cassettes, vectors, and pharmaceutical compositions for the purpose of providing transgene expression in target tissues that is capable of endogenous regulation for treating disorders such as dose-sensitive intellectual ability disorders.

## SUMMARY

The present disclosure is based, in part, on the development of feedback-enabled synthetic genes, polynucleotide target cassettes, vectors, and pharmaceutical compositions for the purpose of providing transgene expression in target tissues that is capable of endogenous regulation for treating disorders such as dose-sensitive intellectual ability disorders such as Rett Syndrome (RTT). This disclosure was made in part with support from the Rett Syndrome Research Trust.

Thus, one aspect of the disclosure relates to a synthetic gene comprising a polynucleotide comprising a coding region encoding MeCP2 and one or more regulatory regions, the polynucleotide further comprising one or more nucleic acid segments each comprising a seed match identified as a binding site for an endogenous miRNA and a 5’ flanking sequence and a 3’ flanking sequence neighboring said seed match, wherein said one or more nucleic acid segments are inserted into a regulatory region of said polynucleotide such that expression of MeCP2 when said synthetic gene is delivered to a cell expressing the endogenous miRNA is reduced relative to expression of a MeCP2 when a synthetic gene that does not comprise the one or more nucleic acid segments is delivered to a cell expressing the endogenous miRNA wherein the one or more nucleic acid segments comprising the seed matches and flanking 3’ and 5’ sequences neighboring the seed matches collectively comprise the nucleotide sequence set forth in SEQ ID NO:1.

A further aspect of the disclosure relates to a vector comprising the synthetic gene of the present disclosure.

A further aspect of the disclosure relates to a pharmaceutical composition comprising the synthetic gene or the vector of the present disclosure and a pharmaceutically acceptable carrier.

Another example of the disclosure relates to a polynucleotide target cassette for providing dose dependent inhibitory feedback to the synthetic gene of the present disclosure.

An additional example relates to a method of preparing a synthetic gene, comprising the step of inserting the polynucleotide target cassette into a regulatory region of the synthetic gene.

A further example of the disclosure relates to a method of making a synthetic gene comprising the steps of inserting one or more nucleic acid segments comprising a seed match and 5’ and 3’ flanking sequences into a regulatory region of the polynucleotide of the synthetic gene.

In one aspect of the present disclosure refers to a polynucleotide target cassette for providing dose dependent inhibitory feedback to a synthetic gene, the synthetic gene comprising a polynucleotide comprising a coding region encoding MeCP2 and one or more regulatory regions, and the cassette

comprising one or more nucleic acid segments comprising a seed match identified as a binding site for an endogenous miRNA and 5’ and 3’ flanking sequences neighboring said seed match, wherein the one or more nucleic acid segments comprising the seed matches and flanking 3’ and 5’ sequences neighboring the seed matches collectively comprise the nucleotide sequence set forth in SEQ ID NO:1.

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Another example of the disclosure relates to a method of identifying one or more seed matches and flanking sequences to be inserted into a synthetic gene, comprising the steps of identifying a seed match and flanking sequences, and inserting said seed match and flanking sequences into a regulatory region of a synthetic gene.

An additional example of the disclosure relates to a method of delivering a synthetic gene to a subject, comprising administering to the subject the synthetic gene, vectors, or pharmaceutical composition of the present disclosure. A further example of the disclosure relates to a method of treating a disease associated with abnormal expression of an endogenous gene, comprising administering the synthetic genes, vectors, or pharmaceutical compositions of the present disclosure, thereby treating the disease. In another aspect, the present disclosure refers to a method of preparing a synthetic gene comprising a polynucleotide comprising a coding region encoding a protein or nucleic acid of interest and one or more regulatory regions, comprising the step of inserting the polynucleotide target cassette as disclosed herein into a regulatory region of the synthetic gene. In another aspect, the present disclosure refers to a method of making the synthetic gene as described herein, comprising the steps of: inserting three or more nucleic acid segments each comprising a seed match identified as a binding site for an endogenous miRNA and 5' and 3' flanking sequences neighboring said seed match into a regulatory region of the polynucleotide comprising a coding region encoding MeCP2 and one or more regulatory regions, wherein the seed matches and 5' and 3' flanking sequences bind to the miRNAs miR-9-5p, miR-26-5p, miR-23-3p, miR-218-5p, miR-27-3p, and let-7-5p. In yet another aspect, the present disclosure refers to a use of the synthetic gene, the vector, or the pharmaceutical composition as disclosed herein in the manufacture of a medicament for treating a disease associated with reduced expression of an endogenous *MECP2* gene or expression of a mutant protein encoded by an endogenous *MECP2* gene in a subject. In another aspect, the present disclosure refers to a synthetic gene comprising: a polynucleotide comprising a coding region encoding MeCP2 and one or more regulatory regions, the polynucleotide further comprising three or more nucleic acid segments each comprising a seed match identified as a binding site for an endogenous miRNA and a 5' flanking sequence and a 3' flanking sequence neighboring said seed match, wherein said one or more nucleic acid segments are inserted into a regulatory region of said polynucleotide such that expression of MeCP2 when said synthetic gene is delivered to a cell expressing the endogenous miRNA is reduced relative to expression of a MeCP2 when a synthetic gene that does not comprise the one or more nucleic acid segments is delivered to a cell expressing the endogenous miRNA, wherein the one or more nucleic acid segments comprising the seed matches and flanking 3' and 5' sequences neighboring the seed matches collectively comprise the nucleotide sequence set forth in SEQ ID NO:2.

