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TWEAK/Fn14 Pathway is Activated by Hypoxia and is a Novel Mediator of Retinal Neovascularization

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TWEAK/Fn14 Pathway is a Novel Mediator of Retinal Neovascularization and is Induced by Hypoxia

by

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Dedication

To my wife, Maryam Dadfarnia, for her tremendous support

To my son, Shayan, for keeping me happy even when my experiments were not going well

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First and foremost, I would like to thank my mentor, Dr. Wenbo Zhang, for his tremendous support and guidance. In addition to patiently teaching me new lab skills, Dr. Zhang continuously educated me on how to develop an idea, plan and execute experiments, and interpret data. Dr. Zhang also performed intravitreal injections for me. My special thanks goes to my committee members Dr. Ronald Tilton, Dr. Massoud Motamedi, Dr. Gerald Campbell and Dr. Gerald Chader for their advice and constructive criticisms throughout this work. Dr. Motamedi and Dr. Tilton were among the first people whom I approached for advice; it was only after talking to them that I felt confident about feasibility of committing to basic science research during my Ophthalmology Residency. I am particularly grateful to Dr. Tilton who offered to meet on a weekly basis and regularly reviewed my work in detail and gave me his honest opinion in every respect in the past year. I would also like to thank Dr. Bernard Godley, Dr. Volker Neugebauer and Dr. Jose Barral for encouraging me to enroll in the PhD program and supporting me along the way. I would like to express my gratitude to my colleagues in the lab, Dr. Rong Liu, Dr. Hua Liu, Dr. Yonju Ha and Dr. Shuqun Hu for helping me with my experiments and for creating a congenial environment. In particular, Dr. Rong Liu taught me many lab skills and Dr. Hua Liu isolated retinal vassels for me. I would like to thank Dr. Adriana Paulucci-Holthauzen and Dr. Leoncio Vergara for their assistance with immunofluorescence microscopy. In addition, I am indebted to Lisa Davis and Dr. Dorian Coppenhaver for their assistance in the past few years. I would like

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TWEAK/Fn14 Pathway is a Novel Mediator of Retinal Neovascularization and is Induced by Hypoxia

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Retinal neovascularization (NV) is a major cause of vision loss in vascular eye diseases such as diabetic retinopathy, retinal vein occlusion and retinopathy of permaturity. Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) and its receptor, fibroblast growth factor inducible 14 (Fn14), have been implicated in angiogenesis but their role in retinal diseases is largely unknown. The purpose of my study was to evaluate the role of TWEAK/Fn14 pathway in NV. I used mouse model of oxygen-induced retinopathy (OIR), which is commonly used for studying NV. OIR was produced by maintaining C57BL/6 wild type neonatal mice in 75% oxygen from postnatal day (P)7 to P12 and room air from P12 to P17. Control mice were kept in room air (RA). Quantitative PCR showed upregulation of Fn14 mRNA in the OIR retina compared to RA. Next, I wanted to see localization of TWEAK and Fn14 in the normal retina (RA) and whether they change in OIR. Immunostaining of retinal sections displayed localization of both TWEAK and Fn14 in neuronal layers in normal retina. However, in the OIR retina, both TWEAK and Fn14 were predominantly expressed in neovascular tufts. This shift in localization was associated with a dramatic overexpression

of Fn14 mRNA in the isolated retinal vessels in OIR compared to RA. Hyperoxia treatment (HT), from P14-P17, has been shown to prevent NV in OIR. Interestingly, HT was associated with normalization of both the TWEAK and Fn14 mRNA to RA levels, further supporting the hypotheses that TWEAK/Fn14 is involved in NV and that hypoxia is an inducer of the TWEAK/Fn14 pathway. To study the mechanism by which hypoxia induces TWEAK/Fn14, I used adenovirus to overexpress hypoxia inducible factor (HIF)-1α in human retinal endothelial (HRE) cells. HIF-1α overexpression was associated with upregulation of both TWEAK and Fn14. Finally, blockade of the TWEAK/Fn14 pathway, by intravitreal injection of Fn14-Fc decoy, was associated in a significant reduction in the extent of NV.

Through my experiments, I have demonstrated that the TWEAK/Fn14 pathway is involved in the initiation and progression of NV, and ischemia-induced upregulation of Fn14 is mediated by HIF-1α.

TABLE OF CONTENTS

List of Figures

List of Abbreviations

BRVO	branch retinal vein occlusion
CRVO	central retinal vein occlusion
DR	diabetic retinopathy
HIF-1α	hypoxia inducible factor-1α
HRE	human retinal endothelial
HT	hyperoxia treatment
NPDR	nonproliferative diabetic retinopathy
OIR	oxygen-induced retinopathy
PDR	proliferative diabetic retinopathy
RA	room air
NV	retinal neovascularization
RVO	retinal vein occlusion
TFBS	Transcription factor binding sites
TWEAK	tumor necrosis factor (TNF)-like weak inducer of apoptosis
VEGF	vascular endothelial growth factor
VO	vaso-obliteration

Chapter 1

TWEAK/Fn14 Pathway is a Novel Mediator of Retinal Neovascularization

INTRODUCTION

In the past few decades, medicine has been revolutionized in many respects. Countless discoveries and innovations have dramatically changed the diagnosis and treatment of numerous diseases. Ophthalmology, in particular, has enjoyed a great deal of fundamental changes in diagnostics, surgical techniques and pharmacological treatments. Despite all these, there are many conditions that continue to cause vision loss, and retinal vascular disease is one of them. Indeed, retinal vascular disease is a major cause of vision loss worldwide. The top three causes of retinal vascular disease are diabetic retinopathy, retinal vein occlusion and retinopathy of prematurity. In the past three decades, significant improvements have been made in the management of these conditions but they still continues to be a major source of concern when it comes to vision loss. A better understanding of the disease process and current treatment strategies will help to develop more effective treatment modalities.

