

Rapid and Stable Knockdown of an Endogenous Gene in Retinal Pigment Epithelium

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ABSTRACT

The selective silencing of target genes in specific cell types by RNA interference (RNAi) represents a powerful approach both to gene therapy of dominantly active mutant alleles, and to the investigation of normal gene function in animal models *in vivo*. We established a simple and versatile *in vitro* method for screening the efficacy of DNA-based short hairpin RNAs (shRNAs), and identified a highly effective shRNA targeting basic fibroblast growth factor (bFGF), a gene thought to play important roles in endogenous neuroprotective responses in the rat retina. We used two viral vectors, based on lentivirus and adeno-associated virus (AAV), to deliver shRNAs and silence bFGF in retinal pigment epithelial cells *in vivo*. The AAV experiments made use of a “stabilized double-stranded” version of these vectors with rapid onset of gene expression. In the rat retinal pigment epithelium, shRNAs delivered by either vector reduced bFGF immunoreactivity to undetectable levels in transduced cells, whereas a nonfunctional control construct incorporating a two-base pair mutation had no measurable effect on bFGF expression. Silencing commenced within a few days after injection of virus and remained stable throughout the period of observation, as long as 60 days. Viral delivery of RNAi constructs offers a powerful and versatile approach for both gene therapy and the analysis of fundamental questions in retinal biology.

INTRODUCTION

RNA INTERFERENCE (RNAi) is an approach for knocking down gene expression *in vitro* and *in vivo*, with considerable promise for human gene therapy. RNAi takes advantage of an endogenous cellular pathway that has evolved for the control of gene expression and as an antiviral defense (Hammond, 2005; Buchon and Vaury, 2006). When triggered by small double-stranded RNAs, this pathway inhibits the expression of genes with sequence similarity to the trigger. Depending on the degree of sequence identity and other incompletely understood factors, the RNAi machinery may cleave target mRNA, inhibit translation of mRNAs without cleaving them, or silence gene expression at the level of transcription (Hammond, 2005; Buchon and Vaury, 2006).

Appropriately targeted RNAi could be used in gene therapy to silence dominantly acting pathogenic mutant alleles, or nor-

mal alleles whose overexpression contributes to disease. Ocular gene therapy is a particularly promising field for RNAi because of the ease and precision with which exogenous nucleic acids can be delivered to the eye, with minimal risk of systemic spread. Small, double-stranded RNA species that trigger RNAi, siRNAs, have been used to induce RNAi in the retina *in vivo*. Their potential value for gene therapy is suggested by animal studies demonstrating suppression of pathogenic ocular neovascularization after knockdown of the angiogenic factor VEGF (vascular endothelial growth factor) or its receptors *in vivo* (Shen *et al.*, 2006).

Despite their broad applicability and rapid onset, a major drawback of siRNAs as therapeutics is their limited lifetime *in vivo*, with knockdown effects typically lasting no longer than 1 week (Arai-Gaun *et al.*, 2004; Shen *et al.*, 2006), an insufficient duration for most human diseases. An alternative approach uses DNA-based RNAi constructs (Brummelkamp *et al.*, 2002;

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Paddison *et al.*, 2002) that drive transcription of small, self-complementary “short hairpin” RNAs (shRNAs) that also function as RNAi triggers. shRNAs hold potential for longer persistence *in vivo* and regulated expression (Fritsch *et al.*, 2004; Gupta *et al.*, 2004), but require specialized delivery methods that can be difficult to target to specific cell types. In this study, we used viral vector-mediated delivery to express shRNAs in retinal cells *in vivo*, to take advantage of the different cellular tropisms and long-term expression observed with viral vectors.

Persistent shRNA expression in retinal cells *in vivo* also has the potential to create loss-of-function animal models of human disease that could be used to test experimental therapies, or to address basic questions in retinal cell biology. Compared with conventional germline knockout technologies, RNAi offers several distinct advantages. It is faster and less expensive than generating knockout rodent lines, and the methodology can be applied to adult animals, avoiding possible effects on development. Gene expression can be perturbed in a single eye while retaining the fellow eye of the same animal as a control. RNAi can be used in nearly all species tested to date, including larger mammals, which have proven to be extremely valuable models of human retinal disease (Acland *et al.*, 2001).

We selected basic fibroblast growth factor (bFGF) as a target gene for proof-of-concept RNAi studies. The first successful neuroprotective studies in the retina employed intravitreal injection of bFGF protein (Faktorovich *et al.*, 1990, 1992). Moreover, a large body of evidence suggests that bFGF functions as an endogenous neuroprotective factor in the rat retina. Several forms of preconditioning stress have been shown to upregulate bFGF expression (Faktorovich *et al.*, 1992; Wen *et al.*, 1995; Liu *et al.*, 1998; Xiao *et al.*, 1998), and subsequently protect photoreceptors from inherited or constant light-induced degeneration. However, no loss-of-function data are available in rats to support the hypothesis that bFGF is an endogenous neuroprotective factor in the retina. Knockout mice are unlikely to offer a fruitful model for the study of endogenous neuroprotection in the retina, because the protective preconditioning responses observed in rats are weak or absent in mice (LaVail *et al.*, 1998; Xiao *et al.*, 1998). Upregulation of bFGF and FGF receptors in response to stress is also much weaker in mice than in rats (Cao *et al.*, 1997; Xiao *et al.*, 1998; Ozaki *et al.*, 2000), and exogenously supplied bFGF protein is ineffective as a neuroprotective agent in mice (LaVail *et al.*, 1998).