Another example of the disclosure relates to a method of treating a disease associated with abnormal expression of an endogenous gene or expression of a mutant protein encoded by an

endogenous gene in a subject, comprising genetically knocking down the endogenous gene in a cell of the subject, and administering the synthetic genes, vectors, or pharmaceutical compositions of the present disclosure, thereby treating the disease.

These and other examples of the disclosure are set forth in more detail in the description of the disclosure below.

## BRIEF DESCRIPTION OF THE DRAWINGS

**FIG. 1** shows a feedback loop depicting how MeCP2-driven miRNAs may attenuate toxic overexpression of exogenous MeCP2.

**FIG. 2** shows sample microarray data. The selected data quantifies miRNA expression levels in MeCP2-null versus wild-type (WT) tissue.

**FIG. 3** shows miRNAs with increased mean expression levels in saline-treated WT mice (compared to saline-treated KO mice). miRNAs with statistically significant increases in expression (in at least 1 of the 3 tissue types) are indicated (*). False positive hits that are also upregulated in response to AAV9/*EGFP* (in any tissue) are indicated (**). n=2 screening replicates per biological sample; n=3 mice per treatment group.

**FIG. 4** shows miRNAs with increased expression in either cervical cord (CC) or medulla in correlation with endogenous MeCP2 expression. Each data point is the average of 2 screening replicates for a single mouse. Groups that are significantly different from each other are boxed in red. n=3 mice per treatment group.

**FIG. 5** shows miRNAs with increased mean expression levels in AAV/*MECP2*-treated KO mice (compared to saline-treated KO mice). miRNAs with statistically significant increases in expression (in at least 1 of the 3 tissue types) are indicated (*). False positive hits that are also upregulated in response to AAV9/*EGFP* (in any tissue) are indicated (**). n=2 screening replicates per biological sample; n=3 mice per treatment group.

**FIG. 6** shows miRNAs with increased mean expression levels in AAV/*MECP2*-treated WT mice (compared to saline-treated WT mice). miRNAs with statistically significant increases in expression (in at least 1 of the 3 tissue types) are indicated (*). False positive hits that are also upregulated in response to AAV9/*EGFP* (in any tissue) are indicated (**). n=2 screening replicates per biological sample; n=3 mice per treatment group.

**FIG. 7** shows miRNAs with increased expression in correlation with exogenous MeCP2 expression in either WT or KO mice.

**FIG. 8** shows that insertion of mir-494-3p targets into the "V2" viral genome slightly (not significantly) decreases exogenous MeCP2 expression in MeCP2-EGFP(+) cells (vs. MeCP2-null cells). After miR-494-3p was identified as a positive hit in a 2016 pilot study, 3 tandem targets were inserted in the 3'UTR of the MeP426-hMECP2-myc-RDH1pA viral genome (targets for miR-132, miR-19, and miR-22 were removed). The modified viral genome was packaged into AAV9 (AAV9/V2-T1) and injected into mosaic MECP2EGFP-fusion/null mice. Transgene expression was slightly decreased in MeCP2-EGFP(+) cells, compared to that observed in the neighboring null cells.

**FIG. 9** shows miRNAs upregulated in correlation with aggregated *MECP2* expression. The *MECP2* (-) group shows data points for saline- and AAV9/*EGFP*-treated KO mice. The *MECP2*(+) group shows data points for saline-, AAV9/*MECP2*-, and AAV9/*EGFP*-treated WT mice as well as AAV9/*MECP2*-treated KO mice. Boxes indicate significant differences.

**FIG. 10 panels A-E** show how the RTT-specific panel reg1 tightly regulates total MeCP2 expression in WT Purkinje cells. Purkinje cells are located close to the intracisternal injection site and are vulnerable to supraphysiological transgene expression. The corrected total cell fluorescence (anti-MeCP2 signal) for each nucleus was normalized to that of the mean MeCP2 signal for myc(-) Purkinje nuclei. The means presented for each mouse in **Panel A** represent the normalized MeCP2 signal averaged across all myc(+) nuclei quantified for the specified host. Iterative averaging across cells within a Z-stack, then across Z-stacks within a single mouse, likewise yields a significant decrease in total MeCP2 expression. **Panels A** and **B** show that after treatment with the published control (AAV9/MeP426-

**What is claimed is:**

1. A synthetic gene comprising:
a polynucleotide comprising a coding region encoding MeCP2 and one or more regulatory regions,
the polynucleotide further comprising one or more nucleic acid segments each comprising a seed match identified as a binding site for an endogenous miRNA and a 5' flanking sequence and a 3' flanking sequence neighboring said seed match,
wherein said one or more nucleic acid segments are inserted into a regulatory region of said polynucleotide such that expression of MeCP2 when said synthetic gene is delivered to a cell expressing the endogenous miRNA is reduced relative to expression of a MeCP2 when a synthetic gene that does not comprise the one or more nucleic acid segments is delivered to a cell expressing the endogenous miRNA,

wherein the one or more nucleic acid segments comprising the seed matches and flanking 3' and 5' sequences neighboring the seed matches collectively comprise the nucleotide sequence set forth in SEQ ID NO:1.

