Endothelin-2-Mediated Protection of Mutant Photoreceptors in Inherited Photoreceptor Degeneration

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Abstract

Expression of the Endothelin-2 (Edn2) mRNA is greatly increased in the photoreceptors (PRs) of mouse models of inherited PR degeneration (IPD). To examine the role of Edn2 in mutant PR survival, we generated Edn2−/− mice carrying homozygous Pde6brd1/rd1 alleles or the Tg(RHO P347S) transgene. In the Edn2−/− background, PR survival increased 110% in Pde6brd1/rd1 mice at post-natal (PN) day 15, and 60% in Tg(RHO P347S) mice at PN40. In contrast, PR survival was not increased in retinal explants of Pde6brd1/rd1; Edn2−/− mice. This finding, together with systemic abnormalities in Edn2−/− mice, suggested that the increased survival of mutant PRs in the Edn2−/− background resulted at least partly from the systemic EDN2 loss of function. To examine directly the role of Edn2 in mutant PRs, we used a scAAV5-Edn2 cDNA vector to restore Edn2 expression in Pde6brd1/rd1; Edn2−/− PRs and observed an 18% increase in PR survival at PN14. Importantly, PR survival was also increased after injection of scAAV5-Edn2 into Pde6brd1/rd1 retinas, by 31% at PN15. Together, these findings suggest that increased Edn2 expression is protective to mutant PRs. To begin to elucidate Edn2-mediated mechanisms that contribute to PR survival, we used microarray analysis and identified a cohort of 20 genes with >4-fold increased expression in Tg(RHO P347S) retinas, including Fgf2. Notably, increased expression of the FGF2 protein in Tg(RHO P347S) PRs was ablated in Tg(RHO P347S); Edn2−/− retinas. Our findings indicate that the increased expression of PR Edn2 increases PR survival, and suggest that the Edn2-dependent increase in PR expression of FGF2 may contribute to the augmented survival.

Introduction

IPDs are genetically heterogeneous disorders characterized by the progressive death of mutant PRs. Although more than 160 IPD-associated genes have been identified in humans, with many of these mutations modeled in mouse, relatively few studies have examined the biochemical mechanisms that promote or resist death in the mutant PR [1,2]. Factors shown to promote the survival of mutant or injured PRs include IL-6 cytokines [3,4,5,6,7], STAT3 [8], and neurotrophic factors including FGF2 [9,10,11,12,13]. In contrast, other molecules including GFAP and vimentin [14,15], complement factor D [16], TNFα [17] and poly-ADP-ribose polymerase-1 [18] have been shown to contribute to the death of mutant or injured PRs.

One gene whose PR expression is strongly induced by PR mutations is Edn2 [19]. Retinal Edn2 transcripts are up-regulated in multiple models of photoreceptor degeneration [19,20,21], as well as in other retina stresses including, for example, retinal detachment [19,22] and retinal hypoxia [23] amongst others. These observations suggest that the increased expression of Edn2 may be a general response to retinal insult.

EDN2 is a vasoactive peptide that binds to two G-protein coupled receptors, EDNR and EDNRB, with equal affinity [24]. EDNRB is expressed in both Müller glia [19] and horizontal cells [25] in neural retina, whereas EDNRA is present in bipolar dendrites [23]. Both receptors are detected in choroidal and retinal vessels [26].

The biological roles of EDN2 are the least well-characterized of the three endothelin family members [27]. EDN2 has been found...
to participate in macrophage chemotraction in breast tumor cell invasion [29,29], keratinocyte differentiation [30], and oviductal contraction during ovulation [31,32]. Within the context of PR injury, the effect of the increased expression of Edn2 on PR survival is unclear. Whereas the intravitreal administration of an EDNRB antagonist increased PR death in one IPD model [5] and reduced the protective effects of Norrin in light damage [39], the subcutaneous administration of a dual EDNRA-EDNRB receptor antagonist after light damage reduced PR layer staining for cleaved caspase-3, a cell death marker [25].

We used genetic analyses to determine whether the increased expression of Edn2 in mutant PRs is a pathogenic or a pro-survival response, in two well-defined mouse models of IPDs, mice carrying a mutant human rhodopsin transgene (Tg(RHO P347S)) [34] associated with human PR degeneration, and Pde6brd1/rd1 mice, with a mutation in the β subunit of the PR-specific phosphodiesterase-6 (PDE-6) gene [35]. We over-expressed Edn2 [9].

At least partly mediated by FGF2, a known neuroprotective factor PRs and found that it plays a protective role in one model of IPD.

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**Results**

**Increased expression of the Edn2 mRNA and peptide in mutant PRs**

To confirm that Edn2 transcripts are up-regulated in the Pde6brd1/rd1, Tg(RHO P347S), and Pde6brd1/rd1 mutant mouse models of IPD we studied, we measured retinal Edn2 mRNA abundance. As reported by Rattner and Nathans in other models [19], we found the Edn2 mRNA to be greatly increased vs. wild-type (WT) controls in Pde6brd1/rd1 (32-fold), Tg(RHO P347S) (70-fold), and Pde6brd1/rd1 (72-fold) retinas, using qRT-PCR (for each model n = 3; p < 0.005) (Fig. 1A).

