Retinal gene therapy with adeno-associated viral (AAV) vectors is safe and effective in humans. However, the limited cargo capacity of AAV prevents their use for therapy of those inherited retinopathies (IRs) due to mutations in large (>5 kb) genes. Viral vectors derived from adenovirus (Ad), lentivirus (LV) and herpes virus (HV) can package large DNA sequences, but do not target efficiently retinal photoreceptors (PRs) where the majority of genes responsible for IRs are expressed. Here, we have evaluated the mouse retinal transduction profiles of vectors derived from 16 different Ad serotypes, 7 LV pseudotypes and from a bovine HV. Most of the vectors tested transduced efficiently the retinal pigment epithelium. We found that LV-GP64 tends to transduce more PRs than the canonical LV-VSVG, albeit this was restricted to a narrow region. We observed more extensive PR transduction with HdAd1, 2 and S/F35++ than with LV, although none of them outperformed the canonical HdAdS or matched the extension of PR transduction achieved with AAV2/8.

INTRODUCTION

Gene therapy has great potential for treatment for many inherited retinopathies (IRs), which are mostly monogenic blinding diseases. Viral vectors based on adeno-associated viruses (AAV) have demonstrated to be safe and efficient in clinical trials for Leber congenital amaurosis type 2 (reviewed in Cideciyan). Leber congenital amaurosis type 2 is an IR due to mutations in the RPE65 gene expressed in the retinal pigment epithelium (RPE). Most of the genes mutated in IRs are expressed in photoreceptors (PRs). Notably, AAV serotypes 5, 7, 8 and 9 target PRs of various species efficiently. However, the cloning capacity of AAV vectors is close to its wild-type genome length (~4.7 kb) and this represents a limitation for gene therapies of several IRs due to defects in larger genes, such as Usher1B (USH1B, OMIM entry #611755). Moreover, AAV limited capacity may prevent the delivery of cassettes with large promoters that maintain transgene expression levels close to physiological as well as cell-type specificity.

Viral vectors that transduce postmitotic cells with transgene capacity higher than AAV include adenovirus (Ad, with a wild-type genome length of ~36 kb), lentivirus (LV, with a wild-type genome length of ~9 kb) and herpes virus (HV, with a wild-type genome length of ~150 kb). The retinal transduction potential of each of them has been evaluated in vivo in animal models: recombinant vectors based on Ad5; the most studied Ad serotype in the context of gene therapy, transduce the RPE primarily when injected subretinally in the mouse retina. LV vectors are enveloped and have been pseudotyped predominantly with the vesicular stomatitis viral glycoprotein (VSVG) since it confers tropism for a broad range of tissues. The LV-VSVG whether based on the human immunodeficiency virus (HIV-1), simian or feline immunodeficiency viruses transduces the RPE mainly when delivered in adult retinas. LV-VSVG vectors based on the equine infectious anemia viruses have been reported to transduce PRs efficiently. However, LV transduction appears mostly limited to PRs in the newborn retina which has a less compact structure than the adult retina.

Here, we aimed at identifying high-capacity Ad, LV and HV vectors with more robust PR transduction efficiency than those described so far. Evolution has modeled the innate ability of viruses to deliver genes to a cell. Ads are divided into subgroups (A to F) and recognize different cellular receptors. In addition, since Ad entry requires high-affinity binding to cell receptors via the knob portion of the fiber, the Ad capsid can be genetically engineered to exchange surface proteins such as the knob (K) or fiber (F) among different serotypes or subgroups, potentially giving rise to modified tropism. Previous works reported that a modified Ad5 (Ad5ΔRGD27,28) and Ad5-based vectors containing heterologous fibers (Ad5/F3729 and Ad5/F3530,31) are efficient for PR targeting. In this work, in addition to naturally-occurring heterologous Ad capsids, we have analyzed PR transduction by eight vectors based on Ad5 mutant capsids (Table 1).

On the other side, heterologous glycoproteins can be easily exchanged among enveloped LV thus generating pseudotyped LV vectors. These may have different transduction properties than vectors with the native envelope glycoproteins. Indeed, it has been shown that HIV may be redirected to the endocytic pathway from its normal route of entry when pseudotyped with VSVG. Thus, we have tested 7 HIV-1 based LV vectors pseudotyped with
Table 1. Viral vectors based on Adenovirus (Ad and HdAd), Lentivirus (LV) or Herpesvirus (HV) evaluated in this study

| Vector | Subgroup | Reference |
|--------|----------|-----------|
| Ad naturally-occurring | HdAd1 | C | 75 |
| n = 8 | HdAd2 | C | 74 |
| | HdAd5 | C | 73 |
| | Ad6 | C | ATCC VR-6 |
| | AdC1 | B | ATCC VR-20 |
| | ChAd7 | E | 42 |
| | ChAd30 | B | 42 |
| | ChAd63 | E | 42 |

