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Citation for published version:
Sinnett, SE, Hector, RD, Gadalla, KKE, Heindel, C, Chen, D, Zaric, V, Bailey, MES, Cobb, SR & Gray, SJ 2017, 'Improved MECP2 gene therapy extends the survival of MeCP2-null mice without apparent toxicity after intracisternal delivery' Molecular Therapy — Methods & Clinical Development, vol 5, pp. 106-115. DOI: 10.1016/j.omtm.2017.04.006

Digital Object Identifier (DOI):
10.1016/j.omtm.2017.04.006

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Molecular Therapy — Methods & Clinical Development

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Improved MECP2 Gene Therapy Extends the Survival of MeCP2-Null Mice without Apparent Toxicity after Intracisternal Delivery

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Intravenous administration of adeno-associated virus serotype 9 (AAV9)/hMECP2 has been shown to extend the lifespan of MeCP2−/− mice, but this delivery route induces liver toxicity in wild-type (WT) mice. To reduce peripheral transgene expression, we explored the safety and efficacy of AAV9/hMECP2 injected into the cisterna magna (ICM). AAV9/hMECP2 (1 x 10^12 viral genomes [vg]; ICM) extended MeCP2−/− survival but aggravated hindlimb clamping and abnormal gait phenotypes. In WT mice, 1 x 10^12 vg of AAV9/hMECP2 induced clamping and abnormal gait. A lower dose mitigated these adverse phenotypes but failed to extend survival of MeCP2−/− mice. Thus, ICM delivery of this vector is impractical as a treatment for Rett syndrome (RTT). To improve the safety of MeCP2 gene therapy, the gene expression cassette was modified to include more endogenous regulatory elements believed to modulate MeCP2 expression in vivo. In MeCP2−/− mice, ICM injection of the modified vector extended lifespan and was well tolerated by the liver but did not rescue RTT behavioral phenotypes. In WT mice, these same doses of the modified vector had no adverse effects on survival or neurological phenotypes. In summary, we identified limitations of the original vector and demonstrated that an improved vector design extends MeCP2−/− survival, without apparent toxicity.

INTRODUCTION

Rett Syndrome (RTT) is an X-linked neurodevelopmental associated with severe motor abnormalities and reduced lifespan in a proportion of patients.1-3 RTT is caused by loss-of-function mutations in methyl CpG-binding protein 2 (MECP2),4 a transcription regulator that is highly expressed in neurons.5,6 Inactivating mutations in MeCP2 can alter the expression of many genes and ultimately disrupt neuronal morphology and circuitry.2,7-10 Adeno-associated virus serotype 9 (AAV9) has been used to transfer wild-type (WT) MECP2 to cells in vivo, extending survival and attenuating neurological deficits in mouse models of RTT.11,12 These models include hemizygous MeCP2−/− males as well as heterozygous MeCP2+−/− females that are mosaic for MeCP2 expression.11,12 Due to their limited survival (~9 to 10 weeks) and severe phenotypes, MeCP2−/− mice are often used for early-stage assessment of putative pharmacological and genetic treatments for RTT.11,12

One study, using a self-complementary (sc) AAV9/MeCP229- hMECP2-myc-BGHpA vector (henceforth termed the first-generation vector AAV9/hMECP2(v1)), extended the survival of MeCP2−/− mice injected intravenously (IV) between postnatal day 28 (PND28) and PND35.13 This IV gene therapy, however, resulted in high transgene expression in the liver as well as significantly elevated levels of alanine aminotransferase (ALT), an indicator of liver toxicity.14 After IV treatment with 1 x 10^11 viral genomes (vg) AAV9/hMECP2(v1), the level of total MeCP2 expression (measured as copies MeCP2 cDNA per β-actin cDNA) in WT liver was ~6x greater than that observed within the brain.14 This liver tropism and hepatic transgene expression observed after IV treatment can be attributed to the AAV9 capsid15 and the use of a small endogenous MeCP2 promoter fragment (MeCP229),11,14 respectively. We therefore concluded that AAV9/ hMECP2(v1) was a successful “proof-of-concept” vector whose design and administration route would need to be optimized before it could be considered for human translation.11

