Superior expression of self-complementary AAV and comparable functionality of mini and full-length MeCP2 support the design of TSHA-102 gene therapy for Rett syndrome

Poster 3481

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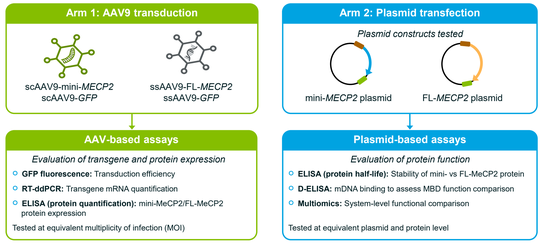
Aims

To investigate expression of transgenes/proteins with self-complementary (sc) adeno-associated virus serotype 9 (AAV9) vs single-stranded (ss) AAV9 vectors, and to compare functionality of miniaturized and full-length methyl-CpG-binding protein 2 (MeCP2) proteins

Background

- Rett syndrome (RTT) is a rare, neurodevelopmental disorder caused by pathologic loss-of-function mutations in the X-linked *MECP2* gene, encoding the MeCP2 protein[1–3]
- For gene regulation, MeCP2 binds to both methylated (m) and unmethylated DNA and functions with partner proteins, including DNA methyltransferases (DNMTs), to modulate chromatin structure, DNA methylation and transcriptional activity[4–7]
- A truncated MeCP2 protein retaining the essential methyl-CpG-binding domain (MBD) and the transcriptional repressor NCoR/SMRT interaction domain (NID) restored a normal phenotype in *Mecp2*$^{-/y}$ mice and showed comparable functionality to full-length MeCP2[8]
  - These findings established RTT is reversible in this model and the mechanistic basis for miniaturized *MECP2* gene therapy to restore expression in MeCP2-deficient cells
- TSHA-102 is a recombinant scAAV9 gene therapy that encodes a functional, miniaturized *MECP2* transgene that retains the essential MBD and NID,[9,10] currently under investigation in the REVEAL pivotal trials[11]
  - In contrast to ssAAV vectors that require conversion of the single-stranded DNA to double-stranded DNA prior to expression, TSHA-102 contains complementary strands of the *MECP2* transgene, delivering a transcription-ready transgene to MeCP2-deficient cells for enhanced stability and efficiency[9,12]
  - A miRNA-Responsive Auto-Regulatory Element (miRARE) further refines expression by silencing transgene expression in MeCP2-healthy cells while permitting expression in MeCP2-deficient cells[9,10]
- scAAV vectors have been shown to drive robust transgene expression across disease models and genetic payloads,[12–14] yet a direct comparison with ssAAV for *MECP2* delivery in RTT models is lacking. Furthermore, although miniaturized (mini-)MeCP2 is sufficient to rescue the disease phenotype in MeCP2-deficient mice,[9,10] a comprehensive comparison of the biochemical and molecular properties of mini-MeCP2 and full-length (FL-MeCP2) versions of MeCP2 have not been fully characterized
- The current study addresses both of these gaps through a multi-level panel of functional assays. Complementary assays compared scAAV vs ssAAV and mini-MeCP2 vs FL-MeCP2 at three levels: vector delivery, transgene expression, and biochemical/molecular function (**Figure 1**)

Figure 1: Overview of experimental design for scAAV9 vs ssAAV9 and mini- vs FL-MeCP2 comparisons



Cell lines: Neuro2a (N2a; wildtype [WT]), N2a-MeCP2 R294X (N2a R294X, RTT model cells)

Methods

scAAV and ssAAV vectors

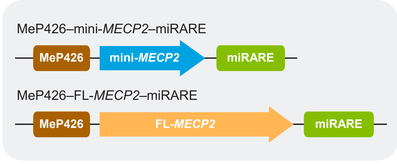
- TSHA-102 (scAAV9-mini-*MECP2*) was evaluated against an analogous ssAAV9 construct in which the mini-*MECP2* and mutant inverted terminal repeat (ITR) were replaced with FL-*MECP2* and WT ITR, while maintaining identical backbone and regulatory elements
- N2a cells expressing either WT *MECP2* or the clinically relevant R294X allele (N2a R294X, RTT model cells) were transduced with either TSHA-102 or the ssAAV9-FL-*MECP2* construct
- Transgene and protein expression were quantified using reverse transcription droplet digital polymerase chain reaction (RT-ddPCR) and enzyme-linked immunosorbent assay (ELISA), respectively. The measured concentration of MeCP2 protein was within 25% of the coefficient of variation
- N2a and N2a R294X cells were also transduced with ssAAV9-*green fluorescent protein (GFP)* or scAAV9-*GFP* vector. Transgene and protein expression were measured by RT-ddPCR and GFP fluorescence from live cells, respectively