In this study, we show that both lentiviral (LV) and adeno-associated viral (AAV) vectors can be used to deliver shRNAs to the rat retinal pigment epithelium (RPE) *in vivo* and mediate knockdown of bFGF. Knockdown of the target protein is effective within a few days and is stable. Our results clearly demonstrate the use of virally encoded shRNAs to achieve long-term knockdown of gene expression in the mammalian RPE *in vivo*.

MATERIALS AND METHODS

Constructs

To create a general target construct for validating shRNA activity *in vitro* (pYT1), the cytomegalovirus (CMV) promoter and DsRed (Discosoma sp. red fluorescent protein) were am-

plified by polymerase chain reaction (PCR) from pCMV-DsRed-Express (Clontech, Mountain View, CA) using primers 5'-TTACGATTCCTTAAGGGCCTTTTGTGGCCTTTT-GCTCA and 5'-GGTTCACGTAGTGGGCCATCGCCC. The product was ligated into the unique *Afl*III site of pEGFP-N1 (Clontech) and its orientation was confirmed by sequencing. To generate a target construct for bFGF (pYT1-bFGF), a segment of rat bFGF cDNA (GenBank accession number NM_019305) was amplified by reverse transcription (RT)-PCR from total retinal RNA, using primers 5'-TTACGATTCAGATCTGG-GGAGCCCCGAGAGCTGCCGCAG and 5'-TTACGATTC-AAGCTTGCTCTTAGCAGACATTGGAAGAAA. The product, corresponding to nucleotides 483–994, was inserted into the multiple cloning site of pYT1 in frame with enhanced green fluorescent protein (EGFP), using *Bgl*III and *Hind*III. These and all other constructs were confirmed to be free of sequence errors before use.

shRNA expression cassettes were generated by PCR using pGEM-Zeo-U6 (gift from G. Hannon, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) containing the human U6 promoter as the template. All reactions used forward primer 5'-CACCTCGAGGATTTAGGTGACACTATAG. Reverse primers incorporated the entire shRNA sequence, with a 25-base stem and an 8-base loop containing an *Hind*III site. The shRNA used for *in vivo* experiments, denoted sh-bFGF, corresponded exactly to the sequence 5'-AGAAGGCCATACTGTTCTTCCAAT in bFGF mRNA. The shRNA base pairs corresponding to the two underlined bases were interchanged in the negative control sh-mut2. We found the GeneAmp XL PCR kit (Applied Biosystems, Foster City, CA) with 1.0 mM magnesium acetate to be most effective for generating error-free PCR products. These were cloned into pENTR/D-TOPO (Invitrogen, Carlsbad, CA) for *in vitro* analysis.

Lentiviral constructs were derived from pLL3.7 (Rubinson *et al.*, 2003). The U6 promoter and multiple cloning site of pLL3.7 were replaced with the sh-bFGF expression cassette that had been tested *in vitro*, or with an empty U6 promoter lacking an shRNA. The CMV promoter was replaced with a 1.6-kb fragment of the mouse CD44 promoter (Hebbard *et al.*, 2000) or with a 487-bp fragment of the chicken glutamine synthetase promoter (Li *et al.*, 1995) (gift from Anthony Young, Ohio State University, Columbus, OH).

To generate sdsAAV proviral plasmids bearing shRNAs, shRNA expression cassettes released from pENTR/D-TOPO by digestion with *Bam*HI and *Asc*I were ligated in a single reaction to a *Bgl*III–*Sal*I fragment of pTR.mOP500.GFP (Flannery *et al.*, 1997) containing a 472-bp fragment of the mouse opsin promoter driving GFP, and a “stabilized double-stranded” AAV (sdsAAV) vector backbone (Grimm *et al.*, 2006) digested with *Asc*I and *Sal*I.

RNA interference in vitro

Plasmid pYT1-bFGF (0.2 μ g) was transiently cotransfected with shRNA constructs into HEK 293 cells grown to 70% confluence in 6-well plates, using Lipofectamine and Plus reagents (Invitrogen) according to the manufacturer's instructions. shRNAs were transfected in amounts ranging from 10 ng to 0.5 μ g. Because pYT1-bFGF is twice the size of an shRNA plasmid, 0.5 μ g of shRNA was equivalent to a 5-fold molar ex-

cess relative to pYT1-bFGF. Various amounts of an empty shRNA plasmid, containing the U6 promoter but lacking an shRNA, were included to ensure that the total amount of DNA was the same in all experiments. After 48 hr, cells were washed and total protein was extracted with lysis buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, pH 8.0). Protein expression was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting with antibodies to GFP (rabbit polyclonal, diluted 1:20,000; Invitrogen Molecular Probes, Eugene, OR) and DsRed (mouse monoclonal, diluted 1:16,000; Clontech). Detection and quantification were performed with horseradish peroxidase (HRP)-conjugated secondary antibodies (diluted 1:10,000; Promega, Madison, WI), Super Signal West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL), and a VersaDoc 1000 imaging system (Bio-Rad, Hercules, CA).

Vector production, injections, and imaging

Packaging, concentration, and titering of viral vectors were performed as described (Grimm, 2002; Greenberg *et al.*, 2007). sdsAAV was pseudotyped with the serotype 5 capsid, and LV with the vesicular stomatitis virus glycoprotein (VSV-G) envelope protein. All animal studies were conducted in accordance with the guidelines of the University of California, San Francisco and Berkeley Committees on Animal Research. Viral vectors were injected into the subretinal space of postnatal day (P)16–P30 Sprague-Dawley rats as described (Greenberg *et al.*, 2007) (2 μ l; sdsAAV, 7×10^{11} genomes/ml; LV, 1×10^9 infectious particles/ml). GFP expression was assessed *in*

in vivo with a RetCam II (Clarity Medical Systems, Pleasanton, CA) to image the fundus of the eye in living anesthetized rats.

