MicroRNA-Restricted Transgene Expression in the Retina

Marianthi Karali1, Anna Manfredi1, Agostina Pupo1, Elena Marrocco1, Annagiusi Gargiulo1, Mariacarmela Allocca1, Michele Della Corte3, Settimo Rossi5, Massimo Giunti5, Maria Laura Bacci4, Francesca Simonelli1,5, Enrico Maria Surace1, Sandro Banfi1,3, Alberto Auricchio1,5

1 Telethon Institute of Genetics and Medicine (TIGEM), Naples, Italy, 2 Medical Genetics, Department of Pediatrics, University of Naples Federico II, Naples, Italy, 3 Medical Genetics, Department of General Pathology, Second University of Naples, Naples, Italy, 4 Department of Veterinary Medical Science (DSMVET), University of Bologna, Bologna, Italy, 5 Department of Ophthalmology, Second University of Naples, Naples, Italy

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.

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 the untranslated region (UTR) of their respective mRNAs [1]. 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 (AF.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 downstream of the WPRE element in the expression plasmid (Figure 1b; see Materials and Methods). The presence of this miRT-bearing vector allows to restrict AAV2/5-CMV-mediated transgene expression to the RPE monolayer (Figure 1a). To restrict the miRT-containing transcript by the endogenous miR-124.

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

To restrict AAV2/5-CMV-mediated transgene expression to the RPE, we exploited miR-204, a miRNA abundantly expressed in differentiating neurons [30]. We and others have shown by ISH that miR-204 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 perfectly complements to the mature miR-124 downstream of the WPRE element in the AAV2/5-CMV-EGFP construct (Figure 1b). The resulting pAAV2.1-CMV-EGFP-miriR124T plasmid was used to produce AAV2/5 vectors that were administered to C57BL/6 mice by subretinal injection. (n = 4) received 2.6 x 10^6 GC (defined as a high dose) of AAV2/5-CMV-EGFP-miriR124T 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-miriR124T (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 exogenous miR204T. 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 x 10^5 GC/e 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-miriR124T (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 electrotroemograms (ERG) on mice injected at a high dose (2.6 x 10^6 GC/e) 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 â­µV (±67,06); miR124T = 350,97 (±132,11) â­µV; miR204T = 320,70 (±105,09) â­µV; max b-wave...
amplitude: EGFP = 667,83 (±616,47) 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]. 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-4xmiR124T and AAV2/5-CMV-EGFP-4xmiR204T 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 2.6×108 GC, in the pig eye 100 μl of a 1:2.6 dilution of the same vector solution, thus containing 1×1010 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 immunolabeling of porcine retinal sections with the Cone Arrestin antibody.

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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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.

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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 × 10^9 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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