Diabetic retinopathy

Diabetic retinopathy (DR) is the leading cause of blindness among working-age adults around the world [1]. DR, which occurs in both insulin-dependent diabetes mellitus (IDDM) and non- insulin-dependent diabetes mellitus (NIDDM), is classified into a mild stage, nonprolifeative DR (NPDR) and a severe stage, proliferative DR (PDR). Among diabetic people, the overall prevalence of DR of any type is 34.6%, and about 7% have

proliferative diabetic retinopathy (PDR), i.e. diabetic retinopathy with pathological retinal neovascularization [2]. Worldwide, there are approximately 93 million people with diabetic retinopathy, 17 million of whom have PDR [2]. In the United States, it is estimated that 28.5% of diabetic patients 40 years or older have diabetic retinopathy and 1.5% have PDR, making the overall prevalence of these conditions 3.8% and 0.2% respectively nationwide [3].

Nonproliferative diabetic retinopathy (NPDR)

Diabetes almost affects all structures of the eye but the cause of vision loss is generally due to retinal microvascular changes, and hyperglycemia is believed to be an important factor in developing DR [4, 5]. Duration of diabetes is the biggest risk factor for developing DR and it is more common in IDDM. In IDDM, the prevalence of mild retinopathy reaches almost 100% after 15 years, whereas in NIDDM, it reaches 60-80% after 30 years [6, 7]. Selective loss of pericytes and thickening of capillary basement membranes are among early structural changes that have been widely reported in DR [8-10]. These changes along with increased permeability of capillaries (vasopermeability), leading to breakdown of the blood retinal barrier, are believed to be the basic mechanism for vision loss in NPDR. Clinically, microaneurysms, sacular protrusions in microcapillaries, are the earliest structural changes visible with an ophthalmoscope. Other visible retinal changes in NPDR include, retinal hemorrhages, cotton wool spots, and hard exudates (Fig. 1).

The main cause of vision loss in NPDR is macular edema. Although many conditions can cause macular edema, DR is by far the most common cause. As a result of breakdown in blood retinal barrier, serum leaks out of capillaries into the retinal tissue and results in swelling of the retina. When this swelling occurs in the peripheral retina it may not have visual consequences but when it occurs in the macula, which is responsible for fine vision, the vision becomes blurry. The vision loss in macular edema is often mild to moderate and depends on the amount of swelling, the extent of macular involvement

and the duration of swelling. Vision loss is usually reversible in short-term edema; however, long-term edema may result in structural changes in the retina and an irreversible vision loss. The standard of care for diabetic macular edema has been laser photocoagulation [11, 12]. Although the goal of this treatment is to prevent vision loss, in most patients it results some improvement in vision. Vascular endothelial growth factor (VEGF) has been implicated in macular edema [13]. In recent years, intravitreal anti-VEGF medications have been frequently used to treat diabetic macular edema with relative success [14-16]. Bevacizumab, which was originally developed for colon cancer treatment and its use in the eye is off lable, is widespreadly used for various retinal vascular diseases because of its low price. Ranibizumab, on the other hand, was developed for the treatment of neovascular age related macular degeneration and has recently been FDA approved for macular edema. Aflibercept is the latest ocular anti-VEGF drug coming to the market. Anti-VEGF medications are often used to treat refractory cases unresponsive to laser photocoagulation but the paradigm is gradually shifting more toward intravitreal pharmacological therapy as a first line treatment.

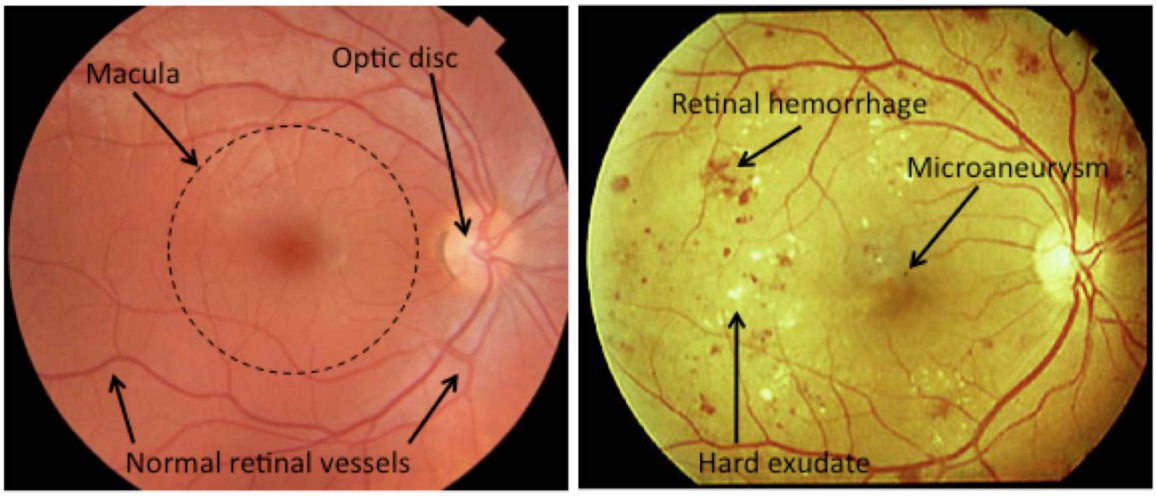

Figure 1: Color fundus photo, comparing normal retina with NPDR

Left: the front view of normal retina, as seen through the pupil, showing optic disc and normal macula. Modified from http://upload.wikimedia.org/wikipedia/commons/3/37/Fundus_photograph_of_normal_right_eye.jpg, 5/18/13. Right: NPDR. Red sposts represent microaneurysms or retinal hemorrhages and yellow/ white spots represent hard exudates. Modified from http://www.rustoneyeinstitute.com/index.cfm/pageid/3796, 5/18/13

Proliferative diabetic retinopathy (PDR)

PDR is the severe form of DR that could potentially result in profound irreversible vision loss. In IDDM, the prevalence of PDR reaches over 50% after 20 years, whereas in NIDDM it reaches 15-30% after 25 years [6,7]. As the name implies, PDR is the vasoproliferative stage of DR and is characterized by pathological neovascularization. This neovascularization may occur anywhere on the retina, on the optic disc, or even on the iris (Fig. 2).

