Fzd4 Haploinsufficiency Delays Retinal Revascularization in the Mouse Model of Oxygen Induced Retinopathy

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Abstract

Mutations in genes that code for components of the Norrin-FZD4 ligand-receptor complex cause the inherited childhood blinding disorder familial exudative vitreoretinopathy (FEVR). Statistical evidence from studies of patients at risk for the acquired disease retinopathy of prematurity (ROP) suggest that rare polymorphisms in these same genes increase the risk of developing severe ROP, implying that decreased Norrin-FZD4 activity predisposes patients to more severe ROP. To test this hypothesis, we measured the development and recovery of retinopathy in wild type and Fzd4 heterozygous mice in the absence or presence of ocular ischemic retinopathy (OIR) treatment. Avascular and total retinal vascular areas and patterning were determined, and vessel number and caliber were quantified. In room air, there was a small delay in retinal vascularization in Fzd4 heterozygous mice that resolved as mice reached maturity suggestive of a slight defect in retinal vascular development. Subsequent to OIR treatment there was no difference between wild type and Fzd4 heterozygous mice in the vaso-obliterated area following exposure to high oxygen. Importantly, after return of Fzd4 heterozygous mice to room air subsequent to OIR treatment, there was a substantial delay in retinal revascularization of the avascular area surrounding the optic nerve, as well as delayed vascularization toward the periphery of the retina. Our study demonstrates that a small decrease in Norrin-Fzd4 dependent retinal vascular development lengthens the period during which complications from OIR could occur.

Introduction

The retina is a thin layer of neural tissue lining the back of the eye responsible for sensing visual stimuli. During development, the retinal vasculature is initiated by endothelial sprouts that lay down the primary vessels that project radially from the optic disc to the retinal periphery to form the inner or primary vascular plexus. From this plexus, secondary vessels penetrate the...
Retina to form the outer or tertiary plexus, thus laying down a vascular supply on either side of the central layer of neurons [1]. Patterning of the retinal vasculature is controlled by guidance cues driven initially by tissue hypoxia that is sensed by tip cells, specialized endothelial cells at the front end of the growing vasculature. Tip cells migrate along a pre-existing astrocyte network with endothelial stalk cells following the tip cells. Once in place, the primary vasculature matures into arteries and veins, the nascent network is pruned, and the blood-retina barrier is formed.

Retinopathy of prematurity (ROP) is a disorder that results in a defect in vascular development of the retinas of infants who are born prematurely. ROP is a leading cause of childhood blindness in developed countries and an emerging problem in developing nations. It is estimated that blindness affects at least ~20,000 prematurely born infants worldwide each year, of which at least 50% of cases are attributed to ROP [2, 3]. Infants with ROP have avascular zones of retina and are at risk of developing secondary neovascularization with subsequent retinal detachment. Treatments aiming to reduce the risk of vision loss in severe ROP include laser and anti-VEGF intraocular injection (e.g. bevacizumab). Recent studies suggest that, despite current treatments, vision of 20/40 or better (mild to no vision loss) occurs in only 1/3 of patients; most will be left with visual impairment while 1/4 will be legally blind [4]. The current visual outcomes in infants with severe ROP underscore the need to improve our understanding of the mechanisms that lead to blinding complications.