To bias transgene expression away from the liver, we decided to assess the safety and efficacy of AAV9/hMECP2(v1) after injection into the cerebrospinal fluid (CSF) of MeCP2−/− and WT mice. In the current study, we identify deleterious side effects (e.g., abnormal gait and hindlimb clamping) observed after cisterna magna (ICM) delivery of AAV9/hMECP2(v1). Importantly, these side effects were not observed after treatment with a control vector delivering EGFp. To address the limitations of the previously published vector, we designed a second-generation vector (AAV9/hMECP2(v2)) that, in
Mecp2−/− mice, increases weight (relative to that of saline-treated Mecp2−/− littersmates) and extends survival without aggravating behavioral phenotypes. In addition, the effective dose of AAV9/ hMECP2(v1) is well tolerated in WT mice after ICM administration.

RESULTS
IntraCSF Administration of AAV9/hMECP2(v1) Extends Survival of Mecp2−/− Mice
To bias transduction toward the central nervous system (CNS), we decided to shift from an IV to an intraCSF route of administration. Prior to conducting extensive safety and efficacy studies, we assessed neuronal tropism and transduction efficiency 3 to 4 weeks after treating Mecp2−/− mice with 1 × 1012 vg AAV9/hMECP2(v1) via the ICM (Figures 1A and 1B). Consistent with the reported CNS expression profile of the MeCP2 promoter,14 we observed predominantly neuronal expression of MeCP2-myc after ICM treatment of juvenile mice, with multiple brain regions showing a modest percentage (~20%-40%) of transduced anti-myc immunopositive cells (Figures 1A and 1B). To evaluate the efficacy of intraCSF MECP2 gene therapy in juvenile Mecp2−/− mice (PND28-35), we injected 1 × 1012 vg AAV9/hMECP2(v1) via the ICM route (a similar intraCSF database for lumbar intrathecal [IT] injections is provided in Figure S1). At this dose, AAV9/hMECP2(v1) extended the median survival of Mecp2−/− mice by 27 days or 47% (p = 0.01, Gehan-Breslow-Wilcoxon test) (Figure 1C).

In addition to reduced survival, Mecp2−/− mice exhibit a well-characterized array of neurological phenotypes (e.g., hindlimb clasping, abnormal gait, and abnormal breathing) and reduced bodyweight compared to their WT littersmates.11,13,15 Body weight and RTT-like phenotypes for Mecp2−/− mice were recorded prior to injection and weekly thereafter using an established observational aggregate phenotypic severity scoring system.15 At 7 to 8 weeks of age, Mecp2−/− mice treated with 1 × 1012 vg/mouse (ICM) had lower body weights than did saline-treated Mecp2−/− mice (Figure 1D). At this dose, RTT-like phenotype severity scores were significantly increased in ICM-treated
mice (p ≤ 0.05, starting at 6 weeks of age) (Figure 1E). In particular, two parameters (limb clamping and gait) were significantly affected at 8 weeks of age (versus pre-ICM clamping and gait scores at 4 weeks of age and versus scores for saline-treated mice at 8 weeks of age) (Figures 1F and 1G). Because most saline-treated Mcp2−/− mice did not survive beyond 9 weeks, the pairwise analyses in Figures 1F and 1G were limited to data obtained at 4 and 8 weeks of age. A central control AA9/hMCP2-Egfp (1 × 1012 vg/mouse, ICM) did not appear to hasten the development of hindlimb clamping in Mcp2−/− mice, indicating that the adverse limb/gait changes were likely caused by Mcp2−/− myofil overexpression (Figure 1H).