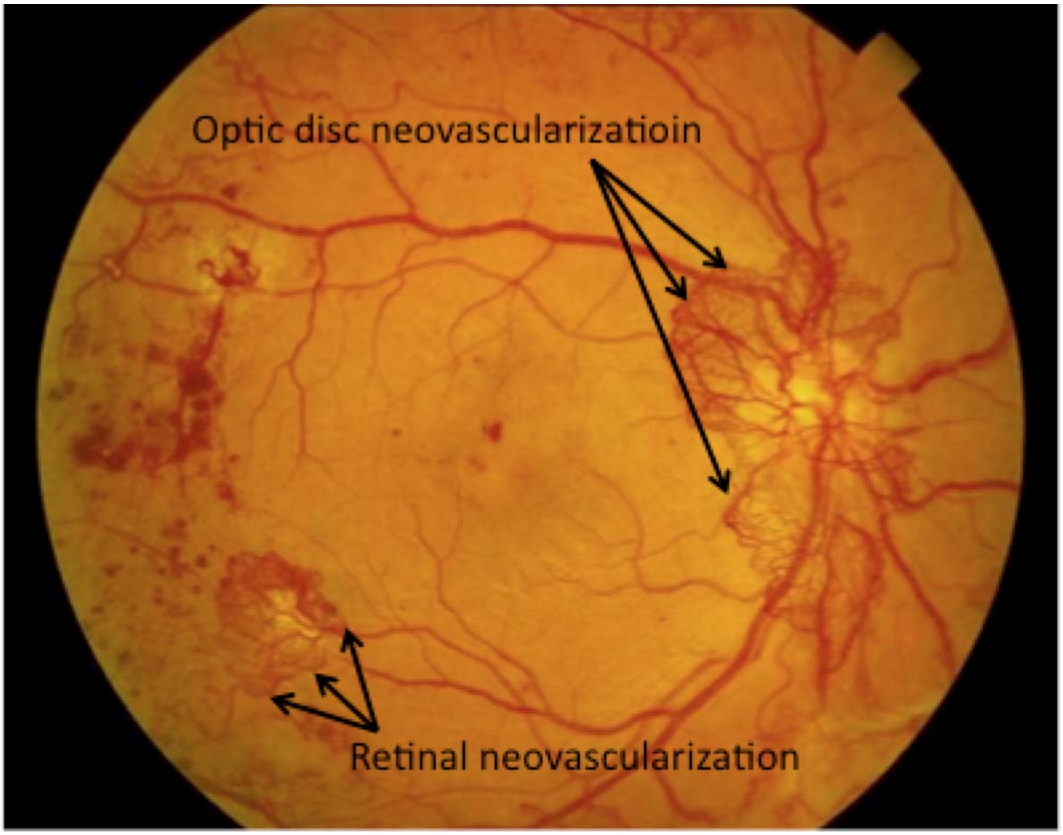

Figure 2. Neovascularization of the retina and optic disc in PDR

Modified from http://retinagallery.com/displayimage.php?album=550&pid=5011; 5/18/13

The development of retinal neovascularization (NV) is a process that involves multiple factors including upregulation of angiogenic factors, inflammation, and breakdown of internal limiting membrane. NV is accompanied by fibrous tissue proliferation, which could cause tractional retinal detachment and irreversible vision loss (Fig. 3). Once the vision is impaired by tractionl retinal detachment, only surgical intervention may stabilize the condition and the vision may not improve.

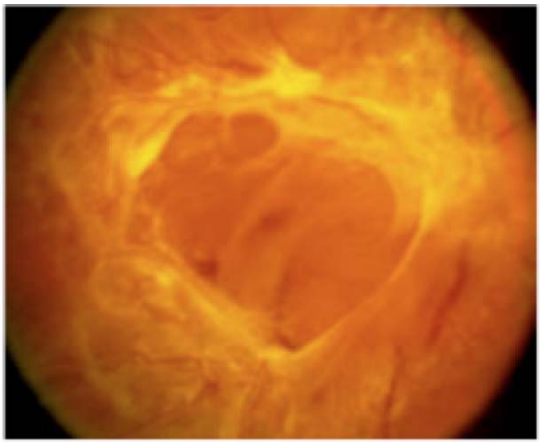

Figure 3. Tractional retinal detachment causing distortion of the retina

From http://www.duncaneye.com/resources/diabetic-retinopathy.php; 5/18/13

Another complication of NV is vitreous hemorrhage. This is because the new vessels grow on the surface of the retina or into the vitreous cavity and lack the structural support of normal capillaries and are susceptible to bleeding. Depending on the severity, vitreous hemorrhage may cause partial or total loss of vision, which usually last weeks to months. Longstanding vitreous hemorrhage may require surgical intervention to recover the vision. Other less common causes of vision loss in PDR include macular ischemia and iris neovascularization.

Current treatment for PDR is directed toward prevention of its devastating complications including tractional retinal detachment and vitreous hemorrhage. This is achieved by panretinal laser photocoagulation, in which the peripheral retina is ablated by laser once certain extent of NV or iris neovascularision is observed [17]. In this treatment, the peripheral retina is covered by laser burns of approximately 500 μm in size and the same distance apart. The laser is absorbed by the retinal pigment epithelium and causes localized destruction of the outer retina.

