Gene Therapy (2015) **22,** 365–373

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ORIGINAL ARTICLE

# Safety and angiogenic effects of systemic gene delivery of a modified erythropoietin

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Erythropoietin (EPO) is critical for red blood cell production and is also an effective neuroprotective agent. However, it may contribute to pathological angiogenesis. Here we investigate the angiogenic potential of EPO and a mutant form with attenuated erythropoietic activity, EPO-R76E, on primary human retinal microvascular endothelial cells (HRMECs) and in the adult retina. Assays of death, proliferation and tube formation were performed on HRMECs exposed to EPO, EPO-R76E or media alone. Postnatal day-9 wild-type mice were injected intramuscularly with adeno-associated virus vectors expressing either enhanced green fluorescent protein or EpoR76E. At 3 months, levels of EPO-R76E in the eye were quantified, and the health of the retinal vasculature was assessed by fluorescein angiography and isolectin immunolabeling. Immunohistochemistry, histology and electroretinogram (ERG) assessments were performed as measures of retinal health. Neither EPO nor EPO-R76E induced proliferation or tube formation in HRMECs under the conditions used. EPO-R76E decreased HRMEC death in a dose-dependent manner. Long-term systemic gene delivery of EPO-R76E was safe in terms of retinal vasculature, histology and the ERG *in vivo*. Our results show that EPO-R76E can block HRMEC death, consistent with its role in erythropoiesis and neuroprotection. In addition, long-term gene delivery of EPO-R76E is safe in the adult retina.

*Gene Therapy* (2015) **22,** 365–373; doi:10.1038/gt.2015.12; published online 26 February 2015

## INTRODUCTION

Erythropoietin (EPO) is a heavily glycosylated cytokine that regulates erythropoiesis by promoting the survival of erythroid progenitor cells in the bone marrow via activation of the EPO receptor (EPOR) homodimer.[1] EPO and its receptors are also expressed at low levels in non-hematopoietic tissues, including the retina.[1,2] Three receptors have been identified: EPOR; soluble EPOR (sEPOR), which sequesters EPO from binding to the cell surface receptors; and the common β chain of the granulocyte macrophages colony-stimulating factor, interleukin-3 and interleukin-5 receptors (CD131).[3–5] In neuronal tissue, EPO activates the phosphatidylinositol 3′-kinase/Akt pathway.[1]

EPO protects retinal neurons from death in models of induced or inherited retinal degeneration.[6–17] However, repeated treatment with systemic EPO may be associated with negative side effects such as polycythemia that could lead to thrombotic complications and increases in blood pressure. One approach to minimize these side effects is the introduction of point mutations in Epo to retain the neuroprotective activity while attenuating the erythropoietic activity.[13–15,18–21] We developed EPO-R76E, which protects neurons in models of Parkinson's disease, retinal degeneration and glaucoma while maintaining the hematocrit within normal limits.[8,13–15,21]

Another concern of long-term systemic treatment with EPO is the potential for neovascularization. Studies in non-retinal developmental systems or tumor-derived cells suggest that EPO is angiogenic.[22] However, the results are controversial and clinical evidence is lacking.[23] There has been one study on the effect of EPO on primary human retinal microvascular endothelial cells (HRMECs). In that study, EPO increased proliferation but not migration or tube formation.[24] Exogenous EPO contributes to neovascularization in development as demonstrated in patients with retinopathy of prematurity and in animal models of oxygen-induced retinopathy.[25–27] The current understanding of the role of EPO in retinal angiogenesis is described in a recent review.[28] In this study, we tested the safety of long-term systemic gene delivery of EpoR76E on the adult retina and compared the angiogenic effects of EPO and EPO-R76E in primary HRMECs using assays of migration, proliferation and cell death.

## RESULTS

### Production and quantification of EPO-R76E

In order to compare EPO and EPO-R76E, each protein needed to be produced and quantified. We utilized Chinese hamster ovary (CHO) cells, which are used to produce commercial EPO. This resulted in the production of EPO and EPO-R76E at a comparable size as commercial EPO (Figure 1a). The concentration of EPO and EPO-R76E was obtained by performing linear regression of the average of the bands at the expected molecular weight of 35 kD in three replicate lanes in a quantitative Coomassie gel of fractionated media from the stably expressing CHO cell lines (Figure 1b). The concentration of EPO and EPO-R76E in the media using this approach was $26.0 \pm 1.3$ ng ml$^{-1}$ (s.d.) and $11.7 \pm 4.5$ ng ml$^{-1}$, respectively. To determine the sensitivity of the commercial enzyme-linked immunosorbent assay (ELISA) in detecting EPO-R76E, known concentrations of EPO or EPO-R76E (based on the quantitative Coomassie) were added to the ELISA. The ELISA accurately detected EPO but not EPO-R76E (Table 1). The ELISA detected 70% of the EPO-R76E that was present, hence for all quantifications, a correction of 1.43 was applied to assure treatment with equal amounts of EPO and EPO-R76E (Table 1).

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Received 10 November 2014; revised 18 December 2014; accepted 15 January 2015; published online 26 February 2015

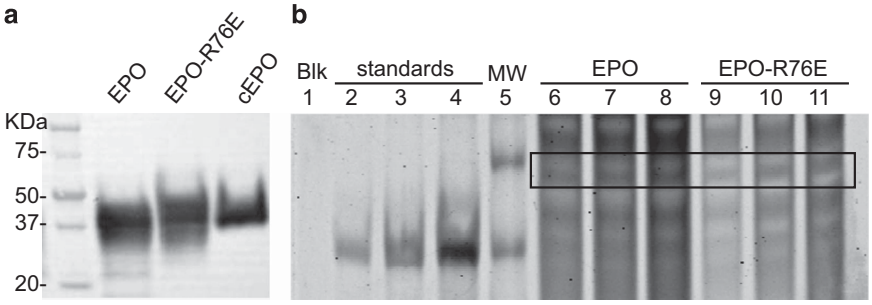


**Figure 1.** Production and quantification of EPO and EPO-R76E from CHO cells. (**a**) Western blotting showing that EPO and EPO-R76E produced from CHO cells are comparable in size to commercial EPO (cEPO). (**b**) Quantitative Coomassie gel. Equal volumes were loaded, alongside three increasing concentrations of standards, and a molecular weight (MW) marker. Lanes are as follows: (1) blank, (2) 200 ng of standard protein, (3) 500 ng of standard protein, (4) 1000 ng of standard protein, (5) MW ladder, (6) 10 μl fractionated media from EPO expressing CHO cells, (7) 15 μl fractionated media from EPO expressing CHO cells, (8) 20 μl fractionated media from EPO expressing CHO cells, (9) 10 μl fractionated media from EPO-R76E expressing CHO cells, and (10) 15 μl fractionated media from EPO-R76E expressing CHO cells, 11) 20 μl fractionated media from EPO-R76E expressing CHO cells. Box indicates location of EPO or EPO-R76E based on size.

