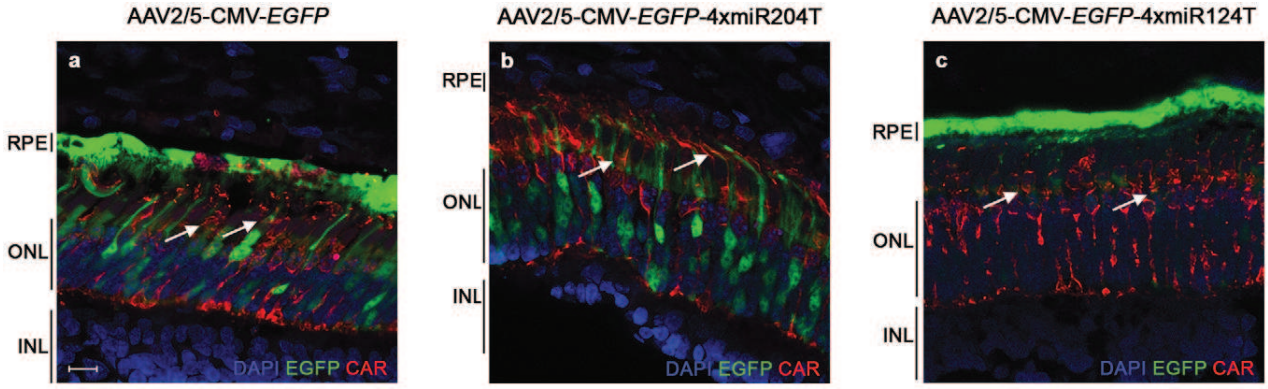

Figure 4. Cone transduction following subretinal administration of AAV vectors bearing miRNA target sites. Retinal cryosections from animals injected subretinally with the various miRNA-regulated vectors as in Fig. 3 were immunolabelled with the cone-specific anti-Cone Arrestin (CAR) antibody (red label). Representative sections of eyes injected with AV2/5-CMV-*EGFP* (**a**), AAV2/5-CMV-*EGFP*-4xmiR204T (**b**) and AAV2/5-CMV-*EGFP*-4xmiR124T (**c**). Colocalization of EGFP and CAR expression is indicated by the arrows. Confocal microscope magnification 63X. Scale bar = 10 μm. For abbreviations, see Fig. 2 legend.
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(anti-hCAR) (arrows in **Figure 4**). A weak EGFP fluorescence was detected by confocal microscopy in the PRs of eyes injected with the AAV2/5-CMV-*EGFP*-4xmiR124T (**Figure 4c**).

With the prospect of using this strategy in animal models of inherited retinal degeneration, we included target sequences for miR-204 in an AAV vector encoding the human *AIPL1* gene, mutated in LCA type 4 (OMIM 604393), with the aim to efficiently transfer *AIPL1* to PRs, its main expression site in the retina. We have recently shown that AAV2/8 vectors target murine [27] and porcine PRs [38] more efficiently than AAV2/5. Therefore, we generated an AAV2/8-CMV-*hAIPL1*-4xmiR204T vector. The AAV2/8-CMV-*hAIPL1*-4xmiR204T vector was injected subretinally in two eleven week-old Large White (LW) female pigs along with the control AAV2/8-CMV-*hAIPL1* that lacks the miR-204 target sites in the contralateral eye. The animals were sacrificed six weeks after injection and their eyes were harvested. Retinal cryosections were then analyzed by immunofluorescence with antibodies directed to human, but not porcine, AIPL1 using confocal microscopy. Transgene expression was detected in both PRs and RPE of the porcine retinas injected with the AAV2/8-CMV-*hAIPL1* control vector, while hAIPL1 expression was efficiently restricted to the PRs in retinas injected with the miR204-regulated vector (**Figure 5**).