Peripheral Transgene Expression is Observed after IntraCSF Delivery of AA9/hMCP2(v1)

IV administration of AA9/hMCP2(v1) (1 × 1011 vg) has previously been shown to cause liver toxicity in WT mice.11 To determine if the transgene was expressed in the liver and other peripheral organs after ICM administration, we conducted biodistribution and gene expression analyses on samples from Mcp2−/− mice treated with 1 × 1012 vg of AA9/hMCP2(v1). Intracerebroventricularly delivered virus escaped to the periphery, with ~30 viral genome copies detected per liver cell (Figure 2A). This observation is consistent with published data demonstrating peripheral transgene biodistribution after intraCSF administration of AAV.13,27 In general, one or more viral genome copies were present per cell across multiple organs (Figure 2A). Gene expression was highest in the liver and brain (~1 Mcp2 cDNA copy per β-actin cDNA in Figure 2B; see Figure S2 for liver toxicity data). By delivering vector directly into the CSF, instead of systemically, we were able to diminish the levels of transgene expression in liver tissue relative to those observed in brain tissue (previously published systemic delivery yielded ~6× greater total expression in the liver than in the brain after IV treatment with 1 × 1011 vg13) (Figure 2B).

IntraCSF Administration of AA9/hMCP2(v1) Induces Deleterious Effects in WT Mice

To thoroughly evaluate the safety of intracisternally delivered AA9/hMCP2(v1), we also treated WT mice in parallel with treated Mcp2−/− mice. The virus-treated (1 × 1012 vg/mouse; ICM) WT cohort had a significantly shorter lifespan compared to that of saline-treated WT mice (p = 0.04) (Figure 3A). At this dose, a significant decrease in body weight was observed between 9 and 20 weeks of age for WT mice treated ICM (Figure 3B). Importantly, administration of AA9/hMCP2(v1) led to a significant and persistent increase (worsening) in aggregate phenotype scores (Figure 3C). In general, WT mice treated with 1 × 1012 vg AA9/hMCP2(v1) (ICM) demonstrated a significant increase in severity scores for hindlimb clamping and abnormal gait within 2 weeks after injection (Figures 3D and 3E; Movie S1). Similarly, WT mice treated intrathecally with 1 × 1012 vg AA9/hMCP2(v1) experienced weight loss and a significant increase in aggregate phenotype severity scores, with significant scores for limb clamping observed at 12 weeks of age (relative to pre-treatment scores) (Figure S1).

Effective Doses of a Second-Generation Vector Appear to Be Well-Tolerated in WT and Mcp2−/− Mice

The severe adverse effects observed in both Mcp2−/− and WT mice led us to conclude that the AA9/hMCP2(v1) vector is not a viable treatment option for RTT. We suspect that many of these symptoms may result from improper hMCP2-myc transgene regulation for two reasons. First, identical treatment with a control vector encoding EGFP (expressed under the truncated Mcp2 promoter Mcp29) does not cause these adverse effects (Figure 1H; Movie S1). Second, transgenic mouse models of loss or overexpression of Mcp2 have been shown to exhibit some overlap in phenotypic abnormalities, including kyphosis, clamping, and decreased locomotor activity.18-22 Thus, it would be strategic for a second-generation MCP2 gene therapy to incorporate additional endogenous control elements to potentially allow for better regulation of transgene expression in a transduced cell.

The Mcp29 core promoter fragment lacks a number of putative regulatory elements (REs) predicted to lie upstream in the Mcp2 promoter region.13,24 These REs may be important in cell-type-specific regulation of Mcp2 expression. The opportunity to engineer additional endogenous repressive elements into the gene expression cassette was particularly appealing in light of the broad peripheral vector distribution observed after ICM injection (Figure 2). Therefore, we designed a second-generation vector containing an extended promoter (MmMcp426), a modified 3’ UTR incorporating the highly conserved distal polyadenylation signal of Mcp2, and a panel of
microRNA (miRNA)-binding sites specific to miRNAs endogenous to, and known to interact with, the MECP2 3' UTR (Figures 4A and S3).26-30 Numerous other vector design variations to the hMECP2(v1) cassette have been carefully tested by our colleagues, leading to the development of the hMECP2(v2) cassette. These other vector designs are described in a companion paper by Gadalla et al.31 in this issue of Molecular Therapy – Methods & Clinical Development. For the remaining data described herein, we focus on intraCSF administration of AAV9/hMECP2(v1) and AAV9/hMECP2(v2). Because IT and ICM injections of AAV9/hMECP2(v1) yielded similar results in WT mice (Figures 3 and S1), we decided to streamline our methods and evaluated AAV9/hMECP2(v2) via ICM administration only.