**Table 1.** Quantification of EPO-R76E

| | *Quantitative Coomassie (mU ml$^{-1}$)* | *ELISA (mU ml$^{-1}$)* | *Correction factor* |
|---|---|---|---|
| EPO | 100 | 112.62 ± 0.67 | — |
| EPO-R76E | 50 | 35.97 ± 0.95 | 1.39 |
| EPO-R76E | 100 | 68.72 ± 0.38 | 1.45 |
| EPO-R76E | 200 | 140.49 ± 2.63 | 1.43 |

Abbreviations: ELISA, enzyme-linked immunosorbent assay; EPO, erythropoietin.

### EPO-R76E activates the EPOR and downstream signaling in HRMECs

Both the EPOR and CD131 were detected on HRMECs (Figure 2). In untreated HRMECs, both receptors were present diffusely throughout the cells (Figures 2a and b). The localization of each receptor changed from a diffuse to a punctate pattern upon stimulation with 20 U ml$^{-1}$ of commercial EPO (Figures 2c and d).

The ability of EPO-R76E to activate the EPOR was assessed by detection of phosphorylation of EPOR and the downstream signaling molecule, Akt. Treatment with EPO, EPO-R76E and commercial EPO all induced phosphorylation of the EPOR (Figures 3a and b) and Akt (Figures 3c and d) comparably. Several concentrations of EPO and EPO-R76E were tested and the level of phosphorylation of EPOR and Akt was comparable between EPO and EPO-R76E at each concentration tested (data not shown).

### Neither EPO nor EPO-R76E induce HRMEC proliferation

Proliferation of the HRMECs was induced by treatment with media containing 10% serum but not with 1% serum (Figure 4). As EPO has a bell-shaped dose curve,[16] a wide range of EPO concentrations were tested. Doses of 5–20 U ml$^{-1}$ of commercial EPO, EPO and EPO-R76E had no effect on HRMEC proliferation (data not shown). Increasing the concentrations to 30, 60 or 120 U ml$^{-1}$ still had no effect (Figure 4). This is in contrast to the efficacy of vascular endothelial growth factor to induce proliferation in HRMECs under the same conditions.[29]

### Neither EPO nor EPO-R76E induce HRMEC tube formation

In the presence of 10% serum-containing media, the HRMECs form tubes through the Matrigel (Figure 5a). No tube formation was detected in the absence of serum (Figure 5b) regardless of treatment with 40 U ml$^{-1}$ of commercial EPO, EPO or EPO-R76E (Figures 5c and e). The length of each tube wall was quantified (Figure 5f). There was no statistically significant difference between the serum-free negative control and those cells treated with EPO, EPO-R76E or commercial EPO. Treatment with 20 U ml$^{-1}$ of commercial EPO, EPO and EPO-R76E was also ineffective (data not shown). This is in contrast to the efficacy of vascular endothelial growth factor to induce proliferation in HRMECs under the same conditions.[29]

### EPO-R76E blocks HRMEC death

Apoptosis was detected in serum-deprived cells but not in cells maintained in 10% serum (Figure 6a). Treatment with 10, 20 or 40 U ml$^{-1}$ commercial EPO or EPO in serum-free media caused a similar amount of apoptosis as in the no serum control. Treatment with EPO-R76E caused a dose-dependent decrease in HRMEC apoptosis, with 80 U ml$^{-1}$ being most effective, but all doses showing statistically significant protection ($P < 0.001$). Additional cells were co-incubated with EPO-R76E and sEPOR to determine whether the effect was due to the activity of EPO-R76E (Figure 6b). Treatment with sEPOR completely blocked the antiapoptotic effect of EPO-R76E at 40 and 80 U ml$^{-1}$ ($P < 0.001$).

### EPO-R76E enters the eye after systemic gene delivery

Hematocrit was in the normal range for the rAAV.eGFP-treated mice, 38 ± 4.7 (s.d.), and was elevated in mice that received rAAV.EpoR76E, 51 ± 5.7, $P < 0.01$. However, as the mice were never phlebotomized, this increase is slight in physiological terms and had no effect on the health of the mice. Western blotting analysis was performed to detect and quantify EPO-R76E in the eye after an intramuscular (IM) injection of rAAV.EpoR76E or rAAV.eGFP (Figure 7). Low levels of EPO are present endogenously in the retina, as seen in the light band from retinas of mice treated with rAAV.eGFP. However, there was an 8.7-fold increase in retina EPO levels in mice injected with rAAV.EpoR76E showing that EPO-R76E was able to enter the eye (Figure 7).

### Gene delivery of EPO-R76E does not alter the retinal vasculature in wild-type mice

The retinal vasculature of wild-type mice had a normal branching pattern 3 months after IM injection with rAAV.EpoR76E ($n = 5$) or rAAV.eGFP ($n = 5$) based on labeling of the whole-mount retina with isolectin B4 (Figures 8a and f) and fluorescein angiography (Figures 8g and h). The vessels also retained their integrity, as demonstrated by lack of fluorescein leakage (Figures 8g and h).