To assess whether the presence of miRNA target sequences perturbs normal retinal function, ERG recordings were performed on all injected pigs. Both rod and cone isolated and combined

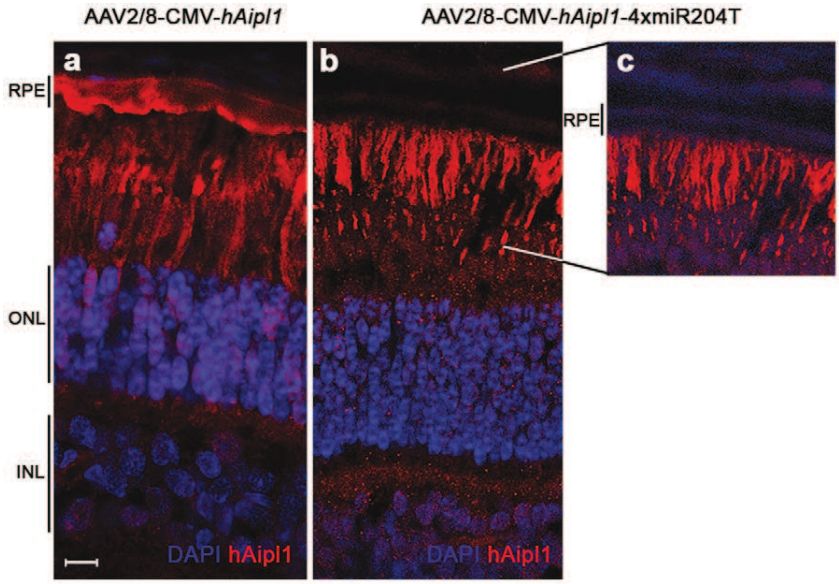

Figure 5. miR204-regulated expression of *hAIPL1* is restricted to the porcine photoreceptors. LW pigs were injected with 1×10^{10} GC/eye with AAV2/8-CMV-*hAIPL1* (**a**) and AAV2/8-CMV-*hAIPL1*-4xmiR204T (**b**) (n = 2 eyes/group). Human AIPL1 immunostaining (red) was performed on pig retinal sections to assess the localization of the transgene expression. The antibody used does not cross-react with the porcine Aipl1. An overexposure of the section in panel (**b**) is shown in (**c**) to highlight that no *hAIPL1* expression was detected in the RPE cells of eyes injected with AAV2/8-CMV-*hAIPL1*-4xmiR204T. Confocal microscope magnification 63X. Scale bar = 10 μm. For abbreviations, see Fig. 2 legend.
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responses of treated eyes (n = 2/vector) showed no statistically significant differences compared to baseline measurements [baseline pre-treatment, photopic = 120 (±4,86) μV; scotopic = 44,7 (±0,5) μV and maximal response 196,6 (±9,21) μV; post-treatment, photopic = 130,5 (±5,5) μV; scotopic = 48 (±2,5) μV and maximal 202 (±5) μV] indicating normal retinal function in the injected animals.

Subretinal administrations of AAV vectors harboring miRNA target sites do not significantly perturb endogenous miRNA activity in the eye

As described before, we detected some scattered and discontinuous EGFP-positive areas within the RPE and PR layers (red arrows in **Figures 2c,d,g,h**) of murine retinas injected with high doses (2.6×10^9 GC/eye) of AAV2/5-CMV-*EGFP*-4xmiR204T and AAV2/5-CMV-*EGFP*-4xmiR124T, respectively. We hypothesized that endogenous miRNAs did not completely eliminate the miRT-bearing EGFP transcripts, thus resulting in unexpected EGFP expression. This could be due to miRNA saturation (*i.e.* depletion of levels of the corresponding endogenous miRNA) caused by the presence of an excessive number of exogenously provided miRNA binding sites. Alternatively, the high number of vector-borne miRTs may have elicited a general deregulation of the miRNA/RISC machinery, resulting in a compromised repressive function. When mice were injected with the same vectors at lower doses, we did not detect any ectopic EGFP fluorescence (**Figures 2e,f,i,j**), suggesting that off-target transgene expression was a dosage-dependent phenomenon.

