The microRNA-responsive autoregulatory element from TSHA-102 for Rett Syndrome modulates therapeutic transgene expression in response to cellular MeCP2 in mouse and human cell lines

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INTRODUCTION

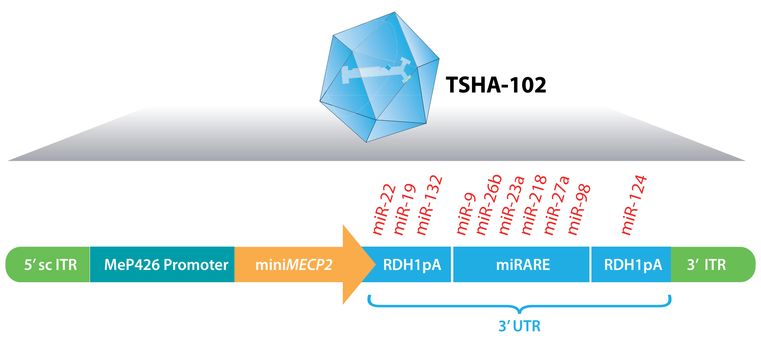
Gene therapy for Rett syndrome (RS) requires tight control of transgene expression:

- RS, an X-linked disorder affecting 5 – 10 per 100,000 women,[1] arises from deficiency in *MECP2*, encoding methyl-CpG binding protein 2[2]
- Symptoms and severity vary, due in part to random X-inactivation
- Duplication of the same locus (*MECP2* duplication syndrome) causes neurodevelopmental defects as severe as those in RS[3]
- Supraphysiologic expression of a *MECP2* transgene can be toxic in mice, consistent with a narrow therapeutic window and the need for negative regulatory elements in RS gene therapy vectors.[4]

TSHA-102 (**Figure 1**):[5]

- Adeno-associated virus (AAV) 9 vector expressing truncated MECP2 cDNA (mini*MECP2*)[6] in the brain, under a *Mecp2* promoter
- Carries in its 3′ untranslated region (3′UTR) a microRNA (miRNA) autoregulatory element (miRARE) designed to prevent mini*MECP2* overexpression.[5,7]

Figure 1: Structure of TSHA-102. A mouse *Mecp2* promoter fragment (MeP426) drives CNS expression of a truncated but functional MeCP2 (miniMeCP2). miRNA target sites (seeds; red) engineered into the miRARE are proposed to silence transgene expression in response to cellular MeCP2 function.

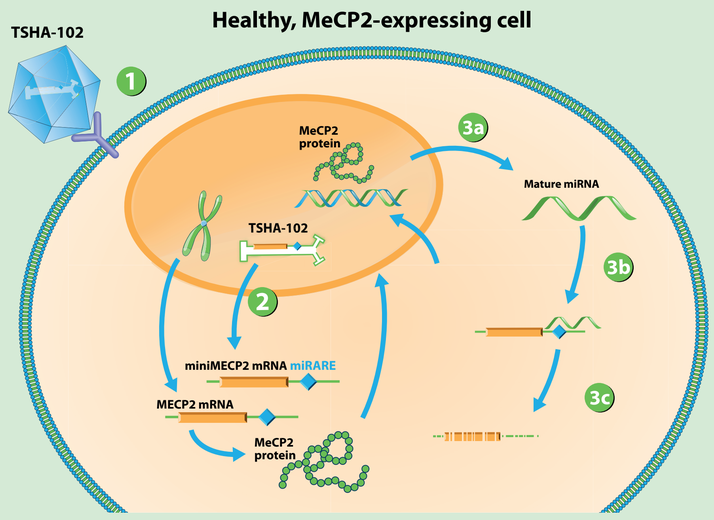


miRNAs are short, non-coding RNAs that bind specific mRNA 'seed' sequences, leading to post-transcriptional silencing by targeting the mRNA for decay and/or restricting translation.[8] The mini*MECP2* 3′UTR carries seeds for at least 10 miRNAs, including 6 in the miRARE. Most seeds were selected based on evidence that MeCP2 induces the corresponding miRNAs.[5]