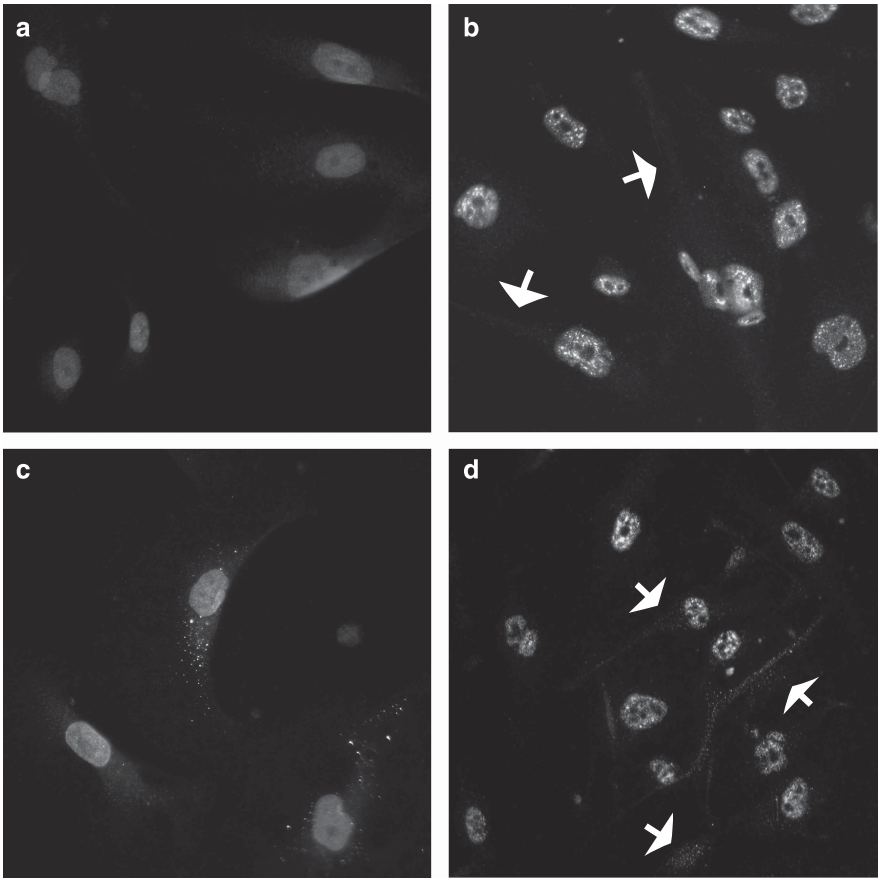


**Figure 2.** HRMECs contain both CD131 and EPOR. Epifluorescence micrographs of HRMECs immunolabeled with anti-CD131 (**a**, **c**) or anti-EPOR (**b**, **d**). Localization of the receptors shifts upon stimulation with EPO (**c**, **d**; arrows).

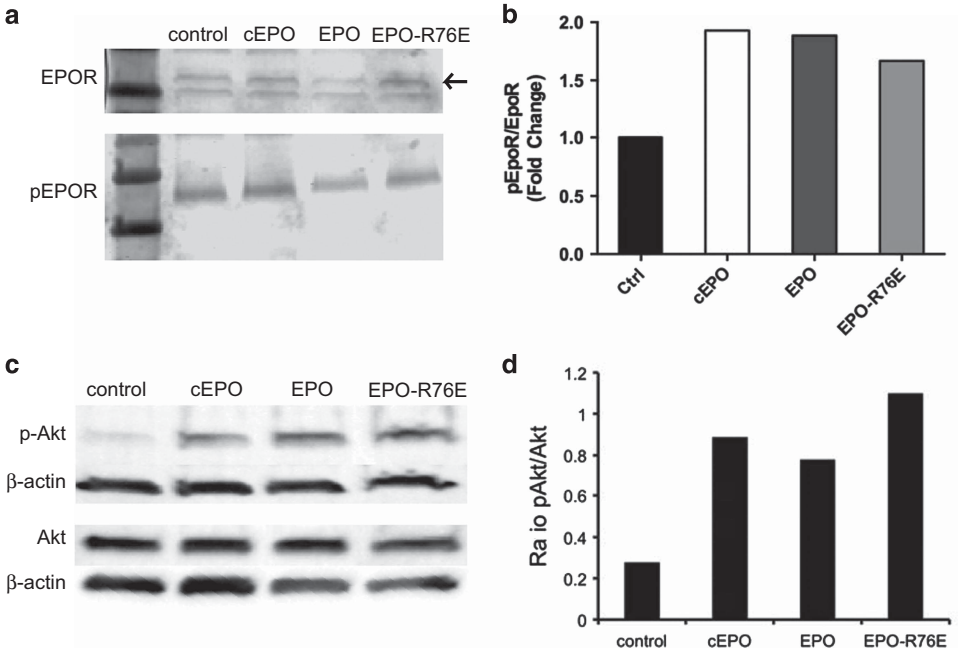


**Figure 3.** EPO, EPO-R76E and commercial EPO (cEPO) activate the EPOR and downstream signaling. (**a**) Western blotting of phosphorylation of the EPOR (pEPOR) in HRMECs treated with EPO, EPO-R76E and commercial EPO. Arrow indicates EPOR. (**b**) Bar graph of quantification of western blotting with pEPOR normalized to β-actin. (**c**) Western blotting of Akt and phosphoAkt after treatment with EPO, EPO-R76E or commercial EPO. (**d**) Bar graph of quantification of western blotting showing the ratio of pAkt to Akt after normalization of each to β-actin.

Long-term treatment with EPO-R76E is safe in the wild-type retina

The retinal histology appears normal in mice 3 and 7 months after injection with rAAV.eGFP ($n=5$) or rAAV.EpoR76E ($n=5$) based on both standard histology (Figures 9a and b) and optical coherence tomography (OCT) imaging (Figures 9c and d). Outer nuclear layer thickness was quantified using calibrated calipers in the OCT software; there was complete overlap between the rAAV.eGFP- and rAAV.EpoR76E-treated groups (Figure 9e). Localization of rhodopsin and glial fibrillary acidic protein (GFAP) also remained normal regardless of treatment (Figures 9f and g). Rhodopsin labeling remained primarily in the outer segments, and GFAP remained restricted to the astrocytes in the ganglion cell layer. In addition, the electroretinogram (ERG) waveform and *a* and *b* wave amplitudes was normal in both the rAAV.EpoR76E and rAAV.eGFP-treated mice (Figure 10). No difference was detected between the 3- and 7-month time points; representative waveforms (Figures 10a and b) and amplitudes (Figures 10c and d) at 2 log cd*s $m^{-2}$ from both time points are shown.

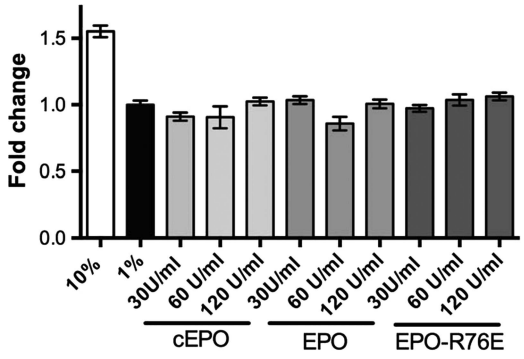


**Figure 4.** Neither EPO nor EPO-R76E induce HRMEC proliferation. Bar graph showing quantification of proliferation, normalized to baseline (1% serum). Proliferation was induced by 10% serum but not by treatment with EPO, EPO-R76E or commercial EPO (cEPO) at three different doses of each in 1% serum. Error bars indicate s.e.m.