Several risk factors have been identified in association with severe ROP. It has been suggested that part of the course of ROP is influenced by genetic factors [5], with evidence of molecular links between ROP and genes that cause familial exudative vitreoretinopathy (FEVR) [6]. FEVR is an inherited developmental blinding disorder with an estimated incidence of 1:10,000. Similar to ROP, babies born with mutations in genes that cause FEVR present with hypovascularization of the retina due to the failure of peripheral retinal vascularization followed by secondary aberrant neovascularization [7–10], with severe forms of FEVR resulting in bilateral congenital retinal folds or retinal detachment [11–16]. FEVR is caused by mutations in six known genes, *FZD4* [17, 18], *TSPAN12* [19–21], *LRP5* [22], *NDP* [23–26], *ZNF408* [27] and *KIF11* [28, 29]. Four of the FEVR genes encode a ligand (norrin encoded by *NDP*), its receptor (encoded by *FZD4*), and two co-receptors (encoded by *LRP5* and *TSPAN12*). Rare polymorphisms in three of the known FEVR genes, *NDP*, *FZD4* and *LRP5* have been identified in patients with severe ROP in various populations [4, 30–38] suggesting that small defects in Norrin-Fzd4 dependent retinal vascular development may affect the development of ROP. Mouse models of FEVR have provided insight into the genotype-phenotype relationships that regulate retinal vascular development. The null mouse models of FEVR are accurate mimics of the human disease, demonstrating a failure of peripheral retina vascularization in addition to abnormal patterning and patency of the formed vessels [39–41].

To approximate ROP, rodents are subjected to ocular ischemic retinopathy (OIR) by placing them in either high oxygen, or high-low oxygen, environments for 5–14 days. The OIR protocol is meant to recapitulate the periods of relative hyperoxic and hypoxic oxygen phases following exposure to the extrauterine environment in prematurely born infants. We determined the extent of vascularization, vaso-obliteration and revascularization, in wild type and Fzd4 heterozygous (*Fzd4*+/−) mice in the absence and presence of exposure to an OIR protocol. *Fzd4*+/− mice had a slight delay in ocular vascular development in room air. Importantly, we determined that *Fzd4*+/− mice subjected to OIR displayed a substantive defect in revascularization following re-exposure to room air compared to wild type mice. This is the first functional demonstration that a small decrease in Norrin-Fzd4 dependent retinal vascular development shortens the period of vascular recovery subsequent to OIR. Our functional results in mice align with the previous genetic studies in patients that observed a greater prevalence of rare
polymorphisms in the genes encoding components of the Norrin-FZD4 pathway in infants that developed more severe ROP.

Materials and Methods

Ethics Statements on Mouse Models and Animal Care

Experiments were carried out using pups bred from Fzd4^+/− male and female breeders (B6;129-Fzd4tm1Nat/J; The Jackson Laboratory, Bar Harbor, ME, USA). Both male and female mice were used in this study. All procedures were performed in accordance with the Canadian Council on Animal Care and approved by the Research Ethics Board for Laboratory Animal Research at Dalhousie University. For OIR studies, mice were exposed to 65% O2 for the first three days followed by room air for 24 hrs and 65% O2 for the final 24 hrs. No mice became ill or died prior to the experimental endpoint. Animals were anesthetized with isoflurane prior to euthanization and retina isolation. For each condition, a minimum of three litters per group (range: 3–5 litters) was compared yielding at least 6 eyes per group (range: 6–11 eyes). Weights for all mice were measured and compared at postnatal day 12 (P12), P17 and P25.

Mouse Genotyping

DNA was extracted (AccuStart™ II Mouse Genotyping Kit, Quanta Biosciences) from ear tissue. Mouse Fzd4 gene primers were obtained from Integrated DNA Technologies, Inc. Primers for the wild type Fzd4 gene were WT F 5’-TGG AAA GCG TAA TGG TCA AGA TCGG and WT R 5’-AGA ATT CAC CAA TCG GTT AGA ACAC. Primers for the Fzd4 gene knockout were Mut F 5’-TGT CTG CTA GAT CAG CCT CTG CCG and Mut R 5’-CAT CAA CAT TAA ATG TGA GCG AGT. Mouse genotype was determined by PCR from genomic DNA.

Retina Isolation

Retinas were isolated and fixed in Dulbecco’s phosphate buffered saline containing 0.1g/l MgCl₂ and 0.133g/l CaCl₂ (Sigma-Aldrich) in 4% paraformaldehyde (Cedarlane) for 2 hours at room temperature. Fixation was quenched with phosphate buffered saline containing 0.3% Triton X-100 and 10% goat serum (Sigma-Aldrich) for 1 hour at room temperature. To visualize the vascular endothelium, retinas were stained with Alexa Fluor 594-conjugated GS-IB4 lectin (20μg/ml; Molecular Probes) at 4°C overnight. Retinas were washed three times with phosphate buffered saline containing 0.1% Triton X-100 and flat-mounted (Fluoromont-G, Sigma-Aldrich).