Cryosections and immunostaining

Eyes were enucleated 3 to 60 days after injection as described (Johnson *et al.*, 2003), cryosectioned at a thickness of 14 μ m, and immunostained as described (Johnson *et al.*, 2003) with antibodies to bFGF (mouse monoclonal, diluted 1:200; Upstate Cell Signaling Solutions, Lake Placid, NY), GFP (rabbit polyclonal, diluted 1:2000; Invitrogen Molecular Probes), or BD Transduction Laboratories glutamine synthetase (mouse monoclonal, diluted 1:1000; BD Biosciences, San Jose, CA). Images were acquired with a Zeiss LSM-510 META confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

RESULTS

Validation of RNAi constructs *in vitro*

We devised a simple and versatile *in vitro* system for rapidly assessing the efficacy of shRNAs targeting any gene of interest. Our target construct contains GFP and DsRed expression cassettes under the control of separate copies of the CMV promoter (Fig. 1A). A multiple cloning site is present upstream of GFP, but not in the DsRed cassette, allowing for the in-frame cloning of the gene of interest to create a GFP fusion. The use of a GFP fusion enables direct assessment of the abundance of the protein target in living cells (Fig. 1C and E); DsRed ex-

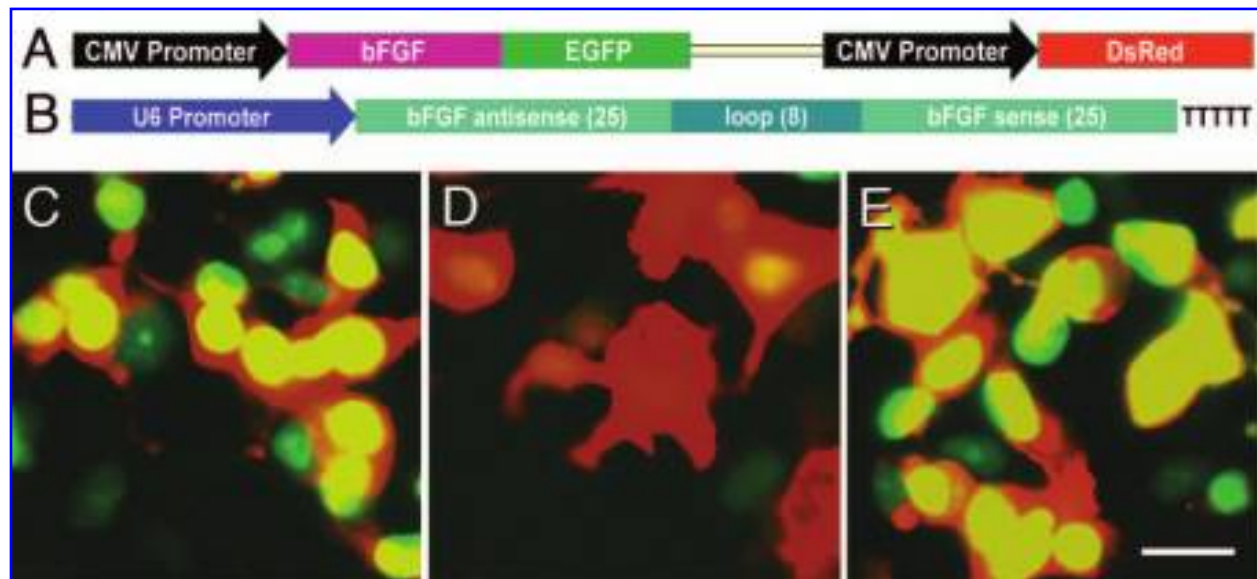


FIG. 1. Validation of an shRNA targeting bFGF in living cells *in vitro*. (A) Target construct. Fusion of bFGF to GFP permits visualization of expressed protein; DsRed marks all transfected cells regardless of bFGF–GFP expression level, and serves as an internal control for transfection efficiency in quantitative experiments. (B) shRNA constructs. All shRNAs have a stem–loop structure with a 25-base stem. Control constructs had mutation of two bases in the stem (sh-mut2), or the U6 promoter with no shRNA (empty vector). The 3' end of the U6 promoter included the 27-mer U6 leader sequence (Paul *et al.*, 2002). TTTT, termination signal. (C–E) Cotransfection of target and shRNA plasmids into HEK 293 cells. (C) Target cotransfected with empty vector. Both bFGF–GFP (green fluorescence) and DsRed (red fluorescence) are present. (D) Target with active shRNA targeting bFGF (sh-bFGF). Expression of bFGF–GFP is mostly silenced, but DsRed is still present. (E) Target with sh-mut2. This control construct is inactive in silencing bFGF–GFP expression. Scale bar: 40 μ m.

pression serves as an internal transfection control by marking all cells that receive the target plasmid, regardless of knockdown of the gene of interest. The GFP fusion also permits straightforward quantitation on immunoblots for any protein, using commercially available anti-GFP antibodies. Target abundance can be normalized to the DsRed signal to control for minor variations in transfection efficiency among experiments. A fusion protein approach similar to ours has been used to assess shRNA-mediated knockdown by flow cytometry, but it employs a transfection control not encoded by the target plasmid (Ho *et al.*, 2006).