## DISCUSSION

Short-term treatment with EPO was protective in patients with optic neuritis, and another trial is ongoing for patients with traumatic optic neuropathy (http://www.clinicaltrials.gov).[30] Animal studies have also shown that long-term gene delivery of EPO is effective in blocking degeneration in progressive retinal or optic

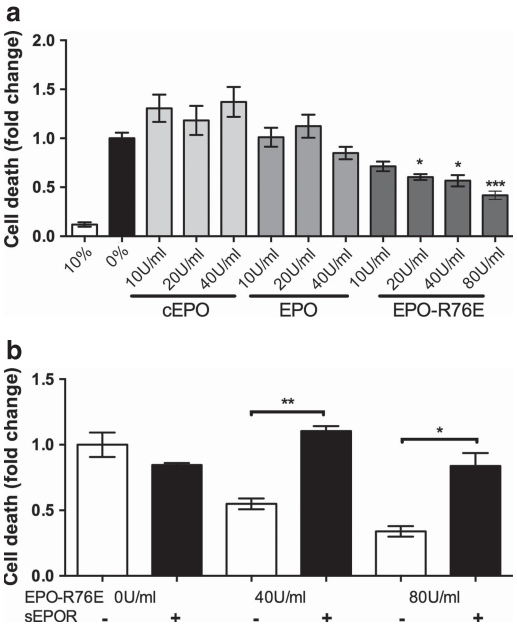


**Figure 6.** EPO-R76E protects the HRMECs from apoptosis. (**a**) Bar graph of quantification of cell death in cultures treated with 10% serum or with 10, 20, 40 or 80 U $ml^{-1}$ commercial EPO, EPO or EPO-R76E in 0% serum, normalized to 0% serum. (**b**) Bar graph of cell death after treatment with 0, 40 or 80 U $ml^{-1}$ EPO-R76E in 0% serum with or without 2 μg $ml^{-1}$ sEPOR. Error bars indicate s.e.m., $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

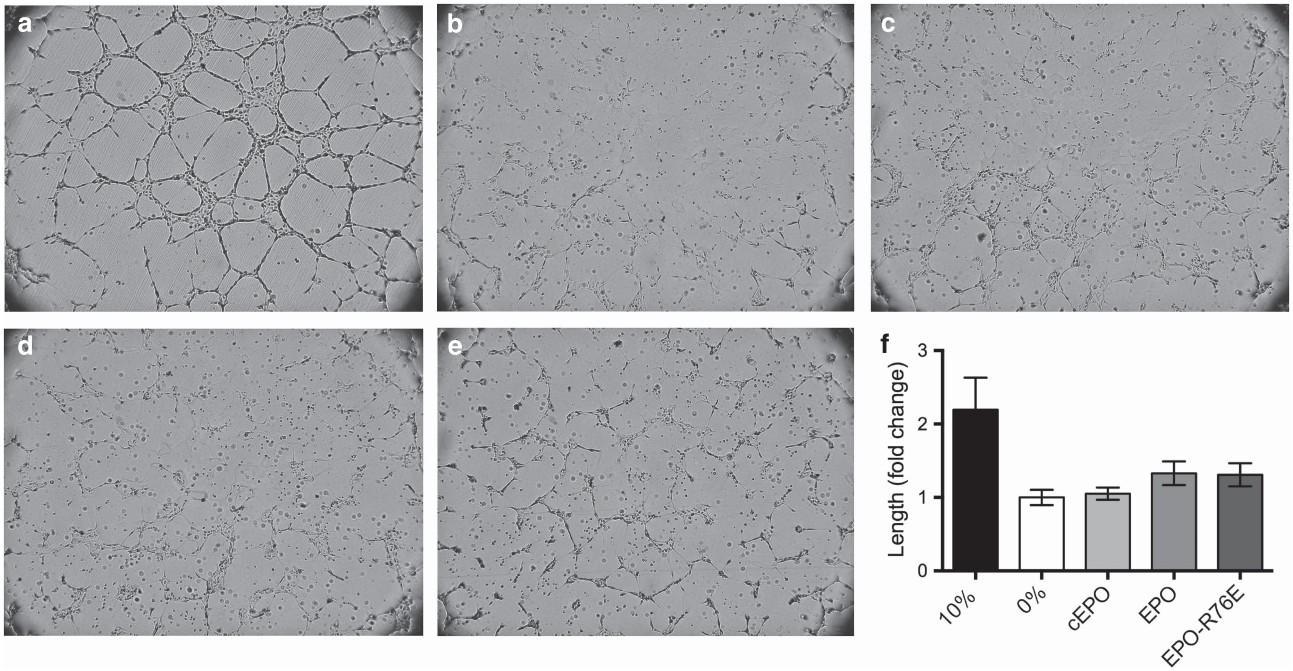


**Figure 5.** Neither EPO nor EPO-R76E induce tube formation in HRMECs. (**a**–**e**) Brightfield micrographs of HRMECs treated with (**a**) 10% serum, (**b**) 0% serum or (**c**) commercial EPO (cEPO), (**d**) EPO or (**e**) EPO-R76E in 0% serum. (**f**) Bar graph quantification of lengths of tube walls in each condition. Error bars indicate s.e.m.

nerve degenerations.[6,7,11] However, long-term systemic treatment with EPO is not safe thus limiting the clinical utility of EPO as a neuroprotective therapy. In contrast, EPO-R76E has attenuated erythropoietic activity, making it safer for long-term systemic treatment.[13–15] In addition, continuous delivery of EPO via rAAV-mediated gene therapy leads to protection from neurodegenerative diseases at lower doses than are needed by repeat bolus protein injections, further increasing the safety of treatment.[13–15] The goal of this study was to determine whether long-term treatment with EPO-R76E caused toxicity to the normal adult retina and whether EPO-R76E has a different angiogenic profile from EPO.

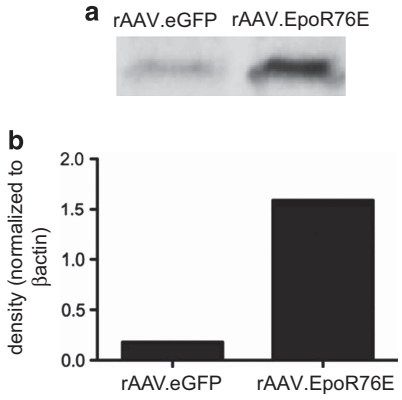


**Figure 7.** EPO-R76E enters the eye after systemic gene delivery. (**a**) Western blotting of EPO-R76E in the eye after IM injection of rAAV.eGFP or rAAV.EpoR76E. (**b**) Bar graph of EPO levels in eyes from mice injected IM with rAAV.eGFP or rAAV.EpoR76E after normalizing to β-actin.