To assess whether expression of exogenous sequences carrying miRTs could interfere with the normal function of the miRNA machinery, we analyzed the endogenous levels of the corresponding miRNAs by qRT-PCR. In particular, we analyzed the expression levels of miR-204 and miR-124, as well as of miR-182. The latter is strongly expressed in the neural retina, predominantly in PRs [25,26], and, to our current knowledge, is unlikely to either be under the direct control of any of the miRNAs tested or have affinity for the miR-204 and miR-124 miRTs. We extracted total RNA either from retinas of AAV2/5-CMV-*EGFP*-4xmiR124T injected mice (n = 4; high dose group) or from the optic cups (including retina, RPE and sclera) of animals injected with AAV2/5-CMV-*EGFP*-4xmiR204T (n = 4; high dose group). As miR-204 is mainly expressed in the RPE and off-target EGFP expression was detected therein, we reasoned that any potential saturation of miR-204 should be assessed in samples that include RPE. We did not observe any significant variation in the endogenous levels of the miRNAs analyzed (**Figure S1a**), implying the absence of a detectable miRNA saturation effect at the level of the whole retinas or optic cups.

To check whether the high number of miRTs could perturb the capacity of miRNAs to regulate their physiological target genes, we analyzed the expression levels of *VAMP3* and *RDH10*, two direct targets of miR-124 [39,40] in retinal samples of animals (n = 4) injected with high doses of either the control AAV2/5-CMV-*EGFP* or the construct bearing the miR-124 binding sites. VAMP3, also known as cellubrevin, is a member of the vesicle-associated membrane protein (VAMP)/synaptobrevin family [41] and is strongly expressed in the retina, according to the BioGPS gene annotation portal (http://biogps.gnf.org/). On the other hand, *RDH10* encodes for retinol dehydrogenase 10 (all-trans) and is primarily expressed in the RPE and the neural retina [42](Mouse Retina SAGE library; [43]). We did not detect any significant variation in the endogenous levels of these target genes (**Figure S1b**), suggesting that the AAV-borne miR124Ts do not interfere with normal miRNA function. Taken together, the above results imply that the exogenously supplied miRTs do not saturate endogenous miRNA levels nor alleviate miRNA repression from its natural targets.

Discussion

Recently, miRNA-mediated regulation of transgene expression has been successfully achieved in the context of somatic gene transfer in specific cell types, lineages or developmental stages [3]. In the present study, we show for the first time that this strategy can be applied to the retina. Subretinal administration of AAV2/5 results in optimal RPE transduction, but also in robust transgene expression in PRs [22]. Here, we demonstrate that subretinal administration of AAV2/5 vectors containing expression cassettes harboring binding sites for miRNAs expressed in specific retinal cell types results in transgene expression tightly restricted to either the RPE or PRs of mice and pigs. In particular, constructs harboring miR-124 binding sites can efficiently de-target reporter expression from the PRs, while the presence of miR-204 sites induces elimination of transgene expression from the RPE. The validation of this approach in the porcine retina confirmed its high clinical potential.

The use of miRNA-regulated vectors can be advantageous in gene therapy for inherited eye diseases: in one instance, when coupled to cell-specific promoters, it adds a layer of regulation to transgene expression. This is desirable as often the promoter elements used in gene therapy vectors do not faithfully recapitulate the expression patterns of the endogenous promoter, probably due to the absence of distant, secondary regulatory elements [9]. For instance, the promoter of *Rhodopsin* - a gene strongly and specifically expressed in rod PRs- induces aberrant reporter expression also in cones [27,44]. In addition, the levels of transgene expression obtained using tissue-specific promoters may not be adequate. For instance, the *Rhodopsin* promoter drives very robust gene expression in PRs. This is not surprising since rhodopsin accounts for more than 70% of PR proteins [45]. While the Rhodopsin promoter element may be desirable if one wants to replace or repress *Rhodopsin* expression, the levels of expression of other transgenes may be supra-physiological or toxic. On the other hand, other retinal-specific promoters, like *RPE65* [23] or *OA1* [46], may provide subtherapeutic levels of transgene expression for some applications in the RPE. Indeed, in one LCA2 clinical trial (ClinicalTrials.gov number, NCT00516477), we have used AAV2/2 vectors expressing *RPE65* from the robust constitutive CBA promoter to obtain therapeutic levels of trangene expression. Assuming that the CBA expression pattern following subretinal administration of AAV2/2 vectors in the human retina is similar to that observed in mice and dogs [47,48], we are presumably misexpressing the *RPE65* in PRs in addition to the RPE. Although this does not appear to be a problem as we did not observe any retinal toxic effect so far [21], ideally we could have tailored *RPE65* expression to RPE if we had included miR-124 target sites in our AAV vector.

