EEV-Conjugated PMO Results in Nuclear Foci Reduction and Aberrant Splicing Correction in Myotonic Dystrophy Cell and Animal Models

321

Mahasweta Girgenrath, Nelsa L. Estrella, Amy N. Hicks, Xiulong Shen, Mark Wysk, Mahboubeh Kheirabadi, Matthew Streeter, Wenlong Lian, Nanjun Liu, Sara L. Blake,Christopher M. Brennan, Ning Li, Vlad Batagui, Keyede Oye, Ningguo Gao, Daniel Wang, Ziqing Leo Qian, and Natarajan Sethuraman

Entrada Therapeutics, Boston, MA

INTRODUCTION

- Myotonic dystrophy type 1 (DM1) is a multi-systemic disease that affects over 40,000 individuals in the US and 50,000 in Europe.
  - Pathology manifests as myotonia, muscle weakness and atrophy, cardiac conduction abnormalities, pulmonary complications, cataracts, and endocrine dysfunction.[1]
  - Currently there are no approved therapies for DM1.
- DM1 is caused by a CUG trinucleotide repeat expansion in the *DMPK* (dystrophia myotonica protein kinase) mRNA that sequesters splicing regulatory proteins such as MBNL (muscleblind-like).[2]
  - Mutant *DMPK* mRNA and MBNL proteins aggregate to form nuclear foci.3 MBNL splicing activity is decreased as a result of nuclear sequestration, thereby inhibiting splicing and expression of many downstream transcripts.[4,5]
- One therapeutic approach for treatment of DM1 is the use of oligonucleotide therapeutics to sterically block CUG repeat expansions.
  - There are significant barriers to the development of oligonucleotide-based therapies such as limited exposure and poor endosomal escape in target tissue after systemic administration.
- We developed an Endosomal Escape Vehicle (EEV™) delivery platform based on cyclic cell-penetrating peptides (cCPPs) to improve cellular uptake and enhance endosomal escape of oligonucleotides and other biologics.[6,7]
  - ENTR-701 is our lead clinical candidate for the treatment of DM1.

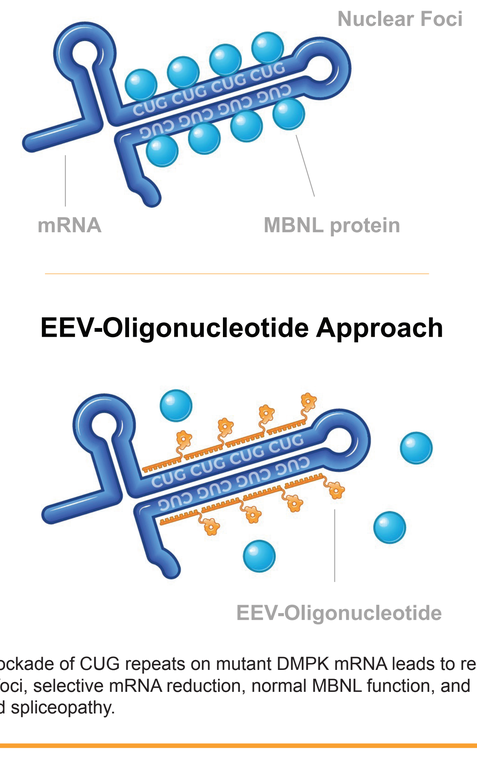
OBJECTIVES

- To examine the therapeutic potential of ENTR-701 in preclinical models of DM1.

MATERIALS AND METHODS

- ENTR-701 consists of a phosphorodiamidate morpholino oligomer (PMO) that blocks CUG repeats in an allele-specific manner, conjugated to our EEV platform (**Figure 1**).
- Nuclear foci reduction, mutant transcript levels, and correction of aberrant splicing were assessed in cellular models and HSA-LR mice.
  - HeLa480 cells stably expressing either (CTG)480 or (CTG)0 DMPK transgenes showed MBNL1-dependent aberrant splicing.[8]
  - Immortalized myoblasts were derived from DM1 patient primary skeletal muscle cells and contain 2,600 CUG repeats within the 3'UTR of *DMPK*.[9]
  - HSA-LR[10] mice carry a transgene with a (CTG)220 repeat expansion in the 3'-UTR of the human skeletal actin gene (ACTA1) which recapitulates molecular pathology and results in a myotonia phenotype.