We did not detect increased HMREC proliferation by treatment with EPO, in contrast to a previous report using a similar dose of EPO.[24] The different results could be due to higher serum conditions, higher passage cells or the particular assay used in the other study. For example, EPO may interact with vascular endothelial growth factor, which is a common component in serum, to enhance angiogenesis.[31] In agreement with the same previous report, we did not detect induction of tube formation.[24] Surprisingly, EPO-R76E blocked HMREC death while commercial EPO and EPO had no effect at the same concentrations. The antiapoptotic effect of EPO-R76E was blocked by co-incubation with sEPOR, indicating that the effect was due to the activity of EPO-R76E. Although the reason for this result is not clear, a potential explanation might be that cell death was blocked by EPO-R76E through an interaction with an EPOR, CD131 heterodimer. As EPO-R76E does not bind to the EPOR homodimer efficiently, it may preferentially bind to the heterodimer, while the opposite could be true of commercial EPO or EPO. In this case, we

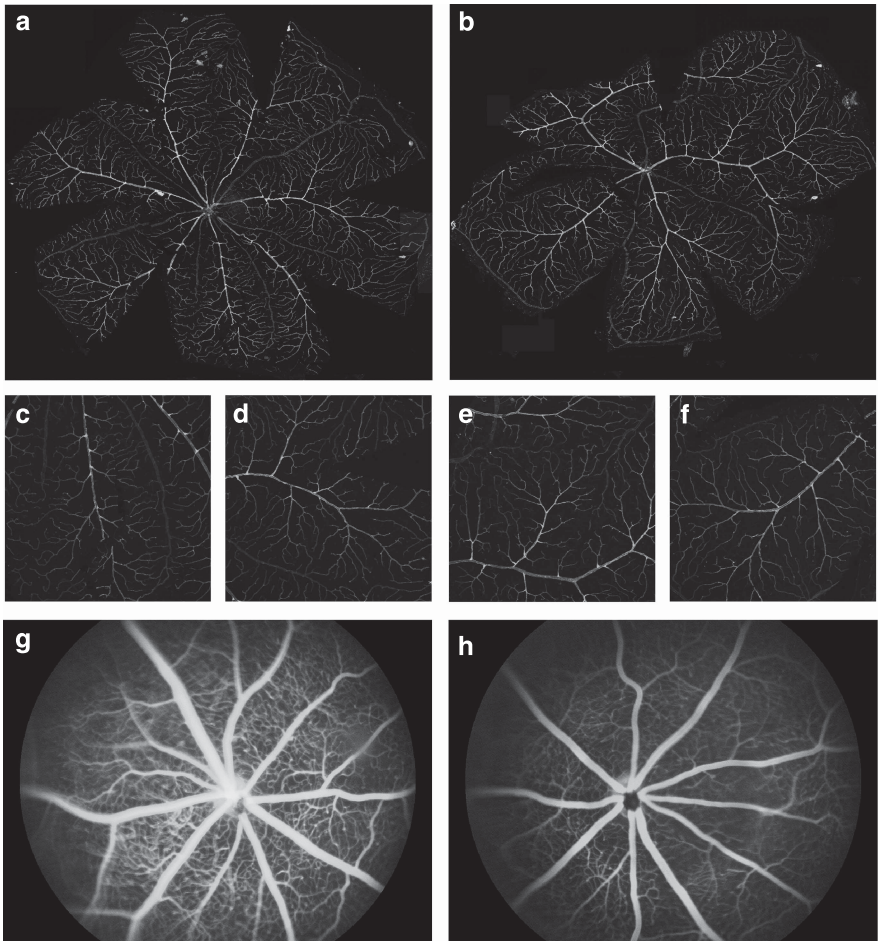


**Figure 8.** Long-term delivery of EPO-R76E has no deleterious effect on the retinal vasculature or histology. (**a**, **b**) Montage of confocal micrographs of representative retina flatmounts from mice treated IM with rAAV.eGFP (**a**) or rAAV.EpoR76E (**b**) and labeled with anti-isolectin 1. (**c**–**f**) Higher magnification images of isolectin labeling from rAAV.eGFP (**c**, **d**) or rAAV.EpoR76E (**e**, **f**) injected mice. (**g**, **h**) Representative fluorescein angiography images from mice treated IM with rAAV.eGFP (**g**) or rAAV.EpoR76E (**h**).

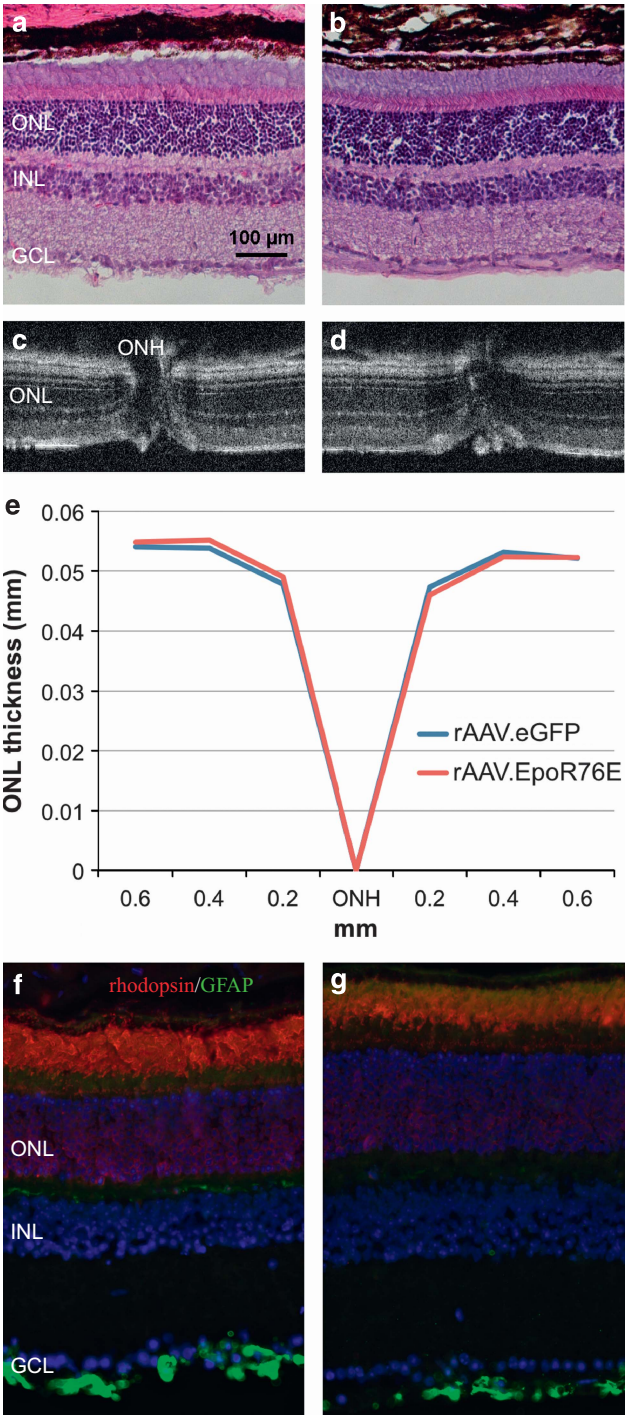


**Figure 9.** Long-term delivery of EPO-R76E has no deleterious effect on retina structure. (**a**, **b**) Light micrographs of retinas from mice treated IM with rAAV.eGFP (**a**) or rAAV.EpoR76E (**b**). (**c**, **d**) OCT B-line scan images through the optic nerve head (ONH) of retinas from a mouse injected with rAAV.eGFP (**c**) or rAAV.EpoR76E (**d**). (**e**) Quantification of ONL thickness. (**f**, **g**) Epifluorescence micrographs of retinas from mice treated IM with rAAV.eGFP (**e**) or rAAV.EpoR76E (**g**) immunolabeled with anti-rhodopsin (red), anti-GFAP (green) and DAPI (blue). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

would expect to detect a protective effect by commercial EPO and EPO at higher doses. This will be explored in future studies.