Functional analyses

- N2a R294X cells were transiently transfected with equimolar plasmids using FuGENE 6 (Promega) to express a similar amount of MeCP2 protein (**Figure 2**)

Figure 2: Plasmid constructs

TSHA-102 vector map was used for mini- and FL-MeCP2



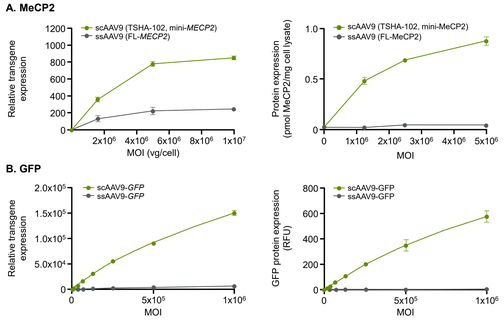
- **Half-life:** Transfected cells were treated with 15 µM cycloheximide, and cellular extracts were prepared at various time intervals (cycloheximide chase experiment[15]). MeCP2 levels were quantified by ELISA
- **Binding to mDNA:** Cell lysates were prepared from transfected cells and DNA-binding (D-)ELISA performed[16,17] using microplates coated with custom biotin-labeled 45 base pair (bp) oligonucleotides containing three methylated CpG sites
- **DNMT inhibition:** DNMT3A/3L complexes (Active Motif) were incubated with different molar amounts of either mini-MeCP2 or FL-MeCP2 recombinant proteins. The Epiquick DNMT Activity/Inhibition Assay Ultra kit (Epigentek) was used to quantify DNMT activity

Global biomolecule rescue analysis

- Multiomics analysis was performed on cell lysates of transfected cells using liquid chromatography/mass spectrometry (LC/MS) to define biomolecule profiles
- Statistical associations between biochemicals and transfection groups were tested using linear regression models. Biomolecules were considered significant hits if the adjusted p-value was ≤0.05
- The fold change (FC) in protein abundance was compared from baseline to post-transfection; biomolecule regulation was considered improved if their FC shifted closer to 1 (indicating no dysregulation) by at least 20%
- To compare mini-MeCP2 and FL-MeCP2, the improvement ratio was calculated by dividing the ΔFC of mini-MeCP2 by the ΔFC of MeCP2, with a ratio of 1 indicating the same molecular activity

Results

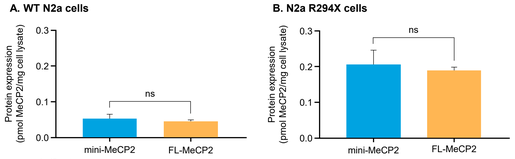
Figure 3: Transduction with scAAV9 vs ssAAV9 vectors demonstrated greater transgene expression and ~30-fold greater protein expression across MeCP2 and GFP in RTT model cells



scAAV vs ssAAV expression

- When looking at MeCP2 expression in RTT model cells, the scAAV9 vector (TSHA-102) produced up to 30-fold more MeCP2 protein than the ssAAV9 vector (ssAAV9-FL-*MECP2*), which expressed an insignificant amount of MeCP2 protein (**Figure 3A, right panel**)
- Using a *GFP* model transgene in RTT model cells, scAAV9 expressed ~30-fold more *GFP* transgene and GFP protein compared with ssAAV9, for which expression was negligible (**Figure 3B**)

Figure 4: Following plasmid transfection, mini-MeCP2 protein expression was comparable to FL-MeCP2 and upregulated 3.5- to 4-fold in RTT model vs WT cells via miRARE-driven regulation

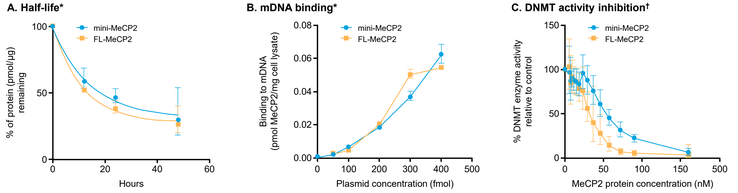


ns, non-significant

MeCP2 protein functional analysis

- mini-MeCP2 and FL-MeCP2 both achieved comparable, stable expression in neuronal cells when transfected with equimolar amounts of plasmid (**Figure 4**)
  - mini-MeCP2 and FL-MeCP2 exhibited the same expression pattern in WT and RTT model cells
  - Protein levels were approximately 3.5- to 4-fold higher in RTT model cells compared with WT cells, consistent with miRARE-driven genotypic regulation of expression