Retinal Vasculature Imaging and Analysis

Retinas were visualized using a Zeiss Axio Imager Z2 microscope containing a digital stage and the MosaiX software program in Axiovision 4.8. All analyses were performed on images captured at 10x magnification with the same image acquisition settings. Avascular and total areas were quantified in CS Photoshop 6 to give a percentage of vaso-obliteration. Vascularization was quantified as a percentage of the total area using Image J software program and NV_Swift plugin [42]. For vessel count, whole mount retinas were analyzed using ImageJ software program. For each quadrant, three line segments were placed at specified locations: adjacent to the optic nerve (proximal), adjacent to the periphery of the retina (distal), and midway between the proximal and distal lines (mid). The number of vessels crossing each line in four different quadrants was counted and averaged at each of the three locations. The width of primary vessels adjacent to the optic disc was calculated using Image J. One eye per animal (the right eye) was used for image analysis. To visualize whether vessels were protruding into multiple layers,
whole mount retinas were imaged using Zeiss LSM 510 Meta Laser Scanning Confocal microscope at 25x. Slices were 1 micron apart. Z-stacks were analyzed using ImageJ Software program.

Statistical Analyses
Imaging analyses are expressed as mean ± standard error of the mean (SEM) and P values were calculated using a one-way ANOVA unpaired t-test. The animal weights were described as mean ± standard error (SE) and compared using the t-test with a two-tailed distribution. Results were considered significant if P < 0.05.

Results
Retina Vascular Development in Wild Type and Fzd4+/- Mice
We determined the status of retinal vascular development in Fzd4+/- mice raised in room air to determine if Fzd4 haploinsufficiency had an effect on vascular development. Comparisons were made with wild type littermates including the average number and caliber of vessels by determining the number of vessels per mm and the percentage of vessels >50 μm (a 50 μm cutoff was chosen to represent the width of major primary vessels (i.e. main arterioles from the inner plexus) based on observation of normal adult retina vessels. Vessel number and caliber were analyzed at three locations (proximal, middle, and distal) based on distance from the optic nerve. Mice were analyzed at P12, P17 and P25, these time points were chosen as they are typically used to evaluate retinal pathology observed in the mouse OIR model.

At P12, wild type mice had more vessels than Fzd4+/- mice with 31 vessels per mm versus 22 in the proximal, 28 versus 20 in the mid periphery, and 28 versus 22 in the distal retinal regions, respectively (Figs 1 and 2). Retinal vessel caliber measured as a percentage of vessels >50 μm was increased in the proximal and mid regions in Fzd4+/- mice compared to wild type mice at P12, while vessel caliber was decreased in the distal region (Fig 2). At P17 and P25, the Fzd4+/- mice were beginning to normalize vessel number and caliber, with vessel caliber fully normalized by P25 and number of vessels approaching normalization. Specifically, in wild type versus Fzd4+/- mice, at P17 there were 30 vessels per mm versus 25 in the proximal, 24 versus 25 in the mid periphery, and 25 versus 21 in the distal region, while at P25 there were 24 vessels per mm versus 23 in the proximal, 22 versus 18 in the mid, and 23 versus 19 in the distal region of the retina (Figs 1 and 2). For vessel caliber, at P17 there was no change in proximal or mid peripheral regions while there was an increase from 6% to 15% in vessels >50 μm in the Fzd4+/- compared to wild type mice. By P25 vessel caliber changes had been resolved in all regions of the retina while there was still a small but statistically significant decrease in the number of vessels/mm across all regions of the retina in the Fzd4+/- mice (Figs 1 and 2).

