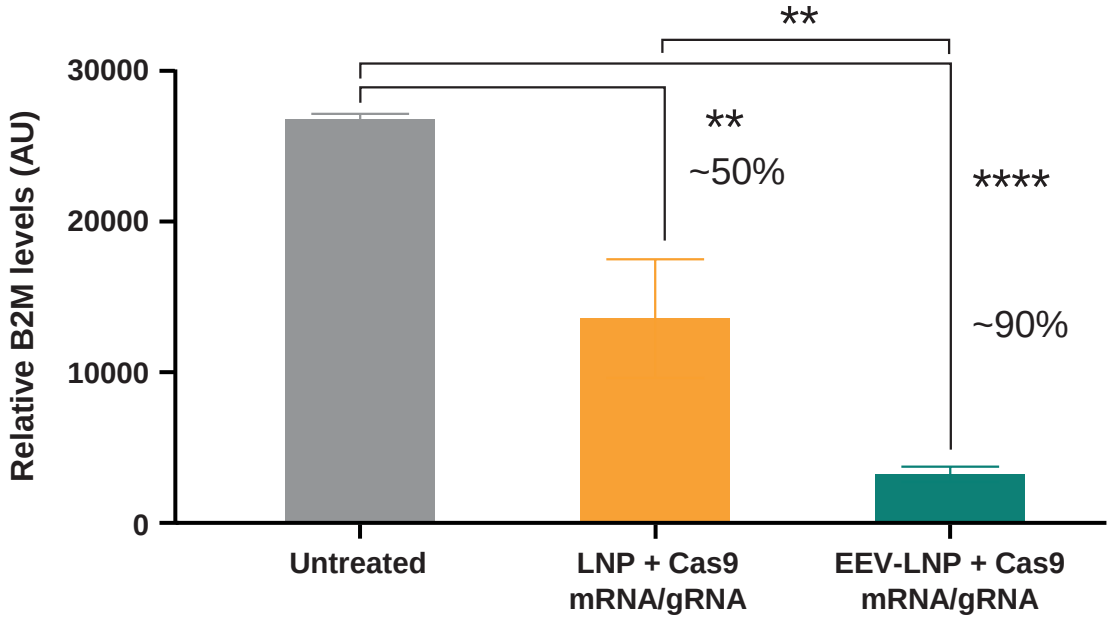
GENE KNOCKDOWN WITH EEV-LNP COMPARED TO LNP ALONE

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)



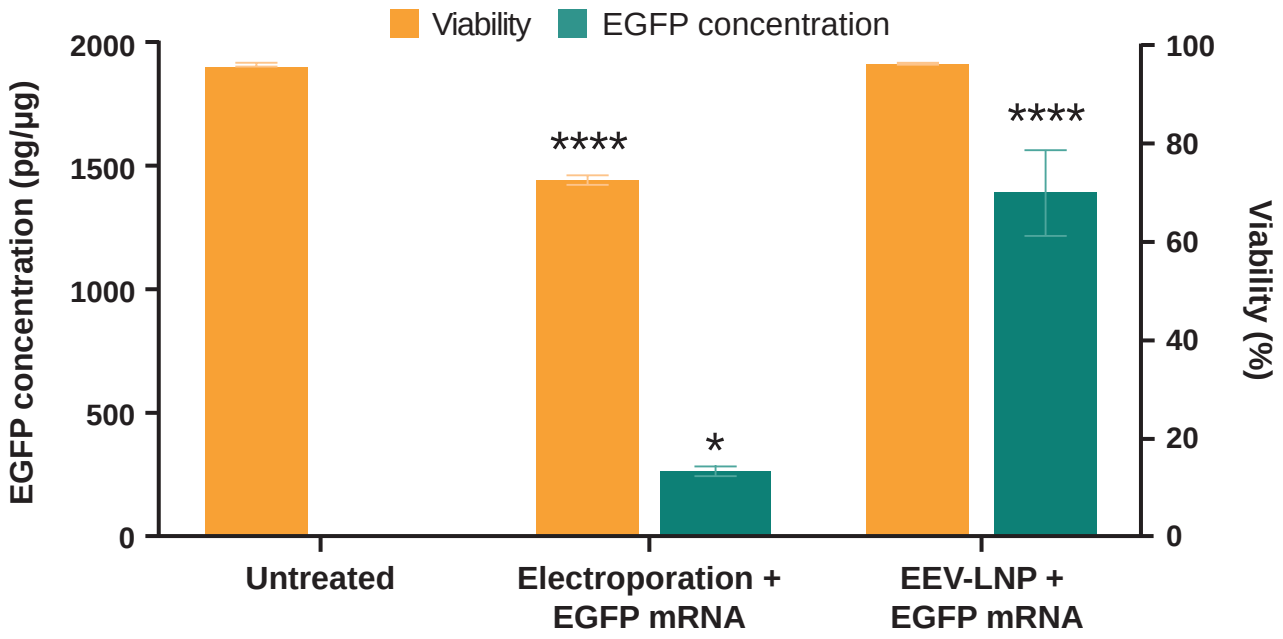
- The beta-2-microglobulin (B2M) gene is a component of the class I major histocompatibility complex involved in the presentation of peptide antigens to the immune system and expressed in human macrophages

