

# Potential applications for RNAi to probe pathogenesis and develop new treatments for ocular disorders

PA Campochiaro<sup>1</sup>

Departments of Ophthalmology and Neuroscience, The Johns Hopkins University School of Medicine, Maumenee, Baltimore, MD, USA

*The eye is a relatively isolated tissue compartment, which provides advantages for utilization of small interfering RNA (siRNA). Feasibility of using siRNA for treatment of choroidal neovascularization has been demonstrated using siRNA directed against vascular endothelial growth factor (VEGF) or VEGF receptor 1 (VEGFR1), and both of these approaches are being tested in clinical trials. The results with VEGFR1 siRNA show that VEGFR1 is proangiogenic in the eye and is not a decoy receptor as it is in developmental angiogenesis. Topical delivery of siRNAs directed against VEGF or its receptors has also been shown to suppress corneal neovascularization. Signaling through transforming growth factor- $\beta$  receptor 2 (TGF $\beta$ R2) has been implicated in*

*excessive ocular scarring and TGF $\beta$ R2 siRNA has shown benefit in a model relevant to excessive scarring after glaucoma filtration surgery. RNAi has been used to identify genes that promote apoptosis or oxidative damage in retinal cells and could be the basis of new treatments for glaucoma or photoreceptor degenerations. In cultured cells derived from ocular tissues, siRNA has become a valuable tool to explore the potential role of various genes in ocular disease processes. Based upon this early experience in vivo and in vitro, it appears that siRNAs may be valuable to help define the pathogenesis and develop new treatments for several ocular diseases.* Gene Therapy (2006) 13, 559–562. doi:10.1038/sj.gt.3302653; published online 29 September 2005

## Introduction

An important way of exploring the function of a gene is to examine effects of eliminating it. Animals with targeted disruption of a gene provide ideal tools to explore the role of the product of that gene during embryonic development. If development is not altered by gene disruption, then adult animals with the disruption can be used to investigate the gene's function in adult animals. However, many gene deficiencies have major impacts during development resulting in embryonic lethality or major abnormalities in structure and function, and even when there are no obvious consequences from deletion of a gene, it is difficult to be certain that its absence during development does not complicate interpretations of its absence in adult animals. Inducible gene disruption techniques circumvent this problem, but are difficult, time consuming, and expensive. One of the major benefits of small interfering RNA (siRNA) is that it provides an efficient way to silence genes in animals at essentially any stage of development.

While it is possible to cause widespread silencing of a gene by systemic administration of siRNA, large amounts of siRNA are needed and effects are transient unless repeated administrations are used, further straining feasibility. The eye is a relatively isolated compartment, which makes it an ideal target organ for gene therapy. Local delivery of viral vector to the eye limits exposure to the rest of the body and reduces the amount of vector that is needed. The same benefits also apply to ocular delivery of siRNA. siRNA injected into the vitreous cavity readily diffuses throughout the eye and is detectable for at least five days,<sup>1</sup> the amounts used for intraocular injections are small compared to those used for systemic application and so as siRNA gets out of the eye it is diluted and is difficult to detect. This allows local silencing of a gene with little chance for an effect on the same gene outside the eye, reducing concern of remote effects in other tissues complicating observations in the eye. The sequence specificity of siRNA resulting in targeting of a single gene combined with local administration in the relatively isolated confines of the eye provide an ideal way to study eye-specific effects of gene disruption. Although experience is still limited, there are several examples suggesting that the theoretical advantages of siRNA utilization in the eye can be translated into tangible benefits.

Correspondence: Dr PA Campochiaro, The Departments of Ophthalmology and Neuroscience, The Johns Hopkins University School of Medicine, Maumenee 719, 600 N. Wolfe Street, Baltimore, MD 21287-9277, USA.

E-mail: pcampo@jhmi.edu

<sup>1</sup>PAC is the George S and Dolores Dore Eccles Professor of Ophthalmology and Neuroscience. PAC is a consultant for Sirna Therapeutics Inc., which is monitored by the Conflict of Interest Committee of the Johns Hopkins University School of Medicine.