Retinal vascular development proceeds three dimensionally into intermediate and deep layers. To determine whether Fzd4+/- mice had a delay in vasculature development into their intermediate and deep layers, whole mount retinas of mice were analyzed by taking Z-stack images of the retina flat mounts at P12, P17, and P25 (Fig 3). Z-stacks were compiled to produce XY, XZ and YZ dimensions. White arrows indicate vessel growth within vascular layers. There was no observable difference between Fzd4+/+ and Fzd4+/- mice at P12, P17 and P25 under normoxic conditions as vessel branching moved through the intermediate layer and into the deep layer of the retina. For both Fzd4+/+ and Fzd4+/- mice at P12 and P17, vessels were detected in the inner and middle layer of the retina with deeper vessels emerging by P25.
Fig 1. Analysis of retinal vasculature during development for wild type and Fzd4+/− mice under normoxic conditions. (A) Wild type and (B) Fzd4+/− mice were raised in room air. At P12, P17, and P25 whole mounted retinas were stained with Alexa Fluor 594-conjugated GS-IB4 lectin and visualized using confocal microscopy. The yellow square in the image on the left is enlarged in the panel on the right.

doi:10.1371/journal.pone.0158320.g001
Effect of Exposure to O₂ in the Mouse OIR Model

The standard mouse OIR model used to mimic ROP exposes mice to 75% O₂ for five days from P7 to P12; this causes vaso-obliteration of the central retina surrounding the optic nerve. At P12, mice are re-exposed to room air resulting in neovascularization at the vaso-obliterated edges (maximum effect at P17), revascularization of the vaso-obliterated area, as well as continued vascularization toward the distal region of the retina [43]. This level of O₂ is used to guarantee all mice in the cohort develop ROP-like vaso-obliteration. The standard mouse protocol did not demonstrate a difference in the effects of OIR between $Fzd4^{+/+}$ and $Fzd4^{+/-}$ mice. Given the small but significant effects of haploinsufficiency in $Fzd4^{+/-}$ mice under normoxic conditions, and to allow for comparison of the wild type and $Fzd4^{+/-}$ mice to human genetic data that suggested a small decrease in Norrin-FZD4 mediated vascular development could increase risk for severe ROP, we wanted to establish the minimum oxygen exposure (the critical tipping point) that elicited vaso-obliteration in mice. At 65% O₂ we observed the same level of vaso-obliteration as was previously seen in wild type mice exposed to 75% O₂ [44, 45] with approximately one third of the retina ablated. Conversely, no retinopathy was detected when mice were exposed to less than 60% O₂. We found that providing an additional insult by introducing one day of exposure to room air at P10, followed by 65% O₂ for one day at P11, prior to returning mice to room air at P12 produced a reliable and consistent vaso-obliteration phenotype (Fig 3). Varying high and low O₂ levels is similar to what is done in the rat model of OIR.

A Delay in Retinal Vascular Recovery in $Fzd4^{+/-}$ Mice Subjected to OIR

The effect of our modified OIR protocol on the area of vaso-obliteration, revascularization of the vaso-obliterated area, continued vascularization toward the periphery, and number and caliber of vessels was assessed at P12, P17 and P25 in wild type and $Fzd4^{+/-}$ mice to determine if the defects observed in vascular development in $Fzd4^{+/-}$ mice would be exacerbated. When mice were removed from the high O₂ environment at P12, there was no difference in the area of vaso-obliteration between $Fzd4^{+/-}$ and wild type mice with both having 34% of the retina ablated. Conversely, no retinopathy was detected when mice were exposed to less than 60% O₂. We found that providing an additional insult by introducing one day of exposure to room air at P10, followed by 65% O₂ for one day at P11, prior to returning mice to room air at P12 produced a reliable and consistent vaso-obliteration phenotype (Fig 3). Varying high and low O₂ levels is similar to what is done in the rat model of OIR.

