A translatable RNAi-driven gene therapy silences \textit{PMP22/Pmp22} genes and improves neuropathy in CMT1A mice

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Charcot-Marie-Tooth disease type 1A (CMT1A), the most common inherited demyelinating peripheral neuropathy, is caused by \textit{PMP22} gene duplication. Overexpression of WT PMP22 in Schwann cells destabilizes the myelin sheath, leading to demyelination and ultimately to secondary axonal loss and disability. No treatments currently exist that modify the disease course. The most direct route to CMT1A therapy will involve reducing PMP22 to normal levels. To accomplish this, we developed a gene therapy strategy to reduce \textit{PMP22} using artificial miRNAs targeting human \textit{PMP22} and mouse \textit{Pmp22} mRNAs. Our lead therapeutic miRNA, miR871, was packaged into an adeno-associated virus 9 (AAV9) vector and delivered by lumbar intrathecal injection into C61-het mice, a model of CMT1A. AAV9-miR871 efficiently transduced Schwann cells in C61-het peripheral nerves and reduced human and mouse PMP22 mRNA and protein levels. Treatment at early and late stages of the disease significantly improved multiple functional outcome measures and nerve conduction velocities. Furthermore, myelin pathology in lumbar roots and femoral motor nerves was ameliorated. The treated mice also showed reductions in circulating biomarkers of CMT1A. Taken together, our data demonstrate that AAV9-miR871–driven silencing of PMP22 rescues a CMT1A model and provides proof of principle for treating CMT1A using a translatable gene therapy approach.
Charcot-Marie-Tooth disease type 1A (CMT1A), the most common inherited demyelinating peripheral neuropathy, is caused by PMP22 gene duplication. Overexpression of WT PMP22 in Schwann cells destabilizes the myelin sheath, leading to demyelination and ultimately to secondary axonal loss and disability. No treatments currently exist that modify the disease course. The most direct route to CMT1A therapy will involve reducing PMP22 to normal levels. To accomplish this, we developed a gene therapy strategy to reduce PMP22 using artificial miRNAs targeting human PMP22 and mouse Pmp22 mRNAs. Our lead therapeutic miRNA, miR871, was packaged into an adeno-associated virus 9 (AAV9) vector and delivered by lumbar intrathecal injection into C61-het mice, a model of CMT1A. AAV9-miR871 efficiently transduced Schwann cells in C61-het peripheral nerves and reduced human and mouse PMP22 mRNA and protein levels. Treatment at early and late stages of the disease significantly improved multiple functional outcome measures and nerve conduction velocities. Furthermore, myelin pathology in lumbar roots and femoral motor nerves was ameliorated. The treated mice also showed reductions in circulating biomarkers of CMT1A. Taken together, our data demonstrate that AAV9-miR871-driven silencing of PMP22 rescues a CMT1A model and provides proof of principle for treating CMT1A using a translatable gene therapy approach.
normally involved in early steps of myelin formation and in the maintenance of myelin and axons in the PNS (12–15). In humans, PMP22 mRNA and PMP22 protein overexpression in nerve biopsies form patients with CMT1A indicates that increased PMP22 dosage is the most likely disease mechanism underlying CMT1A (16–19). This hypothesis is further supported by a recapitulation of numerous CMT1A-associated phenotypes in PMP22-overexpressing rodent models (20–30), including C61-het mice (22), which contain 4 copies of WT human PMP22 on a normal mouse background. The exact mechanisms by which PMP22 overexpression causes CMT1A remain unclear but may involve proteasome dysfunction related to excessive amounts of PMP22 protein. Specifically, in normal myelinating and nonmyelinating SCs, approximately 20% of newly synthesized PMP22 is glycosylated, while the remaining approximately 80% is targeted for proteasomal endoplasmic reticulum–associated degradation (ERAD) (31). Thus, in CMT1A, overexpressed PMP22 is thought to accumulate in perinuclear aggresomes (32, 33) and impair overall proteasome activity (34), resulting in myelin sheath destabilization in SCs and ultimately nerve dysfunction.