External surface protein

| Ad genetically-modified | | |
|-------------------------|--------------------------|------------------|
| Vector | Subgroup | Reference |
| Ad5/F2+pK | Mutant Ad35 fiber with increased affinity for the CD46 ligand in an Ad5 context | 35 |
| Ad5/F2+RGD | RGD motif incorporation in the H-I loop of Ad2 fiber cloned in an Ad5 context | 36 |
| Ad5ΔRGD | RGD motif deleted from penton-base proteins of Ad5 capsid | 37 |
| Ad5/F3 | Fiber from Ad3 cloned in an Ad5 context | 32 |
| HdAd5/K3 | Ad3 knob cloned in an Ad5 context | 38 |
| HdAd5/6 | Ad6 hexon hypervariable regions in Ad5 capsid | 38 |
| Lentivirus | Vesicular stomatitis virus glycoprotein | 85 |
| n = 7 | Jaagsiekte sheep retrovirus | 86 |
| VSVG | Ross River virus | 87 |
| JSRV | Ebola mucin domain-deleted glycoprotein | 77 |
| RRV | Lymphocytic choriomeningitis virus | 85 |
| EBOLAΔO | Mokola virus envelope glycoprotein | 13 |
| LCMV | Baculovirus GP64 envelope glycoprotein | 88 |
| MOKOLA | GP64 | 88 |
| Herpesvirus, n = 1 | BoHV-4 | | 55 |

Various non-native envelope glycoproteins (Table 1), seeking for improved PR transduction. HV vectors present the highest cloning capacity and naturally infect neurons. They are enveloped, but the attachment and entry processes are complex and not fully understood yet, resulting in few pseudotypes available. Here, we have evaluated the ability of a naturally-occurring bovine herpes virus (Table 1), which belongs to a different subfamily (gammaherpesviridae) than HSV-1 (alphaherpesviridae) that was previously investigated in the retina.

RESULTS

We evaluated a total of 16 Ad vectors (eight derived from human and chimpanzee naturally-occurring Ad and eight genetically-modified Ad vectors) for PR transduction after subretinal delivery in the adult mouse retina (see Table 1). The ubiquitous cytomegalovirus (CMV) promoter was included in all vectors to investigate broad cellular tropism on retinal histological sections. The vectors contained either enhanced green fluorescent protein (EGFP) or β-galactosidase (lacZ) as reporter genes, and were subretinally injected in pigmented C57BL/6 or in albino CD-1 or BALB/c mice. Ad vectors were injected at doses ranging from 10^6 to 10^9 viral particles (vp)/eye. Depending on the availability, we used either first-generation (Ad, E1/E3 deleted) or helper-dependent adenoviral vectors (HdAd, deleted of all viral genes and containing only the cis sequences required for vector genome encapsidation), as indicated in Table 1. Because we were interested in vector tropism and eyes were harvested 3–14 days after vector injection, HdAd and Ad can be directly compared for transduction efficiency. This comparison is possible given the rapid onset of transgene expression of Ad vectors, in contrast to AAV vectors. Retinal sections were analyzed for direct EGFP fluorescence or after 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining. We tested the serotype 1 HdAd (HdAd1), serotype 2 HdAd (HdAd2) and serotype 6 Ad (Ad6) vectors from subgroup C that recognize the CAR receptor for initial attachment, followed by internalization through an interaction between the arginine-glycine-aspartic acid (RGD) motif of the Ad penton base with αvβ3 integrins. The genetically-modified HdAd5/F35, HdAd5/F35++, Ad5/F3 and HdAd5/K3 contain fiber or knobs from serotypes reported to recognize the cellular CD46 receptor. Vectors derived from Chimpanzee viruses were classified into subgroup B (AdC1 and ChAd30) or subgroup E (ChAd7 and 63) based on sequence alignments of the hypervariable regions of the hexon gene. Notably, the Ad receptors CAR, αv-integrin and CD46 are expressed by PRs.

Consistent with previous published studies, we found that Ad5 targets mostly the RPE and Müller cells along with some PRs following subretinal injection (Figure 1 and Figure 2). Similar to Ad5, most of the Ad vectors tested targeted efficiently the RPE and some PRs (Figure 1 and Figure 2). However, HdAd1, HdAd2 and HdAd5/F35++ transduced the outer nuclear layer (ONL), which contains PRs, at levels that in some eyes appeared higher and more extensive than with HdAd5 (see Figure 1 and Table 1 for the vector descriptions). No β-galactosidase staining was detected in the retinas of control phosphate-buffered saline (PBS)-injected animals (data not shown).

To confirm that HdAd1, HdAd2 and HdAd5/F35++ transduce adult mouse PRs efficiently as it appears using the CMV-lacZ expression cassette, we produced HdAd vectors containing the PR-specific rhodopsin (Rho) promoter which drives the expression of the EGFP reporter gene. The HdAd vectors were all injected subretinally in C57BL/6 mice at a dose of 5.5 × 10^9 genome copies (GC)/eye, and compared with HdAd5 and AAV2/8, containing the...
same expression cassette and injected at the same dose. Eyes were harvested 14 days or one month later and retinas cryosectioned for further histological analysis. HdAd1, HdAd2 and HdAd5/F35++ showed an extension of PR transduction that was lower than with HdAd5, which was similar to that of AAV2/8 (see Figure 3a). The pattern of PR transduction mediated by HdAd1 and 2 was patchy, as observed in a previous work using a different Ad vector containing a Rho-EGFP cassette.28 HdAd5-mediated PR transduction appeared patchy in some eyes, and continuous in others. In Figures 3a and b, the four best-transduced retinas with each serotype are shown. HdAd1 and 2 seemed to transduce PRs more intensely than HdAd5, although along a narrower area.