Fig 2. Changes in vessel number and calibre under normoxic conditions for wild type and $Fzd4^{+/-}$ mice. Mice were raised in room air and at P12, P17, and P25 whole mounted retinas were stained with Alexa Fluor 594-conjugated GS-IB4 lectin and visualized using confocal microscopy. (A) For vessel count, profile plots were created by drawing three lines in each of the four quadrants at three specified locations: adjacent to the optic nerve (proximal), adjacent to the periphery of the retina (distal) and midway between the proximal and distal lines (middle). The number of vessels crossing each line in each quadrant was counted. (B) The width of vessels at proximal, mid, and distal regions from the optic nerve was calculated using Image J. A 50 µm cutoff was chosen to represent the width of major primary vessels and calculated as a percentage of total vasculature. (C) A schematic representation of vessel number and calibre determination. Line segments were drawn perpendicular to retinal quadrants at the proximal, middle and distal regions. Imaging analyses are expressed as mean ± standard error of the mean (SEM) and P values were calculated using a one-way ANOVA unpaired t-test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. NS, not significant.

doi:10.1371/journal.pone.0158320.g002

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The effect of our modified OIR protocol on the area of vaso-obliteration, revascularization of the vaso-obliterated area, continued vascularization toward the periphery, and number and caliber of vessels was assessed at P12, P17 and P25 in wild type and $Fzd4^{+/-}$ mice to determine if the defects observed in vascular development in $Fzd4^{+/-}$ mice would be exacerbated. When mice were removed from the high O₂ environment at P12, there was no difference in the area of vaso-obliteration between $Fzd4^{+/-}$ and wild type mice with both having 34% of the retina vaso-obliterated (Fig 4). There was a modest decrease in the number of vessels in $Fzd4^{+/-}$ mice in the mid peripheral and distal regions of the retina compared to wild type (Fig 5). Also, vessel caliber was increased in $Fzd4^{+/-}$ mice compared to wild type, from 9% to 13% in the mid periphery and 18% to 25% in the distal region (Fig 5).

The most obvious and important difference between wild type and $Fzd4^{+/-}$ mice exposed to the OIR model was observed at P17, during the recovery phase. Wild type mice had 6% of residual vaso-obliterated retina while $Fzd4^{+/-}$ mice had 10%, a 66% increase compared to wild type (Fig 6). This indicates that, compared to $Fzd4^{+/-}$ mice, $Fzd4^{+/-}$ mice had a substantially decreased ability to revascularize ablated retina. It was also clear that new vessel growth had progressed to the distal periphery in wild type mice at P17 and this was delayed in the $Fzd4^{+/-}$.
Fig 3. Retinal vasculature development into the deep and intermediate layers of wild type and Fzd4<sup>+/−</sup> mice under normoxic conditions. Whole mount retinas of Fzd4<sup>++</sup> and Fzd4<sup>+/−</sup> under normoxic conditions were stained with isolectin-594 and imaged at 25x magnification using a Zeiss LSM 510 Meta laser scanning confocal microscope. Z-stacks representing vascular growth in the deep and intermediate layers at the mid periphery were generated using 1 micron slices. Z-stacks were analyzed using the ImageJ software program. Green bars represent the X-axis, blue bars represent the Y-axis and purple represent the Z-axis. White arrows indicate examples of vessel branching in retinal layers.

doi:10.1371/journal.pone.0158320.g003
Fig 4. Retinal vaso-obliteration of wild type and $Fzd4^{+/+}$ mice after ocular ischemic retinopathy. Wild type and $Fzd4^{+/+}$ mice were exposed to our OIR model from P7 to P12. (A) At P12 whole mounted retinas were stained with Alexa Fluor 594-conjugated GS-IB4 lectin and visualized using confocal microscopy (panel i). Vaso-obliterated areas are shown in white (panel ii). (B). Quantification of the vaso-oblitertated area. Imaging analyses are expressed as mean ± standard error of the mean (SEM) and $P$ values were calculated using a one-way ANOVA unpaired t-test. NS, not significant.