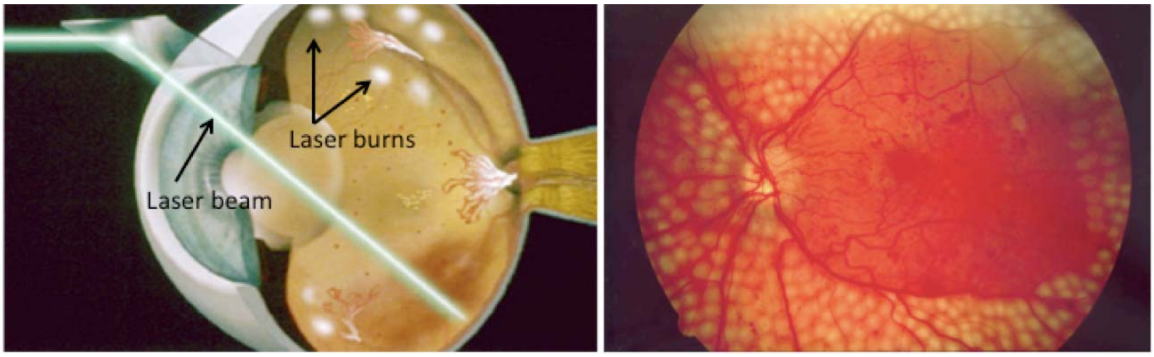

Figure 4. Panretinal laser photocoagulation

Left: an illustration demonstrating how the laser is applied to the retina. Right: a fundus photo taken immediately after panretinal laser photocoagulation showing laser burns (white spots) covering the periphery and sparing the central retina. With time, white spots will change to black scars. Modified from http://www.avclinic.com/Dia_proliferative.htm; 5/18/13

The association between retinal ischemia and NV or iris neovascularization has been known for a long time [18]. Panretinal photocoagulation is thought to cause regression of new vessels by eliminating ischemic cells and by reducing the oxygen demand, even though clinicians started using laser photocoagulation before its mechanism of action was known [19]. The goal of panretinal photocoagulation is not to improve the vision but to prevent further vision loss. Photocoagulation is effective in decreasing the risk of severe vision loss by more than 50% but may result in loss of peripheral vision and night vision. Although panretinal photocoagulation is the standard of care for PDR, it is far from ideal

The notion that ischemic retinal cells must produce a soluble factor that causes NV is several decades old. Detection of higher levels of VEGF in the aqueous of primates with ocular angiogenesis and in the vitreous of human PDR patients supported this idea and now it is widely accepted that VEGF plays a major role in pathological retinal angiogenesis [20, 21]. In recent years, intravitreal injection of anti-VEGF drugs has been shown to cause regression of the retina and iris neovasculrization [22, 23]. A major concern

with these medications, though, is that their effects are short-lived and they require frequent intraocular injections. In addition, suppression of VEGF-mediated cell survival pathways in hypoxic neurons might inhibit neuronal survival and function [24]. Because of these problems, panretinal photocoagulation, with all its shortcomings is the gold standard for PDR.

Retinal vein occlusion

Retinal vein occlusion (RVO) is the second most common cause of retinal vascular disease after DR. The prevalence of RVO is estimated to be 3.7 per 1000 in whites 3.9 per 1000 in blacks, 5.7 per 1000 in Asians, and 6.9 per 1000 in Hispanics. An estimated 16.4 million adults are affected by RVO [25]. Unlike DR, which is part of a systemic disease, RVO is usually the result of a local pathology, although most patients have hypertension. Retinal circulation is responsible for nourishment of the inner two-thirds of the retina. The central retinal artery enters the eye through the optic disc and gives branches to all four quadrants of the retina. The central retinal vein, which is made by union of branch retinal veins exit the eye the optic disc adjacent to the central retinal artery. RVO occurs when there is a blockage in the central retinal vein or one of its branches, as a result of thrombus formation (Fig. 5). Both the branch RVO (BRVO) and the central RVO (CRVO) may be complicated by macular edema, macular ischemia, iris neovascularization, or less commonly NV [26]. Unfortunately, there is no treatment for macular ischemia and once it develops the prognosis is poor. In case of macular edema, grid laser photocoagulation is effective in stabilizing vision in BRVO but has proven ineffective in CRVO [27, 28]. Similar to diabetic macular edema intravitreal injection of anti-VEGF medications has been successfully used to treat macular edema in both BRVO and CRVO but the effects are short-lived [29-32]. In addition, intravitreal injection of steroids has

proven effective in treating macular edema secondary to RVO, although steroids are associated with high risk of glaucoma and cataract formation [33-36].

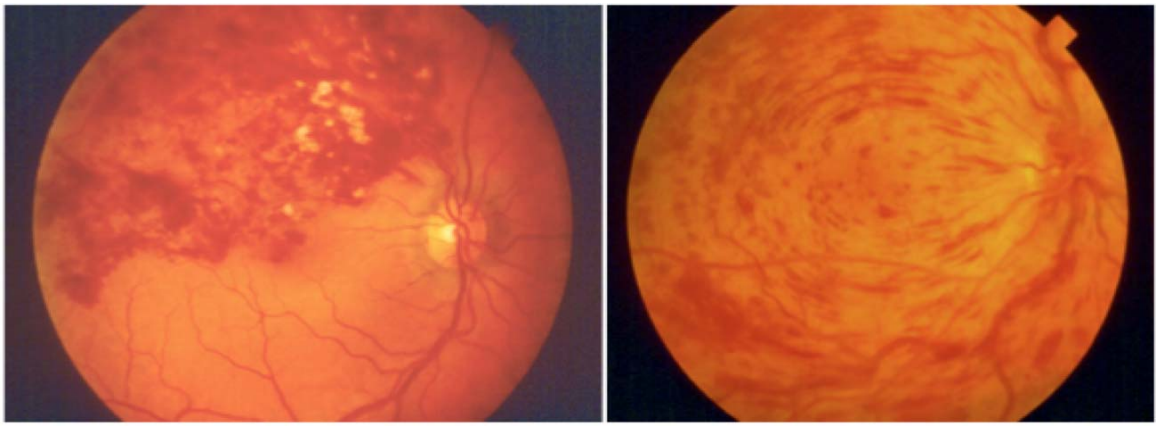

Figure 5. Branch retinal vein occlusion (left) and central retinal vein occlusion (right)

