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MicroRNA-Restricted Transgene Expression in the Retina

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Abstract

Background: Gene transfer using adeno-associated viral (AAV) vectors has been successfully applied in the retina for the treatment of inherited retinal dystrophies. Recently, microRNAs have been exploited to fine-tune transgene expression improving therapeutic outcomes. Here we evaluated the ability of retinal-expressed microRNAs to restrict AAV-mediated transgene expression to specific retinal cell types that represent the main targets of common inherited blinding conditions.

Methodology/Principal Findings: To this end, we generated AAV2/5 vectors expressing *EGFP* and containing four tandem copies of miR-124 or miR-204 complementary sequences in the 3′UTR of the transgene expression cassette. These vectors were administered subretinally to adult C57BL/6 mice and Large White pigs. Our results demonstrate that miR-124 and miR-204 target sequences can efficiently restrict AAV2/5-mediated transgene expression to retinal pigment epithelium and photoreceptors, respectively, in mice and pigs. Interestingly, transgene restriction was observed at low vector doses relevant to therapy.

Conclusions: We conclude that microRNA-mediated regulation of transgene expression can be applied in the retina to either restrict to a specific cell type the robust expression obtained using ubiquitous promoters or to provide an additional layer of gene expression regulation when using cell-specific promoters.

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Introduction

MicroRNAs (miRNAs) are a class of small 20–25-nucleotide long non-coding RNAs that negatively regulate expression of their target genes by binding to specific sequence elements in the 3′ untranslated region (UTR) of their respective mRNAs [1]. MiRNAs predominantly act to decrease target mRNA levels in animal cells [1] and at least one third of them are expressed in a cell type- or developmental-specific manner [2]. Only recently, endogenous miRNAs have been exploited for the tight post-transcriptional regulation of exogenously delivered (trans)genes in therapeutic and experimental applications [3]. Incorporation of target sites for a specific miRNA (miRTs) at the 3′ end of a transgene cassette has been adapted to provide a means of restricting transgene expression domains to specific cell types, lineages or differentiation states [4,5,6,7,8]. This strategy is particularly useful to further improve transgene specificity, when combined with the transcriptional targeting provided by tissue-specific promoters. A detailed knowledge of the cellular and developmental distribution of miRNAs is a major requisite towards the implementation of this strategy. In one of the first therapeutic applications of this approach, Brown and colleagues [9] combined an hepatocyte-specific promoter with target sequences for a hematopoietic-specific miRNA in a lentiviral-based vector to abolish the immune response generated by the off-target expression of clotting factor IX in the antigen-presenting cells (APCs) of Hemophilia B (ΔF.IX) mice. The dynamic expression of miRNAs has also been exploited to ensure an appropriate restriction of transgene expression across development [10].

Inherited retinal degenerations (IRDs) are a group of conditions that result from mutations in genes encoding proteins with critical functions in retinal pigment epithelium (RPE) or photoreceptor (PR) cells and lead to severe visual deficits and ultimately to blindness [11]. IRDs can greatly benefit from gene therapy using adeno-associated virus (AAV)-derived vectors that transduce non-dividing cells and result in long-term transgene expression [12]. The safety and efficacy of AAV-based gene therapy has been verified in various animal models [13] and in humans [14,15,16,17,18,19,20,21]. Recently, AAV2-mediated gene trans-

fer of *RPE65* in patients affected with Leber's Congenital Amaurosis (LCA, type 2; OMIM 204100) achieved stable improvement of visual and retinal function [14,15,16,17,18, 19,20,21].

Tight spatial and temporal control of transgene expression is desirable in the context of gene therapy. In IRDs, gene transfer should be ideally targeted to either RPE or PRs. This can be in part achieved by selecting the appropriate AAV serotype, as AAVs show variable kinetics of transgene expression and differential tropism for a broad range of ocular cell types [22]. Specificity of transgene expression in the retina can be further enhanced using RPE- or photoreceptor-specific promoter elements [23,24]. However, very often the tissue-specific promoters used in gene therapy vectors do not faithfully recapitulate the patterns of the endogenous promoter. In addition, the levels of transgene expression obtained may either be inadequate for therapeutic purposes or supra-physiological and deleterious for retinal function.