When our target construct bearing a bFGF–GFP fusion was transiently transfected into cultured cells in the absence of an shRNA, DsRed and bFGF–GFP were both expressed (Fig. 1C). In some cells that expressed the target construct at low levels, DsRed expression was more difficult to observe (Fig. 1C). Preliminary tests of shRNAs, using this system, suggested that shRNA constructs with 25-mer stems (McCaffrey *et al.*, 2003) were equivalent to or slightly more effective than those with 21-mer or 29-mer stems, and that inclusion of the 27-mer U6 leader sequence (Paul *et al.*, 2002) modestly improved knockdown. These parameters were used in all subsequent experiments. All shRNAs were driven by the human U6 promoter (Paddison *et al.*, 2002) and incorporated an 8-base loop previously shown to be consistent with shRNA function (McCaffrey *et al.*, 2002). Our shRNA design is shown in Fig. 1B. Stem sequences were selected from the rat bFGF-coding sequence based on published guidelines (Khorova *et al.*, 2003; Schwarz *et al.*, 2003; Reynolds *et al.*, 2004).

We tested nine shRNAs, four of which dramatically reduced green fluorescence due to bFGF–GFP (Fig. 1D), demonstrating knockdown of the fusion protein. DsRed expression was unaffected, so that transfected cells fluoresced red (Fig. 1D). One of the effective shRNAs (hereafter sh-bFGF) was selected for further analysis. A negative control, sh-mut2, was generated by reversing the positions of 2 base pairs near the center of the sh-bFGF stem (see Materials and Methods). When tested *in vitro*, sh-mut2 was ineffective in reducing bFGF–GFP fluorescence (Fig. 1E).

We quantified knockdown by immunoblotting (Fig. 2A). When the target construct was cotransfected with an empty RNAi construct lacking an shRNA, the bFGF–GFP fusion protein could be detected as an intense band migrating at approximately 52 kDa, consistent with the combined molecular weights of bFGF and GFP. sh-bFGF markedly decreased bFGF–GFP expression, whereas the level of DsRed was not affected (Fig. 2A). It is likely that our method slightly underestimates the degree of knockdown, because some cells that receive the target plasmid in cotransfection experiments may not also receive the shRNA plasmid (Cashman *et al.*, 2006).

Because expression levels of the target and shRNA constructs *in vitro* may not accurately reflect those in the retina, we assessed the robustness of RNAi over a 20-fold range of concentration of the shRNA plasmid (Fig. 2B). When the target and shRNA constructs were present in equimolar amounts, more than 80% knockdown was observed. Approximately 90% knockdown occurred with a 2:1 excess of sh-bFGF, whereas more than 50% silencing was observed even with a 10-fold excess of the target, conditions under which some cells may not have been transfected with the sh-bFGF construct at all. These

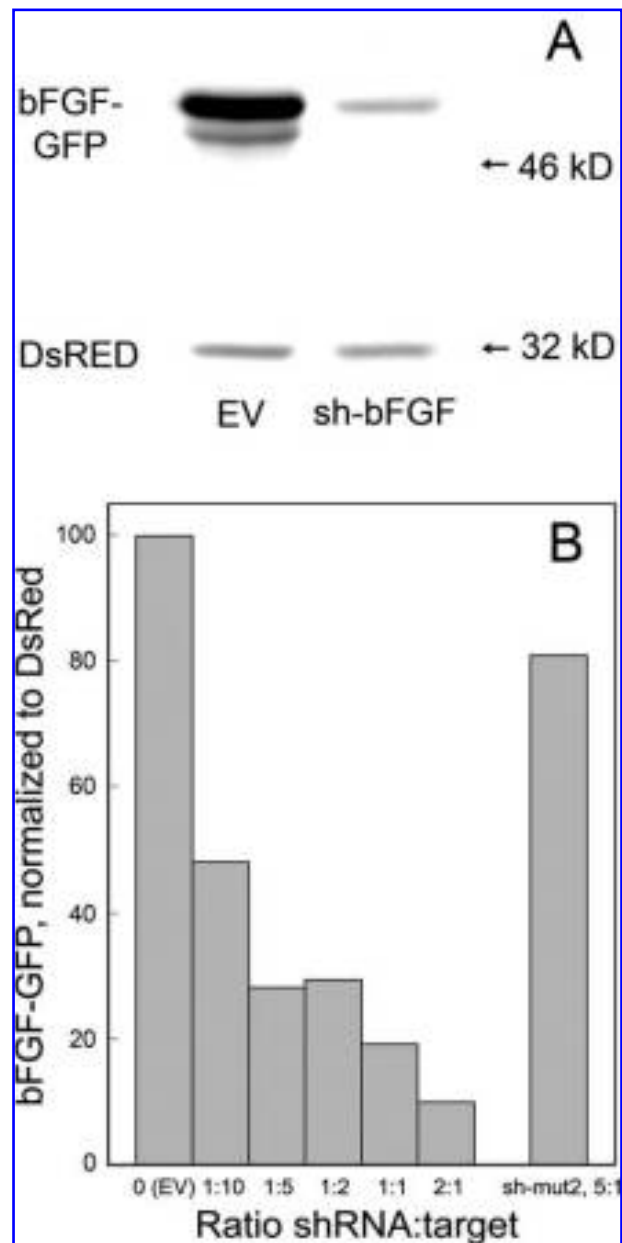


FIG. 2. Quantification of bFGF knockdown. (A) Immunoblot analysis of total protein collected from HEK 293 cells 48 hr after cotransfection of bFGF–GFP target and sh-bFGF or empty vector (EV). The molar ratio of sh-bFGF or EV to target was 2:1. (B) Dilution series. A single representative experiment is shown. Silencing is observed even with a 10-fold excess of target. sh-mut2 produces minimal knockdown even at 5-fold excess to target. A similar degree of silencing was observed at the mRNA level by RT-PCR (data not shown).

results suggested that even low expression of sh-bFGF *in vivo* might be sufficient for silencing. The control construct sh-mut2 produced little or no knockdown even at a 5-fold excess relative to the target (Fig. 2B). RT-PCR analysis gave quantitatively similar results to immunoblotting over the same range of sh-bFGF concentrations (data not shown), suggesting that sh-bFGF acts at the mRNA level.

Knockdown in vivo using lentiviral vectors

Lentiviral (LV) vectors have been widely used to introduce shRNAs into cells in culture and *in vivo* (Rubinson *et al.*, 2003; Tiscornia *et al.*, 2003). When injected subretinally, LV efficiently transduces the RPE (Miyoshi *et al.*, 1997). RPE cells normally exhibit nuclear immunoreactivity for bFGF (Walsh *et al.*, 2001). We examined the effect of a lentiviral vector with the U6 promoter driving sh-bFGF, and GFP under the control of the glutamine synthetase promoter (LV.sh-bFGF; Fig. 3A). This vector supported GFP expression in the RPE (Fig. 3B) at all times examined. As early as 3 days and as late as 28 days after injection, nuclear bFGF immunoreactivity was absent in transduced RPE, but present in adjacent nontransduced RPE (Fig. 3B). Similar results were observed at three postinjection time points, with two separate experimental replicates for each time point and a minimum of three rats in each experiment. A vector lacking sh-bFGF did not cause knockdown (Fig. 3C).

Knockdown in vivo using AAV vectors

We next asked whether a different viral vector, AAV, could be used to drive effective RNAi in the outer retina *in vivo*. The advantages of AAV as a gene transfer vector include its lack of pathogenicity and long-lasting gene expression (Grimm and Kay, 2003). AAV has been used extensively to transduce cells in the retina (Auricchio *et al.*, 2001; Bennett, 2003; Lotery *et al.*, 2003; Acland *et al.*, 2005; Dinculescu *et al.*, 2005; Le Meur *et al.*, 2005; Gorbatyuk *et al.*, 2007). Its tropism in the retina differs from that of LV, raising the possibility of analyzing the effects of target gene knockdown in distinct cell populations. AAV serotypes 2 and 5 transduce both RPE and photoreceptors, whereas AAV-1 and -6 are tropic for RPE (Auricchio *et al.*, 2001; Yang *et al.*, 2002). Most evidence suggests that AAV transduces Müller cells inefficiently (Harvey *et al.*, 2002). A potential drawback of AAV is the relatively slow onset of gene expression, believed to be due to the need to convert the single-stranded DNA (ssDNA) genome to double-stranded DNA

(dsDNA). Conversion can occur by second-strand synthesis or recruitment of a complementary strand delivered by a second viral particle (Fisher *et al.*, 1996; Nakai *et al.*, 2000), both slow processes. To avoid this shortcoming and to promote rapid RNAi, we used a “stabilized double-stranded” AAV (sdsAAV) (Grimm *et al.*, 2006). This vector carries a deletion of the terminal resolution (nicking) site in one of the inverted terminal repeats (ITRs), causing it to retain the structure of a self-complementary intermediate generated during packaging (McCarty *et al.*, 2003). This intermediate is packaged in the viral particles, and need only refold inside the target cell to assume a pseudo-double-stranded conformation competent to drive gene expression. ITRs from two different AAV serotypes were used to prevent repair of the terminal resolution site by recombination (D. Grimm and M.A. Kay, unpublished data). The major drawback of sdsAAV is the limitation of the insert size to half that of standard AAV vectors (~2.2 kb), but the small size of shRNA constructs mitigates this concern.

Our sdsAAV constructs are schematized in Fig. 4A. sdsAAV.sh-bFGF carried the active shRNA, sdsAAV.sh-mut2 the inactive control. Having demonstrated RNAi in RPE using LV, we optimized our AAV vectors for assessment of gene expression and RNAi in photoreceptors. We pseudotyped the vectors with the AAV-5 capsid, which transduces photoreceptors more efficiently than AAV-2 (Yang *et al.*, 2002). The GFP reporter is driven by the mouse opsin (mOPS) promoter (Flannery *et al.*, 1997), which is superior to the “ubiquitous” CMV promoter for photoreceptor gene expression (Miyoshi *et al.*, 1997). The mOPS promoter does not support reporter expression in RPE (Flannery *et al.*, 1997), but because AAV-5 is known to transduce RPE with high efficiency (Auricchio *et al.*, 2001), we hypothesized that bFGF knockdown would be evident in RPE cells in retinal regions with GFP-expressing photoreceptors.

Onset of GFP expression with sdsAAV was rapid, consistent with another study (Yokoi *et al.*, 2007). Green fluorescence was apparent by retinal imaging of live animals 6 days after injection (Fig. 4B) and was even more intense at 16 days postinjection (Fig. 4C; compare with less intense single-stranded