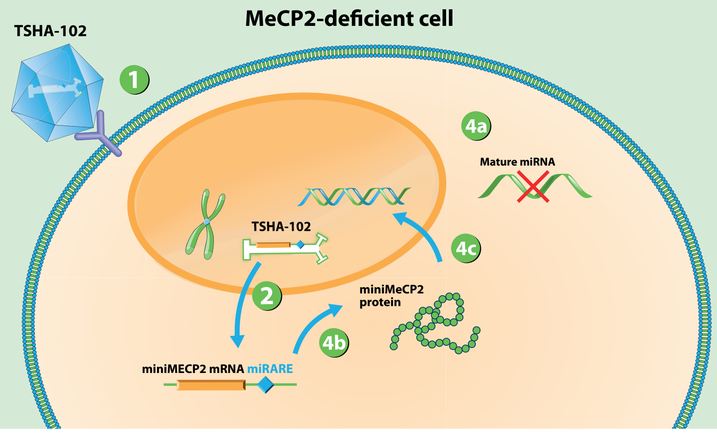
By hypothesis (**Figure 2**), the miRARE in TSHA-102 prevents toxic overexpression of the transgene by establishing a feedback repression system in which MeCP2 expression or restoration induce miRARE-binding miRNAs, which act cell-autonomously to silence transgene expression; miniMECP2 mRNA is translated efficiently in MeCP2-negative cells.

Figure 2. Model for post-transcriptional control of miniMeCP2 expression by the miRARE in TSHA-102.

1. TSHA-102 is endocytosed and its genome is released into the nucleus
2. miniMECP2 mRNA, carrying the miRARE, is transcribed
3. In cells with normal MeCP2 function in the nucleus:
   a. miRNAs are produced
   b. These miRNAs interact with the miRARE element in the miniMECP2 mRNA
   c. This interaction leads to decay of the mRNA and/or suppresses its translation.



4. In cells lacking normal MeCP2 function,
   a. Expression of miRARE-targeting miRNAs is restricted
   b. The transgene mRNA is translated to produce miniMeCP2 protein
   c. The protein is imported into the nucleus, restoring MeCP2 function.



Consistent with this model, in *Mecp2*$^{-/Y}$ mice (a commonly used model for RS), TSHA-102 significantly extends lifespan and delays onset of gait abnormalities. Inclusion of the miRARE in TSHA-102 prevents treatment toxicity in WT animals, including early mortality and gait abnormalities.[5,9]

- To date, the mechanistic basis of this protective effect has not been examined directly. Here, we explore the effects of the miRARE in the presence or absence of cellular MeCP2, using AAV9 and plasmid transfection to drive mini*MECP2* transgene expression in an experimentally tractable cell culture model.

METHODS

- Cell culture, viral vector production, AAV9 transduction and plasmid transfection were per Taysha standard protocols. Recombinant plasmids were produced and characterized at Aldevron (Fargo, ND). miniMECP2 RNA estimation was carried out using a one-step RT-ddPCR assay[10]. Protein estimation employed a sandwich ELISA using miniMeCP2-specific antibodies developed at Taysha.
- CRISPR-mediated knockout of *MECP2* was carried out 2v6.11 in human kidney cells; heterozygous and homozygous KO lines were characterized by sequencing.
- miRNA discovery and quantitative/qualitative analysis by miRNA-Seq were carried out by Azenta Life Sciences (Burlington, MA). miRDB software (http://www.mirdb.org/) was used for *in silico* assessment of miRNA binding.

RESULTS

Cell lines transfected with the pTSHA102^{miRARE} construct (**Figure 3**) or transduced with TSHA-102 (**Figure 4**) expressed mini*MECP2* under control of the MeP246 fragment, with no toxicity seen at any multiplicity of infection (MOI):

- In transfected cells, neuronal cell lines showed comparable miniMeCP2 protein expression; miniMeCP2 level was 4 – 5-fold lower in 2v6.11 cells
- In transduced cells, transgene expression was proportionate to MOI and substantially lower in 2v6.11 cells.

Figure 3. miniMeCP2 protein is expressed in cultured cells. The human non-neuronal cell line 2v6.11 and mouse (Neuro-2a) and human (SH-SY5Y) neuronal cells were transfected with pTSHA102^{miRARE}.

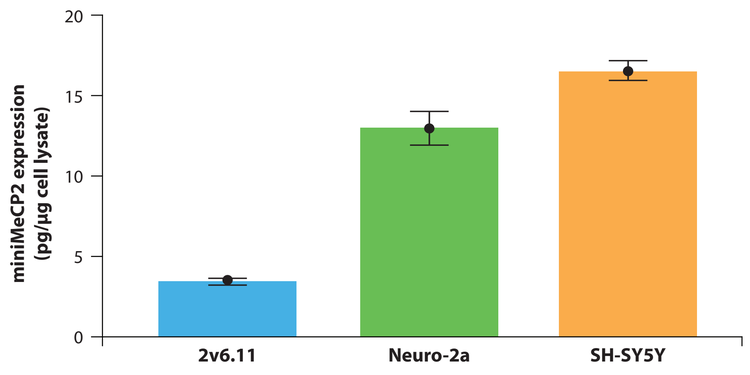
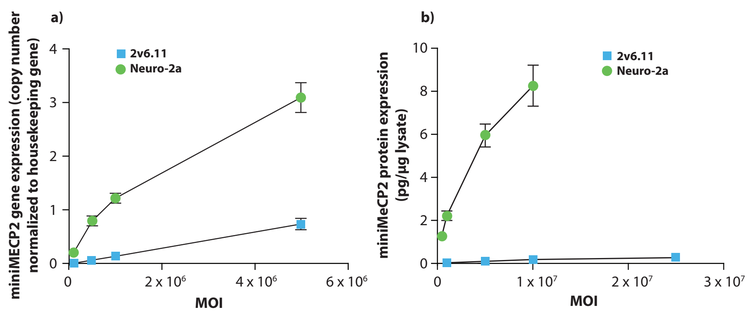