Received 21 June 2005; revised 11 August 2005; accepted 12 August 2005; published online 29 September 2005

## Use of siRNA to investigate gene function in ocular neovascularization

Neovascularization complicates several different eye diseases and constitutes one of the most common and

difficult pathological processes faced by eye physicians.<sup>2</sup> Three types of ocular neovascularization that are extremely common causes of blindness and visual morbidity are choroidal, retinal, and corneal neovascularization. Choroidal neovascularization occurs in diseases of the Bruch's membrane/retinal pigmented epithelial (RPE) cell complex, particularly neovascular age-related macular degeneration, the most prevalent cause of severe vision loss in persons over the age of 60 in developed countries.<sup>3</sup> Retinal neovascularization occurs in ischemic retinopathies, such as diabetic retinopathy, the most common cause of severe vision loss in working age Americans.<sup>4</sup> Corneal neovascularization occurs as a consequence of corneal infection and/or inflammation, such as herpes simplex keratitis.

One of the first uses of siRNA in the eye was related to the study of choroidal neovascularization. Several previous studies had demonstrated that vascular endothelial growth factor (VEGF) is a critical stimulus for choroidal neovascularization.<sup>5-7</sup> Reich *et al.*<sup>8</sup> tested the feasibility of targeting VEGF *in vivo* with siRNA directed against *Vegf* mRNA in two ways. Mice received a subretinal injection of adenoviral vector containing a CMV promoter coupled to *hVegf* cDNA in both eyes combined with siRNA directed against *hVegf* mRNA in one eye and siRNA directed against *green fluorescent protein (GFP)* mRNA in the other eye. ELISA for hVEGF in whole eye homogenates showed significantly less hVEGF in eyes injected with *hVegf* siRNA compared to eyes injected with *GFP* siRNA. In a murine model of choroidal neovascularization induced by rupture of Bruch's membrane with laser photocoagulation,<sup>9</sup> the area of choroidal neovascularization at sites of rupture of Bruch's membrane was significantly less in eyes that received a subretinal injection of *mVegf* siRNA compared to those injected with *GFP* siRNA. Although subsequent experiments investigating the effect of intravitreal injection of siRNA directed against *Vegf* mRNA in a primate model of choroidal neovascularization were inconclusive,<sup>10-13</sup> the murine studies suggest that utilization of siRNA directed VEGF might have therapeutic potential and that possibility is beginning to be explored in a phase I trial testing the safety of intravitreal injection of siRNA directed against *Vegf* mRNA in patients with subfoveal choroidal neovascularization due to neovascular AMD.

There is overlap in the molecular signals involved in the development of corneal neovascularization compared to those involved in choroidal neovascularization, but there are also substantial differences as there are among most vascular beds either within or outside the eye.<sup>14-16</sup> One similarity is that VEGF is an important stimulus for both. Kim *et al.*<sup>17</sup> demonstrated that systemic administration using a polymer delivery system or topical administration of siRNAs directed against VEGF-A, VEGF receptor 1 (VEGFR1), or VEGFR2 caused significant reduction of corneal neovascularization induced by herpes simplex infection or CpG oligodeoxynucleotides. Combining all three siRNAs provided the greatest benefit. In another study, several siRNAs directed against VEGFR1 were evaluated to identify one, Sirna-027, that cleaved *vegfr1* mRNA at the predicted site and maximally reduced its levels in cultured endothelial cells and in mouse models of retinal and choroidal neovascularization.<sup>1</sup> Compared to injec-

tion of an inverted control sequence, quantitative RT-PCR demonstrated statistically significant reductions of 57 and 40% in *vegfr1* mRNA after intravitreal or periocular injection of Sirna-027, respectively. Staining showed uptake of BrdU-labeled Sirna-027 in retinal cells that lasted between 3 and 5 days after intravitreal injection and was still present 5 days after periocular injection. In a choroidal neovascularization model, intravitreal or periocular injections of Sirna-027 resulted in significant reductions in the area of neovascularization ranging from 45 to 66%. In mice with ischemic retinopathy, intravitreal injection of 1.0  $\mu$ g of Sirna-027 significantly reduced retinal neovascularization compared to fellow eyes treated with 1.0  $\mu$ g of inverted control siRNA.