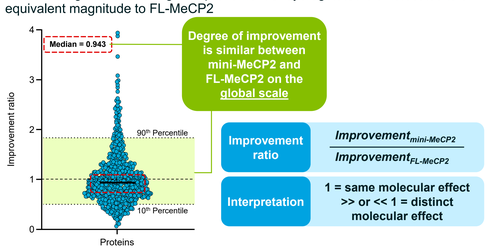
Figure 5: Protein functional analysis confirms mini-MeCP2 is functionally equivalent to FL-MeCP2 demonstrating a comparable half-life, mDNA binding, and DNMT3 inhibition



*Following plasmid transfection in RTT model cells; †In a DNMT3A/3L assay

- mini-MeCP2 and FL-MeCP2 proteins showed similar half-lives in the RTT model cells (12 h vs 9 h, respectively; **Figure 5A**), indicating comparable steady-state abundance and protein synthesis rates
- mini-MeCP2 and FL-MeCP2 proteins both demonstrated binding to mDNA with a similar magnitude (**Figure 5B**)
- Both proteins inhibited DNMT3 activity in a dose-responsive manner, with similar levels of inhibition observed (**Figure 5C**), suggesting that both proteins directly interacted with the DNMT enzyme

Figure 6: RTT model cells demonstrate mini-MeCP2 normalizes the RTT multiomic signature resulting in improvement of dysregulated biomolecules to an equivalent magnitude to FL-MeCP2



Global biomolecule rescue analysis

- RTT model cells displayed highly dysregulated biomolecule profiles, with 5023 proteins, lipids and metabolites dysregulated compared with WT cells. Treatment with either expression plasmid (TSHA-102 mini-*MECP2* or FL-*MECP2*) resulted in improvement of dysregulated biomolecules to an equivalent magnitude (**Figure 6**), suggesting that the activity of mini-MeCP2 protein is comparable to MeCP2 protein across multiple classes of biomolecules on a cellular level
  - Almost no effect on biomolecule dysregulation was observed in WT background (dysregulated 1 and 8 for mini-MeCP2 and FL-MeCP2, respectively), suggesting no off-target activities in WT background as expected due to miRARE

Conclusions

- scAAV9 enables markedly higher (30x) MeCP2 protein expression than an analogous ssAAV9 for MeCP2 delivery, consistent with the literature from other sc- and ss-model vector comparisons[12–14]
- mini-MeCP2 is functionally comparable to FL-MeCP2 across biochemical functions, with both proteins exhibiting similar behavior in RTT model cells:
  - Both mini-MeCP2 and FL-MeCP2 are stably expressed in neuronal cells. miRARE controls protein expression in a genotype-dependent manner, independent of whether the expressed protein is mini- or-FL-MeCP2
  - The half-life of the mini-MeCP2 protein is similar to the FL-MeCP2 protein, suggesting comparable protein stability and supporting the likelihood of similar biological functions
  - Both mini-MeCP2 and FL-MeCP2 similarly bind to mDNA, which is a fundamental process for establishing and maintaining normal gene expression patterns, essential for brain health[18]
  - mini-MeCP2 inhibits DNMT enzymatic activity, further showing that mini-MeCP2 is functionally analogous to FL-MeCP2
  - mini- and FL-MeCP2 proteins produce similar improvements across dysregulated multiomics signatures, indicating preservation of core MeCP2 function, resulting in broad cellular correction with mini-MeCP2 equivalent to that of FL-MeCP2

Key takeaway

The molecular function of mini-MeCP2 and FL-MeCP2 are analogous, with both proteins exhibiting similar, stable expression in neuronal cells. scAAV9 enables superior MeCP2 expression compared with ssAAV9, supporting effective delivery of TSHA-102 to the central nervous system using a minimally invasive lumbar intrathecal administration. Together, the findings provide strong mechanistic support for the scAAV9 + mini-*MECP2* + miRARE design of TSHA-102 and translational support for the ongoing REVEAL trial, indicating TSHA-102 is generally well tolerated with multidomain developmental milestone gain/regain across core RTT domains[11]

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Abbreviations: bp, base pair; DNMT, DNA methyltransferase; (D-)ELISA, (DNA-binding) enzyme-linked immunosorbent assay; FC, fold change; FL, full-length; GFP, green fluorescent protein; h, hour; ITR, inverted terminal repeat; LC, liquid chromatography; mDNA, methylated DNA; MeCP2, methyl-CpG binding protein 2; miRARE, miR-Responsive Auto-Regulatory Element; MOI, multiplicity of infection; MS, mass spectrometry; N2a, Neuro2a cells; NCoR, nuclear receptor corepressor; NID, NCoR/SMRT interaction domain; ns, non-significant; RFU, relative fluorescence units; RT-ddPCR, reverse transcription droplet digital polymerase chain reaction; RTT, Rett syndrome; scAAV(9), self-complementary adeno-associated virus (serotype 9); SMRT, silencing mediator of retinoid and thyroid hormone receptors; ssAAV(9), single-stranded adeno-associated virus (serotype 9); vg, vector genome; WT, wild type

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