A main concern for the application of miRNA-regulated transgene delivery is the potential to saturate their cognate miRNA by exogenously provided miRTs, thus reducing its bioavailability and alleviating control of its natural targets. Interestingly, in all studies that have employed lentiviral platforms for the delivery of miRNA-regulated transgenes, there is no evidence that the excess of exogenously provided miRTs - when engineered according to the principles set for regulated targets [3] - saturates the cognate miRNA [3,4,5,6,9]. Gentner and colleagues showed that only when expression is driven by very strong promoters or when several vector copies are introduced, transgene constructs containing four copies of perfectly complementary miRTs are able to saturate miRNA

regulation following lentiviral delivery [49]. In this study, we designed our vectors according to the principles set for regulated targets to prevent miRNA inhibition in the presence of a strong CMV promoter [3]. Indeed, most of the studies applying these parameters report no evidence of miRNA saturation [3]. We observed some scattered, off-target EGFP expression in murine retinas injected with high doses of miRT-bearing vectors, indicative of miRNA inhibition which was not detected when using a ten-fold lower AAV vector dose. However, we did not measure altered levels of neither miRNAs nor their target genes in the retinas treated with high AAV vector doses suggesting that miRNA saturation, if present, occurs at levels below the detection limit of our assay. As an alternative to lowering the dose of viral genome copies administered, the number of miRTs present in the vector construct could be reduced.

The PR- and RPE-restricted pattern of transgene expression at low vector doses was confirmed in the pig retina, which is more similar to the human one in terms of size and anatomy [32]. We can thus expect that a dose similar to the one successfully used in pigs could be applied to humans. Indeed, we have used a similar dose (1.5×10^{10} GC/eye) of AAV2/2 in patients with LCA2 obtaining improvement of visual function [19,20,21]. Our data suggest that administration of low doses of miRT-harboring vectors enables to tailor transgene expression to specific retinal cell types in the absence of off-target effects and deregulation of endogenous miRNA activity.

Ultimately, the efficacy of AAV-mediated miRNA-regulated gene expression in the retina should be proven in animal models of IRDs and, ideally, in non-human primates which possess a cone-enriched macula. Our data suggest that the addition of miRNA target sites to gene therapy vectors enables fine-tuning of transgene expression in the retina possibly rendering gene therapy safer and more efficient.

Materials and Methods

Plasmid construction, AAV vector production and purification

Recombinant AAV vectors containing the EGFP cDNA under the cytomegalovirus (CMV) promoter and four copies of a sequence (referred to as 'miRT') that is perfectly complementary to the miRNAs of interest in the 3′ UTR were constructed by a two-step cloning protocol. Initially, the cassette containing four copies of a sequence which is perfectly complementary to miR-204 (in capital letters) was constructed by annealing the following two sets of oligonucleotides: 5′-ctagatctAGGCATAGGATGACAAAGGGAAcgataggcatAGGATGACAAAGGGAAaagctt-3′, 5′-TTCCCTTTGTCATCCTATGCCTatcgTTCCCTTTGTCATCCTATGCCTAGAT-3′ and 5′-AGGCATAGGATGACAAAGGGAAtcacAGGCATAGGATGACAAAGGGAAagatc-3′, 5′-tcgagatctTCCCTTTGTCATCCTATGCCTgtgaTTCCCTTTGTCATCCTATGCCTaagctt-3′.