LV vectors have a larger cloning capacity compared with AAV. However, VSVG-pseudotyped LV vectors efficiently transduced newborn14-15 but not adult PRs.12-15 Here, we have evaluated six HIV-1-based vectors pseudotyped with various envelopes (Table 1) and compared them with the more studied VSVG. In previous works, the mokola pseudotype was shown to transduce RPE13,43 after subretinal delivery, and RRV to transduce the RPE of Sprague-Dawley rats.44 LCMV- and GP64-pseudotyped vectors transduced the RPE of Wistar rats, although the vectors were based on a simian and bovine immunodeficiency viruses, respectively.45,46 To our knowledge, neither the EbolaΔO nor the JSRV envelopes had been previously tested in the retina. All vectors contained the CMV-EGFP expression cassette; they were injected subretinally in C57BL/6 mice and retinas were harvested 14 days later. The injected doses range between 2 × 10^3 and 1 × 10^5 transducing units (TU)/eye (Figure 4) and they were the maximum allowed for each viral preparation given that 1 μl is the maximum volume that

![Figure 1.](image)

Mouse retinal transduction after subretinal delivery of adenoviral vectors. Ad vectors containing the CMV-lacZ cassette were delivered subretinally in adult CD-1, BALB/c or C57BL/6 mice. Retinas injected with Ad vectors were analyzed 4–14 days later. Magnification, ×20; scale bar, 100 μm. The vector serotypes used are indicated above each panel. The n of retinas, the dose of each vector and the relative transduction observed in photoreceptors (PR), retinal pigment epithelium (RPE) and Müller cells are indicated below each panel. Abbreviations: rpe, retinal pigment epithelium; onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer; n, number of eyes; vp, viral particles; NT, not transduced.
can be delivered subretinally in adult mice. All vectors, excluding JSRV, transduced the RPE (Figure 4). The baculovirus GP64-pseudotyped LV (6 × 10⁴ TU/eye) appeared to drive a more robust PR transduction (Figure 4, see inset) than the standard VSVG (1 × 10⁵ TU/eye), which transduced PRs in one eye out of five (not shown). However, the transduction was restricted to a narrow area. To evaluate whether LV-GP64 drives PR transduction to levels that can be attractive for therapeutic applications, we generated LV–GP64 vectors containing the Rho–EGFP expression cassette. C57BL/6 retinas were injected subretinally with 4.5 × 10⁵ GC/eye of either LV-GP64 or -VSVG vectors for comparison and analyzed at 2 weeks post injection. No EGFP fluorescence was observed with either vector (not shown).

Among the various high-capacity vectors available, we finally evaluated the herpevirus-based BoHV-4-CMV-EGFP. At 2, 7 and 28 days after subretinal injection of 8 × 10⁶ GC/eye, BoHV-4-CMV-EGFP resulted in efficient RPE but modest PR transduction in adult C57BL/6 retinas (Figure 5). To properly evaluate the specific PR transduction of BoHV-4 vectors, we produced one containing the Rho–EGFP cassette and we injected it subretinally in C57BL/6 mice at a dose of 2.5 × 10⁸ GC/eye. AAV2/8 Rho–EGFP was injected in contralateral eyes at 5 × 10⁸ GC/eye for comparison. Retinal cryosections analyzed one month later by fluorescence microscopy showed that only scattered PRs were transduced (Figure 5) by BoHV-4-Rho-EGFP in contrast to the wide transduction achieved with the AAV2/8 vector.

To test the longevity of mouse retinal transduction mediated by high-capacity viral vectors, we injected subretinally C57BL/6 mice with vectors expressing EGFP under the control of the CMV promoter to monitor transgene expression in vivo by non-invasive fluorescent fundoscopy. Fluorescence was detected for 20 months in HdAd5-injected retinas (Figure 6), which almost equals the mouse lifespan and is the most sustained expression reported thus far in the retina for this vector. EGFP expression was maintained for up to: (i) 14 months post injection in the eyes injected with LV-VSVG (Figure 6), -GP64, -RRV or -EbolaΔO (not shown); (ii) 4 months post injection in those containing BoHV-4. These were the latest time points analyzed (Figure 6).

To investigate the safety of subretinal delivery of high-capacity viral vectors, we monitored retinal electrical activity by Ganzfeld electroretinogram (Figure 7) before killing the animals at the time points of the last ophthalmoscopy (shown in Figure 6). The amplitude of the b-waves was not significantly lower in eyes injected with viral vectors than in control eyes.

**DISCUSSION**

In the present work, we compared a large series of 24 different high-capacity viral vectors for their efficiency at transducing the adult mouse retina following subretinal delivery. Finding an efficient vector, capable of carrying large genes, would be a significant step forward for the development of gene therapy approaches for retinal blinding conditions due to defects in large genes such as STGD1, LCA10 and USH1B, for which no therapy is currently available.