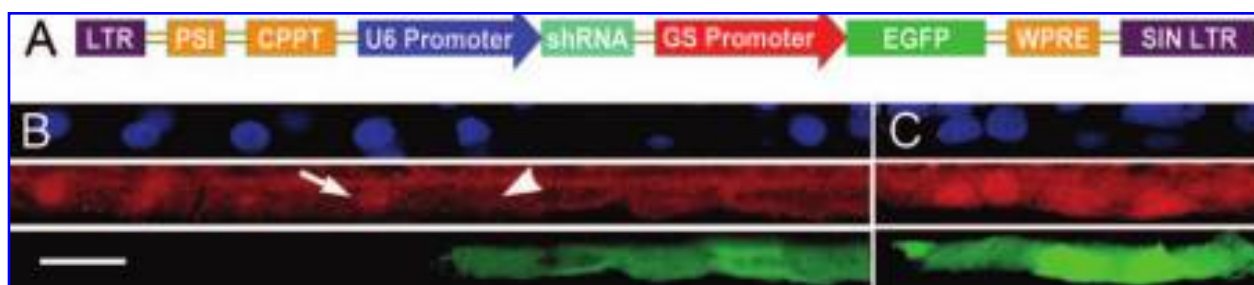


FIG. 3. Knockdown of bFGF in the RPE *in vivo*, using lentivirus (LV). (A) LV construct. LTR, long terminal repeat; PSI, packaging signal; CPPT, central polyurine tract; GS Promoter, chicken glutamine synthetase promoter; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element; SIN LTR, self-inactivating LTR. Constructs had either active sh-bFGF or the U6 promoter with no shRNA. (B) LV with sh-bFGF transduces RPE cells and knocks down bFGF *in vivo*. Top (blue): 4',6-diamidino-2-phenylindole (DAPI). Middle (red): bFGF. Bottom (green): GFP. The GS promoter drives GFP expression in the RPE. Transduced cells (green, all RPE cells on the right side of the microscopic field, bottom panel) have lost nuclear bFGF immunoreactivity 21 days after injection (e.g., arrowhead), whereas adjacent untransduced cells retain it (e.g., arrow). Transduced cells exhibit some artifactual peripheral red color because of bleedthrough from the extremely bright GFP signal, but comparison with the DAPI channel demonstrates that the nuclei lack immunoreactivity. Similar results were obtained from 3 to 28 days after injection, the earliest and latest times examined. (C) LV with no shRNA. Immunoreactivity for bFGF is present in the nuclei of transduced RPE. Scale bar: 50 μ m.

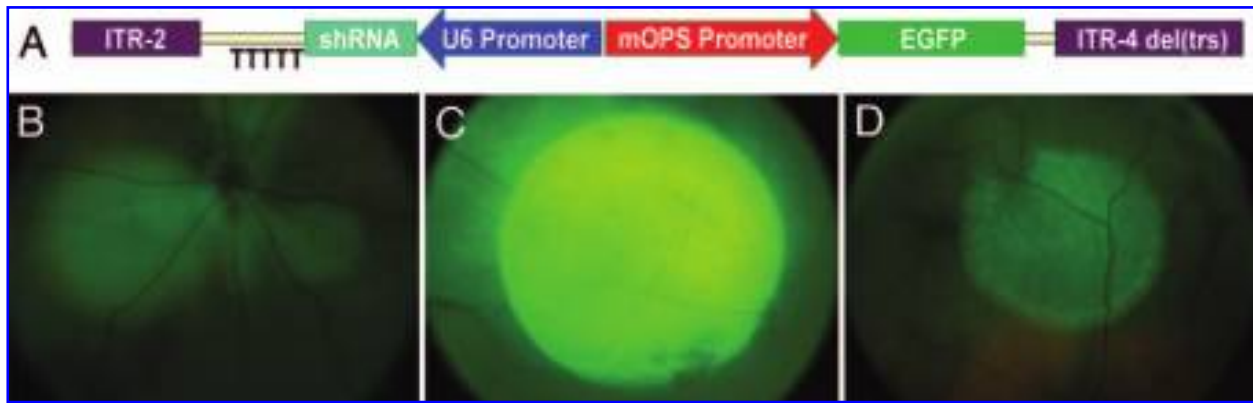


FIG. 4. Rapid kinetics of gene expression driven by sdsAAV *in vivo*. (A) Stabilized double-stranded AAV (sdsAAV) construct. GFP expression is driven by a mouse opsin (mOPS) promoter. Either active sh-bFGF or sh-mut2 was used as the shRNA. (B–D) *In vivo* imaging of GFP expression in the fundus of the eye after subretinal injection of AAV vectors. Identical settings were used to capture all images. Approximately 25% of the retinal area is shown in each image; the optic nerve head is visible in (B), with the retinal vasculature radiating outward from it. (B) GFP expression is already apparent 6 days after injection of sdsAAV-5, the earliest time examined. (C) By 16 days after injection of sdsAAV-5, fluorescence is extremely bright in the transduced region of the retina. A nearly circular transduced region is often seen and is not an aperture illumination artifact, which is prevented by the RetCam II imaging system. Nontransduced retina appears faintly green because of scattering of emitted light. (D) Standard single-stranded AAV-5 (ssAAV-5) carrying an identical mOPS–EGFP reporter shows dimmer fluorescence than sdsAAV-5 (C) even 23 days after injection. ssAAV-5 was injected at the same titer and volume as sdsAAV-5, into an animal of the same age.

AAV-5 at 23 days postinjection, Fig. 4D). Sectioning demonstrated that, as early as 6 days after injection, essentially all photoreceptors in the injected area expressed GFP (Fig. 5B and C). RPE cells in areas injected with sdsAAV.sh-bFGF had lost bFGF immunoreactivity by 6 days after injection, confirming that they were transduced (Fig. 5B). This interval was therefore sufficient for RPE transduction, gene expression, knockdown of bFGF mRNA, and turnover of the existing bFGF protein. RPE outside the injected portion of the retina retained bFGF immunoreactivity (data not shown). sdsAAV.sh-mut2 did not cause bFGF knockdown in RPE (Fig. 5C). We obtained similar results at all five postinjection times examined, ranging from 3 to 60 days, with two separate experimental replicates at each time point and at least three rats in each experiment. There was no evidence of retinal toxicity from sdsAAV injection in any of our experiments.

Photoreceptors exhibit little or no bFGF immunoreactivity under normal circumstances, but acquire it in response to light stress as a putative endogenous protective response (Walsh *et al.*, 2001). We also observed minimal bFGF immunoreactivity in photoreceptors in unstressed retina (Fig. 5A). To assess knockdown of bFGF in light-stressed photoreceptors, we injected sdsAAV.sh-bFGF and waited 30 days to allow the endogenous response to the injury of subretinal injection (Wen *et al.*, 1995) to recede. We then exposed the animals to bright light (115–130 ft-c) for 12 hr, a stress known to upregulate bFGF (Liu *et al.*, 1998). Indeed, bFGF protein expression was increased by light stress in the outer retinas of our animals (Fig. 5D). In transduced areas, we observed loss of nuclear staining in the RPE (Fig. 5D), as we had previously seen in transduced unstressed retinas (Fig. 5B). In contrast, photoreceptors exhibited comparable bFGF immunoreactivity in transduced and nontransduced regions (Fig. 5D).

DISCUSSION

We have shown that two different viral vectors can be used to deliver shRNAs to the rat retina and provide persistent knockdown of endogenous bFGF gene expression in the RPE for at least 1–2 months. Our approach incorporates several features that increase the utility and versatility of the method, including a simple *in vitro* screening assay that correctly predicts the efficacy of shRNAs *in vivo*; rapid and durable knockdown; and a negative control that differs from the active shRNA at only 2 base pairs.

The sdsAAV vector mediated rapid knockdown of bFGF in RPE, evident as early as 6 days after injection. This interval must represent sufficient time for the expression of vector-delivered genes, shRNA processing, RNA interference, and turnover of existing bFGF. In contrast, the onset of observable reporter gene expression with standard ssAAV-2/5 vectors has been reported to occur 2 weeks after injection (Auricchio *et al.*, 2001). AAV vectors mediate stable gene expression lasting at least 3 years (Acland *et al.*, 2005), suggesting that sdsAAV delivery of shRNAs would offer an attractive combination of rapid and long-lasting knockdown.

In contrast to our findings in the RPE, we did not observe loss of bFGF immunoreactivity in photoreceptors transduced with sdsAAV.sh-bFGF. Ineffective RNAi in photoreceptors is one possible explanation, perhaps caused by poor shRNA expression or processing. However, reports of successful RNAi using plasmid-delivered shRNAs in photoreceptors *ex vivo* (Kiang *et al.*, 2005) or AAV-delivered shRNA in photoreceptors (Tessitore *et al.*, 2006; Gorbatyuk *et al.*, 2007) argue against this. An alternative explanation is that bFGF may be produced in neighboring cells (e.g., Müller glia) and internalized by photoreceptors (Harada *et al.*, 2000; Walsh *et al.*, 2001), as has