We also confirmed, by *in situ* hybridization using Pph2 *gob*/* and Tg(RHO P347S) mice, the observation of Rattner and Nathans [19] that the increase in the Edn2 mRNA is PR-specific (Fig. 1B). To determine if Edn2 mRNA up-regulation is a leading or lagging indicator of PR death, we examined the temporal up-regulation of Edn2 mRNA in Pde6brd1/rd1 retinas by qRT-PCR (Fig. 1C,D). The abundance of the Edn2 transcript in Pde6brd1/rd1 retinas at PN10, measured as the ratio of Edn2 mRNA:Gapdh mRNA expression, was comparable to the expression of Edn2 transcripts in WT retinas at all time points examined (data not shown). By PN10, however, a statistically significant increase in Edn2 transcripts was detected in Pde6brd1/rd1 retinas (Fig. 1C,D); n = 3, p < 0.05 vs. WT retinas; at this age, photonic nuclei are present in this mutant [36]. The decrease in Edn2 mRNA expression with time (Fig. 1C) is likely to reflect the decreasing number of PRs in the Pde6brd1/rd1 retina, since the mRNA expression of Edn2 relative to rhodopsin increases with time (Fig. 1D), indicating that the expression of the Edn2 mRNA per PR increased as the number of PRs progressively decreased. Edn2 transcripts remained substantially up-regulated as late as PN18 (data not shown).

To determine whether the increase in Edn2 transcripts in mutant retinas is accompanied by increased levels of EDN2 peptide, we quantified EDN2 in Pph2 *gob*/* retinas by radioimmunoassay after separating the EDN2 peptide from EDN1 and EDN3 by HPLC. The abundance of EDN2 was below the detection limit in WT retinas (<0.05 fmol/retina; n = 10 retinas), making it impossible to determine the maximum fold increase in EDN2 mutant retinas. However, 0.15 fmol/retina (n = 10 retinas) of EDN2 was detected in Pph2 *gob*/* retinas, indicating that the EDN2 peptide is increased by a minimum of three-fold in Pph2 *gob*/* retinas.

**Retinal expression of components of endothelin signaling biology**

To evaluate whether the large increase in Edn2 expression in mutant PRs was accompanied by increases in other components of endothelin biology, we first quantified the expression of the mRNAs for Edn1, Edn3, Edna, and Ednb in WT and mutant retinas (Fig. S1). Overall, there were no significant differences at PN21 between Edn2+/−, Edn2−/−, Tg(RHO P347S);Edn2+/− and Tg(RHO P347S);Edn2−/− retinas in the expression of these genes (all n = 3, p > 0.05) (Fig. S1), with one exception: the mRNA of Edn1 in Tg(RHO P347S);Edn2−/− was modestly increased, by 2.3-fold, compared to Edn2+/− retinas (n = 3, p < 0.05) (Fig. S1).

EDN2 is processed from a prepro peptide that undergoes sequential cleavage events to generate mature EDN2 (mat EDN2) (Fig. 4B). To determine where endothelin-converting enzyme 1 (ECE-1), an enzyme implicated in the processing of big EDN2 to mature EDN2 [37], is expressed in retina, we used immunofluorescence staining. We found that ECE-1 was expressed in Müller cell bodies and radial fibres (Fig. S2A), as shown by comparison with the Müller cell-specific marker glutamine synthetase [38].

To determine whether the expression pattern of the EDNRA and EDNRB receptors was altered in Pde6brd1/rd1 retinas, in the presence or absence of Edn2 expression, we used immunofluorescence staining (Fig. S2B). In mice of all four genotypes, EDNRA expression was low, with only scattered staining in the choroid, OPL, INL and GCL, which may represent retinal microglia [42] that migrate to the mutant PRs in the ONL [39]. There was no significant difference in EDNRA staining in Pde6brd1/rd1, Edn2+/− vs. Pde6brd1/rd1, Edn2−/− retinas (Fig. S2B).

EDNRB expression in WT retina was seen predominantly in the outer plexiform layer (OPL) as well as in cells, possibly astrocytes, in the GCL (Fig. S2B). In the Pde6brd1/rd1 retina, EDNRB expression in the Pde6brd1/rd1 retina was increased throughout Müller cell radial fibres, with stronger staining in the inner limiting membrane (ILM), which contains the end feet of Müller cells. In contrast to the OLM staining observed in light-damaged retinas [19], no outer limiting membrane (OLM) staining of ENDNRB was detected in the Pde6brd1/rd1 retina. ENDNRB expression remained increased in the absence of EDN2 in the Pde6brd1/rd1, Edn2−/− retina (Fig. S2B), indicating that the up-regulation of ENDNRB expression in the mutant retina is not EDN2-dependent.

A simple model consistent with the expression pattern of the endothelin receptors and converting enzyme in the PR mutant retina is therefore that big EDN2 produced in mutant PRs is released into the PR extracellular space, and converted to mat EDN2 by the extracellular moiety of ECE-1 [40] to generate Tg(RHO P347S); Edn2−/− and Pde6brd1/rd1; Edn2−/− animals. We first determined that EDN2 is not required for normal retinal or PR formation or survival by examining the morphology of adult Edn2−/− retinas. No significant difference in gross morphology, or in ONL thickness (n = 5; p > 0.05), was
observed between WT and \( \text{Edn}^{2-/-} \) retinas at PN40 (Fig. 2A). \( \text{Edn}^{2-/-} \) mice were born at normal Mendelian ratios but exhibited running by PN7 and a survival rate of only 25% at PN20 (unpublished observations). \( \text{Edn}^{2-/-} \) mice displayed no overt phenotype. To allow observation of the effects of EDN2 loss in the slower degenerating \( \text{Pde6b}^{rd1/rd1} \) retinas, we were able to extend the lifespan of \( \text{Edn}^{2-/-} \) mice to a maximum of PN50 by using a liquid diet and daily subcutaneous injections of normal saline to maintain fluid and electrolyte balance.