Based on this model, the most direct approach to CMT1A therapy will likely involve reducing overexpressed PMP22 to normal levels in SCs. Prior attempts to accomplish this using drug-based approaches were unsuccessful in human clinical trials (NCT00484510, NCT02600286, NCT05092841, NCT04762758, NCT03023540), and to date, CMT1A remains intractable. Nevertheless, progress continues in the field, with prospective adjunct therapies approaching clinical trials (35–37) and several preclinical strategies to silence PMP22 reported (38–61). For example, CRISPR/Cas9 was used to directly target the PMP22 gene by deleting its regulatory regions with encouraging results in vitro (62) and in vivo (63), and oligonucleotides have been tested to inhibit PMP22 via promoter disruption or through mRNA degradation using RNAase H or RNAi-based mechanisms (using DNA gapmers or siRNAs, respectively) in different CMT1A rodent models (64–67). Among these various silencing approaches, RNAi has so far been used most often as a prospective mechanism to develop a CMT1A therapy (68).

RNAi is a conserved process of gene silencing triggered by endogenous miRNAs, which are encoded in the genomes of eukaryotic organisms. Mature forms of natural miRNAs are small (approximately 22 nucleotides long), noncoding RNA molecules that negatively regulate the expression of a vast fraction of the transcriptome at the posttranscriptional level (69, 70). Importantly, natural miRNAs can be modified in the form of siRNAs, shRNAs, or artificial miRNAs and retargeted to specifically base pair with disease genes, triggering target mRNA degradation through the RNA-induced silencing complex (RISC). siRNAs are chemically synthesized, produce transient effects, and require repeated, lifelong administration to achieve long-term gene silencing. In contrast, shRNAs or artificial miRNAs can be cloned as DNA expression cassettes, delivered to target cells within viral vectors, and transcribed in vivo to produce long-term target gene silencing after 1 administration. As mentioned, siRNAs have been used to trigger RNAi against PMP22 in at least 3 published studies (65, 67), but RNAi treatment of CMT1A in vivo and in vitro models was also achieved using gene therapy vectors expressing natural miRNAs (mir-29a; ref. 71 or mir-318; ref. 72) or by an intraneurally injected AAV2/9 vector expressing rodent Pmp22-targeting artificial shRNAs (73). These shRNAs contained mismatches with the human PMP22 sequence and were not tested in models expressing human PMP22, so it is unclear if these sequences, as well as the invasive and laborious intraneural injection method, can be translated to humans (73).

Here, we designed and tested a translatable AAV9-based gene therapy approach for CMT1A using an artificial miRNA targeting conserved regions on the human PMP22 and mouse Pmp22 transcripts. We demonstrate long-lasting therapeutic effects following a single, clinically relevant lumbar intrathecal injection in a mouse model of CMT1A that expressed both human PMP22 and mouse Pmp22 gene products. Thus, our study provides proof of principle for treating CMT1A with a gene therapy approach that uses artificial miRNA sequences and a route of administration that can be translated to human trials.

Results

Design and in vitro validation of artificial miRNAs to downregulate human and murine PMP22. Full-length human PMP22 and mouse Pmp22 are encoded by 5 exons, with 2 alternatively spliced first exons containing 5′-UTR sequences (ex1a and ex1b). Both variants encode identical 483 bp ORFs and share the same 3′-UTR, which is located in exon 5 (ex5) (ORFs) (Figure 1A). To ensure that we targeted all PMP22 transcripts, we excluded exon 1 from the query sequence and designed artificial miRNAs targeting human PMP22 exons 2 to 5 (1655 nucleotides) using a previously described algorithm (74). This screen identified 117 candidates. Because we intended to use the RNA polymerase III–dependent (pol III–dependent) U6 promoter to drive miRNA expression, we excluded 29 of the 117 candidates because of the presence of RNA pol III termination sequences (5–6 T’s) within the miRNA expression cassettes. The remaining 88 sequences were additionally filtered to ensure that the antisense guide strand of the miRPMP22 miRNAs would equally target human PMP22 and mouse Pmp22 sequences. Only 8 sequences (9%) showed this conservation, and all were located in exon 5, which encodes the 3′-UTR (Figure 1A and Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI159814DS1). Following cloning into a U6T6 expression plasmid, we empirically tested all 8 miRPMP22 miRNAs (mirR68, mirR69, mirR871, mirR872, mirR1706, mirR1740, mirR1741, and mirR1834) for silencing efficacy (Figure 1, B and C). Specifically, we cotransfected HEK293 cells with each individual U6-miRPMP22 plasmid and CMV-driven PMP22 or Pmp22 full-length cDNAs, and then harvested RNA 24 hours later, generated cDNA, and performed real-time quantitative PCR (RT-qPCR) using for PMP22 or Pmp22, normalized to RPL13A. Negative controls included cells transfected with PMP22 or Pmp22 and U6.mirGFP (miRNA targeting EGFP) or an empty U6T6 plasmid (no miR). Data were collected and averaged from 3 independent experiments, with each RT-qPCR assay performed in triplicate. Although 7 of 8 mirRPMP22s (87.5%) showed some level of silencing compared with the “no miR” control, only mirR868 and mirR871 showed...
miPMP22 design and selection criteria:
1) Mature guide strand with 22 nt base pairing with human PMP22 mRNA (1,655 nt query, exons 2-5)
2) Selection based on percentage and distribution of GC content (117 candidates)
3) Absence of RNA pol III termination signal in miRNA sequence (88 of 117)
4) PMP22 target site conservation in human and mouse (8 of 88)
5) In vitro gene silencing efficacy targeting mouse and human PMP22 sequences