After ICM administration of AAV9/hMECP2(v2), we observed a significant increase in MeCP2-/- median survival (p ≤ 0.01, Gehan-Breslow-Wilcoxon test; 42% or 40% increase in median lifespan for mice treated with 1 × 10^10 or 1 × 10^11 vg/mouse, respectively) (Figure 4B). Importantly, these doses were 10- to 100-fold less than the effective dose of the first-generation vector AAV9/hMECP2(v1) (Figure 1C).

Although 1 × 10^10 and 1 × 10^11 vg of AAV9/hMECP2(v2) were both effective at extending lifespan, dose-dependent effects on body weight (p ≤ 0.05, t test comparing saline- and virus-treated mice at each time point) (Figure 4C). Importantly, 1 × 10^12 vg did not affect the mean body weight of treated WT mice. Because WT cohorts treated with saline or 1 × 10^10 vg had nearly identical mean body weights, data for saline-treated mice (Figure 4C) were offset to improve clarity (see Figure 4 caption). WT animals treated with 1 × 10^11 to 1 × 10^12 vg experienced decreased body weight beginning 1 month post-injection (relative to the weight of saline-treated WT mice) (Figure 4C).

At 1 × 10^12 vg (ICM), AAV9/hMECP2(v2) significantly increased aggregate phenotype severity by increasing severity scores specifically for hindlimb clamping and abnormal gait in both MeCP2-/- and WT mice (Figures 4D and 5A). At 1 × 10^10 to 1 × 10^11 vg (ICM), AAV9/hMECP2(v2) had no effect on the mean aggregate scores of WT and MeCP2-/- mice (Figure 4D). This lack of phenotypic rescue in MeCP2-/- mice was surprising in light of previously published data showing that neonatal intracranial injections of AAV9/CBA-hMECP2-myc-5V40Pa could ameliorate the onset of Rett-like abnormalities, including motor dysfunction.11 These results may be due to a number of factors (modest transduction efficiency, age of injection, etc.). To better sort out potential treatment effects in more detail, we conducted additional experiments in randomized, paired...
littermates to eliminate cross-litter variability. Historically, we have observed variability in the phenotypic severity of MeCP2−/− mice from different litters as well as variability in the age at which severe symptoms appear. Thus, we were concerned that noise in the mean behavior scores could obscure meaningful phenotypic differences within pairs of saline- and virus-treated littermates. We therefore decided to run pairwise analyses of saline- and virus-treated MeCP2−/− littermates (Figure S5). In short, AAV9/hMECP2v2 (1 × 10^10 to 1 × 10^11 vg) significantly increased the peak body weight and growth rate (post-treatment) of MeCP2−/− mice relative to that of their saline-treated MeCP2−/− siblings (Figure S5; p = 0.0003 and 0.02, respectively). In addition, the overall health of virus-treated MeCP2−/− mice appeared to decline more gradually (Figure S5; n = 8 pairs, p = 0.03). Finally, early observations on treated mice suggested some possible benefits that may not be captured using a standardized RTT scoring system. To document this qualitatively, we video-recorded pairs of saline- and virus-treated littermates weekly. Videos for these littermate pairs are provided in the Supplemental Information (Movies S2, S3, and S4). A striking feature of these videos is the difference in spontaneous movement between saline- and virus-treated mice. Unlike their saline-treated littermates, AAV9/hMECP2v2-treated mice often walked, climbed, explored their surroundings, and/or approached the camera without prompting. Ultimately, however, all treated mice eventually experienced a decline in overall health and succumbed.

To investigate liver function after ICM administration (1 × 10^12 vg AAV9/hMECP2v2), we quantified levels of albumin, ALT, aspartate aminotransferase (AST), and alkaline phosphatase (ALKP) in the blood serum of treated MeCP2−/− animals (Figure 5). Levels of each marker were indistinguishable between saline- and virus-treated cohorts (p = 0.8, 0.4, 0.4, and 0.9, respectively; Figure 5B). Bio-distribution and gene expression data from treated MeCP2−/− mice showed that virus escaped to the periphery after ICM injection (~33 viral genome copies per liver cell; ~0.07 MeCP2 cDNA copies per β-actin cDNA; Figures 5B and 5C).