Remarkably, the loss of EDN2 function led to dramatically increased PR survival in both \( \text{Pde6b}^{rd1/rd1} \) and \( \text{Tg(RHO P347S)} \) mice. At PN15, the thickness of the ONL increased by 110% in \( \text{Pde6b}^{rd1/rd1}; \text{Edn}^{2-/-} \) vs. \( \text{Pde6b}^{rd1/rd1}; \text{Edn}^{+/+} \) retinas at PN15 (\( n = 6; \ p < 0.05 \)) (Fig. 2B), and by 63% in \( \text{Tg(RHO P347S)} \);
Figure 2. The effect of EDN2 loss on mutant PR survival *in vivo* and in retinal explants. (A) At PN40, the histology and the thickness of the ONL (n = 5; p > 0.05) was normal in toluidine-blue stained Edn2+/+ and Edn2−/− retinas. (B,C) The loss of EDN2 in Tg(RHO P347S) retinas resulted in a mean 63% increase in ONL thickness at PN40 (n = 6; p < 0.005) (C) and a mean 110% increase in ONL thickness in Pde6brd1/rd1 retinas at PN15 (n = 6; p < 0.005). (E) qRT-PCR assays of the Edn2 mRNA, normalized to Gapdh mRNA, in *in vivo* WT, WT explants and Pde6brd1/rd1 explants (n = 3; p < 0.05). Values were compared to the mean Edn2 mRNA levels in *in vivo* WT samples (arbitrarily given a value of 1). Edn2 transcripts were significantly increased in WT as well as Pde6brd1/rd1 explants at PN12 following retinal dissection at PN7, likely as a result of dissection-induced mechanical stress. (F) WT retinal explants cultured *ex vivo* from PN7-PN17 had an average of 7–8 rows of PR nuclei at PN17 (n = 10 retinas, one representative shown). Owing to artifacts in frozen sections, the number of nuclei, instead of ONL thickness, was assessed in retinal explants. The absence of EDN2 in Pde6brd1/rd1; Edn2−/− explants cultivated from PN7-PN17 had an average of 3 rows of PR nuclei at PN17 (n = 4; p < 0.05, one representative shown) (H&E staining). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. (Black bar = 25 μm in A–C, and F). Error bars indicate SEM. doi:10.1371/journal.pone.0058023.g002
**Figure 3. Systemic erythropoietin (EPO) and retinal vascular endothelial growth factor (VEGF) is increased in *Edn2<sup>−/−</sup>* mice.** At PN21, serum EPO was increased 11-fold in *Edn2<sup>−/−</sup>* vs. *Edn2<sup>+/+</sup>* mice (*n* = 7; *p* < 0.005) (A) and retinal VEGF was increased 4-fold (*n* = 4; *p* < 0.005) (B). EPO and VEGF were both measured using ELISA assays. Error bars indicate SEM. doi:10.1371/journal.pone.0058023.g003

**Hypoxia as one mechanism for PR protection in *Pde6brd1/rd1*; *Edn2<sup>−/−</sup>* and *Tg(RHO P347S)*; *Edn2<sup>−/−</sup>* mice**

To identify possible systemically-mediated causes of the increased *in vivo* survival of PRs in *Tg(RHO P347S); Edn2<sup>−/−</sup>* and *Pde6brd1/rd1; Edn2<sup>−/−</sup>* retinas, we examined *Edn2<sup>−/−</sup>* mice for gross morphological abnormalities. Among other phenotypes, we found that *Edn2<sup>−/−</sup>* mice have defective lungs leading to systemic hypoxia (unpublished data). The hypoxia was associated with an 11-fold increase in erythropoietin (EPO) (*p* < 0.005) (Fig. 3A). Moreover, vascular endothelial growth factor (VEGF) levels in *Edn2<sup>−/−</sup>* retinas were 4.2-fold elevated (*n* = 4; *p* < 0.05) (Fig. 3B). The presence of systemic and probable retinal hypoxia in *Edn2<sup>−/−</sup>* mice suggests that hypoxia may be at least partially responsible for the increased *in vivo* PR survival in *Pde6brd1/rd1; Edn2<sup>−/−</sup>* and *Tg(RHO P347S); Edn2<sup>−/−</sup>* retinas, since we have previously demonstrated that hypoxia increases PR survival in *Pde6brd1/rd1* retinal explants [42].

**Over-expression of Edn2 in PRs increases their survival in *Pde6brd1/rd1* mice**

To examine directly the role of increased *Edn2* expression in mutant PRs, we introduced an *Edn2* cDNA into *Pde6brd1/rd1* and *Pde6brd1/rd1; Edn2<sup>−/−</sup>* retinas using the scAAV5-*smCBA* vector [43]. Due to the low survival rate of *Edn2<sup>−/−</sup>* mice, we performed the scAAV5 experiments in *Pde6brd1/rd1* mice. We initially confirmed the PR expression of this vector by assessing the spatial and temporal expression of GFP from a scAAV5-*smCBA-Gfp* (AAV-Gfp) vector in frozen sections (Fig. 4A, panels 1–4). Using paraffin sections, GFP staining was observed in PR nuclei, as confirmed the PR expression of this vector by assessing the spatial and temporal expression of GFP from a scAAV5-*smCBA-Gfp* (AAV-Gfp) vector in frozen sections (Fig. 4A, panels 1–4). Using paraffin sections, GFP staining was observed in PR nuclei, as conf
In subsequent studies, we therefore introduced the AAV vectors at PN8, to assure strong expression by PN12 and later.

To establish that robust retinal expression of a cDNA encoding mature EDN2 could be obtained from a scAAV5-smCBA-matEdn2 vector (AAV-matEdn2), we injected it into Pde6brd1/rd1 retinas at PN8. At PN12, expression of the mature Edn2 mRNA was 2.5- to 11.3-fold greater (mean 6.9-fold; n = 4) than the level of the endogenous Edn2 mRNA in Pde6brd1/rd1 retinas (Fig. 4C, left bar). Similarly, we quantified the expression of a prepro Edn2 cDNA from an scAAV5-smCBA-preproEdn2 (AAV-preproEdn2) vector. Any difference between outcomes in PR survival using the matEdn2 vs. the preproEdn2 vectors could suggest that the processing of prepro EDN2 to mat EDN2 is rate-limiting. We found that expression of the prepro Edn2 mRNA was 1.7- to 7.2-fold (average 4.3-fold; n = 4) greater than the level of the endogenous Edn2 mRNA in Pde6brd1/rd1 retinas (Fig. 4C, left bar). In summary, in Pde6brd1/rd1 retinas both the AAV-matEdn2 and the AAV-preproEdn2 vectors expressed their respective mRNAs at levels on average 4.3- to 6.9-fold above the expression level of the native Edn2 mRNA.

To examine the role of the increased PR expression of Edn2 on PR survival in IPD, we augmented the endogenous increase in Edn2 expression in PRs in Pde6brd1/rd1 mice using AAV vectors.
We used 1X PBS injection as the control injection in the contralateral eye, because when we compared ONL thickness in individual retinas at PN14–15, (a) injection of the scAAV5-smCBA-matEdn2 vector into WT retinas at PN8 did not alter retinal morphology at PN15. (b) Injection of the scAAV5-smCBA-matEdn2 vector at PN8 increased PR ONL thickness of Pde6brd1/rd1 retinas by 31% (n = 9; *p < 0.05) at PN15, and (c) of Pde6brd1/rd1; Edn2−/− retinas at PN14 by 18% (n = 5; *p < 0.05). (d) In contrast, injection of the scAAV5-smCBA-preproEdn2 vector at PN8 had no effect on PR ONL thickness of Pde6brd1/rd1 retinas (n = 6; p > 0.05) at PN15, although (e) this vector improved PR survival in Pde6brd1/rd1; Edn2−/− retinas by 14% (n = 6; *p < 0.05). (B) A bar graph summarizing the effects of AAV vectors expressing Edn2 cDNAs, injected at PN8, on mutant PR survival in Pde6brd1/rd1 and Pde6brd1/rd1; Edn2−/− retinas at PN14–15. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. (Black bar = 25 μm). Error bars indicate SEM.

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Evidence that EDN2-mediated up-regulation of FGF2 expression is a survival response

To identify retinal genes whose expression is directly or indirectly regulated by EDN2 in the presence of the Tg(RHO P347S) mutant allele, we identified mRNAs that were differentially expressed in Tg(RHO P347S); Edn2+/+ vs. Edn2−/− retinas, to mRNAs that were differentially expressed between Tg(RHO P347S); Edn2−/− vs. Edn2+/+ retinas (Fig. 6). This comparison was used, rather than contrasting the mRNAs of Tg(RHO P347S); Edn2−/− vs. Tg(RHO P347S); Edn2−/− retinas, because this latter comparison would fail to distinguish between mRNA changes due the absence of EDN2 alone and indirect changes due to the absence of EDN2 in the presence of the Tg(RHO P347S) mutant allele.

The mRNA populations were examined using Mouse 430_2.0 Affymetrix arrays (two retinas from one mouse/array, four arrays/genotype). Retinal mRNA was collected at PN21, three days after the onset (at PN18) of Edn2 mRNA expression in Tg(RHO P347S) retinas (data not shown). Transcripts with a 2.0-fold difference in expression in these two comparisons (p<0.05 using a two class unpaired t test) are shown in Figure 6.

Of the 20 genes whose expression was most induced (>4-fold; p<0.05) in Tg(RHO P347S); Edn2+/+ vs. Edn2−/− retinas were also induced in Tg(RHO P347S); Edn2−/− vs. Edn2+/+ retinas (Fig. 6). Notably, the expression of all 20 of these Tg(RHO P347S)-induced genes was down-regulated in Tg(RHO P347S); Edn2−/− retinas (Fig. 6). This finding suggests that EDN2 may have broad effects on the transcription of a cohort of retinal genes (including the 20 genes shown in Fig. 6), a cohort that respond to the presence of a PR mutation, in this instance the Tg(RHO P347S) transgene.

An increase in the Muller cell expression of GFAP was also observed in the Tg(RHO P347S); Edn2−/− retina, as has been reported in other IPDs [19,46] (Fig. 7B). GFAP expression in Muller cells decreased significantly in the Tg(RHO P347S); Edn2−/− retina, although not entirely, indicating that the expression of GFAP in Muller cells in the mutant retina is partly regulated, directly or indirectly, by EDN2 (Fig. 7B).

Discussion

Increased PR Edn2 expression appears to be a survival response

We have shown that Edn2 mRNA is induced in Pphp2pel/pel, Tg(RHO P347S), and Pde6brd1/rd1 retinas at early stages of mutant PR degeneration. This finding, combined with those of Rattner and Nathans [19], and others [5,47], establish that the greatly increased expression of Edn2 is a common and possibly general response of PRs (including cones [5]) to the presence of an IPD mutation in either the PRs or in the RPE [21], and is elicited even though the mutations affect proteins with widely diverse functions.

The increased PR expression of Edn2 appears to be an early response imposed by a mutation [19] or other insults, including mechanical and light damage [19,20], rather than an event in a death-signaling cascade, because the increase occurs early in mutant PRs, the majority of which will not die for weeks to months after birth, at least in the Pphp2pel/pel and Tg(RHO P347S) models.

Using the Pde6brd1/rd1 model mouse, we have presented two lines of evidence that the increased expression of Edn2 in mutant PRs is a survival response. First, augmentation of the endogenous increase in Edn2 expression in the Pde6brd1/rd1 mouse with the AAV-matEdn2 cDNA vector improved PR survival, by 31% between PN8 and PN15. Second, the restoration of Edn2 expression with either the AAV-matEdn2 or AAV-preproEdn2 vectors enhanced PR survival in Pde6brd1/rd1; Edn2−/− retinas by 14–18%. Although not large, this latter increase is notable, because it is additive to the enhanced PR survival that we identified in the systemic absence of EDN2 function, in Pde6brd1/rd1; Edn2−/− mice in vivo. The increased survival are also notable given that the rate of PR death in Pde6brd1/rd1; Edn2−/− retinas is the most rapid of all IPD models, with the great majority of PRs being ablated by PN21 [48].

Although the 31% increase in PR survival in Pde6brd1/rd1 mice observed using the AAV-matEdn2 cDNA vector is modest, we suggest that this finding is important. First, it provides the initial evidence that Edn2 gene transfer can improve mutant PR survival. Second, the rate of PR death in the Pde6brd1/rd1 retina is much more rapid than any other mouse model known; the biological insult to the PR is extreme.

The improved PR survival observed with AAV-Edn2 gene transfer suggests future studies to determine if even greater survival can be obtained by additional enhancement of the EDN2 signaling pathway. For example, since the conversion of prepro EDN2 to mat EDN2 by ECEs may be the rate-limiting step in controlling the availability of EDN2, AAV-mediated transfer of the ECE-1 cDNA may increase mutant PR survival. The possibility that ECE-mediated cleavage of big EDN2 (Fig. 4B) is the rate-limiting step in the formation of EDN2 is supported by the finding of Telemaque et al. [49], who found that increased systemic blood pressure following adenovirus-mediated over-expression of prepro EDN1 required the co-injection of an ECE-1 expression construct.

Other strategies to augment EDN2 signaling should also be examined, such as the use of agonists of the EDN2 signaling pathway to increase the response to EDN2. For example, the...
administration of an EDNRB agonist in light damaged retinas has been shown to reduce the number of dying cells [5].

A role for Edn2-dependent increased FGF2 expression in mutant PR survival

The mechanism by which EDN2 mediates its protective effect in mutant PRs remain to be elucidated. One starting point for mechanistic analysis will be to define the roles of the 20 genes we

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**Figure 6. Identification of possible EDN2 regulated genes using microarrays.** Gene expression was examined using the Mouse 430_2.0 Affymetrix array (4 arrays/genotype) and the 20 most differentially expressed mRNAs in Tg(RHO P347S); Edn2<sup>+/+</sup> vs. Edn2<sup>−/−</sup> retinas, and in Tg(RHO P347S); Edn2<sup>+/+</sup> vs. Edn2<sup>−/−</sup> retinas, at PN21 are shown. All differentially expressed genes in Tg(RHO P347S); Edn2<sup>−/−</sup> retinas showed a significant reduction in expression in Tg(RHO P347S); Edn2<sup>−/−</sup> retinas, with the exception of Nuclear protein 1. The fold reduction is shown to the right of each pair of bars. The detection of Edn2 transcripts in Tg(RHO P347S); Edn2<sup>−/−</sup> retinas is due to the expression of partial Edn2 mRNAs from the Edn2<sup>−/−</sup> locus, in which part of exon 1 and all of exon 2 were replaced with the Neo cassette. n.s. = no statistically significant increase in expression. doi:10.1371/journal.pone.0058023.g006
found to have a 4-fold increased expression in Tg(RHO P347S) retinas, including Fgf2. We have shown that the increased expression of Fgf2 may be EDN2-dependent. First, we found that Fgf2 mRNA in whole retina is 3-fold increased in Tg(RHO P347S) mice compared to WT mice. This increase did not occur in the absence of EDN2 in Tg(RHO P347S); Edn2^{+/+} retinas. Second, we demonstrated that FGF2 immunolabelling was increased in Tg(RHO P347S) PRs, but not in the absence of EDN2 in Tg(RHO P347S); Edn2^{-/-} PRs.

Figure 7. FGF2 expression in Tg(RHO P347S) PRs returns to WT levels in the absence of EDN2. (A) qRT-PCR quantification of Edn2, Gm12541, Fgf2 and Gfap expression in Tg(RHO P347S) retinas in the presence or absence of EDN2 function. Bar graphs show the expression of mRNAs in retinas of the indicated genotypes relative to the expression levels seen in Edn2^{+/+} (WT) retinas. Fold down-regulation in Tg(RHO P347S); Edn2^{-/-} retinas vs. Tg(RHO P347S); Edn2^{+/+} retinas is shown to the right of the vertical bars (n = 3; p < 0.05 for all mRNAs). All qRT-PCR values were normalized to Gapdh mRNA. ND (not detected). (B) Immunostaining for FGF2 showed low levels of expression in all three retinal nuclear layers in Edn2^{+/+} and Edn2^{+/+} retinas, but FGF2 expression increased significantly in the PRs of Tg(RHO P347S); Edn2^{+/+} retinas at PN21 (third top panel). In contrast, FGF2 staining was similar to WT retinas in Tg(RHO P347S); Edn2^{-/-} retinas and, most notably, from PRs (fourth upper panel). GFAP expression in Müller cells was increased in Tg(RHO P347S); Edn2^{+/+} retinas (third bottom panel), but reduced in Tg(RHO P347S); Edn2^{-/-} retinas (fourth bottom panel). GFAP expression in Tg(RHO P347S); Edn2^{-/-} retinas was higher than in Edn2^{+/+} retinas. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. (Bar = 25 μm). Error bars indicate SEM.
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Other evidence supports our proposal that the protective effect of increased Edn2 expression in mutant PRs is due, at least in part, to the Edn2-dependent increase in the expression of PR FGF2. First, exogenous FGF2 is an established survival factor for mutant PRs [9,45]. Second, like Edn2, retinal Fgf2 transcripts are induced under conditions of both genetic and environmental PR injury [50]. Third, Fgf2 mRNA [51] and FGF2 protein expression is increased in Muller cells [52] in models of PR injury, and increased FGF2 immunofluorescence has been observed in PR cell bodies after optic nerve section. This increase may represent increased FGF2 expression, increased binding of FGF2 to FGFR1 [53], or both. Fourth, intravitreal administration of an EDNRB agonist increases Fgf2 mRNA levels in whole WT retina, also consistent with FGF2 being part of an EDN2/EDNRB signaling pathway [5]. Finally, EDN1, which exhibits the same selectivity of binding to endothelin receptors as EDN2, is known to induce Fgf2 mRNA expression in vascular smooth muscle cells [54]. Our data highlights a putative EDN2/EDNRB/FGF2 signaling pathway that may be downstream of leukemia inhibitory factor (LIF)-activated signals. A key role for LIF has been identified in PR survival in response to retinal insult. LIF was found to be necessary for the up-regulation of the Edn2 and Fgf2 mRNAs and for increased PR survival in both rhodopsin VPP mutant retinas [5] and light-damaged retinas [4]. Furthermore, intravitreal injection of recombinant LIF into Lf−/− retinas strongly induced the expression of Edn2. Since Lf is expressed in a subset of Muller glia in response to PR injury [5], we propose that one PR survival pathway may involve Muller cell-derived LIF induction of PR EDN2, which then binds to EDNRB on Muller cells; an as yet unidentified Muller cell signal then acts on the PRs to increase FGF2 expression. LIF signaling is mediated through gp130, and PR gp130 has been shown to contribute to PR survival in VPP retinas [55], indicating that PR gp130 may also be a component of the EDN2/EDNRB signaling pathway. Recent work by Braungar et al. suggests that EDN2 may participate in more than one PR survival pathway, depending on the upstream stimulus. In a light damage model, PR protection mediated by the over-expression of Norrin, a Wnt/B-catenin signaling molecule, was also found to be dependent on EDN2/EDNRB signaling [33]. In this model, however, the authors concluded that a LIF/EDN2/FGF2 signaling pathway was likely not associated with the protective effects against light damage observed in Rpe65-Norrin-2 transgenic animals. Taken together, the data suggest that EDN2 signaling may participate in the PR response to retinal injury via multiple survival pathways, including a LIF-independent Norrin-activated pathway and a LIF- and FGF2-dependent pathway.

It is noteworthy that three of the genes whose expression decreased the most in Tg(RHO P547S) retinas in the absence of EDN2: Fgf2, Gm12541 and Nudt6, are all located and overlap on mouse chromosome 3. Fgf2 is sandwiched between Gm12541 and Nudt6, which are transcribed from the opposite DNA strand from Fgf2. Gm12541 overlaps the coding region of Fgf2 at its 5′ end, and Nudt6 overlaps the 3′ UTR of Fgf2 at its 3′ end. This arrangement, together with the coordinate expression of these three genes in the Tg(RHO P547S) retina, suggests that regulatory relationships may exist between them. Indeed, the mRNA of Nudt6 is a known Fgf2 antisense transcript (Fgf2-AS) that inhibits Fgf2 expression [56,57]. The Gm12541 gene predicts a hypothetical protein, but it may also be a non-coding RNA that acts as an Fgf2 regulatory antisense transcript. Some natural antisense transcripts regulate their sense partners positively, whereas others are negative regulators [58,59]. A variety of regulatory models involving Fgf2, Nudt6 and Gm12541 could account for our finding that the expression of these three genes is coordinate regulated in the Tg(RHO P547S) retina.

Systemic loss of EDN2 function also increases the survival of mutant PRs

In addition to the enhanced PR survival observed with AAV-mediated transfer of the maxEdn2 gene to mutant PRs, we also found that systemic loss of EDN2 function was also protective to PRs. We propose that the increased PR survival in Pde6brd1/rd1; Edn2−/− retinas in vivo (vs. in explants) is not due to the loss of PR Edn2 expression from mutant PRs because i) the restoration of PR Edn2 expression using either the AAV-matEdn2 or AAV-preproEdn2 vectors enhanced PR survival in Pde6brd1/rd1; Edn2−/− mice, rather than accelerating PR death; and ii) PR survival was not increased in Pde6brd1/rd1; Edn2−/− retinal explants, consistent with the increased observed in Pde6brd1/rd1; Edn2−/− retinas in vivo being due to extraocular mechanisms.

We propose that the pulmonary abnormalities and consequent systemic hypoxia of Edn2−/− mice (unpublished data), perhaps together with other changes due to the systemic loss of Edn2 expression, may be responsible for the increased PR survival in Pde6brd1/rd1; Edn2−/− and Tg(RHO P547S); Edn2−/− mutant retinas. A role for hypoxia in the increased PR survival is supported by our finding of increased serum EPO and increased retinal VEGF levels in Edn2−/− mice, and by our previous demonstration that the maintenance of Pde6brd1/rd1 retinal explants in 6% O2 increased PR survival [42]. Moreover, Grimm et al. [60] found that preconditioning mice in 6% O2 up-regulated retinal EPO, VEGF and FGF2 and fully protected PRs from light damage. In addition, systemic EPO administration has been shown to increase PR survival in response to both light damage or IPD mutations [60,61]. Increased retinal VEGF expression may also contribute to the increased PR survival observed with hypoxia, since VEGF was found to be protective of ex vivo retinal cultures in a model of ischemia-reperfusion injury [62], and to reduce PR apoptosis in isolated PR cell and outer nuclear layer explants [63]. Altogether, these findings suggest that the >60% increase in PR survival that we observed in Tg(RHO P547S); Edn2−/− and Pde6brd1/rd1; Edn2−/− mice in vivo may be the result, at least in part, of retinal hypoxia and the attendant increases in systemic EPO and intraocular VEGF.

Therapeutic implications of the common Edn2 response in IPDs

We previously demonstrated that, like the increase in Edn2 expression in mutant PRs, a diverse set of IPDs display another common behavior, exponential kinetics of PR death [64,65]. We proposed that the shared kinetics suggest a commonality in the biochemical responses of mutant PRs to a mutation. The biochemical changes that occur downstream of a PR mutation may converge on a relatively small number of shared apoptotic biochemical pathways that resist or mediate PR death [65]. The increase in PR Edn2 expression in multiple models of IPD would appear to be one such shared downstream biochemical response. Consequently, therapies to enhance EDN2 signaling may have potential as a general therapy for IPDs, irrespective of the gene affected.

Other animal models of IPD must now be examined, to determine if AAV-maxEdn2 gene transfer increases mutant PR survival in additional models, and whether the increase is greater with less severe mutational insults than that which occurs in the Pde6brd1/rd1 retina. In addition, tests must be undertaken to establish that the improved PR survival seen with AAV-matEdn2 gene transfer is accompanied by conservation of vision, and that long-term increased Edn2 expression does not have adverse effects.
on retinal function or survival. The development of general therapies that are safe and effective in a broad range of animal models of IPDs has important potential for patients for whom a gene-specific therapy has not yet been developed.

Finally, our studies add to the principle, as shown with FGF2 therapy for IPDs [9,10], that augmentation of the endogenous survival responses of mutant PRs merits on-going examination to identify potential general therapies for this debilitating group of monogenic diseases.

Materials and Methods

Ethics Statement

Animals were raised under 12-hr/ light dark cycles and treated in accordance with the guidelines and principles outlined by the Animal Care Committee at the Hospital for Sick Children (Toronto, ON).

Mice

Mice carrying the Pde6brd1/rd1 (C3H/HeOuJ) and Prph2rd2/J mice (C3A.BLA-Pde6b+/O20-Prph2rd2/J) alleles, including Prph2rd2/J (C3A.BLA-Pde6b+/J) and C57BL/6J controls were obtained from The Jackson Laboratory (Bar Harbor, ME). Prph2rd2/Rd2/J mice were generated by crossing Prph2rd2/J and Pde6brd1/rd1 mice. Tg(RHO P347S) mice were obtained as a gift from Dr. Tiansen Li (Boston, MA). EDN2 null mice (129Sv/Ev Taconic) were generated by mice were obtained as a gift from Dr. Tiansen Li (Boston, MA), and at 75% of this distance in Tg(RHO P347S) retinas. The % change in ONL thickness as indicated refers to (|M - N|/N x100%), where N is the normal condition and M can indicate the loss of EDN2 or introduction of the Edn2 cDNA vector.

RNA preparation, cDNA synthesis and qRT-PCR

Total retinal RNA was extracted with Trizol (Invitrogen, Burlington, ON) and purified using the RNeasy Mini kit (Qiagen, Mississauga, ON) with on-column DNA digestion using the RNase-free DNase set (Qiagen). Purified RNA was resuspended in 30 µl RNase-free ddH2O and cDNA generated using the Omniscript Reverse Transcription Kit (Qiagen). qRT-PCR was performed using the ABI Prism® 7900HT sequence detection system (Applied Biosystems, Foster City, CA), and Sequence Detector System 2.2.1 software (Applied Biosystems) was used to analyze the data.

HPLC, RIA and ELISA

Control peptides (EDN1, EDN2, and EDN3) were obtained from Peptides International (Louisville, KY). Retention times and peak areas were recorded on a tracer (BBC Goerz Metrawatt, Markham, ON). Samples were chloroform: methanol (1:9) solution (1.2 mg/mL) (Roche, Laval, Quebec) for 15–20 minutes at 37°C to separate the sclera and RPE, and dissected retinas were cut and stained with 1% toluidine blue. The width of the ONL was measured using SigmaScan Pro (Systat Software Inc., San Jose, California). To preclude variations in local retinal degeneration rates from influencing ONL measurements, ONL thickness was determined in orthogonal sections, by measuring thickness at a point 25% of the distance from central to peripheral retina in Pde6brd1/rd1 mice, and at 75% of this distance in Tg(RHO P347S) retinas. The % change in ONL thickness as indicated refers to (|M - N|/N x100%), where N is the normal condition and M can indicate the loss of EDN2 or introduction of the Edn2 cDNA vector.

Retinal Explants

Mice were decapitated at PN7 and retinal explants with attached retinal pigment epithelium (RPE) were obtained as previously described [41]. Eyes were incubated in proteinase K solution (1.2 mg/mL) (Roche, Laval, Quebec) for 15–20 minutes at 37°C to separate the sclera and RPE, and dissected retinas were laid flat on Millicell-HA 0.43 µm filter membranes (Millipore, Billera, CA). Explants were maintained under serum and antibiotic-free conditions in R16 basal culture medium (Invitrogen, CA). Media was changed every two days.

AAV production and subretinal delivery

Self-complementary AAV vectors containing the small, hybrid cytomegalovirus-chicken β-actin (smCBA) promoter driving expression of either Gfp, preproEdn2 or matEdn2 were generated and purified by previously described methods [66]. Vector titers was determined by qRT-PCR and final aliquots were resuspended in balanced salt solution (Alcon Laboratories, Forth Worth TX, USA) containing 0.014% Tween 20. qRT-PCR expression of the AAV-specific Edn2 mRNA was measured using a 3′ primer specific to the 3′ tail of the mRNA, including the polyA sequence.

For subretinal injections, mice were anaesthetized with ketamine/xylazine (IP, 150 mg/kg ketamine, 10 mg/kg xylazine) and
the cornea punctured with a 30-gauge needle between the corneoscleral junction and the ora serrata. A 33-gauge blunt needle attached to a 10 μl Hamilton syringe (Hamilton Company, Reno, NV) was used to dispense 1 μl of fluid (viral titer 1–5 × 10^{12} VGF/mL containing 0.1 mg/mL fluorescein (Alcon, Mississauga, ON) to aid visualization of the subretinal injection site. Mice were treated with topical tropicamide ointment (Alcon) following injection and administered 300 μl normal saline S.C. to prevent dehydration.

Microarrays

For microarray studies, retinas were homogenized in GIT buffer (4 M guanidine isothiocyanate, 25 mM sodium acetate (pH 6), 120 mM β-mercaptoethanol). Total RNA was further isolated by cesium chloride density gradient centrifugation and purified using the RNasey Mini kit (Qiagen). RNA quality was assessed using a Bioanalyzer (Agilent Technologies, Mississauga, ON). Briefly, labeled cRNA was hybridized to the Mouse 430_2.0 Array representing over 39,000 mouse transcripts and probe sequences from GenBank®, dbEST, and RefSeq (Alfametrics, Santa Clara, CA). Alfametrics Gene Chip Operating Software (GCOS) was then used to generate cel microarray files which were further analyzed using Robust Multi-chip Averaging (RMA) algorithms (Affymetrix). Significance analysis was performed using the Significance Analysis of Microarrays program (SAM, Stanford University Labs) [67], and a list of differentially expressed genes generated. For the Edn2^{+/+} vs. Tg(RHO P347S) Edn2^{+/+} and Edn2^{+/−} vs. Tg(RHO P347S) Edn2^{+/−} comparisons, an FDR of 5% was used, whereas for the Edn2^{+/+} vs. Edn2^{+/−} analysis, an FDR of 25% was used (NCBI GEO Accession GSE38797).

Supporting Information

Figure S1 Quantification of mRNA expression of Edn1, Edn3, EdnrfA and EdnrfB in mouse retinas. qRT-PCR was used to quantify the expression of Edn1, Edn3, EdnrfA and EdnrfB in Tg(RHO P347S) retinas in the presence and absence of EDN2 function. Bar graphs show the expression of mRNAs in retinas of the indicated genotypes relative to the expression levels seen in Edn2^{+/+} (WT) retinas (arbitrarily assigned a value of 1). There were no significant differences in expression except for a slight increase in the EdnrfA mRNA in Tg(RHO P347S);Edn2^{+/−} vs. Edn2^{+/−} retinas (n = 3, p<0.05) (n = 3;p>0.05 for all other comparisons). All qRT-PCR values were normalized to Gapdh mRNA. (TIF)

Figure S2 Immunostaining of ECE-1, EDNRA and EDNRB in mouse retinas. (A) The localization of ECE-1 immunofluorescence in WT retina overlaps that of the Muller cell marker gluthamine synthetase. ECE-1 expression was observed in both Muller cell extensions and cell bodies. (B) Immunofluorescence localization of EDNRA and EDNRB in mouse retinas. EDNRA staining was sporadically observed in the GCL, INL, OPL and choroid plexus, irrespective of genotype. Although EDNRA staining in choroid, GCL and OPL is indicative of vessels [26], EDNRA expression in the INL and ONL of Pde6b^{+/−}\t^t\tEdn2^{+/−} retinas may represent retinal microglia (42), which migrate to the mutant PRs in the ONL [39]. EDNRB immunoreactivity was predominantly observed in Muller cell radial fibres and in the OPL and GCL, possibly representing horizontal cells and astrocytes, respectively [25]. In the Pde6b^{+/−}\t^t\tEdn2^{+/−} retina, EDNRA expression was significantly increased in Muller cell radial fibres, with stronger staining in the inner limiting membrane; this stronger staining may correspond to the end feet of Muller cells as well as astrocytes [69]. OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer; ILM, inner limiting membrane. (Bar = 25 μm). (TIF)

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Author Contributions

Editing: WH. Conceived and designed the experiments: AB RM IC. Performed the experiments: AB IC. Analyzed the data: AB RM LS ED. Contributed reagents/materials/analysis tools: IC MY WH PD DS. Wrote the paper: AB RM.

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