Similarly, the cassette containing four copies of a sequence which is perfectly complementary to miR-124 (in capital letters) was constructed by annealing the following two sets of oligonucleotides:

5′-ctagatctGGCATTCACCGCGTGCCTTAcgatGGCATTCACCGCGTGCCTTAaagctt-3′, 5′-TAAGGCACGCGGTGAATGCCatcgTAAGGCACGCGGTGAATGCCagat-3′ and ′-GGCATTCACCGCGTGCCTTAtcacGGCATTCACCGCGTGCCTTAagatc-3′5, 5′-tcgagatctTAAGGCACGCGGTGAATGCCgtgaTAAGGCACGCGGTGAATGCCaagctt-3′. In either case, the resulting double-stranded fragments (each one containing two copies of the respective miRT) were ligated thanks to the presence of phosphorylated 5′ ends. The obtained fragments (containing four copies of the respective miRT) were subcloned in pBluescript II SK(+) previously digested with *Xba* I and *Xho* I. The recombinant clones were digested with *Bgl* II to release the fragment containing the four miRT sites with *Bgl* II protruding ends. The fragment was then cloned into the *Bgl* II site of the pAAV2.1-CMV-*EGFP* plasmid [27] and used for the production of AAV2/5 vectors.

To generate the vectors expressing *hAIPL1*, the coding sequence of the *hAIPL1* gene was amplified from human retina cDNA (BioChain, Hayward, CA) using the primers hAIPL1-NotI-forward (5′-ATATGCGGCCGCCATGGATGCCGCTCTGC-TCCT- 3′) and hAIPL1-HindIII-reverse (5′- ACGCGTAAGC-TTTTATCAGTGCTGCAGCGAGTGCC- 3′) and cloned into the pAAV2.1-CMV-*EGFP* following digestion with *Not* I and *Hind* III. The final pAAV2.1-CMV-*hAIPL1*-4xmiR204T plasmid was subsequently produced by cloning the fragment containing four miR-204 target sites (released by *Bgl* II digestion of the pAAV2.1-CMV-*EGFP*-4xmiR204T) in the *Bgl* II site of pAAV2.1-CMV-*hAIPL1*.

AAV vectors were produced by triple transfection of 293 cells, purified by two rounds of $CsCl_2$ ultracentrifugation, and titered (in GC/milliliter) using a real-time PCR-based assay TaqMan® (Applied Biosystems, Foster City, CA) and a dot-blot analysis, as previously described [28]. AAV vector production was carried out by the TIGEM AAV vector core.

Animal procedures and vector administration

Ethics Statement. All studies on mice were conducted in strict accordance with the institutional guidelines for animal research and with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animal in Ophthalmic and Vision Research. All animal treatments were reviewed and approved in advance by the Ethics Committee of the Centre of Biotechnology, Animal Research Unit, Cardarelli Hospital (Naples, Italy). All procedures on mice were then approved by the Italian Ministry of Health (protocol number: 0000667/11/CB; approval date Sept. 11, 2007).

The experiments involving pigs were conducted according to relevant national and international guidelines. All procedures on pigs were reviewed and approved in advance by the Ethics Committee of the Department of Veterinary Medical Science, University of Bologna (Bologna, Italy) and were then approved by the Italian Ministry of Health (protocol number: 23/2009-B, approval date Feb. 04, 2009). All surgery was performed under anesthesia, and all efforts were made to minimize suffering.