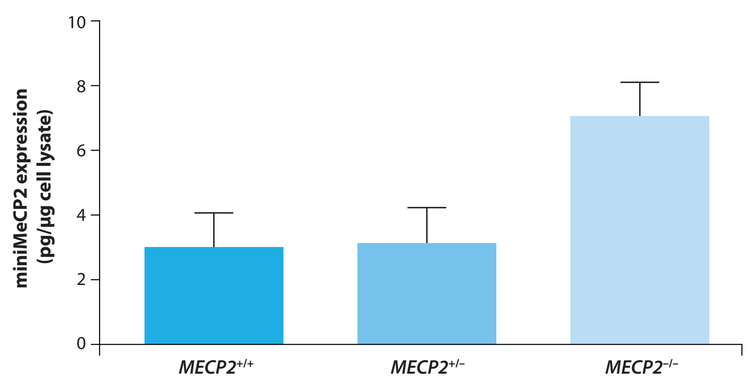
Figure 4. Expression of miniMECP2 RNA (a) and protein (b) in TSHA-102-transduced cells. RNA expression was normalized to that of the housekeeping genes *RPP30* (human cell line 2v6.11) or *Hprt* (mouse neuronal cell line Neuro-2a).



In *MECP2*$^{-/-}$ 2v6.11 cells, miniMeCP2 protein from the pTSHA102^{miRARE} plasmid accumulated at a mean level ~2.3-fold greater than in parental 2v6.11 cells (**Figure 5**).

- This increase occurred despite reduced cell growth and viability of *MECP2*$^{-/-}$ cells
- As expected, heterozygous CRISPR-modified cells (*MECP2*$^{+/-}$) had normal growth and little change in miniMeCP2 expression.

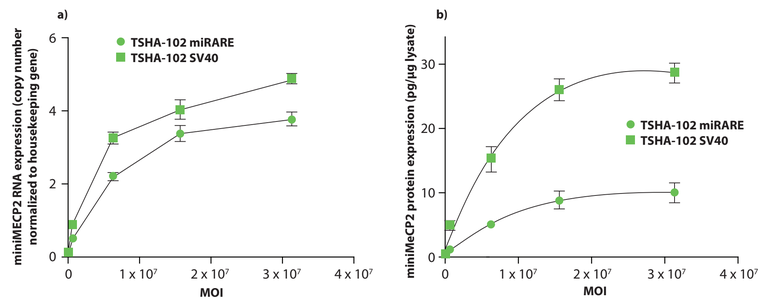
Figure 5. miniMeCP2 protein expression is sensitive to cellular expression of MeCP2. CRISPR was used to generate human 2v6.11 with one or both *MECP2* alleles disrupted. Parental as well as *MECP2*$^{+/-}$ and *MECP2*$^{-/-}$ 2v6.11 were transfected with pTSHA102^{miRARE}. miniMeCP2 protein expression was normalized to luciferase activity derived from a co-transfected reporter construct.



In transduced Neuro-2a cells, presence of the miRARE in TSHA-102 was associated with reduced miniMECP2 mRNA (**Figure 6a**), as well as dramatically reduced miniMeCP2 protein expression (**Figure 6b**). Thus, replacement of the TSHA-102 3′UTR with with an SV40 3′UTR element led to:

- A 49% increase in miniMECP2 mRNA
- A 197% increase miniMeCP2 protein.