We investigated 16 different Ad vectors and found that HdAd5, LV-VSVG, -RRV, -EbolaΔO, -GP64 or BoHV-4 are shown in Figure 7, and compared with the maximum b-wave amplitude of PBS-injected or non-injected controls of similar age. The amplitude of the b-waves was not significantly lower in eyes injected with viral vectors than in control eyes.
Ad5 in terms of transduction efficiency when containing the ubiquitous CMV promoter. The PR transduction levels of HdAd1, HdAd2, HdAd5/F35++ appeared higher than those of HdAd5 when using the CMV-lacZ cassette. Even if HdAd1, 2 and 5 belong to the same C subgroup and recognize CAR receptor for initial binding, it has been reported that subgenus C Ads have differences in the fiber knob region, 48 which could account for different affinities to a target receptor. Differences in transduction capabilities among subgroup C Ads were also found in liver gene transfer studies where Ad2 was found to be a weaker transducer than Ad5. 49 Ad2 retinal transduction had been studied previously, showing β-galactosidase expression in RPE and ganglion cells, 50 but not in PRs. This different outcome could be explained by the short 24-h post-injection time point that was evaluated by the authors, whereas in our experiments PR expression was analyzed four days after injection. However, the use of HdAd1, HdAd2 and HdAd5/F35++ vectors containing the Rho-EGFP cassette resulted in levels of ONL transduction which were overall similar to the most commonly used HdAd5. The transduction differences observed using the CMV-lacZ and the Rho-EGFP expression cassettes could be due to the presence of 'pseudotransduction' after injection of lacZ-expressing vectors. Some β-galactosidase enzyme may diffuse from the RPE to the inter-photoreceptor matrix and to the ONL, as previously suggested. 13,19,51

Figure 3. Mouse photoreceptor transduction after subretinal delivery of HdAd5, HdAd1, HdAd2, HdAd5/F35++ and AAV2/8. Vectors expressing EGFP from the photoreceptor-specific rhodopsin promoter where injected subretinally in adult C57BL/6 mice (5.5 × 10^8 GC/eye), and analyzed 14 days or one month post injection. (a) Montage of whole retinal cross-sections from the four best transduced eyes for each serotype. The extension of photoreceptor transduction is indicated by arrows. The n of retinas showing a transduction pattern similar to those shown in the pictures are n = 8/12 for AAV2/8, n = 6/8 for HdAd5, n = 8/9 for HdAd1, n = 6/9 for HdAd2 and n = 5/8 for HdAd5/F35++. In the remaining retinas, the ONL transduction was either significantly lower than in the retinas shown in the pictures or undetectable. Magnification, ×10. (b) High magnification images of the retinas shown in a. Magnification, ×40; scale bar, 25 μm. A faint, off-target EGFP signal is observed in the retinal pigment epithelium of all retinas (arrows). Abbreviations: rpe, retinal pigment epithelium; onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer.
Among the various LV pseudotypes tested, LV-GP64 appeared to have a greater PR transduction efficiency than LV-VSVG. However, this was limited to a narrow area. The interaction of GP64 with phospholipids on the cell surface was first reported to have a role in baculovirus infection into mammalian cells and lately, binding to heparan sulfate was also found to be involved. Both pathways may have played a role in LV-GP64-mediated retinal transduction. In our case LV-Mokola failed to transduce PRs as it has been previously shown. In the case of LV-LCMV, even if the RPE was transduced, it is possible that we have underestimated its PR transduction properties since its infectious titer could not be assessed on HT-1080 cells, the target cells used to titer the LV vectors used in this study.

Vectors derived from herpes virus are interesting for PR targeting because they naturally infect neurons. Studies with HSV-1 containing the CMV-EGFP expression cassette showed that only the RPE was transduced in most cases, although in one report PR outer segments were also found to be involved. Both pathways may have played a role in LV-GP64-mediated retinal transduction. In our case LV-Mokola failed to transduce PRs as it has been previously shown. In the case of LV-LCMV, even if the RPE was transduced, it is possible that we have underestimated its PR transduction properties since its infectious titer could not be assessed on HT-1080 cells, the target cells used to titer the LV vectors used in this study.

Figure 4. Mouse retinal transduction after subretinal administration of LV vectors. LV vectors containing the CMV-EGFP expression cassette were injected subretinally in adult C57BL/6 mice, and retinas were analyzed 2 weeks post injection. The dose used was the maximum possible for each LV vector based on the prep titers. The n of retinas, the dose of each vector and the relative transduction observed in photoreceptors (PR) and retinal pigment epithelium (RPE) are indicated below each panel. RPE was the only target of most LV pseudotypes but LV-GP64, which transduced some PRs (see inset). Abbreviations: rpe, retinal pigment epithelium; onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer; n, number of eyes; TU, transducing units; NT, not transduced. Magnification, ×20; scale bar, 50 μm.