Hypothetically, the divergent safety and efficacy profiles of intracranially administered AAV9/hMECP2v1 and AAV9/hMECP2v2 could potentially be due to differences in how these two viral genomes regulate transgene expression in the brain, liver, and/or other organs. After all viral genomes were found throughout the entire body 3 to 4 weeks after ICM injection (Figures 2, 5, and S2). In MeCP2−/− mice, AAV9/hMECP2v2 generated fewer cDNA copies per viral genome in the heart, liver, and spinal cord (Figure 6). Interestingly, a 10-fold dose increase (from 1 × 10^11 vg to 1 × 10^12 vg) for AAV9/hMECP2v2 resulted in decreased normalized transgene expression in the heart and liver, suggesting the presence of a dose-dependent inhibitory feedback mechanism in these tissues (1E11 vg AAV9/hMECP2v2/ mouse: **p ≤ 0.004 for heart, liver, and lumbar spinal cord; *p = 0.04 for cervical spinal cord; 1E12 vg AAV9/hMECP2v2/ mouse: **p = 0.001 for liver; *p ≤ 0.05 for heart, cervical spinal cord, and thoracic spinal cord) (Figure 6). Immunofluorescence analyses also showed that transgene expression is more tightly regulated by AAV9/hMECP2v2 (versus that of AAV9/hMECP2v1))

![Figure 4. IntraCSF Delivery of AAV9/hMECP2v2](image-url)
in WT liver tissue after ICM administration (1 × 10^{11} vg/mouse; Figure 7A). Interestingly, ICM administration of AAV9/hMECP2 (v2) (1 × 10^{11} vg/mouse) resulted in more anti-myc immunopositive hepatic cells in Mecp2⁻/⁻ mice than in WT mice (1.2% versus 0.2%, respectively; p = 0.04; Figures 7A–7C). Mecp2⁻/⁻ mice treated with 1 × 10^{11} AAV9/hMECP2(v1) reached their natural endpoints before these immunofluorescent analyses were conducted. Figure 6, however, shows that the ratio of transcript to viral genome is more tightly regulated by AAV9/hMECP2(v2) (versus that of AAV9/ hMECP2(v1)) in Mecp2⁻/⁻ liver tissue. Finally, our examination of brain tissue provided fewer clues explaining the improved safety of AAV9/hMECP2(v2). On a WT background, 1 × 10^{11} vg of either AAV9/hMECP2(v1) or AAV9/hMECP2(v2) resulted in low apparent transduction efficiencies, with predominantly neuronal tropism (Figures 7D–7G).

**DISCUSSION**

IV administration of AAV9/hMECP2(v1) has been previously shown to extend the lifespan of Mecp2⁻/⁻ mice, but overexpression of MeCP2 in the liver induced liver toxicity.¹¹ We have now shown that ICM administration of AAV9/hMECP2(v1) extends the lifespan of Mecp2⁻/⁻ mice, with a more favorable biodistribution toward CNS transduction compared to IV. Importantly, however, intracisternal administration of 1 × 10^{12} vg AAV9/hMECP2(v1) still resulted in potentially problematic peripheral transgene expression (Figure 2), a trend toward elevated blood serum toxicity indicators (Figure S2), and deleterious neurological side effects (Figures 1 and 3), which were not seen in mice treated with an AAV9/EGFP control vector. Increased severity of hindlimb clasp and abnormal gait were unexpected, given the absence of these side effects in previously published MeCP2 gene transfer studies.¹²,¹³,¹⁴ In light of these data, our conclusion is that AAV9/hMECP2(v1) cannot move forward in translational studies. Thus, we developed a second-generation MeCP2 viral genome that included additional putative regulatory elements from the endogenous MECP2 promoter and 3’ UTR. By modifying the viral genome, we were able to extend Mecp2⁻/⁻ survival with a 10- to 100-fold lower dose compared to that used for the AAV9/hMECP2(v1) vector (Figures 1 and 4). This dose range is especially beneficial for a gene therapy because it increases the likelihood of administering a safe, effective dose in vivo. This low dose (1 × 10^{10} to 1 × 10^{11} vg injected ICM) is well tolerated by the liver and does not increase severity scores for limb clasp and abnormal gait in treated Mecp2⁻/⁻ or WT mice (Figures 5 and S4). Our second-generation vector significantly improved peak body weight and acute growth rate (post-treatment) in Mecp2⁻/⁻ mice (Figure S5). The mechanism underlying the increased body weight is unclear. In addition, our second-generation vector significantly attenuated the deterioration in overall health of MeCP2-null mice (Figure S5). Our overall conclusion is that the AAV9/hMECP2(v2) design provides a substantial improvement over the original design we previously published,¹¹ conferring some benefit to Mecp2⁻/⁻ mice over a
10-fold dynamic range (1 × 10^10 to 1 × 10^11 vg injected ICM), which is also well-tolerated.