With this study, we aimed to improve controlled transgene expression in the retina by exploiting the post-transcriptional regulation offered by the endogenous miRNA machinery. To this end, we integrated our knowledge on the cellular distribution of miRNAs within the mouse eye [25,26] with the use AAV-mediated strategies for gene transfer to the retina [22]. Here we describe the first paradigm of harnessing retinal-specific miRNAs to delimit transgene expression to the RPE monolayer or the PRs of the adult retina using AAV vectors. We show that efficient restriction of transgene expression can be obtained even at low vector dosages. These findings have implications for the design of gene therapy approaches for hereditary retinopathies as they may improve safety and efficacy of gene transfer.

Results

Use of miR-204 target sites restricts transgene expression to murine PRs *in vivo*

We sought to assess whether post-transcriptional restriction of AAV-mediated transgene expression to PRs could be achieved by exploiting endogenous miRNAs. For this purpose we selected miR-204, a miRNA that is strongly expressed in the RPE from as early as E10.5 to adulthood [25,26] (**Figure 1a**). miR-204 expression was absent from the PRs and was detected by *in situ* hybridization (ISH) at low levels in the inner nuclear layer of the neural retina as well in the ganglion cell layer [26] (**Figure 1a**).

Based on the miR-204 expression pattern in the adult murine retina, we inserted four copies of a sequence that is perfectly complementary to the mature miR-204 (miR204T) immediately downstream of the Woodchuck hepatitis virus Post-transcriptional Regulatory Element (WPRE) in the pAAV2.1-CMV-*EGFP* plasmid (**Figure 1b**; see Materials and Methods). The presence in this plasmid of the ubiquitous CMV promoter drives robust transgene expression in both RPE and PRs [27,28]. The resulting pAAV2.1-CMV-*EGFP*-4xmiR204T plasmid (**Figure 1b**) was used to produce AAV2/5 vectors that efficiently transduce RPE and PRs upon subretinal administration in several species, including mice [29]. Four-week-old C57BL/6 mice (n = 4) were injected with 2.6×10^9 genome copies (GC) (defined as "high dose") of AAV2/5-CMV-*EGFP*-4xmiR204T in one eye, and the same dose of AAV2/5-CMV-*EGFP* as control in the contralateral one. Four weeks after injection, eyes were harvested, retinas were sectioned and retinal sections were analyzed by direct fluorescence microscopy to assess localization of EGFP expression.

The number of EGFP-positive RPE cells in the eyes injected with AAV2/5-CMV-*EGFP*-4xmiR204T (**Figures 2c,d**) was dramatically reduced, compared to contralateral eyes injected with AAV2/5-CMV-*EGFP* (**Figures 2a,b**), indicating efficient suppression of the *EGFP*-miR204T mRNA by endogenous miR-204. Despite the strong reduction of EGFP expression in the RPE, occasional EGFP-positive RPE cells were detected (red arrows in **Figures 2c,d**). We hypothesized that loss of miR-204-mediated regulation in these EGFP-positive RPE cells could result from the saturation of the miRNA activity due to an excess of exogenous miR204Ts. To indirectly test this, we used a 10-fold lower vector dose (2.6×10^8 GC/eye; defined as "low dose"). Analysis of EGFP fluorescence in the low-dose group (n = 4) showed specific restriction of transgene expression to the PRs, while no EGFP expression could be detected within the RPE in any of the sections from the four eyes injected with the miR204T-containing vector (**Figures 2e,f**).