Note that retinal hemorrhages are limited to one area in BRVO and scatterd throughout retina in CRVO. From http://www.bethesdaretina.com/library.htm; 5/18/13

A devastating complication of RVO is iris neovascularization known as rubeosis iridis, which may cause intractable glaucoma culminating in a blind painful eye (Fig. 6). Similar to a ball that a certain air pressure is required to maintain its shape, the eyeball has to maintain a certain fluid pressure to prevent its structural collapse. However, unlike a ball in which the air is trapped inside, in the eye the fluid is constantly produced and drained out of the eyeball. If for any reason the drainage is blocked the intraocular pressure increases causing glaucoma and irreversible damage to the optic nerve. The intraocular fluid is drained at the angle between iris and cornea. When there is iris neovascularization, the new vessels that grow on the iris often grow over the angle and block the drainage, causing a sudden rise in intraocular pressure damaging the cornea and optic nerve. This type of glaucoma, known as rubeotic or neovasscular glaucoma, is refractory to treatment and despite all measures many patients go on to develop a constantly painful eye with profound vision loss. The standard treatment is panretinal

photocoagulation and most patients require surgical intervention. Recently intravirtreal injection of anti-VEGF drugs has been shown to cause rapid regression of new vessels [37]. However, once the fluid drainage is blocked regression of new vessels is not able to restore the drainage and the glaucoma persists. To this date, prevention is the best treatment for neovascular glaucoma. Studies have shown that patients with large areas of retinal ischemia are more prone to developing rubeotic glaucoma [26]. To prevent neovascular glaucoma, RVO patients are followed closely and receive sectoral or panretinal photocoagulation as soon as any sign of iris neovascularization is observed [38].

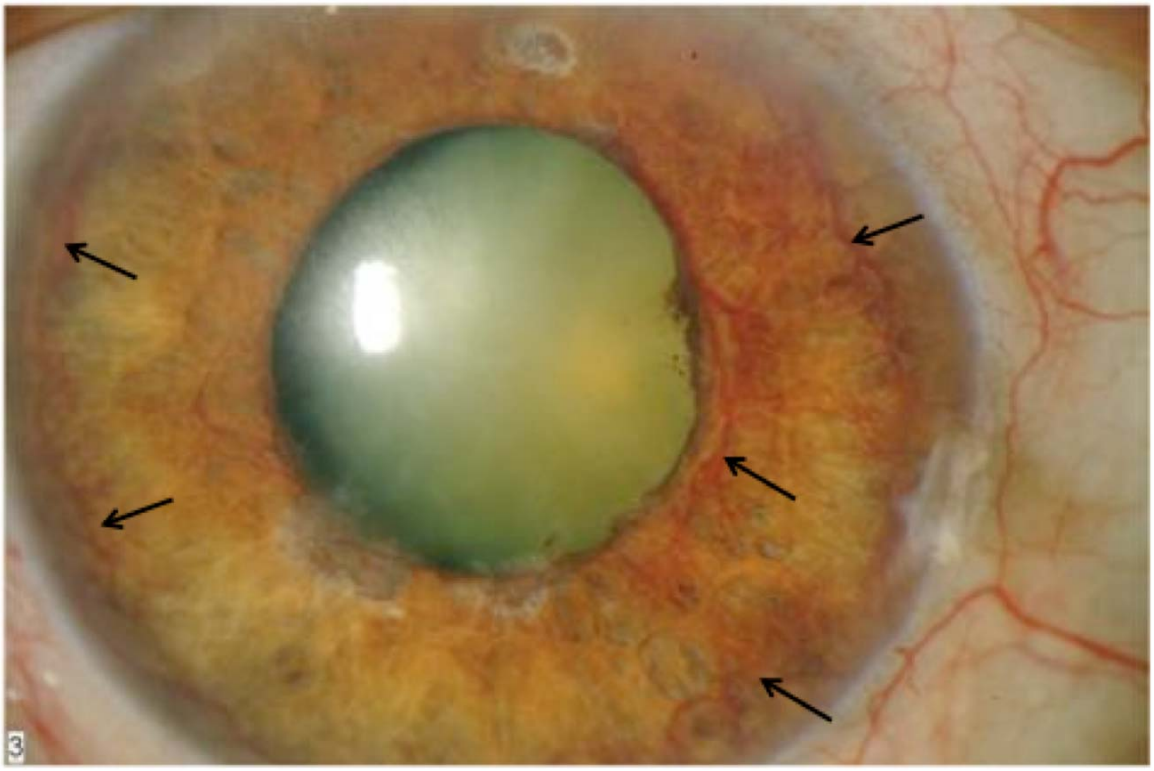

Figure 6. Iris neovascularization in RVO

Normally iris vessels are not visible. Arrows point to pathological neovasculrization areas. Modified from http://eyewiki.aao.org/File:NVI.jpg; 5/18/13

Retinopathy of prematuriy

In the United States, retinopathy of prematurity (ROP) is the second most common cause of blindness in the pediatric population [39]. So far, there have been two ROP epidemics; the first epidemic started in 1940-50 when high dose oxygen therapy was used for premature infants. The second epidemic started in 1980 as the survival rate of low birth weight infants increased in developed countries [40, 41]. In fetus, normal vascularization of the retina starts at the optic disc and grows toward the periphery becoming complete at approximately full term pregnancy. Therefore, in premature infants the peripheral retina is still avascular at birth, and physiological angiogenesis continues for weeks to months after birth. In ROP, this normal retinal vascular development is altered and abnormal neovascularization occurs (Fig. 8).