The mechanism behind the improved therapeutic index of AAV9/hMECP2(v2) versus AAV9/hMECP2(v1) is unclear, although our data points toward it providing generally more regulated expression. We currently know that the modified viral genome reduces transgene expression (as detected by immunofluorescence) in WT liver tissue at the dose (1 × 10^11 vg) and route in our study (Figure 7). In addition, in Mecp2−/− mice, AAV9/hMECP2(v2) appears to regulate transgene expression more tightly (than that of AAV9/hMECP2(v1)), with fewer cDNA copies generated per viral genome in the heart, liver, and spinal cord (AAV9/hMECP2(v1) compared against AAV9/hMECP2(v2)) (Figure 6). Indeed, when we evaluated the liver safety profile of 1 × 10^12 vg AAV9/hMECP2(v2), we observed no difference in the mean levels of liver toxicity markers (versus those of saline-treated mice; Figure 5). Tighter regulation of transgene expression is especially important in light of the broad peripheral biodistribution observed for both vectors after ICM administration (Figures 2 and 5). This tighter regulation is consistent with the inclusion of additional silencing elements in the second-generation vector. However, the exact mechanism (such as a specific miRNA target) underlying the tighter transgene regulation for AAV9/hMECP2(v2) remains to be determined.

Importantly, neither AAV9/hMECP2(v1) nor AAV9/hMECP2(v2) were able to completely and permanently reverse the symptoms of RIT in mice. Furthermore, at higher doses (1 × 10^12 vg injected ICM), the AAV9/hMECP2(v2) vector induced deleterious behavioral effects in WT mice, indicating an upper tolerated dose between 1 × 10^11 and 1 × 10^12 vg in mice by this route. Thus, third-generation vectors should be developed with a goal of expanding this upper tolerated dosing threshold and/or increasing the therapeutic effect at lower doses. Given the dosing limits determined in mice and the known side effects associated with overdosing, dose-ranging studies in large animals that are carefully monitored for side effects would be warranted before human translation should be considered. In closing, this study suggests that the inclusion of regulatory elements that help control levels of transgene expression in the CNS and periphery may prove to be critical for intraCSF delivery of MeCP2 gene therapy.

MATERIALS AND METHODS

Vectors

The complete names for hMECP2(v1) and hMECP2(v2) are self-complementary MeP229-human MeCP2-myc-BHpa and self-complementary MeP426-human MeCP2-myc-RDH1pA, respectively. Both gene expression cassettes encode the c1 isofrom of MeCP2, which is composed of exons 1, 3, and 4. The MeP426 promoter includes additional putative regulatory elements that are absent from the shorter truncated promoter MeP229. RDH1pA is a synthetic 3' UTR containing 110 bp of the highly conserved MeCP2 distal polyadenylation signal and an upstream miRNA-binding panel containing sites for three additional miRNAs endogenous to the MeCP2 3' UTR: miR-19, miR-22, and miR-132. The sequences for MeP426 and RDH1pA are provided in Figure S3. The

Figure 7. The hMECP2(v2) Viral Genome Tightly Regulates Transgene Expression in WT Liver Tissue after ICM Administration (A) In WT mice, the hMECP2(v2) viral genome tightly regulates transgene expression in the liver. Arrows point to myc+ cells. (B and C) hMECP2(v2) drives transgene expression in MeCP2 null liver tissue. (C) Percentage of myc+ liver cells (versus at DAPI-liver cells), n = 3-5 mice and 9-15 imaged sections per group. (D-G) AAV9/ hMECP2(v1) and AAV9/hMECP2(v2) yield similar transduction efficiencies and neuronal transgene in treated WT mice. (A-C) A single dose (1 × 10^11 vg/mouse) was evaluated for AAV9/hMECP2(v1) and AAV9/hMECP2(v2). (D-G) Data points are mean ± SEM.
self-complementary AAV9/Mcp225-GFP-SV40/pA vector has been previously described.15

Animals
Animal studies at University of North Carolina (UNC) Chapel Hill were conducted according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC). Mice were weaned at PND28 and were provided food and water ad libitum. Both WT and Mcp2<sup>2/2</sup> mice were weaned at PND28 so that Mcp2<sup>2/2</sup> mice could achieve a viable weight (~10 g) before weaning. Euthanasia criteria have been previously described.15

Virus Production
Vectors were produced by triple transfection, iodixanol gradient centrifugation, and ion exchange chromatography, as described for the UNC Vector Core.16 Purified vectors were dialyzed in PBS (350 mM final NaCl concentration) containing 5% D-sorbitol and stored at ~80°C until use. Thawed aliquots were subsequently stored at 4°C. A filter-sterilized solution of PBS (350 mM final NaCl concentration) containing 5% D-sorbitol was used as vehicle and virus dilution buffer.

Injections
Mice were randomized into treatment groups and injected (ICM or IT, vector or vehicle treated) between 28 and 35 days of age. For littermate comparisons, vehicle and virus treatments were randomly assigned without any prior knowledge regarding each sibling’s body weight or general physical condition. Percutaneous IT injections were performed on non-anesthetized mice as described.16 Mice assigned to ICM cohorts were injected intraperitoneally with a sterile-filtered solution of 1.25% Avertin (also known as tribromoethanol; 0.02 mL/g body weight) dissolved in 1x PBS prior to inhalational isoflurane anesthesia. Mice remained on inhalational anesthesia for the duration of the surgery, which was completed using a sterile technique. An incision was made in the scalp, exposing the skull and neck muscles. A 50-μL Hamilton syringe was used to deliver a 10-μL bolus injection into the cisterna magna. Venous tissue adhesive (3M) was then used to close the incision. Injected mice were returned to a recovery cage, where they remained until they were ambulatory. Injected mice had access to acetaminophen (~200 mg/kg body weight) in their drinking water (~100 mL) for the next 48 hr.

Behavior Scoring
Behavior scoring was completed by an observer blinded to treatment according to previously published guidelines.16 In short, mobility, gait, hindlimb clamping, breathing, tremor, and general appearance were each assessed on a scale of 0–2, with 12 being the maximum aggregate score attainable. Videos of mice provided as supplemental data were taken by an observer that was not blinded to treatment.

Immunofluorescent Analyses
Mice were perfused 3 to 4 weeks post-injection with 1x PBS, followed by filtered 4% paraformaldehyde (PFA) in 1 x PBS, 2 mM NaOH, pH 7.4. Brains were then fixed for an additional 48 hr at 4°C. A vibrating microtome (Leica VT1000S) was used to prepare 40-μM sections from liver and brain tissue. Sections were washed in 50% ethanol, followed by three washes in 0.3 M PBS with 0.3% Triton X-100. Sections were then transferred to 10 mM sodium citrate (pH 6, 85°C, 30 min) for antigen retrieval, and then washed (three times) prior to blocking for 1 hr at room temperature in 5% goat serum (in 0.3 M PBS, 0.3% Triton X-100). Tissue sections were then incubated with primary antibodies at 4°C for 48 hr. Primary antibody mixtures were chicken anti-myc antibody (Novus; 1:500) mixed with either rabbit anti-mouse McCP2 (Cell Signaling; 1:500) or rabbit anti-mouse NeuN (Millipore; 1:500) in 5% goat serum. Sections were then washed three times and incubated with secondary antibodies for at least 4 hr at room temperature (goat anti-chicken Alexa Fluor 488 [1:1,000]; goat anti-rabbit Alexa Fluor 594 [1:1,000] in 0% goat serum). After immunolabeling, sections were incubated with DAPI (12,000, v/v) in 0.3 M PBS and 0.3% Triton X-100 for 1 hr at room temperature. Sections were washed 3x prior to mounting with ProLong Gold Antifade Mountant with DAPI. Neuronal tropism (or % NeuN<sup>+</sup> cells) = 100 x (NeuN<sup>+</sup> cells / all myc<sup>+</sup> cells). Transduction efficiencies were calculated as the percentage of total nuclei that are also myc+. Apparent transduction efficiencies (or % myc<sup>+</sup> cells) = 100 x (myc<sup>+</sup> cells / all DAPI<sup>+</sup> cells). Cells within an 80x field of view were counted, with multiple brain sections imaged per mouse.

Microscopy
All immunolabeled sections were imaged with a Zeiss 710 confocal microscope at the UNC Confocal and Multiphoton Imaging Facility.

Biodistribution and Gene Expression
Biodistribution of viral genomes and gene expression analyses (using mRNA as a source target) for saline- and virus-treated Mcp2<sup>2/2</sup> mice were performed according to previously published methods16 using primers specific for human McCP2. Primer sequences were published by Gadalla et al.12 Tissue was harvested 3 to 4 weeks post-ICM. To assess how tightly transgene expression was regulated, we calculated the following ratio per organ per mouse: (HMcCP2<sub>2</sub> DNA copies per β – actin DNA/viral genome copies per host DN)A. We then calculated the mean ratio across four to five mice per cohort.

Blood Serum Collection
Blood serum was collected 3 to 4 weeks post-ICM according to previously published methods16. Serum testing of blinded samples was completed by the Animal Clinical Chemistry Core at UNC.

Statistical Analyses
Graphpad Prism was used to generate Kaplan-Meier survival plots and calculate p values for survival cohorts. Unpaired t tests or paired t tests were performed as appropriate for all other statistical calculations.

SUPPLEMENTAL INFORMATION
Supplemental Information includes five figures and four movies and can be found with this article online at http://dx.doi.org/10.1016/j.omtm.2017.04.006.
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AUTHOR CONTRIBUTIONS
S.S.E. injected mice, analyzed data, conducted immunofluorescence analyses, and prepared the manuscript text and figures. K.K.E.G. optimized the immunofluorescence protocol, consulted on the project, and provided feedback on the manuscript. R.D.H. designed AAV9/hMECP2(v2). C.H. scored behavior blind to genotype and treatment. D.C. processed tissue samples for biodistribution and gene expression analysis. V.Z. validated primer design and PCR conditions for biodistribution and gene expression analyses and carried out those analyses. S.R.C., M.E.S.E., and S.J.G. led the collaboration, provided guidance on experimental designs, analyzed data, and helped with manuscript preparation.

CONFLICTS OF INTEREST
S.J.G. declares a conflict of interest with Asklepios Biopharma, from which he has received patent royalties for IP that are not used in this study.

ACKNOWLEDGMENTS
This work was funded by NIH grant 4T12HD040127-15 (to S.S.E.) and a grant from the Retin Syndrome Research Trust (to S.J.G., S.R.C., and M.E.S.E.). Indirect administrative support for S.J.G. was provided by Research to Prevent Blindness to the UNC-CH Department of Ophthalmology. The authors thank the UNC Animal Histopathology and Laboratory Medicine Core Facility (which is supported in part by an NCI Center Core Support Grant 2P30CA016086-40 to the UNC Lineberger Comprehensive Cancer Center) and the UNC Confocal and Multiphoton Imaging Facility (supported by NINDS Center grant P30 NS05892 for assistance with blood serum testing and imaging, respectively). We thank Alexandra Rozenberg and Mary Kate Crawford for technical assistance with IT injections and colony maintenance, respectively. We thank Bethany Wagner and Emma Hoffman for proofreading this manuscript. We are grateful for constructive discussions of the project direction and results with Dr. Brian Kaspar ( Nationwide Children’s Hospital) and Dr. Gail Mandel ( Oregon Health and Science University) as part of a consortium focused on developing gene therapy for Retin syndrome.

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