doi:10.1371/journal.pone.0158320.g004
Fig 5. Decreased vessel number and increased vessel caliber in Fzd4+/− mice after ocular ischemic retinopathy. Comparison of vessel number and caliber for wild-type and Fzd4+/− mice after ocular ischemic retinopathy at P12, P17 and P25. (A) For vessel count, the number of vessels at lines in each quadrant at proximal, mid, and distal regions from the optic nerve was determined. (B) The width of vessels at proximal, mid, and distal regions from the optic nerve was determined was calculated using Image J. A 50 μm cutoff was chosen to represent the width of major primary vessels. Imaging analyses are expressed as
mice (Figs 5 and 6A), as determined by number of vessels/mm and the intensity of Alexa Fluor 594-conjugated GS-IB4 lectin staining. There was very little neovascularization in either wild type or Fzd4+/− mice exposed to our modified OIR protocol. At P25, the small decrease in vessel number measured in Fzd4+/− mice was retained across all areas of the retina while vessel caliber was similar to wild type (Figs 5 and 7), consistent with delayed revascularization in both the proximal and distal regions of the retina in Fzd4+/− mice exposed to OIR.

We also determined whether retinal vessel growth in the intermediate and deep layers was altered in Fzd4+/+ and Fzd4+/− mice under OIR conditions (Fig 8). Fzd4+/+ and Fzd4+/− mice were compared to mice raised under normoxic conditions (Fig 3), and to each other, following mean ± standard error of the mean (SEM) and P values were calculated using a one-way ANOVA unpaired t-test. * P<0.05, ** P<0.01, *** P<0.001. VO, vaso-obliterated. NS, not significant.

doi:10.1371/journal.pone.0158320.g005

doi:10.1371/journal.pone.0158320.g006

Fig 6. Delayed revascularization in Fzd4+/+ mice after ocular ischemic retinopathy. Wild type and Fzd4+/− mice were exposed to our OIR model from P7 to P12 and returned to room air. At P17 (A) whole mounted retinas were stained with Alexa Fluor 594-conjugated GS-IB4 lectin and visualized using confocal microscopy (panel i), vaso-obliterated areas are shown in white (panel ii). (B). Quantification of the vaso-obliterated area. Imaging analyses are expressed as mean ± standard error of the mean (SEM) and P values were calculated using a one-way ANOVA unpaired t-test. ** P<0.01.

doi:10.1371/journal.pone.0158320.g006
OIR treatment. At P12, both the \( Fzd4^{+/-} \) and \( Fzd4^{+/-} \) mice displayed a delay in intermediate and deep layer development after OIR treatment compared to retinas from normoxic mice. At P17 and especially at P25, the \( Fzd4^{+/-} \) and \( Fzd4^{+/-} \) mice did form intermediate and deep layers, however, most vessels were within a single plane compared mice reared under normoxic conditions, suggesting a delay in the formation of these deeper layers resulting from prior exposure to OIR conditions.

As weight has been known to affect vaso-obliteration and vascular recovery in mice [46, 47] we compared weight of wild type and \( Fzd4^{+/-} \) mice at P12, P17, and P25 under normoxic and the modified OIR conditions. We did not observe differences in weight between the wild type and \( Fzd4^{+/-} \) mice (S1 Table).

Discussion

In this study we sought to determine if haploinsufficiency of \( Fzd4 \) affects ocular vascular development in the mouse, and if \( Fzd4 \) haploinsufficiency alters the course of vascular changes observed during OIR. \( Fzd4^{+/-} \) mice had a small delay in vascular development in room air that
resolved as the mice matured. Specifically, at P12 there was a decrease in vessel number in all areas of the retina and increased caliber in the proximal and mid peripheral areas in Fzd4+/− mice. By P17 vessel number resolved slightly in Fzd4+/− mice while there remained increased distal vessel caliber measured as a percentage of vessels > 50 μm. This is possibly due to a compensatory response to hypoxic conditions in the peripheral retina, or a relative delay in development of the microvascular bed (as opposed to the larger primary vessels in the inner plexus) resulting from delayed peripheral vascularization due to Fzd4 haploinsufficiency. By P25, retinal vessel patterning and caliber was similar between wild type and Fzd4+/− mice.