Mice. Four-week old C57BL/6 mice (Harlan, S. Pietro al Natisone, Italy) were anesthetized with an intraperitoneal injection of avertin (1.25% w/v of 2,2,2-tribromoethanol and 2.5% v/v of 2-methyl-2-Butanol; Sigma–Aldrich, St. Louis, MO) at 2 ml/100 g of body weight, and viral vectors were delivered via a trans-scleral transchoroidal approach, as previously described [50]. Mice were injected in the right eye with 2.6×10^{9} GC of AAV2/5-CMV-*EGFP*-4xmiRT in the high dose experiments and 2.6×10^{8} GC of AAV2/5-CMV-*EGFP*-4xmiRT in the low dose. The same doses of AAV2/5-CMV-*EGFP* were injected in the left eye, as control. Following injection, the extent of transduction was assessed by ophthalmoscopy at days 7 and 28. Eyes were harvested at day 28 after injection.

Pigs. The Large White pigs (LW) used in our study were registered as purebred in the LW Herd Book of the Italian National Pig Breeders' Association. Pigs were starved overnight leaving water *ad libitum*. The anesthetic and surgical procedures for pigs were previously described [38].

Histological analysis

Mice were sacrificed, and their eyeballs were then harvested and fixed overnight by immersion in 4% paraformaldehyde (PFA). Before harvesting the eyeballs, the temporal aspect of the sclerae was marked by cautery in order to orient the eyes with respect to the injection site at the moment of the inclusion. The eyeballs were cut so that the lens and vitreous could be removed leaving the eyecup intact. Mice eyecups were infiltrated with 30% sucrose for cryopreservation, and embedded in tissue freezing medium (O.C.T. matrix, Kaltek, Padua, Italy). For each eye, 150 to 200 serial sections (10 µm-thick) were cut along the horizontal plane and the sections were progressively distributed on 10 slides so that each slide contained 15 to 20 sections, each representative of the whole eye at different levels. The sections were stained with 4′,6′-diamidino-2-phenylindole (Vectashield, Vector Lab Inc., Peterborough, UK) and EGFP was monitored with a Zeiss Axiocam (Carl Zeiss, Oberkochen, Germany) at different magnifications.

Pigs were sacrificed and their eyeballs were harvested and fixed overnight by immersion in 4% PFA. The eyeballs were cut so that the lens and vitreous could be removed, leaving the eyecups in place. The eyecups were cryoprotected by progressive infiltration with 10%, 20% and 30% sucrose. Before embedding, the swine eyecups were analyzed under a fluorescence stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany) in order to localize the transduced region, whenever an EGFP-encoding vector was administered. Embedding was performed in tissue-freezing medium (O.C.T. matrix, Kaltek, Padua, Italy). For each eye, 200 to 300 serial sections (12 mm-thick) were cut along the horizontal meridian and the sections were progressively distributed on glass slides so that each slide contained 6 to 10 sections. Section staining and image acquisition was performed as described for mice.

Immunofluorescence staining

Frozen retinal sections were washed once with PBS and then fixed for 10 min in 4% PFA. Sections were then permeabilized for 1 hour in PBS containing 0.1% Triton® X-100. Blocking solution containing 10% normal goat serum (Sigma–Aldrich, St. Louis, MO) was applied for 1 hour. Primary antibodies were diluted in PBS and incubated overnight at 4°C. The secondary antibody (Alexa Fluor® 594, anti-rabbit, 1:1000; Molecular Probes, Invitrogen, Carlsbad, CA) was incubated for 45 min. The primary antibodies used were rabbit anti-hAIPL1 (1:700; kindly provided by Michael E. Cheetham, University College London, London, UK) and rabbit anti-hCAR [51] (1:10000; kindly provided by Cheryl M. Craft, University of Southern California, Los Angeles, Ca). Vectashield (Vector Lab Inc., Peterborough, UK) was used to visualize nuclei. Sections were photographed using either a Zeiss Axioplan microscope (Carl Zeiss, Oberkochen, Germany) or a Leica Laser Confocal Microscope System (Leica Microsystems GmbH, Wetzlar, Germany).