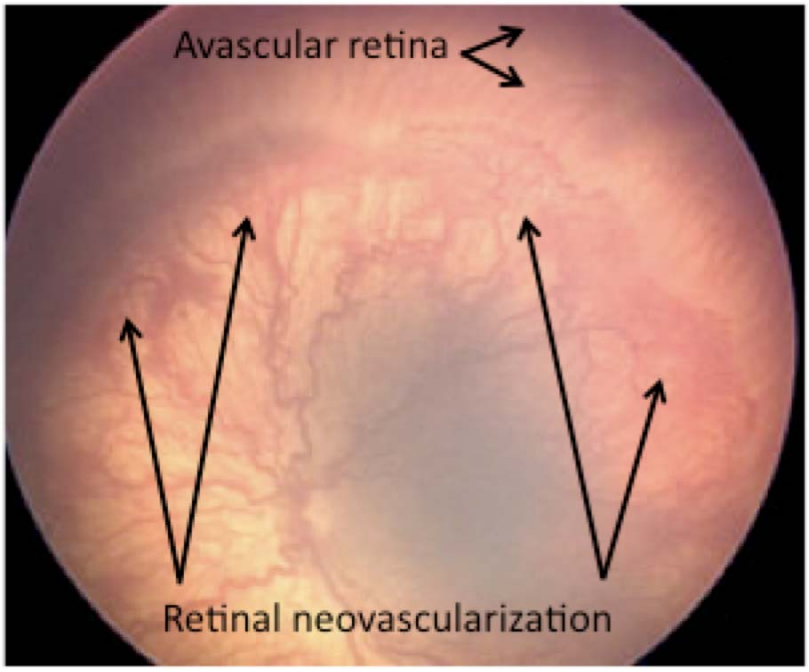

Figure 7. Retinopathy of prematurity

Modified from http://www.retinatexas.com/Retinopathy%20of%20Prematurity.html; 5/19/13

Several decades ago, the biggest risk factor for ROP in premature infants was high dose of supplemental oxygen. This problem has been recognized, and currently oxygen is delivered in a control manner. As a result, the most important risk factors at present are gestational age and birth weight. The shorter the gestational age and the lower the birth weight the higher the risk of ROP [42]. Newborns with a gestational age of 30 weeks or less or a birth weight of less than 1500 g are particularly at high risk of ROP. In majority of cases ROP regresses spontaneously and normal vascularization of the retina is resumed and completed. However, in some cases NV continues to grow into the vitreous cavity causing complications such as retinal distortion and tractional retinal detachment. Once the disease reaches this point, the visual prognosis is poor. At present, the standard of care for ROP prevention of complications by screening of high risk infants with frequent fundus examination, and laser or cryotherapy treatment of NV once certain criteria are met (Fig. 8) [43]. Although application of laser burns or cryotherapy to the avascular retina reduces the chance of profound vision loss, sight threatening complications may manifest months to years later [44]. Similar to other retinal vascular disease, anti-VEGF drugs have been used in the treatment of ROP both as monotherapy as well as in combination with laser photocoagulations[45, 46]. Given the systemic absorption of these drugs, even when injected intravitreally, their use in pediatric population is controversial. It will take years before their long-term ocular effects and possible systemic complications are known.

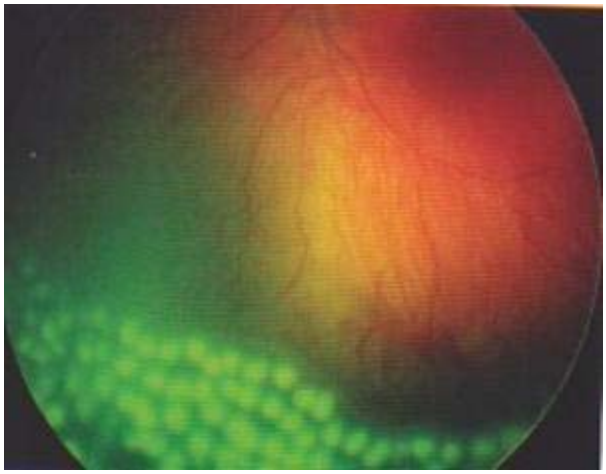

Figure 8. Laser treatment for ROP

Laser burns are applied to the peripheral retina, which is avascular. From http://www.physicianbyte.com/RetinopathyOfPrematurity.aspx; 5/21/13

TWEAK/Fn14 pathway and angiogenesis

TWEAK is initially synthesized as a 249-amino-acid type II transmembrane protein. It is proteolytically processed in the C-terminal region to a 156-amino-acid soluble cytokine (sTWEAK) [47]. It has been shown that, in some diseases, the level of sTWEAK is low, and it has been suggested that it could be a potential biomarker for predicting the prognosis in cardiovascular and peripheral arterial diseases [48-51]. Fn14, the smallest member of the TNFR superfamily, has been recognized as the TWEAK receptor. It is initially synthesized as a 129-amino-acid type I transmembrane protein that is then proteolytically processed by signal peptidase into a 102-amino-acid cell-surface receptor [47].

The angiogenic function of TWEAK/Fn14 was recognized soon after TWEAK was identified [52, 53]. These studies have shown that activation of Fn14 by TWEAK induces endothelial cell migration, proliferation and formation of capillaries in both in vitro and in vivo angiogenesis models [52, 54-56]. Although subsequent studies further

identified TWEAK/Fn14 as a key mediator in kidney injury, neuronal damage, and muscle atrophy, its role in pathological or physiological retinal angiogenesis has not been determined [57-59].

The development of retinal neovascularization is a complex process that involves multiple factors including upregulation of angiogenic factors, inflammation, and breakdown of internal limiting membrane. TWEAK/Fn14 has been shown to induce expression of many angiogenic factors such as VEGF, IL-6 and IL-8 [60, 61]. TWEAK/Fn14 pathway also promotes production of matrix metalloproteinase proteins, which may facilitate the breakdown of internal limiting membrane [62, 63].