We show that, in a healthy, adult retina, EPO-R76E does not induce neovascularization. However, EPO or EPO-R76E may exacerbate pathological angiogenesis as has been reported in the hypoxic and diabetic retina.[27,28,32] A future avenue of investigation will be to compare the effects of EPO and EPO-R76E on retinal neovascularization in the oxygen-induced retinopathy model. The results of this study provide evidence of a good safety profile for EPO-R76E by demonstrating a lack of retinal neovascularization or toxicity in primary HRMECs or the adult mouse retina at doses we commonly use to induce neuroprotection. These results are consistent with other reports showing a lack of angiogenesis by EPO.[23] We also demonstrate that the lack of effect is not due to the inability of EPO-R76E to enter the eye. We show that systemic gene delivery of EPO-R76E causes an over eightfold increase in EPO levels in the eye. This supports studies showing that EPO can cross the blood–brain barrier.[33]

## MATERIALS AND METHODS

### Epo and EpoR76E expression plasmids

Human Epo cDNA was cloned into pCMV6-XL4 from pCMV6.EPO (Origene, Rockville, MD, USA). To generate EpoR76E, site-directed mutagenesis was performed with a Quick Change Multi Site Kit according to manufacturer instructions (Agilent Technologies, Santa Clara, CA, USA). Primers were: sense 5′-GGAAGCTGTCCTGGAGGGCCAGGCCCTG-3′ and antisense 5′-CAGG GCCTGGCCCTCCAGGACAGCTTCC-3′. Epo and EpoR76E were sub-cloned into pBluescript with NotI and XbaI restriction enzymes and transferred to pcDNA3.1 (Life Technologies, Carlsbad, CA, USA) in NotI and BamHI sites.

### Generation of stable, high-expressing cell lines

CHO cells (provided by the Vanderbilt Antibody and Protein Resource, VAPR) were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 20 mM HEPES, 1 mM pyruvate, 2 mM L-glutamine, 20 µg ml$^{-1}$ proline and antibiotic–antimicotic (Life Technologies). Cultures were maintained at 37 °C, 5% $CO_2$ and 95% relative humidity. The CHO cells were transfected with the expression plasmids and high-expressing colonies were identified and selected on the ClonePix FL by the VAPR (Life Technologies) using a fluorophore labeled mouse anti-EPO (MAB2871, R&D Systems, Minneapolis, MN, USA).

### Production of EPO and EPO-R76E for *in vitro* experiments

Stably expressing CHO cells lines were grown in full Dulbecco's modified Eagle's medium media to confluency and then washed and grown in media without serum for 3 days. Media was concentrated with Amicon Ultra 10 K devices (Millipore, Billerica, MA, USA) centrifuged at 5000 *g* for 20 min. EPO and EPO-R76E were quantified using the Human EPO Quantikine IVD ELISA Kit per manufacturer's instructions (R&D Systems).

### Quantification of EPO-R76E

Stable lines were cultured in FreeStyle CHO Expression Medium (Life Technologies) for 72 h. The media was concentrated to 2 ml using Amicon ultra filtration units with a MWCO of 3K (Millipore), filtered and injected on to a HiPrep 26/60 Sephacryl S-100 HR column connected to an AKTAxpress unit (GE Healthcare Life Sciences, Piscataway, NJ, USA). Standard column procedures outlined in the Unicorn 5.11 software wizard (GE Healthcare Lifesciences) were followed by the VAPR. Fractions were analyzed with the EPO ELISA kit (R&D Systems).

To determine the sensitivity of the ELISA kit for EPO-R76E, a quantitative Coomassie was performed by the VAPR. Three volumes of media fractions from EPO or EPO-R76E-expressing CHO cells were loaded into a Bis/Tris gel and infrared-Coomassie stained. Known concentrations of His-tagged GFP were used as standards. The amount of stained protein was quantified using an Odyssey Scanner system (LI-COR, Lincoln, NE, USA). Linear regression analysis was performed to identify the concentrations of EPO and EPO-R76E (identified by size on the gel). Then equal concentrations of EPO and EPO-R76E were used in the EPO ELISA. As the ELISA reports in mU ml$^{-1}$, a conversion was performed based on the datasheet for commercial EPO: 50 µg ml$^{-1}$ equals 7500 U ml$^{-1}$.

### Primary HRMEC cell culture

Passage 3 primary HRMEC (Cell Systems, Kirkland, WA, USA) were cultured in plates coated with Attachment Factor in 131 Medium supplemented

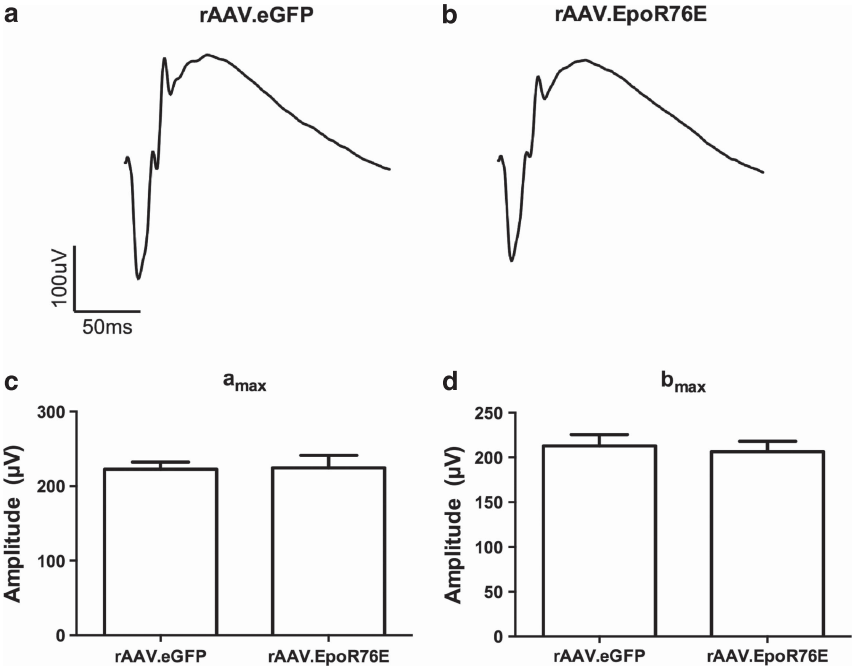


**Figure 10.** Long-term delivery of EPO-R76E has no deleterious effect on retina electrophysiology. (**a**, **b**) Average waveforms of flash ERGs from mice treated IM with rAAV.eGFP (**a**) or rAAV.EpoR76E (**b**). (**c**, **d**) Quantification of $a_{max}$ (**c**) and $b_{max}$ (**d**) at 2 log cd*s $m^{-2}$.

with Microvascular Growth Supplement (MVGS) and antibiotic–antimycotic solution (Life Technologies).