miR-PMP22-871 (miR871)

miR871 binding sites
Human
Mouse

miR871 guide strand

miR871 binding site, human
GGGGGUCCUGUGUUGUGUAGAG

miR871 binding site, mouse
GGGGGUCCUGUGUUGUGUAGAG

miR871 guide strand

Figure 1. Design and in vitro screen of artificial miRNAs targeting PMP22. (A) Full-length PMP22 mRNA was transcribed from 5 exons (ex), producing 2 major transcripts with identical ORFs (black shading). We designed 8 candidate miRNAs to equally target both human PMP22 and murine Pmp22 mRNAs. (B) Gray and black arrowheads show miR871 cut sites by Drosha and Dicer, respectively. dsRNAs form G:U wobble base pairs (indicated by gray shading). Underlined sequence represents the mature miR871 antisense guide strand. Bottom panel shows alignment of the miR871 binding site on human PMP22 and murine Pmp22 mRNAs. Gray asterisk indicates a G:A mismatch at the miR871 binding site, but each nucleotide at this location can form 2 hydrogen bonds with the miR871 guide strand as a G:U wobble (human) or A:U wobble (mouse). (C) RT-qPCR was performed to measure in vitro human PMP22 or murine Pmp22 silencing by the indicated miRPMP22s (n = 3/group). Gene expression was normalized to human RPL13A. *P < 0.05, by unpaired, 1-tailed Student’s t test. Values represent the mean ± SEM. Rel, relative. (D) Schematic of scAAV9, which was used to deliver miR871 or miRLacZ expression cassettes in vivo. The U6 promoter drives the transcription of miR871 or miRLacZ, and the CMV promoter drives the EGFP gene with the SV40 polyadenylation sequence.

statistically significant (P < 0.05) silencing of PMP22 and Pmp22 sequences. Because miR871 consistently silenced both genes by approximately 60%, we chose the miR871 sequence as our lead. The U6-miR871 sequence was then subcloned into a self-complementary adeno-associated virus (AAV) (scAAV. CMV.EGFP) backbone containing a separate CMV.EGFP reporter gene (Figure 1D), and we generated AAV9 particles using triple transfection in HEK293 cells (hereafter referred to as AAV9-miR871). Lysates were purified by iodixanol-gradient ultracentrifugation and fast protein liquid chromatography (FPLC), as previously described (75). Similarly, we generated a control scAAV9.CMV.EGFP vector expressing a U6
SCs in immunostained tissue sections reached an average of 54.78% ± 4.53% in anterior lumbar roots, 44.07% ± 2.96% in sciatic nerves, and 40.18% ± 4.93% in femoral nerves (n = 4 mice; Figure 2B). VGCNs in DNA extracted from PNS tissues reached 2.44 in anterior lumbar roots, 1.23 in sciatic nerves, and 0.69 in femoral nerves (n = 4 mice; Figure 2C).

In vivo validation of AAV9-miR871–mediated silencing of the PMP22 gene in CMT1A mice. Prior to any treatment studies, we performed a detailed characterization of baseline functional and morphological deficits of the C61-het CMT1A mouse line, which contains 4 copies of the human PMP22 gene and 2 normal copies of murine Pmp22, compared with WT mice at 2, 4, 6, 8, and 10 months of age. We confirmed progressive functional impairment associated with early-onset demyelination (Supplemental Figures 2–7). We also assessed the potential toxicity...
of the AAV9-miRLacZ vector after injection into 2-months-old CMT1A mice that were examined 6 weeks (3.5 months of age) or 4 months (6 months of age) later. AAV9-miRLacZ caused no significant increase in the numbers of immunofluorescent cells in spinal roots, sciatic nerves, or dorsal root ganglia (DRGs) beyond the baseline (Supplemental Figures 8, 9, and 11). However, injection of AAV9-miRLacZ increased the number of CD20+ and CD3+ cells in CMT1A mouse livers 6 weeks after injection (3.5 months of age), but this reaction subsided by the 4-month post-injection time point (6 months of age; Supplemental Figure 10). Interestingly, inflammatory infiltrates increased with age in the PNS of noninjected CMT1A mice (Supplemental Figures 8 and 9).