Using whole genome gene expression array in the mouse model of oxygen-induced retinopathy (OIR), Fn14 was noted to be upregulated by 4.27 fold, putting it among the top 24 factors that were upregulated (courtesy of W. Zhang; unpublished data). This is an interesting finding, as VEGF was upregulated by 2.45 fold.

All of these suggest that TWEAK/Fn14 may play a role in retinal angiogenesis and could potentially be a target for novel treatment modalities.

Animal model

There is no perfect animal model to mimic human diabetes mellitus leading to PDR and NV; the same is true about RVO, ROP and other retinal vascular diseases. However, there is a mouse model in which pathological neovascularization of the retina is produced by exposing the animal to a high level of oxygen. This model is called oxygen-induced retinopathy (OIR). It was first described by Smith et al. and the method of creating it is discussed in detail in Materials and Methods [64]. Although OIR resembles ROP, it is also a good model for NV, which is a complication of all retinal vascular disease. A better understanding of this model is essential because it is extensively used for studies on NV and I have used it for most of my experiments [65, 66]. Unlike in human

that the retina is almost fully vascularized at birth, in mouse retinal vascularization occurs after birth (Fig. 9) [67]. However, the pattern of vascularizaiton is similar in both.

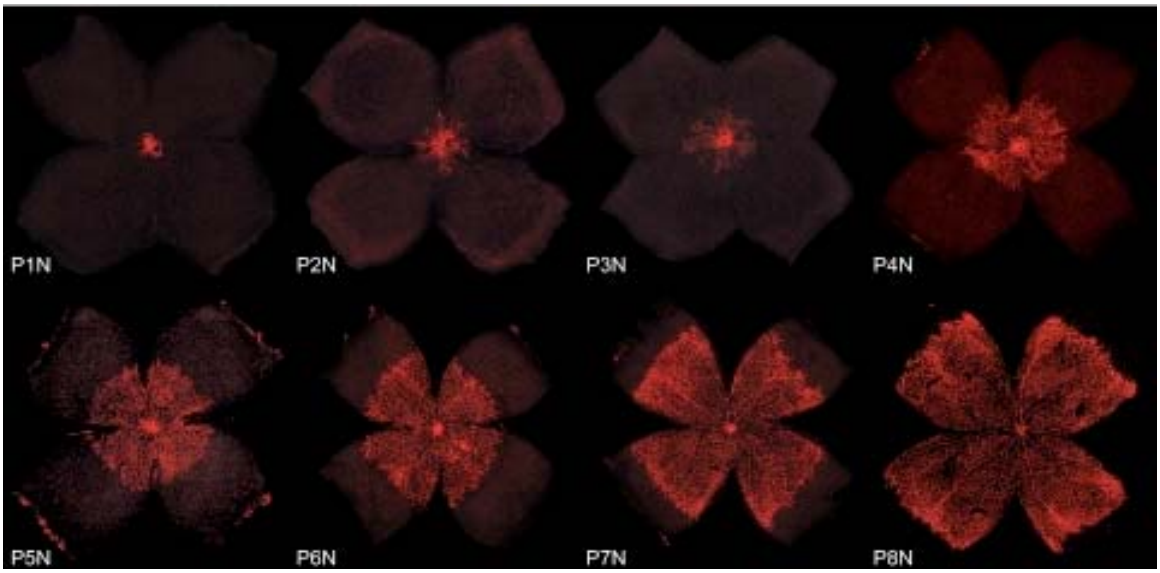

Figure 9. Normal retinal angiogenesis in C57BL/6J mouse

Retinal flat mount stained with isolectin B4 shows progression of vascularization of the retina (bright red) in a daily basis. At post-natal day 1 (P1N) the entire retina is avascular and the vessels are only visible at disc (red spot). Retinal vascularization occurs in a fast pace and becomes complete approximately eight days after birth (P8N). Used with permission from The Association for Research in Vision and Ophthalmology [67]

In OIR, when the mouse is placed in hyperoxia condition, vaso-obliteration occurs in the posterior part of the retina and after five days of hyperoxia a significant part of the retina becomes avascular. When the animal is returned to room air, the avascular area partially revascularizes but relative hypoxia causes pathological retinal neovascularization (NV) on the surface of the retina, mostly at the intersection of vascular and avascular areas (Fig. 10). This model is very useful for studying

mechanisms of NV and evaluating the effects of various interventions in preventing or treating NV.