It has been postulated that VEGFR1 is a decoy receptor that negatively regulates the activity of VEGFR2,<sup>18</sup> and during developmental angiogenesis this appears to be the case, because *vegfr1*  $-/-$  mice show excessive proliferation of angioblasts, a sign of VEGF-A over action.<sup>19</sup> However, the two studies cited above<sup>1,17</sup> have utilized siRNA which allows specific inhibition of VEGFR1 with no inhibition of highly homologous VEGFR2 to demonstrate that VEGFR1 plays an important stimulatory role in three kinds of ocular neovascularization. The second study<sup>1</sup> also illustrates the kinds of controls needed to definitively prove an RNAi mechanism and emphasizes the need to test multiple sequences; while bioinformatics provides a starting point for target sequence selection, expectations are often unfulfilled and identification of an optimal target sequence requires empirical testing. Identification of a target gene and optimal target sequence within that gene are simply the first two steps needed for development of siRNA-based therapy, and must be followed by optimization of delivery to the cytosol of target cells. Chemical modifications that slow degradation such as inclusion of unpaired deoxythymidines, a phosphorothioate linkage, and two inverted 2'-deoxy abasic nucleotides<sup>20</sup> and as noted above injection into the relatively isolated ocular compartment can help to sustain siRNA levels, but the duration of activity from a single injection is unknown. A phase I study to investigate the safety of a single intravitreal injection of Sirna-027 in patients with subfoveal choroidal neovascularization due to AMD is underway.

## Use of siRNA to investigate gene function in ocular fibrosis

Exaggerated wound repair resulting in fibrosis and scarring is a major cause of ocular disease. After retinal reattachment surgery, roughly 10% of patients develop a processes called proliferative vitreoretinopathy in which scar tissue occurs on the surface of the retina and exerts traction which in many instances redetaches the retina requiring additional surgery and substantially reducing visual prognosis.<sup>21</sup> Filtration surgery for glaucoma involves creation of an opening between the anterior chamber and the periocular space to allow egress of fluid and reduce intraocular pressure. The opening is covered by flaps of sclera and conjunctiva, which are loosely sutured to allow for slow percolation of fluid from the eye, because rapid exit of fluid causes excessively low

intraocular pressure that can be just as problematic as excessively high intraocular pressure. The major cause of failure of filtration surgery is excessive scarring causing the flaps of sclera and conjunctiva to seal down preventing exit of fluid. Transforming growth factor- $\beta$  (TGF $\beta$ ) and its receptors have been implicated in wound repair and fibrotic diseases. Nakamura *et al.*<sup>22</sup> used siRNAs directed against TGF $\beta$  receptor II to explore its role in ocular fibrosis. Two of four TGF $\beta$  receptor II siRNAs tested reduced TGF $\beta$  receptor II expression, production of fibronectin, and migration in cultured human corneal fibroblasts. In a mouse model in which subconjunctival scarring is induced by injection of latex beads beneath the conjunctiva, coinjection of TGF $\beta$  receptor II siRNA significantly reduced the number of inflammatory cells and matrix deposition. This model is relevant to the subconjunctival scarring that occurs after glaucoma filtering surgery and suggests that targeting TGF $\beta$  receptor II with siRNA may have therapeutic potential.

### Use of siRNA to investigate gene function in apoptosis of retinal ganglion cells

Apoptosis plays a central role in photoreceptor degenerations and glaucoma. This has prompted attempts to define the proapoptotic genes involved in these processes to develop retinal cell survival therapies. Sectioning of the optic nerve results in massive apoptosis of retinal ganglion cells and is felt to be relevant to the gradual apoptosis of retinal ganglion cells that occurs due to high intraocular pressure in glaucomatous eyes.<sup>23</sup> Lingor *et al.*<sup>24</sup> used siRNAs directed against *c-Jun*, *apoptotic protease-activating factor-1 (Apaf-1)*, or *Bax* to investigate their role in axotomy-induced apoptosis of retinal ganglion cells. They used a clever strategy of injecting siRNA into the proximal stump of the optic nerve, and using Cy-3-labeled siRNA they demonstrated that this technique results in specific transduction of retinal ganglion cells. A single injection of *cJun* or *Apaf-1* siRNA into the stump of the optic nerve significantly increased the number of retinal ganglion cells in the retina 14 days after transection, implicating *cJun* and *Apaf-1* in the death of retinal ganglion cells. Injection of *Bax* siRNA had no significant effect. These data support consideration of additional studies to further define genes involved in retinal cell death to determine if targeting them with siRNAs has therapeutic potential.