After we confirmed sufficient biodistribution, transduction of PNS tissues, and safety, we evaluated the efficacy of AAV9-miR871 in silencing PMP22/Pmp22 gene expression and reducing overall human and mouse PMP22 protein levels, compared with the expression of other myelin-related genes and proteins. We injected AAV9-miR871, which targets both the PMP22 and Pmp22 transcripts, or the AAV9-miRlacZ negative control, which expresses a functional but nontargeting miRNA, into adult CMT1A mice and then analyzed gene expression by RT-qPCR and Western blotting 6 weeks after injection. At the mRNA level, early treatment with AAV9-miR871 in CMT1A mice reduced human and murine PMP22 levels in spinal roots (43% human PMP22; 45% murine PMP22), sciatic nerves (51% human PMP22; 74% murine PMP22), and femoral nerves (87% human PMP22; 38% murine PMP22) (Figure 3, D–I). In contrast, we found that murine MPZ protein levels were increased in roots (63%) and femoral nerves (102%), reflecting improved myelination, whereas they remained unchanged in sciatic nerves (Figure 3, D–I).

We assessed motor performance in all groups before injection and until the end of the observation period by rotarod (5 and 17.5 rpm), grip, and hang tests (Figure 3, J–M, and Supplemental Figure 13). Time-course analysis of the above tests showed that AAV9-miR871 treatment improved the motor performance of CMT1A mice, reaching WT levels, whereas AAV9-miRLacZ-treated CMT1A mice performed similarly to noninjected CMT1A mice and significantly worse than WT mice (Figure 3, J–M, and Supplemental Figure 13). Moreover, early treatment with AAV9-miR871 completely rescued the hind limb clasping phenotype of CMT1A mice (Figure 3N and Supplemental Figure 14).

Electrophysiological examination in 6-month-old mice (4 months after vector injection) (Figure 3, O and P) showed a significantly improved MNCV score for AAV9-miR871-treated CMT1A mice (36.87 ± 5.60 m/s) compared with that of the AAV9-miRLacZ-treated group (25.89 ± 1.99 m/s), approaching WT values at the same age (41.61 ± 5.06 m/s). Although the amplitude of the compound muscle action potential (CMAP) was also significantly improved in the treated mice (3.52 ± 0.18 mV) compared with AAV9-miRLacZ-treated controls (1.44 ± 0.59 mV), it did not reach WT levels (6.89 ± 1.76 mV).

As with other CMT blood biomarker studies, we found that circulating NF-L (76–79) and Gdf15 (80, 81) levels, associated with axonal degeneration, were significantly ameliorated after early treatment of CMT1A mice with AAV9-miR871 (NF-L: 321.37 ± 51.68 pg/mL; Gdf15: 56.25 ± 14.84 pg/mL) compared with their AAV9-miRLacZ vector-treated littermates (540.65 ± 134.49 pg/mL; Gdf15: 81.93 ± 23.12 pg/mL) (Figure 3, Q and R). This reduction in NF-L and Gdf15 levels in the AAV9-miR871 treatment group is consistent with improved motor function following gene-silencing treatment. Thus, NF-L and Gdf15 blood...
levels may be useful as treatment-responsive and clinically relevant biomarkers for future gene therapy in patients with CMT1A.

We performed morphometric analysis of myelination in transverse semithin sections of anterior lumbar spinal roots attached to the spinal cord and (F and G) femoral motor nerves at low (upper panels) and higher (lower panels) magnification from CMT1A-AAV9-miR871 and CMT1A-AAV9-miRLacZ mice. Thinly myelinated (t) or demyelinated (red asterisk) fibers as well as onion bulb formations (red arrowhead) are indicated. Quantification of abnormally myelinated fibers in (C–E) lumbar motor roots and (H–J) femoral motor nerves from 6-month-old noninjected WT and CMT1A mice (n = 5/group), as well as from CMT1A-AAV9-miR871 and CMT1A-AAV9-miRLacZ (n = 16/group). Values represent the mean ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001, by 1-way ANOVA with Tukey’s multiple-comparison test. Scale bars: (A and B) 50 μm and 10 μm (enlarged insets); (F and G) 40 μm and 25 μm (enlarged insets).