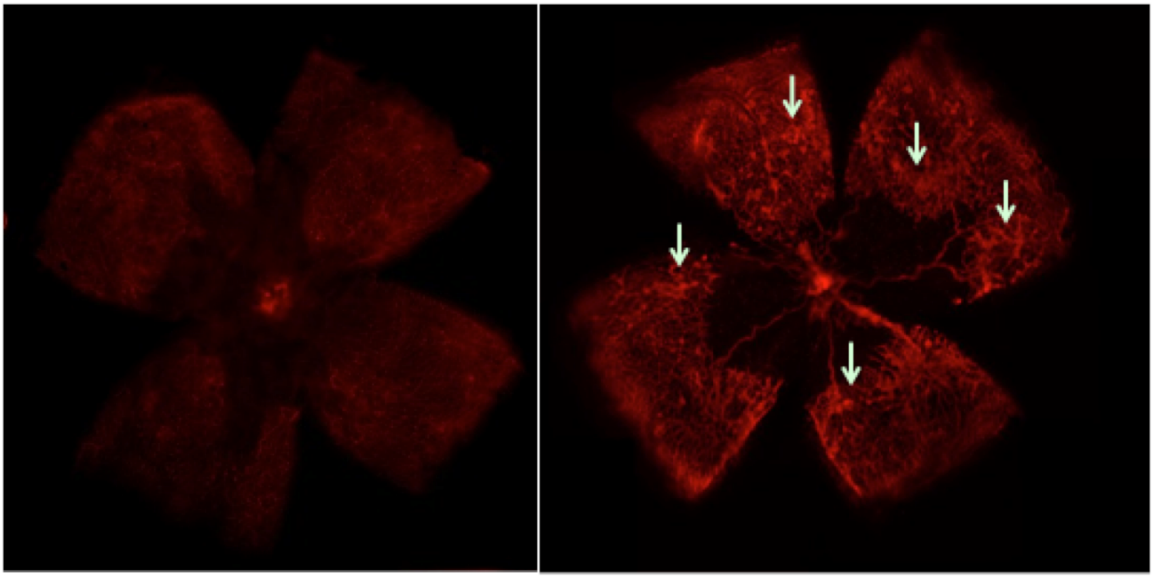

Figure 10. vaso-obliteration and NV in OIR

At P12, after five days of hyperoxia, at there is a significant area of vaso-obliteration at the central retina (left). At P17, after five days of relative hypoxia, neovascular tufts develop on the surface of the retina (right).

Objective

The brief review I presented here shows that NV is a major complication of retinal vascular disease, and unfortunately current treatments are far from satisfactory. Although panretinal laser photocoagulation is the standard of care for treating NV in retinal vascular disease, in recent years anti-VEGF medications have been added o the treatment strategies. The effects of these medications last only few weeks and they require frequent intravitreal injections. In addition, there are legitimate concerns about their safety. We have previously developed an animal model of NV in rabbit by

intravitreal injection of VEGF and have shown that intravitreal injection of bevacizumab, an anti-VEGF medication, in the presence of florid NV, could result in closure of normal retinal capillaries and further increase the area of retinal ischemia (Fig. 11) [68]. Our findings are supported by human studies that have shown enlargement of foveal avascular zone following intravitreal injection of bevacizumab [69, 70]. In addition, there are reports of macular ischemia following intravitreal injection of bevacizumab in patients with central retinal vein occlusion, diabetic retinopathy, retinal vasculitis or ocular ischemic syndrome [71-78].

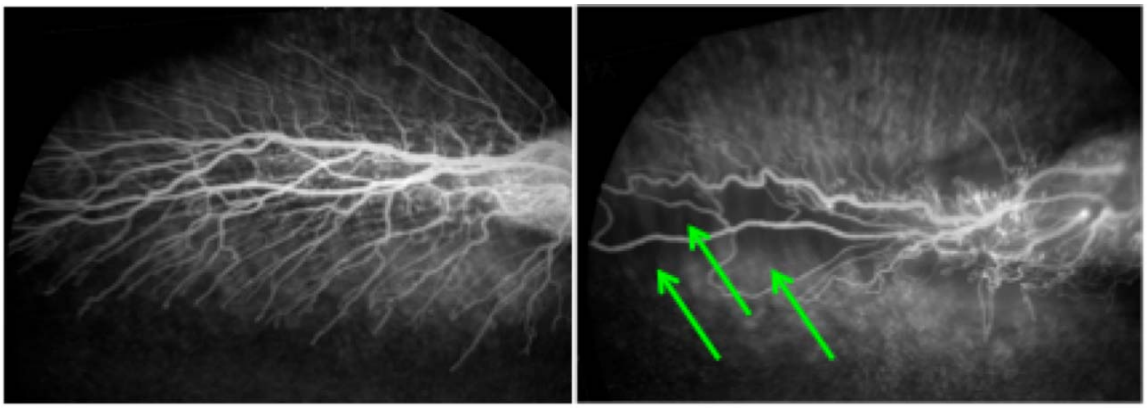

Figure 11. Capillary drop out following intravitreal injection of bevacizumab

Fundus fluorescein angiogram showing normal retinal capillaries in rabbit (left) and severe capillary drop out (green arrows) following intravitreal injection of bevacizumab after VEGF-induced retinal neovascularization (right).

As described above, current treatment strategies for retinal vascular diseases are only effective in a subset of patients and are associated with side effects that occasionally exacerbate vision loss. Therefore, there is an urgent need for finding other treatments that could benefit patients who are unresponsive to current treatments. The advent of Anti-VEGF medications has raised the prospect of pharmacological treatments for retinal vascular diseases. It is plausible that future pharmacological agents may be superior to

anti-VEGF drugs in terms of efficacy and side effects. After all, VEGF is not the only growth factor that is known to be involved in retinal neovascularization; some other examples include fibroblast growth factor, transforming growth factor beta and angiopoietin [79]. In addition to these factors, TWEAK/Fn14 has been shown to be involved in angiogenesis both in vitro and in vivo [52, 80, 81]. Although the angiogenic function of the TWEAK/Fn14 pathway was recognized soon after TWEAK was identified, its role in physiological or pathological angiogenesis has not been explored and its role in retinal neovascularization is completely unknown. The goal of my study was to investigate the role of TWEAK/Fn14 in NV, in order to find new treatment modalities for retinal vascular diseases.

MATERIALS AND METHODS

Oxygen-induced retinopathy (OIR)

All animal experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch.

C57BL/6J mice (Jackson Laboratory) were used for the study. OIR was achieved by following established methods. A custom designed, leak proof, hyperoxia chamber that could maintain constant desired O2 level using an oxygen sensor and injector (ProOx 110; BioSpherix), was utilized. The sensor was calibrated periodically, according to the company's instructions, to ensure its accuracy. Mice were mated and new litter was let to grow in their cage kept in room air. At postnatal day (P)7, six pups and their feeding mother were moved to a new cage and the whole cage was placed in the hyperoxia chamber. The body weight of a pup influences the extent of retinal NV and for this

reason six pups were used at all times to reduce variation in body weight. Next, O2 was injected into the chamber and the sensor was set to maintain the level at 75% O2 at all times. After five days, at P12, the cage was returned to room air and kept until P17. Age-matched mice maintained in room air, from birth to P17, served as room air control (RA) (Fig. 11). