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 electoretinogram (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.

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. EPO and its receptors are also expressed at low levels in non-hematopoietic tissues, including the retina. Three receptors have been identified: EPOR, soluble EPO (sEPOR), which sequesters EPO from binding to the cell surface receptors; and the common β chain of the granulocyte macrophage colony-stimulating factor, interleukin-3 and interleukin-5 receptors (CD131). In neuronal tissue, EPO activates the phosphatidylinositol 3-kinase/Akt pathway. EPO protects retinal neurons from death in models of induced or inherited retinal degeneration. 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 that could block EPO R76E activity while attenuating the erythropoietic activity. We developed EPO-R76E, which protects neurons in models of Parkinson’s disease, retinal degeneration and glaucoma while maintaining the hematocrit within normal limits.

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. However, the results are controversial and clinical evidence is lacking. 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. Exogenous EPO contributes to neovascularization in development as demonstrated in patients with retinopathy of prematurity and in animal models of oxygen-induced retinopathy. The current understanding of the role of EPO in retinal angiogenesis is described in a recent review. 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 determined 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 ± 1.3 ng ml\(^{-1}\) (s.d.) and 11.7 ± 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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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, 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.

Neither EPO nor EPO-R76E induce HRMEC tube formation
In the presence of 10% serum-containing media, the HRMECs formed 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.

Table 1. Quantification of EPO-R76E

| Quantitative Coomassie (μg/ml) | ELISA (mU/ml) | 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 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.

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).\(^3\) Animal studies have also shown that long-term gene delivery of EPO is effective in blocking degeneration in progressive retinal or optic...
nerve degenerations. 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. 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.

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. We did not detect increased HMREC proliferation by treatment with EPO, in contrast to a previous report using a similar dose of EPO. 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. In agreement with the same previous report, we did not detect induction of tube formation. 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 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.

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).
hypoxic and diabetic retina. 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. 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.

**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 GCTCGGCCCTCCAGGAGACAGCTG-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% CO₂ 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 to confluence 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 AKTApex 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...
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-protein 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 donkey anti-rabbit (H+L IRDye-800CW conjugate, Promega, Madison, WI, USA), p-Akt (Ser473, Cell Signaling) or β-actin (AM4302, Life Technologies). 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 680LT conjugate; 926-68023) and donkey anti-mouse (H+L IRDye-800CW conjugate; 926-62212, LI-COR).

Long-term delivery of rAAV.EpoR76E is safe
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Proliferation assay
A 96-well plate was coated with Attachment Factor and seeded with 1.0 × 10⁴ 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 MS, 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 × 10⁴ 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 eEPOR. 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 × 10⁴ cells per well and incubated for 24 h. The cells were preserved with 4%
parafomaldehyde 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-EPO, 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-phenylinodole).

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 adenovirus-associated viral vectors (rAAV) were produced and purified by the University of Pennsylvania Vector Core. Using a beveled Hamilton syringe, 5 × 1010 genome copies (gc) of rAAV2/8.CMV.rhesusE-pOR76E or rAAV2/8.CMV.eGFP was delivered into the quadriceps of anesthetized with 2.5% isoflurane, the eyes were dilated with 1% phenylindole). Immediately after performing ERG, anesthetized mice were imaged on a Nikon Eclipse epi fluorescence 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.

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REFERENCES

1 Ogunsola O, Bogdanova A. Epo and non-hematopoietic cells: what do we know? Meth Mol Biol 2013; 982: 13-41.

2 García-Ramírez M, Hernández C, Simó R. Expression of erythropoietin and its receptor in the human retina: a comparative study of diabetic and non-diabetic subjects. Diabetes Care 2008; 31: 1189–1194.

3 Leist M, Ghezzi P, Grasso G, Bianchi R, Villa P, Fratelli M et al. Derivatives of erythropoietin that are tissue protective but not erythropoietic. Science 2004; 305: 239–242.

4 Mennini T, De Paola M, Bigini P, Mastrotto C, Fumagalli E, Barbera S et al. Nonhematopoietic erythropoietin derivatives prevent motoneuron degeneration in vitro and in vivo. Mol Med 2006; 12: 153–160.

5 Brines M, Cerami A. The receptor that tames the innate immune response. Mol Med 2012; 18: 486–496.

6 Grimm C, Wenzel A, Grozer M, Meyser H, Seeliger M, Samardzija M et al. HIF-1-induced erythropoietin in the hypoxic retina protects against light-induced retinal degeneration. Nat Med 2002; 8: 718–724.

7 Grimm C, Wenzel A, Stanescu D, Samardzija M, Hotop S, Grozer M et al. Constitutive overexpression of human erythropoietin protects the mouse retina against induced but not inherited retinal degeneration. J Neurosci 2004; 24: 5651–5658.

8 Hines-Beard J, Desai S, Haag R, Esumi N, D'Sourney L, Parker S et al. Identification of a therapeutic dose of continuously delivered erythropoietin in the eye using an inducible promoter system. Curr Gene Ther 2013; 13: 275–281.

9 Kilic U, Kilic E, Soliz J, Bassetti C, Gassmann M, Hermann D. Erythropoietin protects from axotomy-induced degeneration of retinal ganglion cells by activating ERK1/2. FASEB J 2005; 19: 249–251.

10 King C, Rodger J, Bartlett C, Esmailli T, Dunlop S, Beazley L. Erythropoietin is both neuroprotective and neuroregenerative following optic nerve transection. Exp Neurol 2007; 205: 48–55.

11 Rex T, Allocca M, Domenici L, Surace E, Maguire A, Lyubarsky A et al. Systemic but not intracaular Epo gene transfer protects the retina from light-and genetic-induced degeneration. Mol Ther 2004; 10: 855–861.

12 Rex T, Wong Y, Kodali K, Merry S. Neuroprotection of photoreceptors by direct delivery of erythropoietin to the retina of the retinal degeneration slow mouse. Exp Eye Res 2009; 89: 735–740.

13 Sullivan T, Geisert E, Hines-Beard J, Rex T. Systemic AAV-mediated gene therapy preserves retinal ganglion cells and visual function in DBA/2 J glaucomatous mice. Hum Gene Ther 2011; 22: 1191–1200.

14 Sullivan T, Geisert E, Templeton J, Rex T. Dose-dependent treatment of optic nerve crush by exogenous systemic mutant erythropoietin. Exp Eye Res 2012; 96: 36–41.

15 Sullivan T, Kodali K, Rex T. Systemic gene delivery protects the photoreceptors in the retinal degeneration slow mouse. Neurochem Res 2011; 36: 613–618.

16 Weishaupt J, Rohde G, Polking E, Siren A-L, Ehrenreich H, Bahr M. Effect of erythropoietin on axotomy-induced apoptosis in rat retinal ganglion cells. Invest Ophthalmol Vis Sci 2004; 45: 1514–1522.

17 Zhong L, Bradley J, Schubert W, Ahmed E, Adamis A, Shima D et al. Erythropoietin promotes survival of retinal ganglion cells in DBA/2 J glaucoma mice. Invest Ophthalmol Vis Sci 2007; 48: 1212–1218.

18 Villa P, van Beek J, Larsen A, Genwen J, Christensen S, Cerami A et al. Reduced functional deficits, neuroinflammation, and secondary tissue damage after treatment of stroke by nonerythropoietic erythropoietin derivatives. J Cereb Blood Flow Metab 2007; 27: 552–563.

19 Gan Y, Xing J, Jing Z, Stefler R, Zhang F, Luo Y et al. Mutant erythropoietin without erythropoietic activity is neuroprotective against ischemic brain injury. Stroke 2012; 43: 3071–3077.

20 Colella P, Iodici C, Di Vicino U, Annunziata I, Surace E, Auricchio A. Non-erythropoietic erythropoietin derivatives protect from light-induced and genetic photoreceptor degeneration. Hum Mol Genet 2011; 20: 2251–2262.

21 Dhanuskodi A, Akano E, Roguski E, Xue Y, Rao S, Matta S et al. A single intramuscular injection of rAAV-mediated mutant erythropoietin protects against MPTP-induced Parkinsonism. Genes Brain Behav 2012; 13: 224–230.

22 Ribatti D. Angiogenic activity of classical hematopoietic cytokines. Leuk Res 2012; 36: 537–543.

23 Jelkmann W, Elliott S. Erythropoietin and the vascular wall: the controversy continues. Nutr Metab Cardiovasc Dis 2013; 23: 537–543.

24 Yoshida T, Gong J, Xu Z, Wei Y, Duh E. Inhibition of pathological retinal angiogenesis by the integrin αvβ3 antagonist tetraidothyroacetic acid (tetrac). Exp Eye Res 2012; 94: 41–48.

25 Kandasamy Y, Kumar P, Hartley L. The effect of erythropoietin on the severity of retinopathy of prematurity. Eye 2014; 28: 814–818.

26 Heidary G, Vanderveen D, Smith L. Retinopathy of prematurity: current concepts in molecular pathogenesis. Semin Ophthalmol 2009; 24: 77–81.

27 Morita M, Ohneda O, Yamashita T, Takahashi S, Suzuki N, Nakajima O et al. HIF-HIF-2αpalp is a key factor in retinopathy of prematurity in association with erythropoietin. EMBO J 2003; 22: 1134–1146.

28 Caprara C, Grimm C. From oxygen to erythropoietin: relevance of hypoxia for retinal development, health and disease. Prog Ret Eye Res 2012; 31: 89–119.

29 Capozzi M, McCollum G, Penn J. The role of cytochrome P450 epoxygenases in retinal angiogenesis. Invest Ophthalmol Vis Sci 2014; 55: 4253–4260.

30 Suh K, Hein K, Sattler M, Görlich A, Ciupka C, Scholz K et al. A randomized, double-blind, phase 2 study of erythropoietin in optic neuritis. Ann Neurol 2012; 72: 199–210.

31 Yang Z, Wang H, Jiang Y, Hartnett M. VEGFA activates erythropoietin receptor and enhances VEGFR2-mediated pathological angiogenesis. Am J Pathol 2014; 184: 1230–1239.

32 Watanabe D, Suzuma K, Matsui S, Kurimoto M, Kiyu J, Kita M et al. Erythropoietin as a retinal angiogenic factor in proliferative diabetic retinopathy. New Engl J Med 2005; 353: 782–792.

33 Banks W, Jumbe N, Farrell C, Niehoff M, Heatherington A. Passage of erythropoietic agents across the blood-brain barrier: a comparison of human and murine erythropoietin and the analog darbepoeitin alfa. Eur J Pharmacol 2004; 505: 93–101.

34 Elliott S, Busse L, Bass M, Lu H, Sarosi I, Sinclair A et al. Anti-Epo receptor antibodies do not predict Epo receptor expression. Blood 2006; 107: 3454.