Late treatment compared with extended early treatment. After assessing the effectiveness of early treatment with AAV9-miR871 in CMT1A mice, we further examined its effectiveness when injected later in the disease course. We injected mice either at 6 months (late treatment) or 2 months (extended early treatment) of age and analyzed various outcomes at 10 months of age. We evaluated both mice that received late treatment (4 months after injection) and mice that had extended early treatment (8 months after injection) using a VGCN calculation, behavioral testing, blood NF-L and Gdf15 testing, electrophysiological examination, as well as by morphometric analysis of myelination and IHC, while real-time PCR and Western blot analysis were performed only in the late treatment groups (Figure 6A). Vector biodistribution in older animals was confirmed by VGCN in PNS and non-PNS tissues (Supplemental Figures 19A and 20). In the late treatment group, we confirmed vector biodistribution with EGFP expression levels in lumbar roots and sciatic nerves (Supplemental Figure 19B).

At the mRNA level, as with early treatment, late treatment with AAV9-miR871 in CMT1A mice downregulated PMP22 and
At the protein level, late treatment improved Pmp22 levels in roots and sciatic and femoral nerves (Figure 6, B and P). AAV9-miR871 late treatment improved the hind limb clasp phenotype of CMT1A mice, but without reaching WT levels, in contrast to mice given the extended early treatment, in which the phenotype was completely rescued, reaching WT levels (Figure 6N and Supplemental Figure 23).

Electrophysiological examination of 10-month-old mice showed that the sciatic MNVC was significantly improved in CMT1A mice in both the late (36.92 ± 3.94 m/s) and extended early (38.74 ± 5.30 m/s) AAV9-miR871 treatment groups compared with the AAV9-miRLacZ-treated group (24.82 ± 2.58 m/s) (Figure 6O). Interestingly, only the CMT1A mice that received extended early treatment reached age-matched WT values (43.50 ± 2.72 m/s). CMAP amplitudes were not improved in any of the AAV9-miR871 treatment groups (Figure 6P). Similarly, NF-L and Gdf15 levels remained elevated in the animals that were treated late compared with age-matched WT mice (Figure 6, Q and R).

We performed morphometric analysis of myelination in transverse semithin sections of PNS tissues from 10-month-old CMT1A mice that received late or extended early treatment. With this analysis, we showed that the anterior lumbar roots (Figure 7, A–F) and femoral motor nerves (Figure 7, G–L) had significantly reduced percentages of thinly myelinated and demyelinated fibers in the mice that received late treatment, but without reaching WT levels. In contrast, these morphological abnormalities were fully rescued in the mice that underwent extended early treatment, reaching WT levels (Figure 7, A–L). The degree of myelin pathology remained too mild in the sciatic nerves of 10-month-old CMT1A mice to be considered as a treatment readout (Supplemental Figure 24).

As in the early treatment group, analysis of inflammation by immunofluorescence revealed that late treatment with AAV9-miR871 decreased the numbers of CD20+ and femoral motor nerves (Figure 7, G–L) had significantly reduced percentages of thinly myelinated and demyelinated fibers in the mice that received late treatment, but without reaching WT levels. In contrast, these morphological abnormalities were fully rescued in the mice that underwent extended early treatment, reaching WT levels (Figure 7, A–L). The degree of myelin pathology remained too mild in the sciatic nerves of 10-month-old CMT1A mice to be considered as a treatment readout (Supplemental Figure 24).

Discussion

CMT1A is the most common inherited demyelinating neuropathy, resulting from a PMP22 gene dosage effect in SCs. Ideally, CMT1A therapies should reduce overexpressed PMP22, while avoiding excessive knockdown that could lead to the milder phenotype of hereditary neuropathy with pressure palsies (HNPP). We accomplished that here with our study, which, to our knowledge, presents the first translatable AAV9-mediated PMP22 gene–silencing approach leading to phenotypic improvement in a CMT1A mouse model. Although this is a preclinical study, we designed our approach from the outset with an eye toward translation to prospective human clinical trials, in 2 ways. First, the therapeutic construct is applicable to animal models and patients with CMT1A alike. The most relevant animal models, such as the C61-het mouse we used here, express transgenic copies of human PMP22 on a normal mouse background. Thus, both murine Pmp22 and human PMP22 genes contribute to AAV9-miRLacZ late treatment performed similarly to noninjected CMT1A mice and significantly worse than WT mice (Figure 6, J–M, and Supplemental Figures 21 and 22). AAV9-miR871 late treatment improved the hind limb clasp phenotype of CMT1A mice, but without reaching WT levels, in contrast to mice given the extended early treatment, in which the phenotype was completely rescued, reaching WT levels (Figure 6N and Supplemental Figure 23).

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As in the early treatment group, analysis of inflammation by immunofluorescence revealed that late treatment with AAV9-miR871 decreased the numbers of CD20+, CD45+, CD68+, and CD3+ cells in PNS tissues (Figure 8 and Supplemental Figures 25 and 26). Injection with the therapeutic vector did not cause any inflammatory responses in the liver 4 months after injection (Supplemental Figure 27).

Discussion

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excessive gene dosage, leading to CMT1A-like phenotypes, and testing a translatable approach in mice requires targeting both transcripts. Importantly, the artificial miRNA we designed, miR871, targets both human PMP22 and murine Pmp22 transcripts. Second, we delivered AAV9-miR871 through a clinically applicable method of lumbar intrathecal injection into C61-het CMT1A mice, which reproduces the clinical course, severity, and symptoms of patients with CMT1A.

The translatable and effectiveness of the intrathecal administration route have been demonstrated in larger animals (82, 83) and in human trials (NCT03381729, NCT02362438), which showed effects in nerves distal to the injection site, including transduction of SCs in the tibial nerve of dogs after intrathecal injection of AAV9 (83). Another route of administration that could be easily translated to the clinic is intravenous injections. However, our studies in mice showed that intrathecal injection provides adequate biodistribution throughout the PNS, with much lower vector amounts injected compared with intravenous delivery (84). Intraneural injections of AAV shRNA were also proposed to treat a CMT1A model (73), however, the translatability of this delivery method is considered challenging in the clinic.

In addition to incorporating species conservation and a feasible route of administration into our study design, we also demonstrated efficacy at multiple levels. First, we confirmed in vitro and in vivo the PMP22/Pmp22 silencing efficiency of miR871 and its effects on other myelin-related genes and proteins, while also assessing the transduction efficiency of AAV9 in PNS tissues after lumbar intrathecal injection (Figures 1 and 2). We then demonstrated by multiple outcome measures the therapeutic effects of AAV9-miR871 after treatment at both early and later stages of the neuropathy, supporting the relevance of this approach for direct clinical translation to treat CMT1A. As demonstrated through our detailed baseline longitudinal, functional, and morphological analyses, the C61 het model of CMT1A used in this study develops an early-onset, progressive demyelinating pathology that reproduces human disease features (Supplemental Figures 2–7). Thus, already at the early intervention time point, the model presented significant pathological features and slowing of nerve conduction velocities that progressed with aging. Therefore, both early and late treatments represent post-onset interventions, reproducing the clinical scenario of treating younger or older patients with CMT1A, in whom demyelination is already present in childhood (85, 86), followed by slowly progressive axonal loss (8, 9, 87, 88). Our mouse data suggest that earlier treatment is effective, as several outcome measures were corrected to WT levels (Figures 3–5). A direct comparison of CMT1A mice that received extended early or late treatment, injected at 2 or 6 months of age, respectively, and analyzed at 10 months of age, confirmed that treatment more efficiently reversed disease manifestations if given earlier (Figures 6 and 7). This could be explained by the fact that later stages of the neuropathy were characterized by already advanced axonal degeneration. While it appears feasible to stimulate remyelination by transduced demyelinating SCs, increasing axonal loss found at later stages is irreversible. Nevertheless, our data suggest that the ability to improve CMT1A-like symptoms in mice with preexisting pathology is promising for the translation of this strategy to patients who may already be experiencing the effects of CMT1A. Indeed, our work is consistent with the findings of a tetracycline-inducible Pmp22-transgenic mouse study (89). Pmp22 overexpression occurred in the absence of tetracycline, causing demyelination and numerous neuropathic phenotypes. Importantly, when mice were given tetracycline, thereby shutting off the Pmp22 transgene, myelin normalization began to occur within 1 week, with nearly normal myelin observed by 4 months. Together, these data and ours suggest that some CMT1A phenotypes may be reversible. It is also possible that we will see an even greater reversal of phenotypes in these mice as they age 4 months after treatment.

Another question we considered, regarding translation, was the necessity to restrict miR871 expression to SCs alone. In our previous studies (84), we demonstrated that an AAV9 vector expressing a transgene through the SC-specific Mpz promoter efficiently transduced myelinating SCs throughout the PNS following a single lumbar intrathecal injection. In the current study, we used AAV9 to deliver a U6.miR.CMV.EGFP construct, in which both sequences (EGFP and miR871) were driven by ubiquitous promoters (CMV and U6, respectively) (Figure 1D). We calculated transduction rates via immunofluorescence using the CMV.EGFP reporter gene and VGCN analysis (Figure 2). Not surprisingly, expression was more widespread, with both SCs and other cell types transduced, including motor and sensory neurons, leading to axonal expression. We also detected VGCNs in many non-PNS tissues typically transduced by AAV9, with the highest numbers detected in the liver (Supplemental Figures 12 and 19), but without any apparent toxic effects in lumbar roots, sciatic nerves, liver, or DRGs (Figures 5 and 8, and Supplemental Figures 8–11, 16–18, and 25–27). Given that PMP22 expression levels are normally very low and do not have any known effects in other cell types besides myelinating SCs.
(11, 90–92), we do not expect any adverse effects from ubiquitous silencing of PMP22 expression. It is also important to mention that transduction evaluation through a reporter gene may not directly correlate with miR expression, when using 2 different ubiquitous promoters. An emerging area of study involves the ability of miRs to travel outside transduced cells through exosomes and potentially act at distant sites and neighboring cells. We did not directly measure exosome packaging of miR871, but future work should determine the potential for incomplete transduction leading to broader correction in adjacent, nontransduced cells.

The $5\times10^{11}$ vg/mouse vector dose used for intrathecal injection in this study corresponds to approximately $2.3\times10^{13}$ vg/kg. With this dose, we achieved sufficient SC transduction and PMP22 silencing to improve molecular, histopathological, and functional deficits. As such, this dose is comparable to those used in prior clinical AAV9 gene therapy studies that targeted motor neurons in the spinal cord, including the AveXis SMA trial (NCT03381729) for intrathecal delivery of Zolgensma $1.2\times10^{13}$ vg/kg (93) and the giant axonal neuropathy (GAN) clinical trial (NCT02362438), in which intrathecal doses ranged from $3.5 \times 10^{13}$ to $3.5 \times 10^{15}$ total vg/patient ($\approx 1.75 \times 10^{12}$ vg/kg to $1.75 \times 10^{13}$ vg/kg) (94). Given that our dose was slightly higher than those currently used in clinical trials and the fact that our potential treatment population would be mostly adults with CMT1A, a dose escalation study would be useful for identifying the optimal vector concentration that would provide robust therapeutic benefit and minimal risk of PMP22 haploinsufficiency.

To examine the potential side effects of PMP22/Pmp22 oversilencing, we tested AAV9-miR871 in WT mice (Supplemental Results and Supplemental Figures...
Several therapeutic approaches for CMT1A have been suggested through the years, most of them are symptomatic, require repeated treatment sessions, or have potential long-term side effects. For example, intravenously delivered squalenoyl siRNA PMP22 nanoparticles (67) have been shown to provide therapeutic benefit in JP18/JY13 mice overexpressing the human PMP22 gene. However, potential toxicity with repeated dosing and long-term stability, as well as the effects of this treatment on PMP22 mRNA or protein levels, remain to be shown. On the contrary, a gene therapy approach like ours would provide a one-off treatment option. In a previously reported preclinical gene therapy approach, AAV2/9 vectors expressed shRNAs specifically designed to target murine Pmp22 (73). Because the shRNAs contain potentially disruptive mismatches with the human transcript, their direct translatability in humans was untested and remains unclear. Moreover, the shRNA vectors were delivered through direct intraneural injection, a method that is more difficult to translate to clinical practice for treating CMT1A and carries more risks because of the toxic nature of concentrated anesthesia and the risk of direct fiber damage (96). In contrast, the lumbar intrathecal injection used in our study is considered a routine procedure that can be easily applied in the clinic, providing widespread biodistribution in the PNS. Compared with intravenous delivery, intrathecal delivery also requires a much lower viral volume to provide beneficial effects and hence results in lower toxicity (84, 97). It remains to be shown that adequate biodistribution can also be achieved in larger animals before clinical translation.