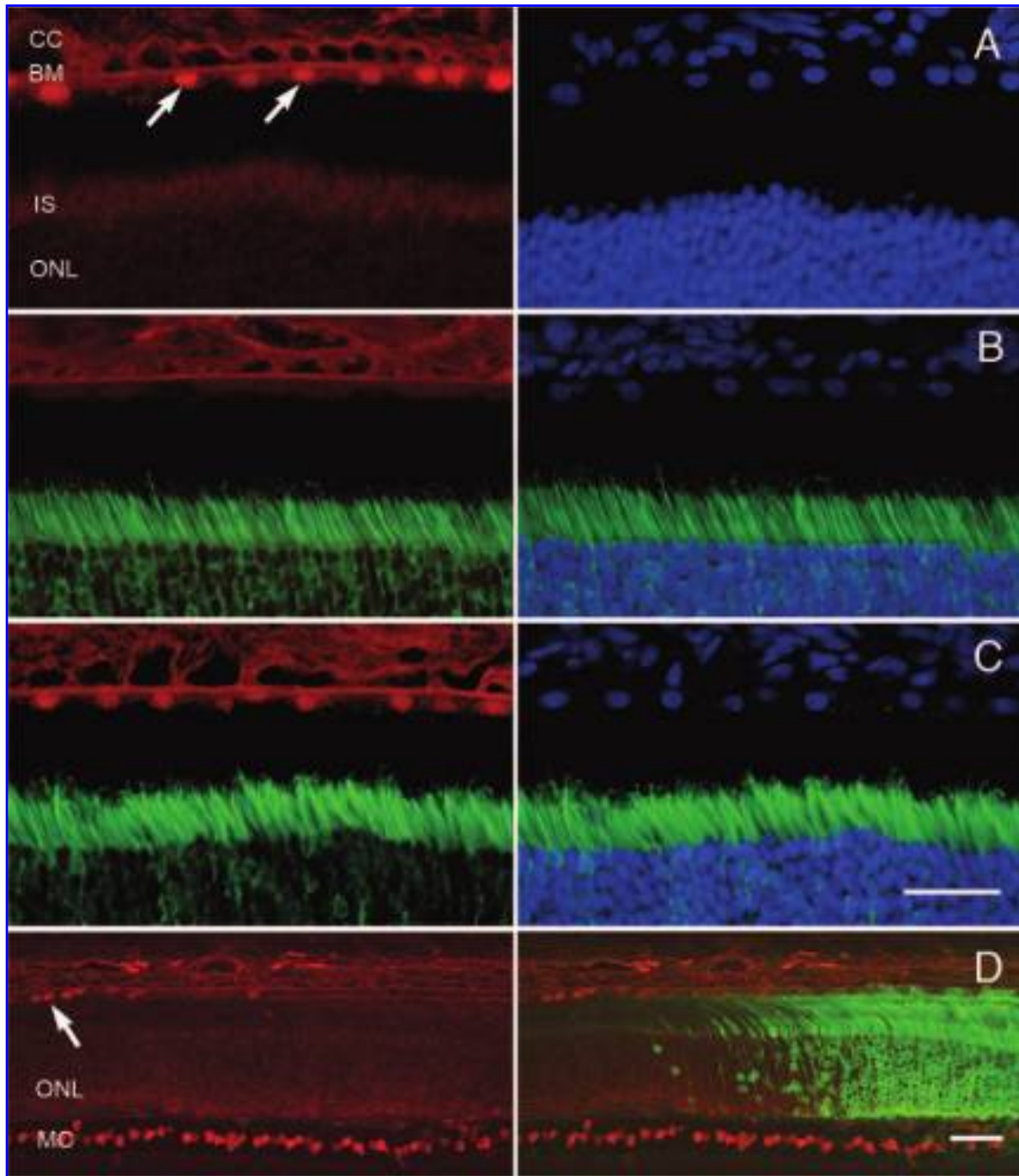


FIG. 5. Knockdown of bFGF *in vivo*, using sdsAAV. Red, bFGF; green, GFP; blue, DAPI. For each field in (A–C), bFGF and GFP are shown together at *left*, DAPI and GFP at *right*. (A) Uninjected retina. Immunoreactivity for bFGF is observed in RPE nuclei (arrows), Bruch’s membrane (BM), and choriocapillaris (CC). IS, photoreceptor inner segments, exhibiting slight autofluorescence; ONL, outer nuclear layer. Photoreceptor outer segments are artifactually absent. (B) Subretinal injection of sdsAAV with sh-bFGF. GFP, under the control of the mOPS promoter, is expressed in essentially all photoreceptors. RPE cells are also transduced (Flannery *et al.*, 1997) (mOPS promoter is not active in RPE). RPE cells have lost bFGF immunoreactivity 6 days after injection, the earliest time examined. Similar results were obtained up to 60 days after injection, the latest time examined (data not shown). (C) sdsAAV-5 with sh-mut2 does not silence bFGF expression in RPE. (D) Retina stressed by bright light for 12 hr shows upregulation of bFGF protein in the photoreceptors in the ONL [compare with (A)], as shown previously by Walsh *et al.* (2001). GFP and bFGF are shown on the *right* and bFGF alone on the *left*. The region of retina not transduced by sdsAAV (*left* side of field) shows bFGF immunoreactivity of RPE nuclei (arrow), whereas the region of retina transduced by sdsAAV, on the *right* side of the field, as indicated by the GFP, shows loss of RPE nuclear immunoreactivity. Both transduced and non-transduced regions show similar degrees of upregulated bFGF immunoreactivity in the photoreceptor nuclei of the ONL. The Müller cell nuclei (MC) in the inner nuclear layer are intensely positive for bFGF immunoreactivity throughout the retina. Scale bars: 25 μ m.

been reported for other cell types (Roghani and Moscatelli, 1992).

Studies of gene function using RNAi require careful controls to reduce the probability that an observed phenotype derives from “off-target” knockdown of an unintended gene. We have shown that a control shRNA, differing from the experimental construct sh-bFGF by the exchange of only 2 base pairs, is inactive against the target both *in vitro* and *in vivo*. This control is preferable to the more common use of an unrelated siRNA or shRNA (Arai-Gaun *et al.*, 2004; Kiang *et al.*, 2005), because our closely related construct is likely to share many of the off-target effects of sh-bFGF. Off-target silencing is often driven by the 5′ end of the antisense strand (Jackson *et al.*, 2006), which is identical between sh-bFGF and sh-mut2.

Viral delivery of shRNAs has several advantages over conventional knockout technology for the study of gene function. It is faster and less expensive, and can be administered to animals that have completed embryonic development, and is applicable to species other than mouse. Dogs with spontaneous mutations have emerged as valuable models of human retinal disease (Acland *et al.*, 2001; Cideciyan *et al.*, 2005) with their relatively large eyes and long life spans, and may be preferable to mice for preclinical evaluation of experimental therapies (Acland *et al.*, 2001; Tao *et al.*, 2002; Lai *et al.*, 2004). Durable RNAi could provide models of human diseases for which no corresponding loss-of-function mutant is available. Although studies using synthetic RNA duplexes (siRNAs) have already established the utility of RNAi in retinal research (Arai-Gaun *et al.*, 2004; Shen *et al.*, 2006), virally delivered shRNAs are potentially much more powerful (Michel *et al.*, 2005; Tessitore *et al.*, 2006; Gorbatyuk *et al.*, 2007). Their longer duration of action may be better suited to the study of retinal degeneration, and could avoid the need for repeated intraocular injections.

Our study complements the demonstration of knockdown of adenovirus-encoded VEGF in the RPE of mice by coinjection of a separate adenovirus expressing an anti-VEGF shRNA (Cashman *et al.*, 2006). In this earlier study, duration of knockdown beyond 1 week was not assessed. In contrast, we achieved knockdown using both lentiviral and AAV vectors that was stable for at least 1–2 months. The AAV vector was a stabilized double-stranded version, which also conferred rapid shRNA expression and knockdown. Together, these studies serve as a proof of concept for knocking down gene function in the postnatal RPE.

The RPE is the site of expression of a number of mutant genes responsible for dominant eye diseases such as Sorsby fundus dystrophy and Best vitelliform macular dystrophy, which may be amenable to treatment by RNAi. Moreover, the RPE is of substantial interest because of its central role in age-related macular degeneration, an epidemic disease of the elderly for which nonviral siRNA therapy is currently being tested in clinical trials. Rapid and stable knockdown of endogenous gene expression in the RPE, as demonstrated here, would be optimal for the application of RNAi to human gene therapy.

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