Figure 1. Mechanism of EEV-PMO Conjugate in DM1.



Steric blockade of CUG repeats on mutant DMPK mRNA leads to reduced nuclear foci, selective mRNA reduction, normal MBNL function, and corrected spliceopathy.

RESULTS

ENTR-701 in HeLa480 Mutant *DMPK* Cell Line

ENTR-701 reduced nuclear foci in HeLa480 cells (**Figures 2A, 2B**). In addition, free uptake of ENTR-701 selectively reduced (CUG)480 containing *DMPK* mRNA (**Figure 2C**).

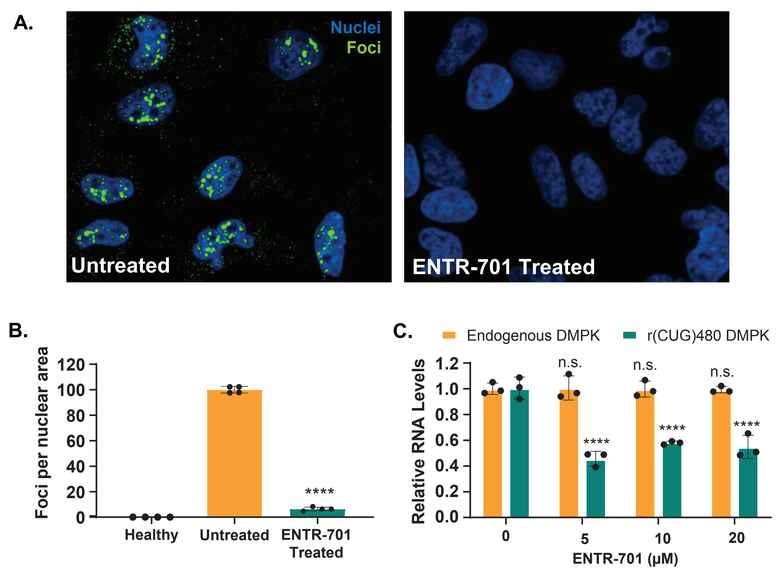
Treatment of DM1 Patient-Derived Myotubes with ENTR-701

ENTR-701 treatment in DM1 patient-derived muscle cells resulted in significant nuclear foci reduction (**Figures 3A, 3B**) and correction of aberrant splicing (**Figure 3C**).

Efficacy of ENTR-701 in HSA-LR Mouse Model of DM1

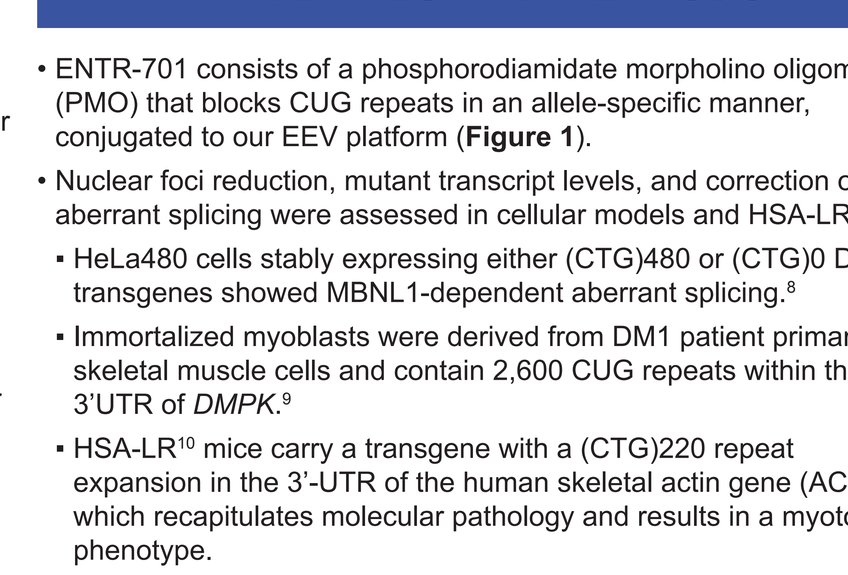
ENTR-701 treatment reduced the number of nuclear foci (**Figure 4A, 4B**), corrected aberrant splicing of *Atp2a1* (**Figure 4C**) and reduced HSA-LR(CUG)220 mRNA in HSA-LR mice (**Figure 4D**).