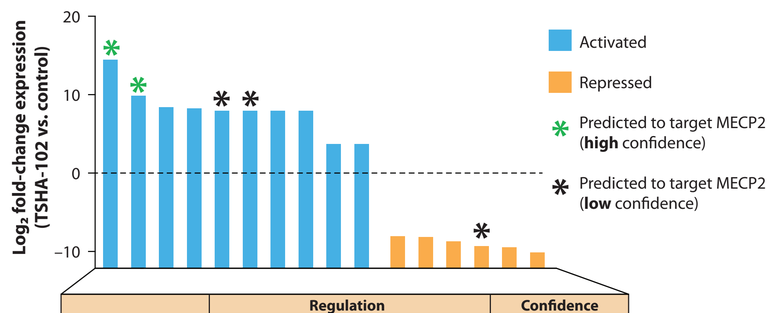
Figure 6. Silencing of miniMECP2 RNA (a) and protein (b) expression by miRARE in Neuro-2a cells. Cells were transduced with TSHA-102 or a modified version of the viral vector carrying the SV40 3′UTR sequence in place of the RDH1pA-miRARE sequence.



miRNA expression levels in culture cells were complex and dynamic, varying between cell types and altering in response to transfection with pTSHA102^{miRARE}. In all, 16 mouse and 41 human miRNAs had significantly altered expression following transfection. Thus, compared with mock-transfected cells, pTSHA102^{miRARE} -transfected Neuro-2 cells had (**Figure 7**):

- 10 miRNA species (9 novel, 1 previously characterized) with significantly higher expression
- 6 novel miRNA species with significantly lower expression.

Figure 7. Dynamics of miRNA expression in Neuro-2a cells following transfection with the pTSHA102^{miRARE}. Multiple miRNAs show significant induction (blue) or repression (orange). Of the induced miRNAs, two (NovelmiRNA-583 and -967) are strongly predicted to bind seed sequences in miniMECP2 mRNA.



	miRNA	Regulation: Fold change (Log_2)	Regulation: Fold change	Regulation: Δ TPM	Confidence: *p*	Confidence: FDR
*	**NovelmiRNA-967**	**14.31**	**20252.8**	**139.21**	**1.19E-07**	**0.0002**
*	**NovelmiRNA-583**	**9.76**	**867.5**	**5.97**	**0.003**	**0.27**
	NovelmiRNA-780	8.32	320.5	2.20	0.026	1
	Nommi-miR-6932-3p	8.17	288.8	1.98	0.028	1
*	**NovelmiRNA-770**	**7.96**	**249.4**	**1.71**	**0.041**	**1**
*	**NovelmiRNA-713**	**7.91**	**240.4**	**1.65**	**0.042**	**1**
	NovelmiRNA-636	7.87	233.9	1.60	0.045	1
	NovelmiRNA-22	7.82	226.1	1.55	0.049	1
	NovelmiRNA-1047	3.64	12.49	13.99	0.040	1
	NovelmiRNA-67	3.64	12.49	13.99	0.040	1
	NovelmiRNA-284	–8.01	0.00387	–1.77	0.038	1
	NovelmiRNA-590	–8.07	0.00372	–1.84	0.033	1
	NovelmiRNA-448	–8.63	0.00252	–2.72	0.017	0.99
*	**NovelmiRNA-216**	**–9.23**	**0.00166**	**–4.13**	**0.007**	**0.50**

Human neuronal (SH-SY5Y) and non-neuronal 2v6.11 cells also showed dynamic effects on miRNA populations, with no overlap evident among miRNA species regulated. None of the previously characterized miniMECP2 3′UTR-binding miRNAs showed >1.6-fold differential expression, but some regulated miRNAs are predicted to bind miniMECP2 or MECP2 mRNA.

CONCLUSIONS AND FUTURE DIRECTIONS

The miRARE, designed as a MeCP2-responsive negative regulator, permits expression of mini*MECP2* in TSHA-102-treated animals while preventing toxic effects.[5] This protection is proposed to be due to MeCP2-regulated miRNAs binding seed sequences in the miRARE.

Here, we show that post-transcriptional gene silencing by the miRARE can be recapitulated in cell culture. In initial studies, we show:

- **The miRARE partially silences transgene expression in neuronal and non-neuronal cell lines**
- **Consistent with the miRARE's proposed sensitivity to cellular MeCP2, transgene product expression increases in cells with both endogenous *MECP2* copies disrupted**
- **Silencing appears to occur partly by inducing mRNA decay, but more substantially by reducing miniMeCP2 protein accumulation, suggesting that the miRARE acts in *cis* to prevent translation.**

Finally, we confirmed that miRNA populations are dynamic, with some miRNA species modulated by >10^4-fold after mini*MECP2* cDNA transfection.

To date, we have not established whether the miRNA species whose seed sequences were engineered into the miRARE mediate transgene silencing or respond to restoration of MECP2 function. These effects might only be evident in a MeCP2-deficient neuronal cell; in addition, they might be cell-type specific or sensitive to extracellular environment.[5]

A mechanistic understanding of the miRARE will offer new tools for precision targeting of molecular therapeutics. Further cell culture experiments are under way:

- **To confirm the functional interaction between the miRARE and the cellular *MECP2* genotype**
- **To characterize miRNA dynamics following restoration of MeCP2 function.**

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