Use of miR-124 target sites restricts transgene expression to murine RPE *in vivo*

To restrict AAV2/5-CMV-mediated transgene expression to the RPE, we exploited miR-124, a miRNA abundantly expressed in differentiated neurons [30]. We and others have shown by ISH that miR-124 stains strongly all retinal cell layers, but is not detected in the RPE [26,31] (**Figure 1a**). Therefore, we cloned four tandem copies of a sequence that is perfectly complementary to the mature miR-124 downstream of the WPRE element in the pAAV2.1-CMV-*EGFP* plasmid (**Figure 1b**). The resulting pAAV2.1-CMV-*EGFP*-4xmiR124T plasmid was used to produce AAV2/5 vectors that were administered to C57BL/6 mice by subretinal injection. Mice (n = 4) received 2.6×10^9 GC (defined as "high dose") of AAV2/5-CMV-*EGFP*-4xmiR124T in one eye, and the same dose of the AAV2/5-CMV-*EGFP* as control in the contralateral one. The animals were sacrificed four weeks after injection. Reporter expression in the transduced retina was evaluated by fluorescence microscopy of retinal sections.

We observed a dramatic reduction in the number of EGFP-positive PRs in eyes injected with the AAV2/5-CMV-*EGFP*-4xmiR124T (**Figures 2g,h**) compared to eyes injected with the control vector (**Figures 2a,b**), suggesting efficient elimination of the miRT-containing transcript by the endogenous miR-124. However, similarly to what observed with the miR204T-containing construct, few scattered EGFP-positive PR cells could be seen in the neural retina (red arrows in **Figures 2g,h**), which implies loss of miRNA-mediated regulation therein. EGFP expression in these cells could result from the saturation of miRNA activity due to an excess of exogenously provided miR124Ts. We then tested a 10-fold decrement in vector dose to assess if off-target expression of the miRT-containing transcript in the transduced PRs would be eliminated. C57BL/6 mice (n = 4) were injected with 2.6×10^8 GC/eye of either virus, and their eyes were analyzed four weeks after injection. We did not observe any EGFP-positive cells in the neural retina of eyes administered with the low dose of AAV2/5-CMV-*EGFP*-4xmiR124T (**Figures 2i,j**), suggesting that at this dose, the presence of the miR124Ts tightly restricts transgene expression to the RPE.

Finally, to exclude that the presence of exogenous miRNA target sequences can interfere with the physiological function of the PRs, we performed electroretinograms (ERG) on mice injected at a high dose (2.6×10^9 GC/eye) with the AAV2/5 vectors harboring the miR-124 or miR-204 target sequences and the control *EGFP* construct. ERG recordings of eyes injected with the miRT-bearing vectors showed no statistically significant differences compared to eyes injected with the *EGFP* control [max a-wave amplitude: EGFP = 336,18 μV (±67,06); miR124T = 350,97 (±132,11) μV; miR204T = 320,70 (±105,09) μV; max b-wave

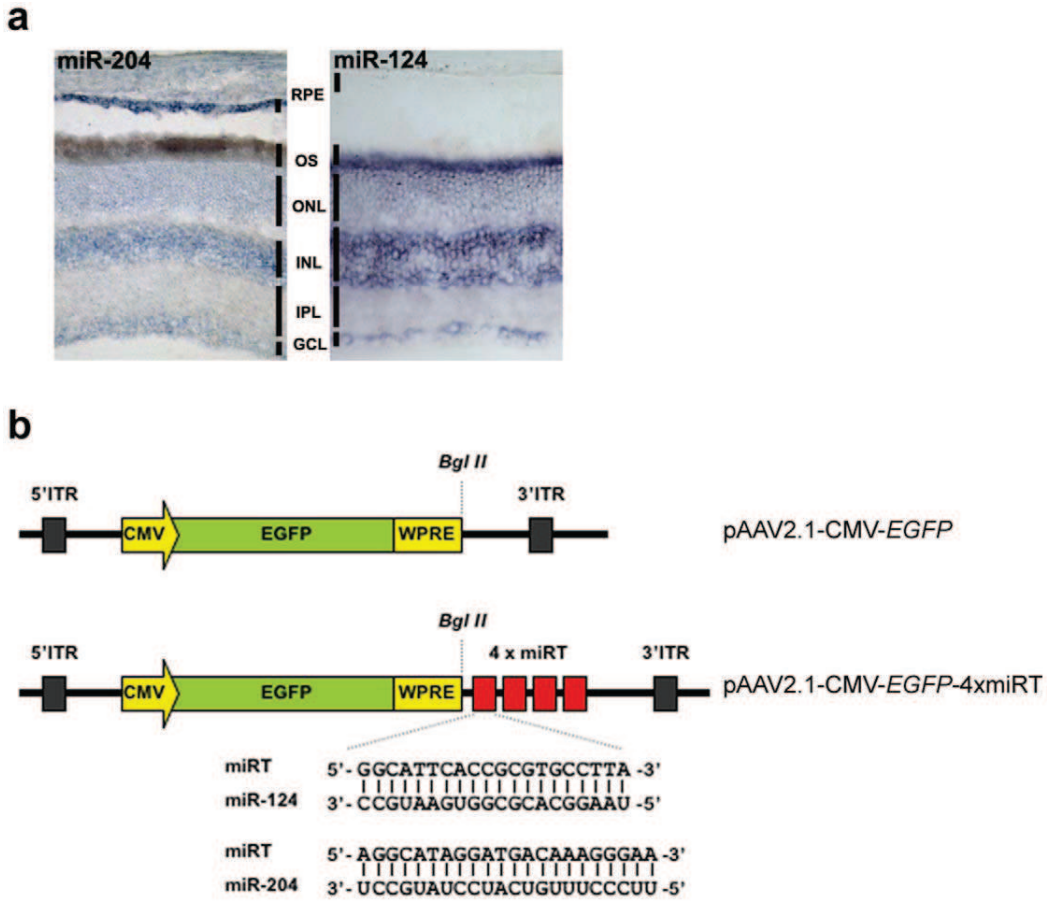

Figure 1. miR-124 and miR-204 expression in retina supports the use of AAV vectors harboring corresponding miRTs. (**a**) Expression profile of miR-204 and miR-124 in retina sections of adult, albino (CD1) mouse as revealed by ISH using LNA-modified probes. miR-124, a neuronal-specific miRNA, is expressed in all layers of the neural retina but is not detected in the RPE. miR-204 is expressed in the GCL and the INL of the neural retina and stains strongly the RPE. RNA ISH for the detection of mature miRNAs was performed using miRCURY LNA™ microRNA Detection Probes (Exiqon, Vedbaek, Denmark) as previously described [26]. (**b**) Schematic representation of the AAV vectors harboring the miRT sites. Four tandem copies (4xmiRT) of a sequence perfectly complementary to the sequence of the mature miR-124 or miR-204 (see alignments) were cloned downstream of the WPRE element in the pAAV2.1-CMV-*EGFP* plasmid. Abbreviations are as follows: CMV, Human Cytomegalovirus promoter; EGFP, Enhanced Green Fluorescence Protein; GCL, Ganglion Cell Layer; INL, Inner Nuclear