### Use of siRNA to investigate gene function in oxidative damage in the retina

Oxidative damage has been implicated in several ocular diseases including age-related macular degeneration<sup>25</sup> and cone cell death in retinitis pigmentosa.<sup>26</sup> Wu *et al.*<sup>27</sup> used siRNA directed against the p66 isoform of Shc adaptor protein to investigate its role in oxidative damage in the retina. Transfection of cultured RPE cells with p66Shc siRNA reduced H<sub>2</sub>O<sub>2</sub>-induced generation of reactive oxygen species and apoptosis. Intravitreal injection of paraquat in mice provides an acute model of oxidative damage-induced retinal degeneration.<sup>28</sup> Subretinal injection of p66Shc in BALB/c mice prior to

injection of paraquat preserved retinal function assessed by electroretinograms. These data suggest that siRNA directed against p66Shc should be considered for treatment of oxidative damage in the retina.

### Use of siRNA for gene knockdown in cultured ocular cells

Gene knockdown by siRNA transfection of cultured cells or organ cultures is a useful way to explore gene function, and cultures derived from ocular tissues are no exception. This strategy has been used to explore the function of microsomal glutathione S-transferase 1 in protection of RPE cells from oxidative stress,<sup>29</sup> the effect of loss of mitochondrial DNA sequences on RPE cells,<sup>30</sup> the role of *NeuroD* in photoreceptor formation,<sup>31</sup> the role of stem cell factor/*c-Kit* in tumorigenesis of uveal melanocytes,<sup>32</sup> the role of aldehyde dehydrogenase isozymes or methionine sulfoxide reductase A in oxidative defense of lens cells,<sup>33,34</sup> the role of eukaryotic translation initiation factor 5A on tumor necrosis factor- $\alpha$ -induced apoptosis of lamina cribosa cells,<sup>35</sup> survival-promoting effects of hepatocyte growth factor on corneal epithelial cells,<sup>36</sup> the role of 1Na<sup>+</sup>-3HCO<sub>3</sub><sup>-</sup> cotransporter in HCO<sub>3</sub><sup>-</sup> flux in corneal endothelial cells,<sup>37</sup> TGF $\beta$ 2-induced production of extracellular matrix by astrocytes derived from optic nerve,<sup>38</sup> and regulation of eye development by transcriptional control of CCCTC binding factor.<sup>39</sup>

### Conclusions

Gene knockdown by RNAi provides an extremely useful tool for investigation of the role of various genes in pathologic processes including those involving the eye. While the development of siRNA-based therapies has promise for all tissues, the unique characteristics of the eye provide advantages and two siRNAs are being investigated in phase I clinical trials in patients with neovascular AMD. Advances that allow for sustained siRNA levels in the eye and/or expression of siRNA in ocular cells could further improve feasibility and yield benefits in many types of ocular diseases.

### References

- 1 Shen J, Samul R, Lima e Silva R, Akiyama H, Liu H, Saishin Y *et al.* Suppression of ocular neovascularization with siRNA targeting VEGF receptor 1. *Gene Therapy* [E-pub ahead of print, 29 September 2005; doi:10.1038/sj.gt.3302641].
- 2 Campochiaro PA. Retinal and choroidal neovascularization. *J Cell Physiol* 2000; **184**: 301–310.
- 3 Klein R, Klein BEK, Linton KP. The Beaver Dam Eye Study: the relation of age-related maculopathy to smoking. *Am J Epidemiol* 1993; **137**: 190–200.
- 4 Klein R, Klein BEK, Moss SE, Davis MD, DeMets, DL. The Wisconsin Epidemiologic Study of Diabetic Retinopathy. II. Prevalence and risk of diabetic retinopathy when age at diagnosis is less than 30 years. *Arch Ophthalmol* 1984; **102**: 520–526.
- 5 Kwak N, Okamoto N, Wood JM, Campochiaro PA. VEGF is an important stimulator in a model of choroidal neovascularization. *Invest Ophthalmol Vis Sci* 2000; **41**: 3158–3164.