Regarding the safety of AAV9-based vectors in humans, follow-up studies in AAV9-treated patients with spinal muscular atrophy (SMA) suggested stable beneficial effects of Zolgensma with no major adverse reactions or long-term toxicity (93, 98, 99). However, more recent studies suggest that long-term overexpression of proteins (100) or miRNAs (101) via AAV9 viral vectors may dysregulate endogenous mechanisms, causing toxic side effects. Our data suggested that AAV9-miR871 treatment did not cause inflammation in PNS tissues, as had been previously suggested in another study using the AAV9 serotype carrying a different payload (102), but in fact acted to reduce inflammation native to the CMT1A animal model. Moreover, injection with the therapeutic vector did not cause any chronic inflammatory responses in the liver 4 months after injection (Supplemental Figures 18 and 27). Although our approach was shown to improve the baseline inflammatory status of the CMT1A model without causing any systemic or liver toxicity, it will be important to consistently demonstrate its safety with more detailed toxicity studies across different species. Potential cellular and humoral immune responses can be stimulated against the AAV capsid or protein-coding gene product. Since our one-off therapeutic payload is a noncoding RNA, our vector should be inherently less immunogenic than those used in gene replacement strategies.

To design successful clinical trials involving patients with CMT1A, it is important to establish relevant and sensitive outcome measures. The gene-silencing approach described here provides functional improvements that can be easily evaluated in patients through electrophysiological testing. Since previous clinical trials suggested a lack of sensitivity of standard CMT clinical scores to detect treatment response (45, 103), more detailed clinical functional and patient-reported outcome measures will also be necessary (104–107), along with MRI-based quantification of...
Muscle atrophy (108). Here, we also demonstrate, for the first time to our knowledge, the responsiveness of NF-L (109, 110) and GdD15 (80, 81) plasma biomarkers in a CMT1A model. Responsiveness of these translatable biomarkers is highly encouraging for their utility in parallel clinical trials of miRNA therapies. Although, a recent study (79) showed a lack of correlation of NF-L plasma levels with disease progression over time in patients with CMT1A, this might be due to the older age of the patients tested (mean age, 46 years). In this regard, validation of additional clinically relevant plasma and skin biomarkers as indicators for future gene therapy efficacy would be essential (19, 111, 112, 113).

In conclusion, we developed and characterized an artificial miRNA designed to specifically target human PMP22 and murine Pmp22 transcripts and evaluated the therapeutic benefit in a CMT1A mouse model that reproduces CMT1A-associated phenotypes. Our results indicate that a single lumbar intrathecal injection of AAV9-miR871 at early and late stages of the neuropathy, and always after onset, can correct the functional, morphological, and inflammatory abnormalities of CMT1A without causing any apparent side effects. Taken together, these results constitute an important step toward the development of a clinically relevant and translatable gene therapy to treat CMT1A.

**Methods**

All materials and methods are presented in the Supplemental Methods.

**Statistics.** Each set of data is presented as the mean ± SD or SEM, with n equal to the number of biological repeats for in vitro experiments or independent samples from individual animals for in vivo experiments. For comparison of means between 2 independent groups, an unpaired, 1-tailed Student’s t test was performed. For comparison of means between 3 or more independent groups, 1-way ANOVA was performed. Statistical significance for all experiments was defined as a P value of less than 0.05. When ANOVA tests suggested significant difference among groups, Tukey’s multiple-comparison post hoc test was applied. When a sample group was used for more than 1 comparison, Bonferroni’s correction of P values was additionally applied. All statistical analyses were performed using GraphPad Prism, version 6 (GraphPad Software).

**Study approval.** All animal procedures were approved by the Cyprus Government’s chief veterinary officer (project license CY/EXP/PR.L3/2017) according to national law, which is harmonized with European Union guidelines (EC Directive 86/609/EEC).

**Author contributions**

MS co-designed and conducted or directed all experiments, acquired and analyzed data, created figures and legends, and drafted and reviewed the manuscript. AK performed electrophysiology experiments. SGC performed and analyzed in vitro screening of artificial miRNAs. MJJ and RH standardized, performed and analyzed ELISAs for serum GdD15 levels. LMW contributed to in vitro screening of artificial miRNAs. AMF assisted with viral vector production. AH and HZ performed and analyzed plasma NF-L levels. J.R., CT, and CC performed and analyzed VGCNs. SQH designed artificial miRNAs and supervised in vitro screening, created figures and legends, and drafted and reviewed the manuscript. KAK co-designed and supervised all experiments and drafted and reviewed the manuscript. All authors read and approved the final manuscript.

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