2. A vector comprising the synthetic gene of claim 1.

3. The vector of claim 2, wherein the vector is a plasmid, a viral vector, an expression cassette, a transformed cell or a nanoparticle.

4. A pharmaceutical composition comprising the synthetic gene of claim 1 or the vector of claim 2 or 3 and a pharmaceutically acceptable carrier.

5. A polynucleotide target cassette for providing dose dependent inhibitory feedback to a synthetic gene, the synthetic gene comprising a polynucleotide comprising a coding region encoding MeCP2 and one or more regulatory regions, and the cassette comprising one or more nucleic acid segments comprising a seed match identified as a binding site for an endogenous miRNA and 5' and 3' flanking sequences neighboring said seed match, wherein the one or more nucleic acid segments comprising the seed matches and flanking 3' and 5' sequences neighboring the seed matches collectively comprise the nucleotide sequence set forth in SEQ ID NO:1.

6. The polynucleotide target cassette of claim 5, wherein the seed matches and 5’ and 3’ flanking sequences bind to the miRNAs miR-9-5p, miR-26-5p, miR-23-3p, miR-218-5p, miR-27-3p, and let-7-5p.

7. The polynucleotide target cassette of claim 5, wherein the seed matches and 5’ and 3’ flanking sequences bind to the miRNAs miR-690, miR-451a, and let-7-5p.

8. A method of preparing a synthetic gene comprising a polynucleotide comprising a coding region encoding a protein or nucleic acid of interest and one or more regulatory regions, comprising the step of inserting the polynucleotide target cassette of any one of claims 5-7 into a regulatory region of the synthetic gene.

9. A method of making the synthetic gene of claim 1, comprising the steps of:
inserting three or more nucleic acid segments each comprising a seed match identified as a binding site for an endogenous miRNA and 5’ and 3’ flanking sequences neighboring said seed match into a regulatory region of the polynucleotide comprising a coding region encoding MeCP2 and one or more regulatory regions,
wherein the seed matches and 5’ and 3’ flanking sequences bind to the miRNAs miR-9-5p, miR-26-5p, miR-23-3p, miR-218-5p, miR-27-3p, and let-7-5p.

10. The method of claim 8, wherein the seed matches and 5’ and 3’ flanking sequences bind to the miRNAs miR-9-5p, miR-26-5p miR-23-3p, miR-218-5p, miR-27-3p, and let-7-5p.

11. The method of claim 8, wherein the seed matches and 5’ and 3’ flanking sequences bind to the miRNAs miR-690, miR-451a, and let-7-5p.

12. The method of any one of claims 8-11, further comprising the steps of:
screening for miRNAs with increased expression when the protein or nucleic acid of interest is expressed in a cell relative to when the protein or nucleic acid of interest is not expressed;
identifying a seed match and 5’ and 3’ flanking sequences for three or more miRNAs having increased expression; and
preparing the nucleic acid segment comprising said seed match and 5’ and 3’ flanking sequences to be inserted into a regulatory region of said polynucleotide.

13. The method of any one of claims 9-12, wherein the seed matches and flanking 3’ and 5’ sequences neighboring the seed matches comprise the nucleotide sequence set forth in SEQ ID NO:1.

14. Use of the synthetic gene of claim 1, the vector of claim 2 or 3, or the pharmaceutical composition of claim 4 in the manufacture of a medicament for treating a disease associated with reduced expression of an endogenous *MECP2* gene or expression of a mutant protein encoded by an endogenous *MECP2* gene in a subject.

15. The use of claim 14, wherein the disease is Rett syndrome.

16. A synthetic gene comprising:
a polynucleotide comprising a coding region encoding MeCP2 and one or more regulatory regions,
the polynucleotide further comprising three or more nucleic acid segments each comprising a seed match identified as a binding site for an endogenous miRNA and a 5’ flanking sequence and a 3’ flanking sequence neighboring said seed match,
wherein said one or more nucleic acid segments are inserted into a regulatory region of said polynucleotide such that expression of MeCP2 when said synthetic gene is delivered to a cell expressing the endogenous miRNA is reduced relative to expression of a MeCP2 when a synthetic gene that does not comprise the one or more nucleic acid segments is delivered to a cell expressing the endogenous miRNA, wherein the one or more nucleic acid segments comprising the seed matches and flanking 3’ and 5’ sequences neighboring the seed matches collectively comprise the nucleotide sequence set forth in SEQ ID NO:2.

17. A vector comprising the synthetic gene of claim 16.

18. The vector of claim 17, wherein the vector is a plasmid, a viral vector, an expression cassette, a transformed cell or a nanoparticle.