Layer; IPL, Inner Plexiform Layer; ITR, Inverted Terminal Repeat; miRT, miRNA target site; ONL, Photoreceptor Outer Nuclear Layer; OS, Photoreceptor Outer Segments; RPE, Retinal Pigment Epithelium; WPRE, Woodchuck hepatitis virus Post-transcriptional Regulatory Element.
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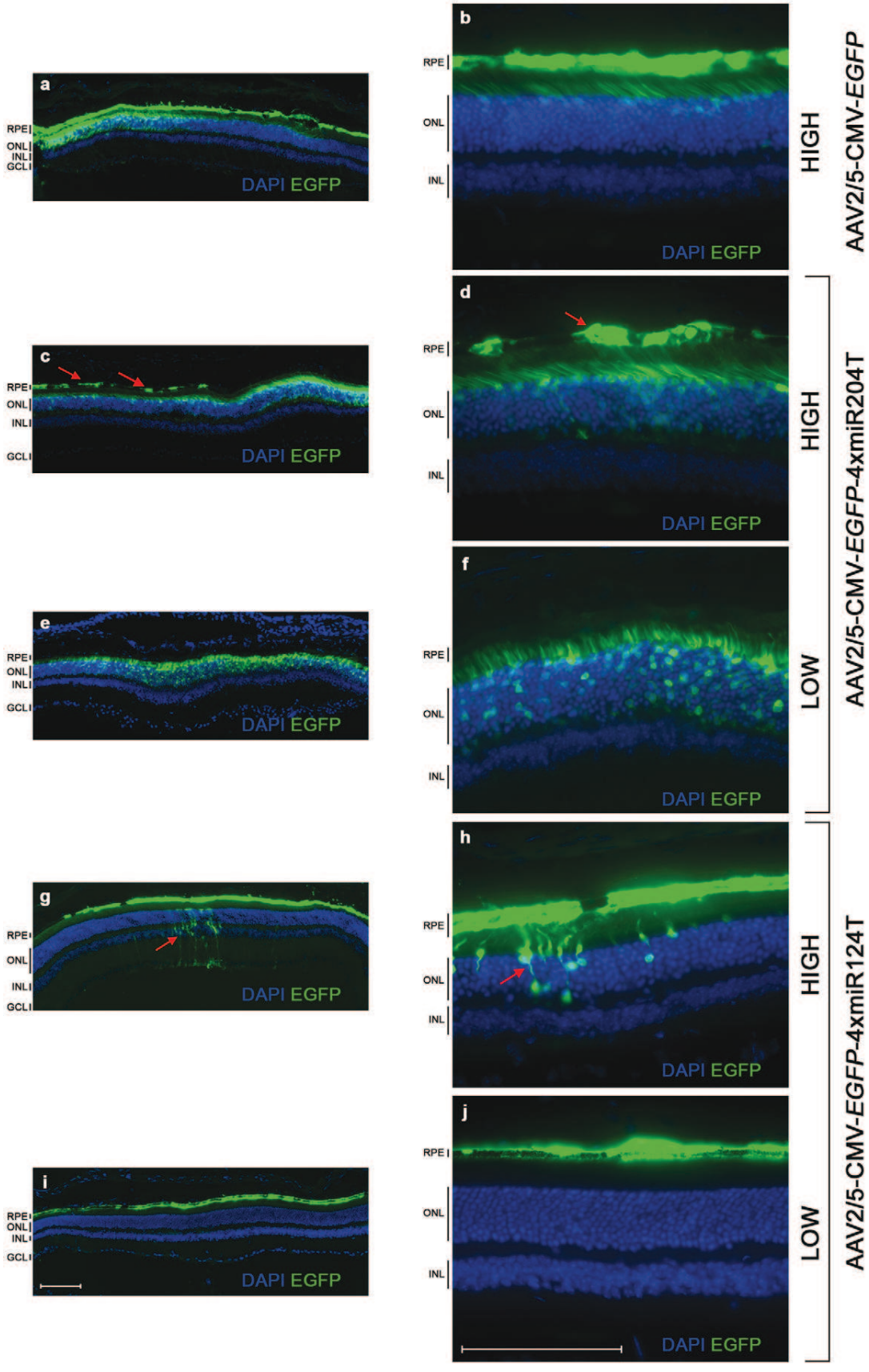
amplitude: EGFP = 667,83 (±165,47) µV; miR124T = 598,18 (±90,28) µV; miR204T = 690,83 (±143,96) µV].

miRNA-regulation of transgene expression in the porcine retina

We extended our studies to the pig (*Sus scrofa*) as, among non-primate mammals, the porcine retina most closely resembles the human one in terms of size, anatomy, cellular composition and physiology, rendering it a valuable preclinical model system for eye disease and therapy [32]. The mature sequence of miR-124 and miR-204 is identical in pigs and mouse (miRBase, http://www.mirbase.org/; [33]). Given the highly conserved cellular distribution of these two miRNAs across species [26,34,35], we assumed that miR-124 and miR-204 are likely to be expressed in the same porcine retinal layers.