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

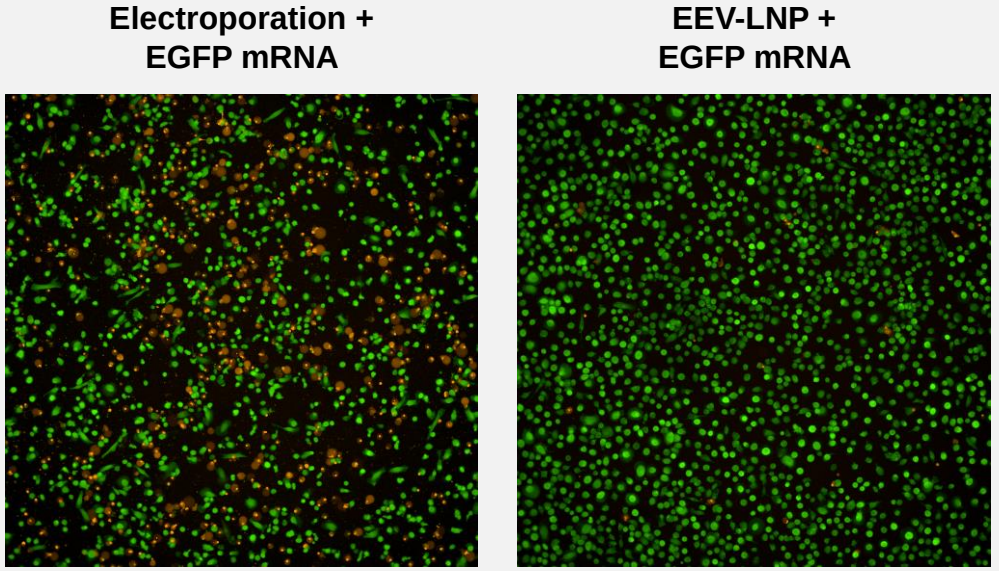
EEV-LNP demonstrated 5-fold improvement in mRNA delivery compared to electroporation, with reduced impact on cell viability

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)

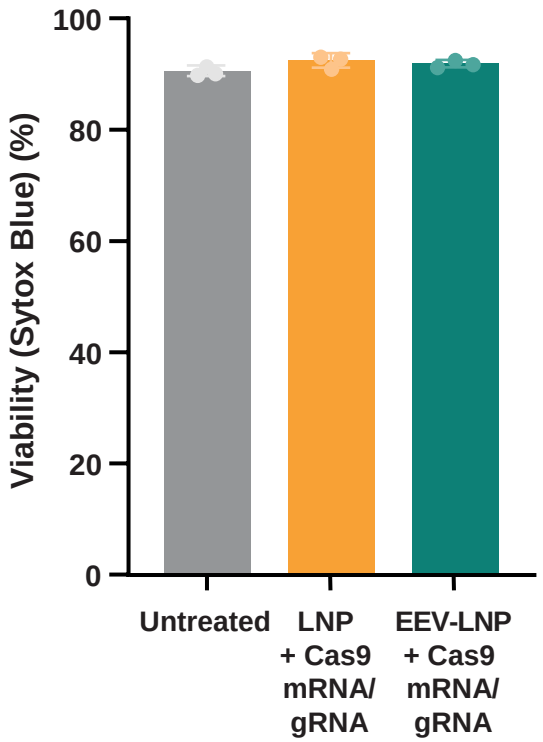
Red: Necrotic/Dead Cells (Propidium Iodide)

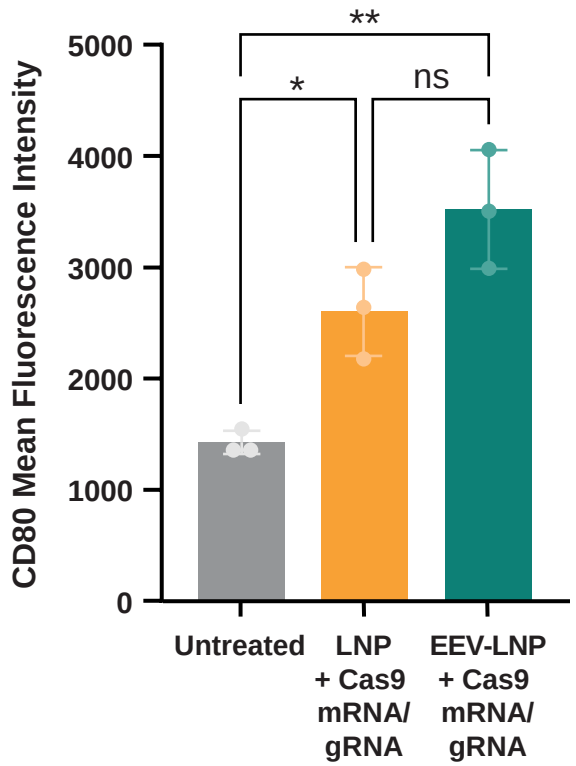
Human primary macrophages were differentiated from monocytes isolated from PBMCs and treated with EEV-LNP or electroporated (EGFP mRNA dose 2500 ng/10^6 cells) using Lonza nucleofection method. EGFP expression was measured by ELISA after 24 hours (left). Cell viability quantified by LIVE/DEAD™ Cell Imaging 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.