### Western blotting analysis of HRMECs

When 90% confluent, the HRMECs were deprived of MVGS for 4 h and treated with 10 U $ml^{-1}$ commercial EPO (ProSpec, East Brunswick, NJ, USA; or gift of Amgen, Thousand Oaks, CA, USA), 10 U $ml^{-1}$ EPO or EPO-R76E in media from CHO cells, or media from untransfected cells as a control, for 20 min. Cells were washed and scraped in Tris-buffered saline (TBS) containing 0.5% Triton-X-100 and protease and phosphatase inhibitor cocktails (P8340 and P5726, Sigma, St Louis, MO, USA). Lysates were passed through a 27-G needle, sonicated, centrifuged at 12 000 r.p.m. for 20 min, and protein quantification was determined using a BCA protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL, USA).

Equal amounts of protein were resolved by electrophoresis on 0.1% sodium dodecyl sulfate in a 4–20% Mini-protean TGX gel (Bio-Rad, Hercules, CA, USA) and transferred to a 0.45-μm polyvinylidene difluoride membrane. For detection of Akt and pAkt, the membrane was blocked for 3 h in TBS containing 0.1% Tween20 (v/v), 5% (w/v) nonfat dry milk or 5% (w/v) bovine serum albumin (BSA) to detect phospho-proteins. The membrane was incubated in anti-Akt (pan, 11E7, Cell Signaling, Danvers, MA, USA), p-Akt (Ser473, Cell Signaling) or β-actin (AM4302, Life Technologies). After washing, the membrane was incubated in secondary antibodies, goat anti-rabbit (H+L, W4011) and goat anti-mouse (H+L, HRP conjugate, Promega, Madison, WI, USA) or donkey anti-goat IgG-HRP (sc2020, Santa Cruz, Dallas, TX, USA). The bands were developed in Western Lightning Plus-ECL (PerkinElmer, Waltham, MA, USA, imaged on a Bio-Rad imaging system and Image Lab software and quantified with the Quantity One software (Bio-Rad).

For detection of EPOR and pEPOR, the membrane was blocked for 1 h in Odyssey Blocking Buffer TBS (LI-COR) with 0.1% Tween20. The membrane was incubated with anti-p-EpoR (Tyr456; sc-20236 R, Santa Cruz), anti-EpoR (M-20; sc-697, Santa Cruz), or β-actin. This antibody was shown to be specific for EpoR in a comparative study.[34] After washing, the membrane was incubated in secondary antibodies donkey anti-rabbit (H+L IRDye-680LT conjugate; 926-68023) and donkey anti-mouse (H+L IRDye-800CW conjugate; 926-32212, LI-COR). Bands were imaged on an Odyssey Scanner system and quantified using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

### Proliferation assay

A 96-well plate was coated with Attachment Factor and seeded with $1.0 \times 10^3$ HRMEC cells/well overnight. Cells were incubated in 1% MVGS for 12 h and then treated with commercial EPO, EPO or EPO-R76E in 131 media containing 1% MVGS according to previously published protocol.[29] At 48 h, proliferation was assessed by incubating in tetrazolium salt WST-1 (Millipore) for 4 h and measuring absorbance at 450nm with a reference of 600 nm (Spectramax M5, Molecular Devices, Sunnyvale, CA, USA). The assay was repeated twice with six replicates per assay. ANOVA and a Bonferroni post hoc test was performed.

### Tube-forming assay

A 24-well plate was coated with growth factor reduced Matrigel Basement Membrane Matrix (BD Biosciences, Franklin Lakes, NJ, USA) and incubated at room temperature for 30 min and at 37 °C for 1 h. Primary HRMECs were serum-starved overnight and then re-suspended in 131 media with 0.5% serum according to previously published protocol.[29] Each Matrigel-coated well was plated with 12 000 cells and incubated for 15 min at room temperature and 30 min at 37 °C and then switched to serum-free 131 media containing 40 U $ml^{-1}$ of commercial EPO, EPO or EPO-R76E. Images were taken 24 h later with an AZ Plan Apo ×0.5 objective on a Nikon AZ100 microscope (Nikon, Melville, NY, USA). The assay was performed twice with three replicates per assay. ANOVA and a Bonferroni post hoc test was performed.

### Cell death assay

A 96-well plate was coated with Attachment Factor and seeded with $7.0 \times 10^3$ HRMEC cells/well and attached overnight. The next day, cells were serum deprived and treated with commercial EPO, EPO or EPO-R76E with or without 2 μg $ml^{-1}$ sEPOR. After 18 h, levels of DNA in the cell cytoplasm were quantified using the Cell Death Detection ELISA assay following the manufacturer's instructions (Roche, Indianapolis, IN, USA). Absorbance was measured at 409 nm, with a 490-nm reference. The assay was performed twice with three replicates per assay. ANOVA and a Bonferroni post hoc test was performed.

### Immunocytochemistry

Sterile 12-mm coverslips were placed in the wells of a 24-well plate and coated with Attachment Factor. Primary HRMEC were seeded at $2.5 \times 10^3$ cells per well and incubated for 24 h. The cells were preserved with 4%

paraformaldehyde for 10 min, rinsed with phosphate-buffered saline (PBS) and incubated in blocking buffer (0.5% Tween-20 and 5% BSA in TBS) for 1 h. Primary antibody (1:100; anti-EPOR, sc-697; or anti-CD131, sc-678; Santa Cruz) was added to the blocking solution, and cells were incubated overnight at 4 °C. The cells were then rinsed with PBS and incubated in secondary antibody in the blocking solution at room temperature for 1 h, washed with PBS and mounted in Vectashield-DAPI (4,6-diamidino-2-phenylindole).

### Mice

Wild-type C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Mice were maintained on a 12-h light/dark cycle in the Vanderbilt University animal facility with food and water provided *ad libitum*. The protocol was approved by the Animal Care and Use Committee of Vanderbilt University and was in accordance with the ARVO statement for the use of animals in vision research.