We injected subretinally eleven week-old Large White (LW) female pigs (n = 2 eyes/group) with AAV2/5-CMV-*EGFP*-4xmiR204T and AAV2/5-CMV-*EGFP*-4xmiR124T and compared them with eyes injected with the AAV2/5-CMV-*EGFP* as control. Considering the size proportions of murine and porcine eyes, the dose administered in pigs was equivalent to the low dose injected in mouse (in the mouse eye we administered 1 µl containing 2.6×10^8 GC, in the pig eye 100 µl of a 1:2.6 dilution of the same vector solution, thus containing 1×10^{10} GC). Retinal sections were analyzed, following animal sacrifice six weeks after injection. As shown in Figure 3, the use of target sequences for miR-204 (**Figures 3c,d**) and miR-124 (**Figures 3e,f**) efficiently restricted AAV2/5 mediated *EGFP* expression to the PRs and RPE of the porcine retina, respectively.

Cones are important targets of gene therapy since several blinding conditions, either inherited as monogenic or as complex traits, are due to mutations in genes expressed in cones or are characterized by progressive cone degeneration [36]. Since the porcine retina has a high number of cones compared to the murine one [37], we checked whether both rod and cone PRs were equally transduced following AAV2/5-mediated delivery. Expression of EGFP in cone PRs was confirmed by immunolabelling of porcine retinal sections with the Cone Arrestin antibody


a
RPE
ONL
INL
GCL
DAPI EGFP
b
RPE
ONL
INL
DAPI EGFP
HIGH
AAV2/5-CMV-EGFP
c
RPE
ONL
INL
GCL
DAPI EGFP
d
RPE
ONL
INL
DAPI EGFP
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e
RPE
ONL
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RPE
ONL
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DAPI EGFP
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AAV2/5-CMV-EGFP-4xmiR204T
g
RPE
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AAV2/5-CMV-EGFP-4xmiR124T
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RPE
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Figure 2. miRNA-regulated *EGFP* expression in the mouse retina. C57BL/6 adult mice (n = 4 eyes/group) were injected subretinally with: 2.6×10^9 GC/eye of AAV2/5-CMV-*EGFP* (**a** and **b**; high dose); 2.6×10^9 GC/eye (**c** and **d**; high dose) or 2.6×10^8 GC/eye (**e** and **f**; low dose) of AAV2/5-CMV-*EGFP*-4xmiR204T; 2.6×10^9 GC/eye (**g** and **h**; high dose) or 2.6×10^8 GC/eye (**i** and **j**; low dose) of AAV2/5-CMV-*EGFP*-4xmiR124T. Mice were sacrificed four weeks after injection, and retinal sections were analyzed by direct fluorescence microscopy. Images at 10X (panels on the left) and 40X magnification (panels on the right) are shown. At high vector doses ectopic EGFP expression (red arrows) was observed in few PR and RPE cells, respectively. Scale bar = 100 μm. Abbreviations: RPE, retinal pigment epithelium; ONL, photoreceptor outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.
doi:10.1371/journal.pone.0022166.g002