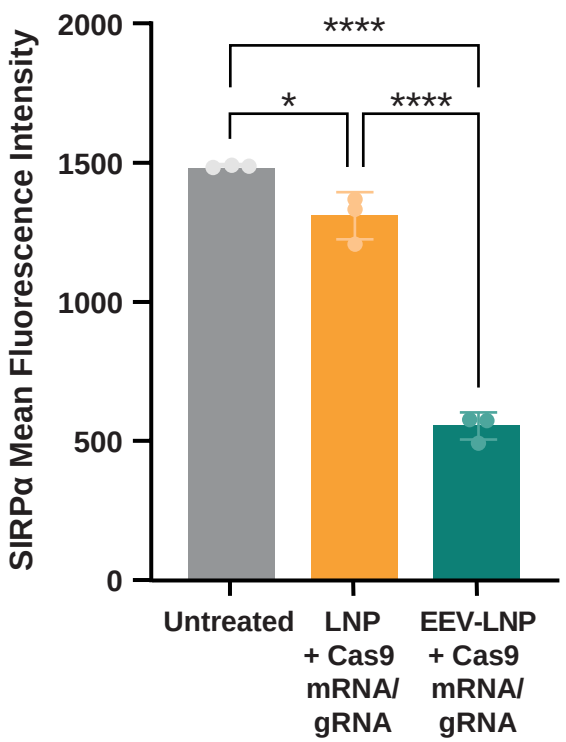
SIRPα KNOCKDOWN IN PRIMARY MACROPHAGES WITH EEV-LNP

EEV-LNP demonstrated greater knockdown of SIRPα by FACS or NGS compared to LNP alone, with no significant impact on cell viability

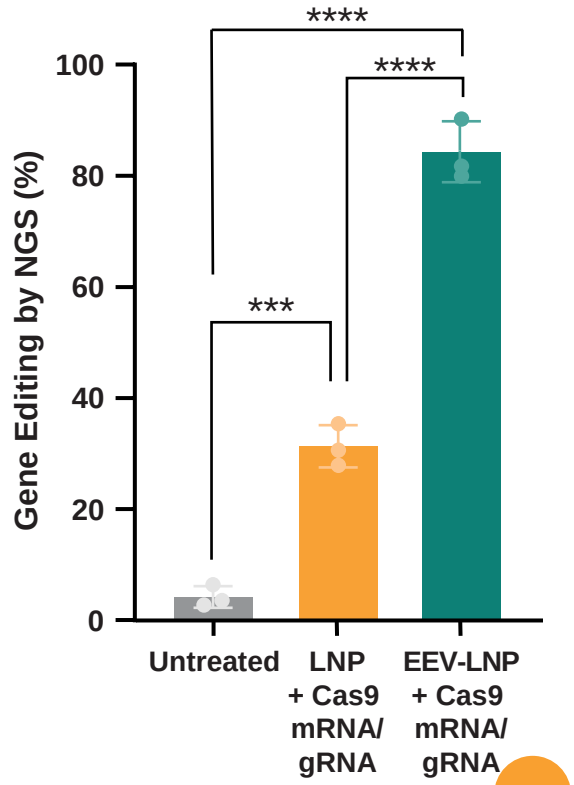
SIRPα Knockdown in Primary Macrophages (FACS)







SIRPα Knockdown (NGS)



Human primary macrophages were differentiated from monocytes isolated from PBMCs with GM-CSF and treated with 1000 ng/ml EEV-LNP or LNP containing Cas9 mRNA and multiple gRNAs targeting SIRPα gene. Cell viability quantified by Sytox Blue™ Dead Cell Stain. SIRPα level was quantified using CD147. Percent Gene Editing was determined by NGS and analyzed by CRISPResso2. SIRPα is a membrane glycoprotein that inhibits macrophage function. CD80 is a co-stimulatory marker expressed on M1 macrophages. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. One-way ANOVA was used for statistical comparison. **ANOVA**, analysis of variance; **EEV**, Endosomal Escape Vehicle; **FACS**, fluorescence activated cell sorting; **FITC**, fluorescein isothiocyanate; **LNP**, lipid nanoparticle; **PBMC**, peripheral blood mononuclear cell; **FACS**, fluorescence-activated cell sorting; **NGS**, next-generation sequencing; **ns**, not significant; **SIRPα**, signal regulatory protein alpha.

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