- 6 Saishin Y, Saishin Y, Takahashi K, Lima Silva R, Hylton D, Rudge JJWS *et al.* VEGF-TRAP<sub>R1R2</sub> suppresses choroidal neovascularization and VEGF-induced breakdown of the blood-retinal barrier. *J Cell Physiol* 2003; **195**: 241–248.
- 7 Krysztolik MG, Afshari MA, Adamis AP, Gaudreault J, Gragoudas ES, Michaud NM *et al.* Prevention of experimental choroidal neovascularization with intravitreal anti-vascular endothelial growth factor antibody fragment. *Arch Ophthalmol* 2002; **120**: 338–346.
- 8 Reich SJ, Fosnot J, Kuroki A, Tang W, Yang X, Maguire AM *et al.* Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. *Mol Vis* 2003; **9**: 210–216.
- 9 Tobe T, Ortega S, Luna JD, Ozaki H, Okamoto N, Derevanik N *et al.* Targeted disruption of the *FGF2* gene does not prevent choroidal neovascularization in a murine model. *Am J Pathol* 1998; **153**: 1641–1646.
- 10 Tolentino MJ, Brucker AJ, Fosnot J, Ying GS, Wu IH, Malik G *et al.* Intravitreal injection of vascular endothelial growth factor small interfering RNA inhibits growth and leakage in a nonhuman primate, laser-induced model of choroidal neovascularization. *Retina* 2004; **24**: 51–56.
- 11 Tolentino MJ, Brucker AJ, Fosnot J, Ying G, Wu IH, Malik G *et al.* Erratum. *Retina* 2004; **24**: 660.
- 12 Tolentino MJ, Brucker A, Wan S, Reich SJ, Gordon J, Duh YJ. Letter to the Editor. *Retina* 2004; **24**: 661.
- 13 Maguire M, Fine SL, Ying GS. Intravitreal injection of VEGF siRNA. *Retina* 2005; **25**: 101–102.
- 14 Oshima Y, Deering T, Oshima S, Nambu H, Reddy PS, Kaleko M *et al.* Angiopoietin-2 enhances retinal vessel sensitivity to vascular endothelial growth factor. *J Cell Physiol* 2004; **199**: 412–417.
- 15 Oshima Y, Oshima S, Nambu H, Kachi S, Hackett SF, Melia M *et al.* Increased expression of VEGF in retinal pigmented epithelial cells is not sufficient to cause choroidal neovascularization. *J Cell Physiol* 2004; **201**: 393–400.
- 16 Oshima Y, Oshima S, Nambu H, Kachi S, Takahashi K, Umeda N *et al.* Different effects of angiopoietin 2 in different vascular beds in the eye; new vessels are most sensitive. *FASEB J* 2005; **19**: 963–965.
- 17 Kim B, Tang Q, Biswas PS, Xu J, Schiffelers RM, Xie FY *et al.* Inhibition of ocular angiogenesis by siRNA targeting vascular endothelial growth factor pathway genes. *Am J Pathol* 2004; **165**: 2177–2185.
- 18 Park JE, Chen HH, Winer J, Houck KA, Ferrara N. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, *in vitro* and *in vivo*, and high affinity binding Flt-1 but not to Flk-1/KDR. *J Biol Chem* 1994; **269**: 25646–25654.
- 19 Fong GH, Zhang L, Bryce DM, Peng J. Increased hemangioblast commitment, not vascular disorganization, is the primary defect in *flt-1* knockout mice. *Development* 1999; **126**: 3015–3025.
- 20 Peracchi A, Beigelman L, Usman N, Herschlag D. Rescue of abasic hammerhead ribozymes by exogenous addition of specific bases. *Proc Natl Acad Sci USA* 1996; **93**: 11522–11527.
- 21 Campochiaro PA. Pathogenic mechanisms in proliferative vitreoretinopathy. *Arch Ophthalmol* 1997; **115**: 237–241.
- 22 Nakamura H, Siddiqui SS, Shen X, Malik AB, Pulido JS, Kumar NM *et al.* RNA interference targeting transforming growth factor-beta type II receptor suppresses ocular inflammation and fibrosis. *Mol Vis* 2004; **10**: 703–711.
- 23 Berkelaar M, Clarke DB, Wang YC, Bray GM, Aguayo AJ. Axotomy results in delayed death and apoptosis of retinal ganglion cells in adult rats. *J Neurosci* 1994; **14**: 4368–4374.