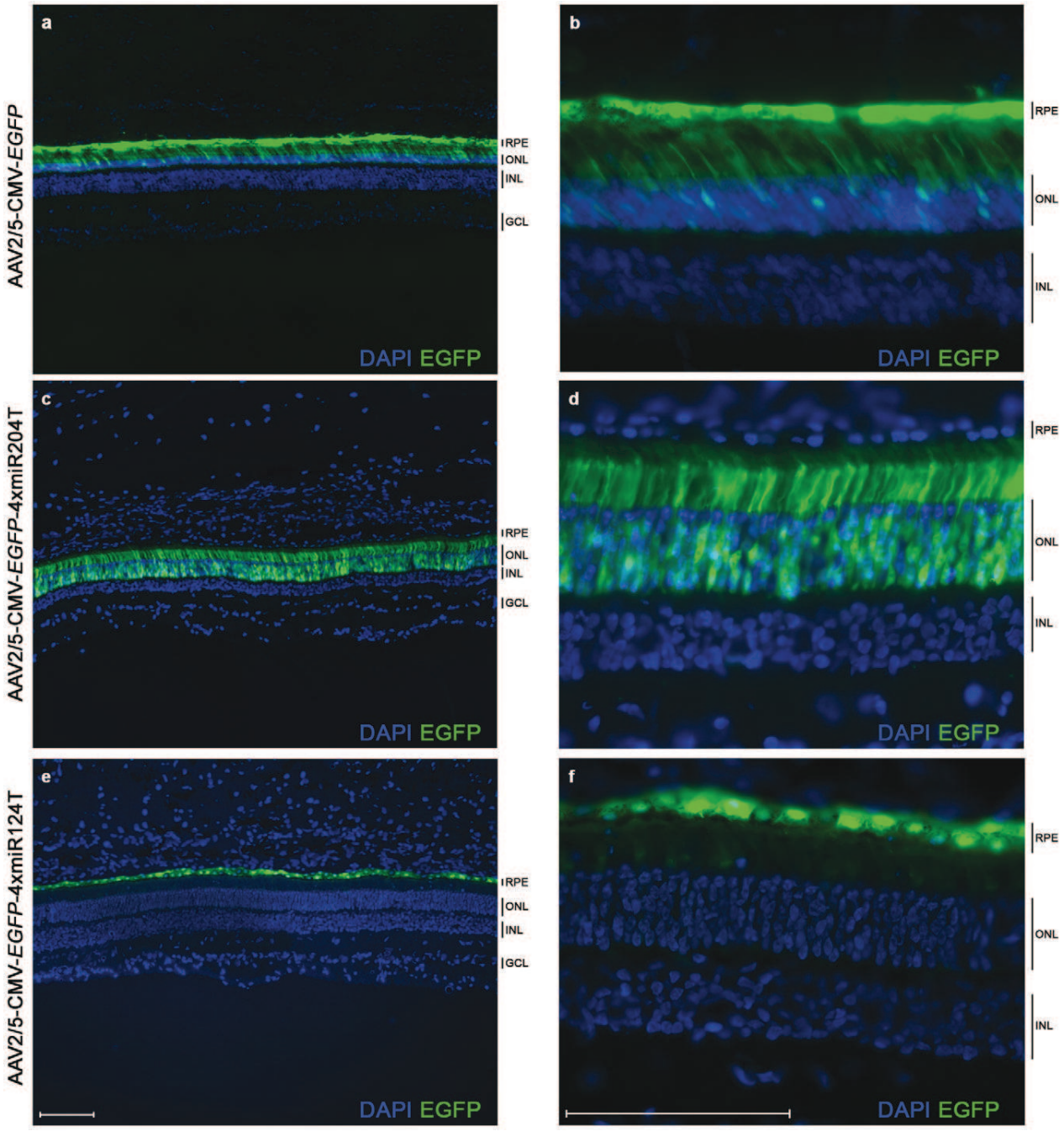

Figure 3. miRNA-regulated *EGFP* expression in the pig retina. Large White (LW) female pigs (n = 2 eyes/group) were injected subretinally with: AAV2/5-CMV-*EGFP* (**a** and **b**); AAV2/5-CMV-*EGFP*-4xmiR204T (**c** and **d**); AAV2/5-CMV-*EGFP*-4xmiR124T (**e** and **f**). All eyes were injected with 1×10^{10} GC/eye of each vector. Retinal cryosections were obtained six weeks after injection and analyzed by direct fluorescence microscopy. Magnification 10X (**a–c**) and 40X (**d–f**). Scale bar = 100 μm. For abbreviations, see Fig. 2 legend.
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