Figure 5. Mouse retinal transduction mediated by BoHV-4. The BoHV-4-CMV-EGFP vector was injected subretinally (8 × 10⁶ GC/eye) in adult C57BL/6 mice (left panel). Histological analyses performed one month post injection revealed that both the retinal pigment epithelium and some photoreceptors (arrows) were targeted (n = 2/4 retinas with a transduction pattern similar to that shown in the picture, the remaining retinas show fewer transduced photoreceptors). The BoHV-4 vector containing the photoreceptor-specific Rho-EGFP expression cassette was injected subretinally (2.5 × 10⁶ GC/eye, middle panel, n = 10/10) in adult C57BL/6 mice. AAV2/8 containing the same expression cassette was injected alongside at 5 × 10⁸ GC/eye (right panel, n = 4/4), showing that AAV2/8 targets photoreceptors more efficiently than BoHV-4. Abbreviations: rpe, retinal pigment epithelium; onl, outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer. Magnification, ×20; scale bar, 50 μm.

Adeno, lenti and herpesviral vectors in the retina
When PR-specific Rho-EGFP cassette was used, very rare PRs were positive for transgene expression (Figure 5). It is possible that the strong transcriptional activity of the CMV promoter is requested for PR transduction by a BoHV-4-based vector.

The large-capacity vector platforms we have used result in long-term transgene expression in the retina. We show that EGFP expression mediated by HDAd5 is present by fundus ophthalmoscopy for 20 months after injection (Figure 6), while first-generation Ad5 transduction shuts off at 3 months (not shown). Retinal transduction mediated by HDAd5 was previously found to be sustained for up to 12 months. Here, we report a duration that largely exceeds prior studies (20 months) that is close to the lifespan of a mouse and is consistent with previous studies showing multi-year HDAd-mediated expression in the liver of animal models. Consistent with previous studies, we also show that both LV-VSVG and -GP64 express EGFP long-term in the mouse retina for up to 14 months post-injection. In vivo transduction transduction mediated by HSV-1 vectors has been transient with loss of transgene expression by 6 weeks post injection, representing a drawback for their applications in genetic diseases requiring long-term expression of the therapeutic gene. In contrast, we show that subretinal delivery of BoHV-4-based vectors results in stable EGFP fluorescence detectable up to 4 months post injection by ophthalmoscopy. This finding is the first report of long-term retinal transduction mediated by an HV vector.

Subretinal injection of HDAd5, LV-VSVG, -GP64, -RRV- and EbolaΔO or BoHV-4 was not associated with significant reduction of the electroretinogram b-wave amplitude when compared with control eyes (see Figure 7). However, additional studies including a careful histopathological examination are required to rule out potential toxicity resulting from subretinal delivery of high-capacity vectors.

It is somehow puzzling that none of the high-capacity vectors investigated significantly outperforms the others, despite the wide difference in their capsid proteins. In the case of Ad, we have tested capsids recognizing different receptors, covering almost all the reported repertoire (CAR, αβ3.5 integrins, CD46 and others) which are localized in the adult mouse ONL yet we were not able to detect levels of PR transduction dramatically higher than those achieved with the canonical HDAd5. The larger size of the high-capacity particle we have investigated compared with AAV might play a role. Indeed, several AAV serotypes (AAV5, AAV7, AAV8 and AAV9) efficiently transduce PRs and while the diameter of an AAV particle is around 25 nm, the Ad, LV and HV viral vector particles all have diameters >80 nm. Thus, diffusion of larger viral particles towards PRs may be limited by anatomical barriers, which might prevent their interaction with their cognate receptors. In the retina, hindrance may be exerted by the inter-photoreceptor matrix and the outer limiting membrane, which may limit access to PR bodies from the subretinal space where the large vectors are injected. This is indirectly suggested by the higher levels of PR transduction observed for both Ad and LV vectors in the developing mouse retinas where these barriers may be more permissive to the vectors. Indeed, Ad5 transduces PRs efficiently when delivered to neonatal retinas and LV-VSVG transduced PRs efficiently when delivered to neonatal retina and rescued PR degeneration when administered to neonatal but not to mature mouse PRs. As a matter of fact, the inter-photoreceptor matrix was found to be an effective barrier that impaired LV-VSVG from transducing the adult PR layer. Notably, a LV vector based on the equine infectious anemia virus, which was originally shown to transduce and rescue neonatal mouse retinas, has been recently reported to transduce adult rabbit and macaque PRs with satisfactory safety profile. However, so far there is no evidence that these levels of adult PR transduction are therapeutically relevant. The two clinical trials which are currently being carried out for both Usher1B (NCT01505062, NCT02065011) and STGD1 (NCT01367444, NCT01736592) using equine infectious anemia virus vectors will help to understand whether this class of vectors effectively
transduces adult human PRs. Weakened physical barriers due to PR loss are observed in animal models of retinal degeneration such as rd and Rho−/−.84,85 PR transduction mediated by Ad5 and LV-VSVG was shown to be more efficient in rd than in wild-type retinas,5 while more Müller cells were transduced by LV-Mokola in Rho−/− than in control retinas.86 These results suggest that disruption of anatomical barriers may improve Ad- and LV-mediated penetration and thus transduction,85,86 or as well indicate that retinal remodeling during disease may modify the tropism of high-capacity vectors.86 A recent work carried out in adult mouse retinal explants, which lack physical barriers as they are compromised during the process of explantation, have shown that LV-VSVG is not able to transduce mouse PRs.70 Surprisingly, when a human retinal explant was transduced with LV-VSVG, 70 limited PR transduction was observed, which suggests that transduction may also be species dependent.70

In summary, among the 24 different high-capacity viral vectors investigated, we found HdAd5, 2 and S/F35++ to transduce mouse PRs with similar efficiencies to HdAd5, but with an overall PR transduction efficiency that appears lower than the one of AAV2/8 that remains the golden standard among naturally occurring AAV serotypes for inherited PR diseases. Recently, effective gene transfer to mouse PRs has been shown with dual AAV vectors, which significantly expand AAV cargo capacity in the retina.72

In the future, it will be interesting to compare side-by-side the PR transduction efficiency of high-capacity HdAd-based vectors to that of dual AAV vectors, which allow efficient PR transduction although at levels which are lower than those obtained with normal size AAV vectors.71,72

MATERIALS AND METHODS
First-generation Ad vectors
Chimpanzee ChAd7, ChAd30 and ChAd63 preAd plasmid were obtained as described previously.5,6,75 Ad5, AdC1 and Ad6 plasmids were constructed by following the same procedure as described in35 starting from wild-type viruses obtained from ATCC. These plasmids were first digested with Pmel to release the viral ITRs. Then, 3–5 × 10⁶ HEK293/PER.C6 cells were transfected with 10 µg of Ad viral plasmids using Lipofectamine (Invitrogen, Life Technologies, Monza, Italy). This generated recombinant Ad vectors which were then expanded using 2 × 10⁶ cells. Purification was performed by two-step cesium chloride gradient.35 The Ad5, Ad6, AdC1, ChAd7, ChAd30 and ChAd63 vectors containing the CMV-EGFP expression cassette were provided by SC.

Ad5/F2+pK,36 Ad5/F2+RCD36 and AdSARG37 vectors were constructed using a combination of conventional cloning and bacterial RecA-mediated recombination. Ad5-, Ad5/F2+pK- and Ad5/F2+RCD-CMV-lacZ plasmids were digested with Psel and recovered as viruses in 293 cells. Viruses were propagated, purified and titered as described.36 RJP provided these vectors. AdSARG37 was transfected and recovered as a virus in 293 cells, banded in CsCl gradients and collected, dialyzed and aliquotated as described.37 This vector was provided by DMS.

The Tigem Adenoviral Vector Core (PP and NB-P) expanded in 293 cells seed vectors provided by other collaborators, which included Ad5-CMV-lacZ, AdSARG-CMV-EGFP, Ad5/F2+pK-CMV-lacZ and Ad5/F2+RCD-CMV-lacZ.

The E1-deleted Ad vector FGA dsF35-CMV-LacZ24 was a generous gift from Dr David T. Curiel, Washington University, St Louis, MO, USA (and provided to us by PN).

The viral particles (vp)/ml titer of the first-generation Ad vectors, obtained by measuring the absorbance at 260 nm following virion lysis and correction for vector genome size, was provided for each vector by our collaborators.

Helper-dependent Ad vectors
All HdAd vectors were produced in 116 cells as previously described.41 The helper virus AdNG16373 was used to produce HdAd5-Ad5/K3-CMV-LacZ and HdAd5-Rho-EGFP-WPRE-bGHpA. The helper virus Ad2LC8cCARP21 was used to produce Hdad2-CMV-LacZ and Hdad2-Rho-EGFP-WPRE-bGHpA. The helper virus Ad1LC8cCEVS1-1 was used to produce Hdad1-CMV-LacZ and Hdad1-Rho-EGFP-WPRE-bGHpA. The helper virus Ad1-PBGF3533 was used to produce HdadSF35-CMV-LacZ. The helper virus AdNg163-5F35++ (modified as described in Wang et al36) was used to produce HdadSF35++-CMV-LacZ and HdadSF35++-Rho-EGFP-WPRE-bGHpA. The helper virus Ad3S-NG16332 was used to produce Hdad3S-CMV-LacZ. The helper virus AdNg177-28 was used to produce Hdad5S-6-CMV-LacZ. The Hdad vectors were provided by PN. The Tigem Adenoviral Vector Core supplied HdAd5-CMV-lacZ and expanded Hdad5-Rho-EGFP-WPRE-bGHpA.

For the generation of Hdad-Rho-EGFP-WPRE-bGHpA vectors, the Rho-EGFP-WPRE-bGHpA cassette was PCR-amplified from the pAAv2.1-Rho-EGFP77 as well as an envelope plasmid that eGFP)77 as well as an envelope plasmid that

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Figure 7. Electroretinogram (ERG) responses following subretinal injection of HdAd5, LV or BoHV-4. Ganzfeld ERG maximum b-wave amplitudes (± s.e.) in retinas injected with: HdAd5, LV or BoHV-4-CMV-EGFP vectors. The b-wave ERG values of eyes injected with HdAd5 at 1·3 × 10⁸ vp/eye were compared with those of non-injected C57BL/6 mice of similar age. LV-VSVG, -RRV, -EbolaΔO, -GP64 were pooled together in the LV column (n = 2 eyes for each LV pseudotype). LV were injected at 1·0 × 10⁷–1 × 10⁷ TU/eye and the ERG values were compared with those of non-injected C57BL/6 mice. ERG values of eyes that received BoHV-4 at 8 × 10⁹ GC/eye were compared with those of eyes injected with PBS at the same age. Abbreviations: NI, not injected.
confluent HEK293T cells using calcium phosphate transfection. After 12 h, the cells were washed and fresh medium was added (Dulbecco’s modified Eagle’s medium, 2% (vol/vol) fetal bovine serum (FBS), 1% (vol/vol) penicillin–streptomycin). Supernatants were collected at 24, 36, 48, 60 and 72 h post transfection and frozen at –80 °C. The supernatants were thawed, filtered through a 0.45-μm pore-size filter, and pelleted by a 16-h centrifugation step (7700 g at 4 °C) in a Sorvall GSA rotor. Thermo Fisher Scientific, Waltham, MA, USA). The viral pellet was resuspended in Dulbecco’s modified Eagle’s medium for an approximate 200-fold concentration, and the virus was stored at –80 °C until use. Titers were calculated on HT-1080 cells (ATCC) and given in transducing units (TU/ml).

The Rh-oEGF lentiviral plasmid was generated as follows: Rh-oEGF was amplified from the plasmid pAAV2-Rho-oEGFP and using the primer Rv 5′-GCG TGG TGG GCC GGC CCA GCA G-3′ and the primer Fl 5′-AAA TGA TCA ATG AGC AGA TCT TCC CCA CCT AGC AAC CAC-3′ which contained the MfeI restriction site and the primer Rv 5′-TTA ACT CGA GCT TGT ACA GCT CGT CCA TGC CG-3′ that contained the XhoI site.

The PCR product was subcloned in pCR2.1-TOPO (Invitrogen, SRL, Milan, Italy) and sequenced. The Rho-oEGFP fragment was then digested with MfeI/ XhoI, purified and ligated with the LV plasmid backbone. The backbone consisted of the pSWMU Universal Lentiviral Expression Vector (promoterless) VPK211 (Cell Biologics, San Diego, CA, USA) digested with EcoRI/SalI (sharing compatible cohesive ends with MfeI and XhoI of the insert, respectively). The obtained LV-Rh-oEGFP plasmid was then sequenced and sent to the Gene Transfer Vector Core (University of Iowa, Iowa City, IA, USA) for production of LV vectors pseudotyped with either the VSVG or the GP64 envelope glycoproteins or with viral particles harboring Env.CD46. The resulting clones were obtained using the Lentivirus qPCR Titre Kit (Applied Biological Materials, Richmond, BC, Canada). Transducing/infected titers on HT-1080 cells could not be calculated, as the Rho promoter is inactive in this cell type. The LV vectors were provided by BLD.

BoHV-4 vectors

BoHV-4-A,78 BoHV-4-A-Rho-EFGPA75 and BoHV-4-A-Rho-EFGP-EWPREΔTK were propagated by infecting confluent monolayers of Madin Darby bovine kidney (ATCC, CCL-22), bovine embryo kidney (IBEK) provided by Dr. Filomena István, István Tüsköcsi Sperimentale, Brescia, Italy; (BS CL-94) or BEK-expressing cre recombinase (BEKcre)76 at a multiplicity of infection (MOI) of 50 (50% tissue culture infectious dose) per cell and maintained in minimal essential medium (MEM, Sigma, Milan, Italy) with 2% FBS for 2 h. The medium was then removed and replaced with fresh MEM containing 10% FBS. When approximately 90% of the cell monolayer exhibited cytopathic effect (72 h post-infection), the virus was prepared by freezing and thawing cells three times and pelleting the virions through 30% sucrose, as described previously.79 Virus pellets were resuspended in cold MEM without FBS. TCI5 were determined by limited dilution in Madin Darby bovine kidney cells. The physical titers of the CMV-EGFP vector were calculated as GC/ml by TaqMan PCR with a probe recognizing EGFPA.

The sequence of the FAM-labeled TaqMan probe used is:

| FAM-fl | Rv 5′-GCT CAG TAC ACA AGC CTA GCA-3′ |
|---|---|

For the BoHV-4-A-Rho-EFGP-EWPREΔTK cloning, Rho-EFGP-EWPRE-bGHpA was excised from the pAAV2.1-Rho-EFGP plasmid76 with NheI/XhoI, blunted with T4 DNA polymerase and subcloned in a Smal-digested pNST2 shuttle vector75; a plasmid vector containing two BoHV-4 TK genes, to obtain pNNK-Rho-EFGP-WPRE-TK. Next, TK-Rho-EFGP-WPRE-TK was cut out from the plasmid backbone and electrotransfected in SW102 Escherichia coli containing the pBAC-BoHV-4-A-KanaGalkATK.78 pBAC-BoHV-4-A-Kana- 

GaKATK is a BoHV-4 genome clone coming from a non-pathogenic strain of BoHV-4 isolated from the milk cell fraction of an healthy cow, whose genome was cloned as a bacterial artificial chromosome (BAC), pBAC-BoHV-4-A, and where its TK locus was targeted with a KanGalk selectable cassette (GC/m) of vectors with the Rho-EFGFPA containing the E. coli containing the pBAC-BoHV-4-A-KanaGalkATK were first heat induced, then negatively selected on deoxygalactose minimal plates. The resulting clones were additionally negatively selected with medium containing kanamycin. Then, to obtain pBAC-BoHV-4-A-Rho-EFGP-WPREΔTK, the retargeting was performed to the same site to replace the KanGalk cassette with the TK-Rho-EFGP-WPRE-TK cassette. Retargeted clones (pBAC-BoHV-4-A-Rho-EFGP-WPREΔTK) were distinguished from the unretargeted control clone (pBAC-BoHV-4-A-KanaGalkATK), by HindIII digestion. The selected clones’ stability was assessed by serially passing over 25 days and analysis by HindIII restriction enzyme digestion. Infectious BoHV-4-A-Rho-EFGP-WPREΔTK virus was reconstituted in both BEK and BEKcre cells,78 which enabled the deletion of the floxed BAC cassette from pBAC-BoHV-4-A-Rho-EFGP-WPREΔTK. Furthermore, BoHV-4-A-Rho- 

EFGP-WPREΔTK, BoHV-4-A-EFGPΔTK and BoHV-4-A were compared in terms of replication and no differences were observed among them. The Rho-EFGP vector physical titer was calculated by TaqMan PCR with a probe recognizing the bGHpA sequence, as explained in the Materials and Methods section for the HAd vectors. All BoHV-4 vectors were provided by GD.

AAV Vectors

AAV2/8 vectors were produced by the TIGEM AAV Vector Core (Napoli, Italy) using the pAAV2.1-CMV-EFGP70 and -Rho-EFGP expression plasmids, and the packaging pAAV2/8 plasmid.81 Vectors were produced by triple transfection of 293 cells followed by cesium chloride purification.81 For each viral preparation, physical titers (GC/ml) were determined by both PCR quantification using TaqMan (PerkinElmer Life and Analytical Sciences, Boston, MA, USA) and dot-blot analysis.82

Animal procedures and vector administration

Ethics Statement. All studies on mice were conducted in strict accordance with: (i) the institutional guidelines for animal research; (ii) the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research; (iii) the Italian Ministry of Health regulation for animal procedures. All procedures on mice were submitted to the Italian Ministry of Health; Department of Public Health, Animal Health, Nutrition and Food Safety on 17th October 2011. The Ministry of Health approved the procedures by silence/consent, as per article 7 of the 116/92 Ministerial Decree. Surgery was performed under anesthesia and all efforts were made to minimize suffering.

Mice. Four- to five-week-old male C57BL/6, CD-1 or BALB/c mice (Harlan, S. Pietro al Natisone, Italy) were anesthetized with an intraperitoneal injection of 2 ml per 100 g body weight of avertin (1.25% w/v of 2,2,2-tribromoethanol and 2.5% w/v of 2-methyl-2-butanol (Sigma-Aldrich, Milan, Italy)),83 then viral vectors were delivered subretinally via a trans-scleral/choroidal approach as described.47 A volume of 1 μl of viral vectors was injected into PBS was delivered subretinally.

Histological analysis

Mice were killed and their eyeballs were harvested and fixed overnight by immersion in 2 or 4% paraformaldehyde. Before harvest, the temporal aspect of the sclera was marked by cautery 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 eyes were infiltrated with 30% sucrose for cryopreservation and embedded in tissue-freezing medium (O.C.T. matrix, Kaltek, Padua, Italy). For each eye, 150–200 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 15–20 sections, each section representing the whole eye at different levels. The sections were stained with 49,69-diamidino-2-phenylindole (Vectorshied, Vector Lab, Peterborough, UK) if the vector contained EGFP as a reporter protein. If the viral vector contained lacZ as the reporter gene, the cryosections were treated for X-gal staining (see below). EGFP and X-gal were monitored with a Leica DM 5000B microscope (Leica Microsystems, Wetzlar, Germany) at various magnifications.

X-gal staining

Cryosections were fixed in 0.5% glutaraldehyde in PBS+ buffer (1 mM CaCl2 and 0.5 mM MgCl2 in PBS (pH 7.4)) for 10 min at room temperature. After fixation, three 5-minute rinses were performed with PBS+ buffer. Sections were stained using X-gal solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mM MgCl2 and 0.5 mM mg- powder in PBS+ buffer) at room temperature with protection from light. The reaction was stopped when staining was observed in the sample cryosections, and was analyzed only if the negative control (cryosections from non-injected or PBS-injected retinas) were X-gal negative. After they were X-gal stained, the sections were counterstained with eosin, dehydrated and coverslipped.

Funding photography

Funding imaging was performed from 4 days to 20 months post viral injection (PI) using a Topcon TRC-50IX retinal camera connected to a

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Adeno, lenti and herpesviral vectors in the retina

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charge-coupled-device NikonD1H digital camera (Topcon Medical System, Oakland, NJ, USA). Mice ocular fundi were photographed using a 300 W flash with a fluorescein filter, after dilating the pupils with a drop of tropicamide 1% (Visufarma, Roma, Italy).

Electroretinogram recordings
Electrophysiological recordings in mice were performed as detailed.\textsuperscript{64}

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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