### IM injection of viral vectors

The recombinant adeno-associated viral vectors (rAAV) were produced and purified by the University of Pennsylvania Vector Core. Using a beveled Hamilton syringe, $5 \times 10^9$ genome copies (gc) of rAAV2/8.CMV.rhesusEpoR76E or rAAV2/8.CMV.eGFP was delivered into the quadriceps of postnatal day-7 mice.

### Electroretinograms

Flash ERG recordings were performed 3 ($n = 15$ mice per group) and 7 ($n = 10$ mice per group) months after injection of viral vectors using the Diagnosys Espion System (Diagnosys LLC, Lowell, MA, USA). Briefly, mice were dark-adapted overnight, anesthetized with an intraperitoneal injection of ketamine and xylazine and their pupils dilated with 1% tropicamide. Eyes were kept moist with Systane ultra eye drops (Alcon, Fort Worth, TX, USA). The reference needle electrode was placed in the middle of the forehead and the ground electrode was placed in the rump, both intradermally. A gold wire loop corneal electrode was used. Seven light intensities were tested ($-4$, $-3$, $-2$, $-1$, 0, 1 and 2 log cd*s $m^{-2}$). A Student's *t*-test was performed to compare the two treatment groups.

### Optical coherence tomography

Immediately after performing ERG, anesthetized mice were imaged on a Bioptigen ultra-high resolution OCT system (Bioptigen, Durham, NC, USA). The retinas were imaged with a mouse retina bore, and measurements of outer nuclear layer thickness were made using digital, calibrated calipers.

### Fluorescein angiography

Three months after vector injection, mice ($n = 9$ per group) were anesthetized with 2.5% isoflurane, the eyes were dilated with 1% tropicamide and 2.5% phenylephrine and kept moist with 2.5% methylcellulose eye drops (Goniovisc Eye Care and Cure, Inc., Tuscon, AZ, USA). The cornea was positioned in contact with the Micron III lens (Phoenix Research Laboratories, Pleasanton, CA, USA). Fluorescein (0.1 ml of AK-Fluor-10%; Akorn, Inc, Lake Forest, IL, USA) was injected intraperitoneally, and images were collected using appropriate filters on the Micron III microscope.

### Western blotting analysis of mouse eyes

Mice ($n = 5$ per group) were euthanized and enucleated, and eyecups were fast frozen in liquid nitrogen. The same lysis buffer used for cells was added to the eyecups, and tissue was mechanically disrupted and the sclera was removed. Remaining lysate was sonicated and incubated with 0.1% sodium dodecyl sulfate on ice for 30 min followed by 20 min centrifugation at 14 000 r.p.m. Protein quantification was performed as mentioned above.

Equal amounts of protein were resolved by electrophoresis on 0.1% sodium dodecyl sulfate in a 4–20% Mini-protean TGX gel (Bio-Rad) and transferred to 0.45-μm polyvinylidene difluoride membrane. The membrane was blocked for 3 h in TBS containing 0.1% Tween20 (v/v), 5% (w/v) nonfat dry milk or 5% (w/v) BSA to detect phospho-proteins. The membrane was incubated in anti-EPO and β-actin. After washing, the membrane was incubated in goat anti-rabbit and goat anti-mouse, followed by washing and incubation in Western Lightning Plus-ECL. Imaging was performed on a Bio-Rad imaging system and Image Lab software, and quantification was performed with the Quantity One software.

### Whole-mount Labeling

For analysis of retinal vasculature, mice were analyzed 3 months postinjection ($n = 2$ per group). Enucleated eyes were preserved in 4% paraformaldehyde at 4 °C for at least 15 min and transferred to cold 2 × PBS on ice for 10 min. After that, retinas were dissected and rinsed with PBS, and radial incisions were performed to flatten the retina. The retinas were incubated in cold (−20 °C) methanol for 20 min and then blocked in PBS containing 0.3% Triton and 0.2% BSA for 1 h. The retinas were incubated overnight at 4 °C with isolectin B4 (isolectin GS-IB4-alexa 488, Invitrogen, Carlsbad, CA, USA) diluted in blocking solution. The next day, retinas were washed with PBS+0.3% Triton and mounted in Prolong mounting media and imaged on an Olympus confocal microscope (Olympus, Center Valley, PA, USA).

### Immunohistochemistry

Retinal histology and immunohistochemistry was performed at 3 and 7 months after vector injection ($n = 15$ per treatment group). Enucleated eyes were preserved in 4% paraformaldehyde for 2 h, cryo-protected in 30% sucrose at 4 °C overnight and embedded in tissue-freezing media (Triangle Biomedical, Durham, NC, USA). Ten-micron thick sections were collected on a cryostat (Fisher, Pittsburgh, PA, USA). Slides were rinsed with PBS and incubated at room temperature for 2 h in normal donkey serum (1:20) in 0.1 M phosphate buffer with 0.5% BSA and 0.1% Triton X 100 (PBT). The slides were incubated overnight at 4 °C in anti-rhodopsin (1:50, Abcam, Cambridge, MA, USA) or anti-GFAP (1:400, Dako, Carpinteria, CA, USA) in PBT, then rinsed with PBS and incubated for 2 h at room temperature in the appropriate fluorophore-conjugated secondary antibody (donkey anti-rabbit or mouse -Alexa Fluor 488 or 568, Life Technologies). Slides were rinsed with PBS, mounted in Vectashield Mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA), and imaged on a Nikon Eclipse epifluorescence microscope (Nikon). Images were collected at the same magnification, gain and exposure settings.

### Histology

Additional cryo-sections were stained with hematoxylin and eosin (Fisher) and brightfield microscopy was performed on an Olympus Provis AX70 microscope ($n = 15$ per treatment group). Images were collected near the optic nerve head using a Nikon DS-Fi2 camera and the NIS elements Advanced Research software (Nikon).

## CONFLICT OF INTEREST

TSR is a co-inventor on a patent application (US No. 13/979,451) regarding neuroprotective action of EPO-R76E. No licensing has occurred. The other authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

We thank Megan Capozzi in Dr John Penn's laboratory for assistance with the tube-formation assay, Dr Ashwath Jayagopal for assistance with fluorescein angiography and Kristi Wynn for technical assistance. Stable cell lines were generated and EPO and EPO-R76E were quantified by the VAPR, which is supported by the Vanderbilt Institute of Chemical Biology and the Vanderbilt Ingram Cancer Center (P30 CA68485). This work was supported by grants from Research to Prevent Blindness Career Development Award; Research to Prevent Blindness Unrestricted Funds (P. Sternberg); CTSA award UL1TR000445 from the National Center for Advancing Translational Sciences; Department of Defense W81XW-10-1-0528; P30-EY008126; NEI EY022349; and Fight for Sight.

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