Electroretinography

Electrophysiological recordings in mice were performed as detailed in [52] and bilateral ERG evaluations in pigs were carried out as previously described [38].

miRNA and gene expression analysis

MiRNA and gene expression analysis in mice administered with the AAV2/5-CMV-*EGFP*-4xmiR124T and AAV2/5-CMV-*EGFP*-4xmiR204T constructs was performed on samples from whole retinas and optic cups, respectively. Total RNA was extracted using the miRNeasy kit (Qiagen, Inc., Hilden, Germany) according to the manufacturer's instructions and quantified using the NanoDrop 1000 (Thermo Fischer Scientific, Waltham, MA). RNA quality was assessed by gel electrophoresis.

Quantitative (q) Reverse Transcriptase (RT-) PCR-based detection of mature miR-124, miR-182 and miR-204 was performed using the TaqMan® microRNA assays (Applied Biosystems, Foster City, CA). All reactions were performed in triplicate. The qRT-PCR results, recorded as threshold cycle numbers (Ct), were converted to absolute copy number (*i.e.* copies of miRNA per ng of RNA) using a standard curve. To generate the standard curve, serial amounts (ranging from 10^2 to 10^8 copies) of a synthetic RNA oligonucleotide corresponding to miR-124 (5′-UAAGGCACGCGGUGAAUGCC-3′; Sigma–Aldrich, St. Louis, MO) were mixed with 10 ng of total yeast RNA. The samples were analyzed using the TaqMan® microRNA assay and the correlation between threshold cycle numbers (Ct) and copies of miRNA was established.

For the expression analysis of target genes, cDNA synthesis was performed using the Quantitect Reverse Transcription kit (Qiagen, Inc., Hilden, Germany) starting from 1 µg of DNase-treated RNA. In order to unambiguously distinguish spliced cDNA from genomic DNA contamination, exon-specific primers were designed to amplify across introns of the genes tested. All primers were previously tested by reverse transcription (RT)-PCR and no RT control reactions were performed. Primer sequences are the following: MmRdh10_For: 5′-CTAGAGATTAAT-CATGGCCAC-3′; MmRdh10_Rev: 5′-CTCGTGAAAACCCA-CAACTC-3′; MmVamp3_For: 5′-CAGACACAAAATCAAG-TAGATG-3′; MmVamp3_Rev: 5′-CAGTGCATCTGCGCGG-TC-3′. qRT-PCR experiments were performed using the ABI Prism 7900HT Fast Sequence Detection System with ABI Power SYBR Green reagents (Applied Biosystems, Foster City, CA). Real-time PCR results were analyzed using the comparative Ct method normalized against the housekeeping genes *GAPDH* and *ACTB*. The range of expression levels was determined by calculating the standard deviation of the ΔCT.

Supporting Information

Figure S1 AAV vectors harboring miRTs do not detectably perturb miRNA expression and activity in the eye. (**a**) miRNA expression profile analysis in retinas and optic cups of animals injected subretinally with AAV (n = 4 samples/group). Expression levels were determined by qRT-PCR on RNA extracted from retinas injected with AAV2/5-CMV-*EGFP*-4xmR124T and from optic cups of eyes treated with AAV2/5-CMV-*EGFP*-4xmR204T following delivery of a high AAV vector dose (2.6×10^9 GC/eye). Subretinal administration of AAV vectors harboring miRTs does not detectably perturb endogenous miRNA expression in the eye. (**b**) Expression levels of *RDH10* and *VAMP3*, two direct targets of miR-124 in retinas injected with high doses of AAV2/5-CMV-*EGFP*-4xmR124T animals (n = 4). The contralateral eyes injected with the AAV2/5-CMV-*EGFP* control were used as reference. *ACTB* and *HPRT* were used as internal controls. Subretinal administration of AAV vectors harboring miRTs does not detectably perturb endogenous miRNA activity in the eye. Error bars represent the mean plus or minus SEM.
(TIF)

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Author Contributions

Conceived and designed the experiments: AA SB MK. Performed the experiments: MK AM AP EM AG MA MDC SR MG. Analyzed the data: MK AM AA SB EMS. Wrote the paper: MK AM MLB FS SB AA.

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