Figure 2. ENTR-701 Reduces Nuclear Foci and Mutant mRNA in HeLa480 Cells.



HeLa480 cells were treated with ENTR-701 and number of nuclear foci were analyzed by were quantitated relative to nuclear area and normalized to untreated cells (**A, B**). *DMPK* transcript levels were measured by qPCR. Mutant r(CUG)480 levels were normalized to untreated samples (**C**). Data shown as mean ± standard deviation. ****p<0.0001 vs. untreated (**B**) and vs. 0 μM (**C**), n.s., not significant compared to 0 μM (panel C).

Figure 3. ENTR-701 Reduces Nuclear Foci and Correct Aberrant Splicing in DM1 Patient-Derived Cells.



Immortalized DM1 patient-derived myotubes were treated with ENTR-701. Nuclear foci area was quantitated relative to total nuclear area and normalized to untreated cells (**A, B**). *MBNL1* exon 5 and *SOS1* exon 25 inclusion were analyzed by RT- PCR and Labchip (**C**). Data shown as mean ± standard deviation. ****p<0.0001 vs. untreated.

Figure 4. Efficacy of ENTR-701 in HSA-LR Mice.

Reduction of nuclear foci in tibialis anterior by immunofluorescence (**A, B**). Correction of *Atp2a1* (sarcoplasmic/endoplasmic reticulum calcium ATPase) splicing (**C**).Reduction of HSA-r(CUG)220 with repeat expansion (**D**). Data shown as mean ± standard deviation. **p<0.01, ***p<0.001, ****p<0.0001 vs. HSA-LR vehicle.

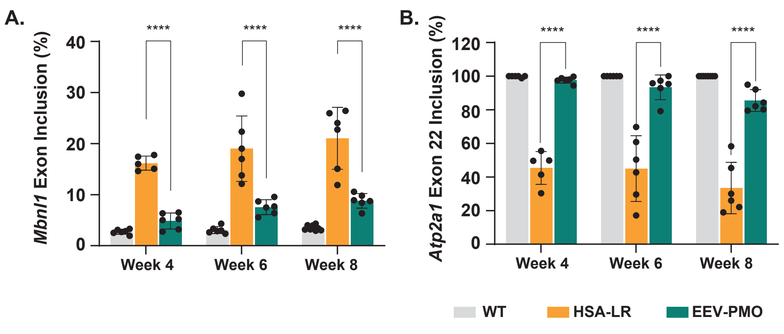
Durable Efficacy of ENTR-701 in HSA-LR Mice

A single dose of ENTR-701 corrected aberrant splicing of *Mbnl1* (**Figure 5A**), *Atp2a1* (**Figure 5B**), and *Nfix* (**Figure 5C**) for at least 8 weeks post-dose.