- 24 Lingor P, Koeberle P, Kugler S, Bahr M. Down-regulation of apoptosis mediators by RNAi inhibits axotomy-induced retinal ganglion cell death. *Brain* 2005; **128**: 550–558.
- 25 The Age-Related Eye Disease Study Research Group. A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E, beta carotene, and zinc for age-related macular degeneration and vision loss. *Arch Ophthalmol* 2001; **119**: 1417–1436.
- 26 Shen J, Yan X, Dong A, Petters RM, Peng Y-W, Wong F *et al.* Oxidative damage is a potential cause of cone cell death in retinitis pigmentosa. *J Cell Physiol* 2005; **203**: 457–464.
- 27 Wu Z, Hackett SF, Kachi S, Rogers B, Campochiaro PA. p66shc regulates redox-sensitive NF- $\kappa$ B activation and oxidative stress-induced apoptosis in human RPE cells. *Invest Ophthalmol Vis Sci* 2005; **46**: E-Abstract 1615.
- 28 Cingolani C, Rogers B, Shen J, Lu L, Campochiaro PA. Intraocular injection of paraquat: a new model of oxidative damage-induced retinal degeneration. *Invest Ophthalmol Vis Sci* 2005; **46**: E-Abstract 1599.
- 29 Maeda A, Crabb JW, Palczewski K. Microsomal glutathione S-transferase 1 in the retinal pigment epithelium: protection against oxidative stress and a potential role in aging. *Biochemistry* 2005; **44**: 480–489.
- 30 Miceli MV, Jazwinski SM. Common and cell type-specific responses of human cells to mitochondrial dysfunction. *Exp Cell Res* 2005; **302**: 270–280.
- 31 Yan RT, Wang SZ. Requirement of NeuroD for photoreceptor formation in the chick retina. *Invest Ophthalmol Vis Sci* 2004; **45**: 48–58.
- 32 Lefevre G, Glotin AL, Calipel A, Mouriaux F, Tran T, Kherrouche Z *et al.* Roles of stem cell factor/c-kit and effects of glivec/STI571 in human uveal melanoma cell tumorigenesis. *J Biol Chem* 2004; **279**: 31769–31779.
- 33 Choudhary S, Xiao T, Vergara LA, Srivastava S, Nees D, Piatigorsky J *et al.* Role of aldehyde dehydrogenase isozymes in the defense of rat lens and human lens epithelial cells against oxidative stress. *Invest Ophthalmol Vis Sci* 2005; **46**: 259–267.
- 34 Kantorow M, Hawse JR, Cowell TL, Benhamed S, Pizarro GO, Reddy VN *et al.* Methionine sulfoxide reductase A is important for lens cell viability and resistance to oxidative stress. *Proc Natl Acad Sci USA* 2004; **101**: 9654–9659.
- 35 Taylor CA, Senchyna M, Flanagan J, Joyce EM, Cliche DO, Boone AN *et al.* Role of eIF5A in TNF-alpha-mediated apoptosis of lamina cribosa cells. *Invest Ophthalmol Vis Sci* 2004; **45**: 3568–3576.
- 36 Kakazu A, Chandrasekher G, Bazan HEP. HGF protect corneal epithelial cells from apoptosis by the PI-3K/Akt-1/Bad- but not the ERK1/2-mediated signaling pathway. *Invest Ophthalmol Vis Sci* 2004; **45**: 3485–3492.
- 37 Li J, Sun XC, Bonanno JA. Role of NBC1 in apical and basolateral HCO<sub>3</sub>-permeabilities and transendothelial HCO<sub>3</sub>-fluxes in bovine corneal endothelium. *Am J Physiol Cell Physiol* 2004; **288**: C739–C746.
- 38 Fuchshofer R, Birke M, Wige-Lussen U, Kook D, Lutjen-Drecoll E. Transforming growth factor-beta2 modulated extracellular matrix component expression in cultured human optic nerve head astrocytes. *Invest Ophthalmol Vis Sci* 2005; **46**: 568–578.
- 39 Li T, Lu Z, Lu L. Regulation of eye development by transcription control of CCTC binding factor (CTCF). *J Biol Chem* 2004; **279**: 27575–27583.