We went on to use an OIR model to determine if the small defect we observed in the Norrin-Fzd4 mediated retinal vascular development in the Fzd4+/− mice affects vaso-obliteration and subsequent revascularization. A main driver of our mouse OIR study was the previous observations that rare polymorphisms in the known FEVR genes NDP, FZD4 and LRP5 were enriched in patients with severe ROP [4, 30–38, 48]. The human data implied that a small decrease in Norrin-FZD4 signaling could alter the course of ROP. We determined that the vaso-obliterated area was unaffected in Fzd4+/− mice compared to wild type mice, however, following re-exposure to room air Fzd4+/− mice displayed a substantive delay in revascularization.
toward the proximal region of the retina, as well as a delay in vascularization toward peripheral retina. Exposure of Fzd4+/− mice to the modified OIR model does not increase neovascular development nor does it affect the emergence of the deeper retina layers compared to wild type mice. ROP in premature infants is due to disruption of normal vascularization of the developing retina, a process that normally requires full gestation for completion in humans. The two-phase hypothesis for the development of ROP consists of an initial period of relative hyperoxia (phase 1) with associated delays in vascularization, followed by retinal hypoxia due to absence of vessels in the developing retina (phase 2). This leads to aberrant neovascularization at the developing ROP edge putting the infant at risk of developing tractional retinal detachment. The lengthening of the period of vascular recovery we observed in Fzd4+/− mice would allow for a longer time frame for which infants would be at risk of developing neovascularization. This is consistent with the observed rare polymorphisms identified in Norrin-FZD4 genes in premature infants with severe ROP, and suggests that in these infants a small decrease in Norrin-FZD4 mediated vascular development results in an increased risk for the development of blinding complications of ROP.

In support of our results are two studies where Norrin dose was increased in mouse models of OIR to drive Norrin-Fzd4 signaling. Ectopic overexpression of Norrin in transgenic mice (βB1-crystallin-norrin mice) exposed to the OIR protocol promoted vascular regrowth at P14 and P16 in a dose dependent fashion [49]. It also reduced vaso-obliteration suggesting increased norrin-Fzd4 signaling may offer protection against hyperoxic damage. More recently, Tokunaga et al [50] were able to rescue OIR in mice following a single intravitreal injection of Norrin at P14. They observed a significant decrease in the avascular area subsequent to the OIR protocol, and to a lesser degree a decrease in neovascularization at P17. They noted a progression of vascular recovery between P15 and P20 suggesting faster remodeling in Norrin treated animals.

Characterizing the effects of stressors on developmental angiogenesis improves our understanding of retinal vascular disease processes and can help identify potential therapeutic targets. ROP is a blinding disorder that affects prematurely born infants and is a leading cause of blindness, leaving an estimated 20,000 infants blind or severely visually impaired worldwide annually.[2] Current treatments for severe ROP including laser and anti-VEGF agents are helpful in decreasing the risk of blindness. Nevertheless, vision loss [51, 52], reduced visual fields [53, 54], persistent vascularization defects with risks for late complications [55–60] and concerns for potential systemic side effects [61–65] remain significant problems, highlighting the need to develop better predictors for infants at risk for more severe ROP to aid in prioritizing treatment to reduce lifelong visual impairment. Our studies suggest that screening for rare polymorphisms in FEVR genes could be a worthwhile endeavour to determine their predictive value for the development of more severe ROP. Treatments that aim to alter Norrin-Fzd4 signaling may decrease the risk of blindness in prematurely born infants by reducing the vulnerable time period during which blinding complications can occur.

Supporting Information
S1 Table. Weights of mice used in this study.
(PDF)

Acknowledgments
The authors thank Yanli Zhou and Dr. Anna-Maria Szczesniak for their assistance with immunohistochemistry and animal care.
Author Contributions
Conceived and designed the experiments: JMR SN MEK.
Performed the experiments: MHN JBF SN.
Analyzed the data: MHN GH CRM JMR.
Contributed reagents/materials/analysis tools: MEK CRM JMR.
Wrote the paper: MHN CRM JMR.

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