Spliceopathy in HSA-LR mice corrected with ENTR-701

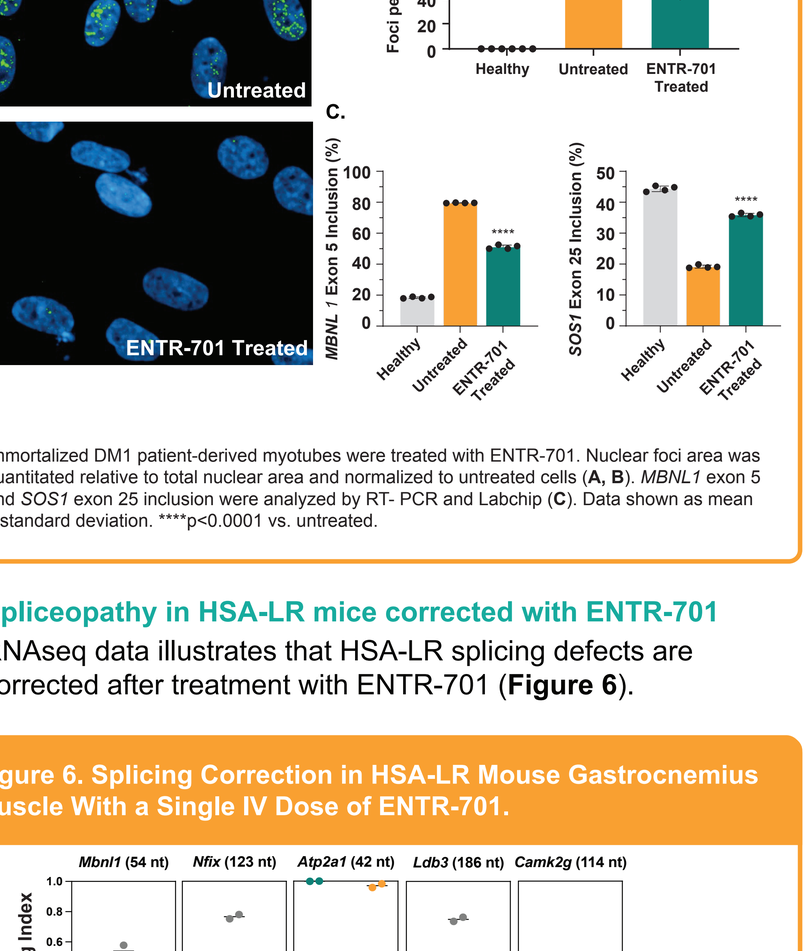
RNAseq data illustrates that HSA-LR splicing defects are corrected after treatment with ENTR-701 (**Figure 6**).

Figure 5. Durable Splicing Correction in HSA-LR Mouse Tibialis Anterior Muscle With a Single Dose of ENTR-701.



Mbnl1 (muscleblind-like splicing regulator 1) exon 5 (**A**). *Atp2a1* (sarcoplasmic/endoplasmic reticulum calcium ATPase) exon 22 (**B**). *Nfix* (nuclear factor I X) exon 7 (**C**). Data shown as mean ± standard deviation following a single 60 mg/kg (PMO equivalent) IV dose.
***p<0.001,
****p<0.0001.

Figure 6. Splicing Correction in HSA-LR Mouse Gastrocnemius Muscle With a Single IV Dose of ENTR-701.



HSA-LR mice administered 60 mg/kg PMO equivalent ENTR-701 IV. RNAseq data was normalized to transcriptome.

ENTR-701 Rescues the Myotonia Phenotype in HSA-LR mice

A single 60 mpk (PMO equivalent) dose of ENTR-701 ameliorated observable pinch-induced myotonia symptoms for at least 8 weeks post-dose.

CONCLUSIONS

- Our DM1 clinical candidate, ENTR-701, reduces nuclear foci and CUG-repeat expansion containing transcript levels in the HeLa480 cell model and the HSA-LR mouse model of DM1.
- DM1 patient-derived muscle cells with 2600 CUG repeats also showed a reduction in nuclear foci when treated with ENTR-701.
- ENTR-701 corrected aberrant downstream splicing in all three models.
- A single dose of ENTR-701 demonstrates durable splicing correction and ameliorates myotonia for at least 8 weeks post-dose.
- These results illustrate the therapeutic potential of the EEV-oligonucleotide approach for DM1 and support further study of ENTR-701 in patients with DM1.

ACKNOWLEDGEMENTS

This research was funded by Entrada Therapeutics, Inc. (Boston, MA). The authors would like to thank Vishi Agarwal (Entrada) for her assistance developing the pinch-induced myotonia observation assay, Aji Nair (Entrada) for assistance with poster development, and Eric T. Wang's lab at the University of Florida for providing the HeLa cell line. References: 1. Thornton C.A. *Neurol Clin.* 2014. 2. Philips, A.V. et al. *Science.* 1998. 3. Pettersson, O.J. *NAR.* 2015. 4. Wagner, S.D. et al. *PLoS Genet.* 2016. 5. Tanner, M. et al. *Nucleic Acids Res.* 2021. 6. Qian Z. *Biochemistry.* 2016. 7. Sahni A. *ACS Chem Biol*. 2020. 8. Reddy, K. et al. *Proc Natl Acad Sci.* 2019. 9. Arandel, L. et al. *Dis Model Mech.* 2017. 10. Mankodi, A. et al. *Science*. 2000.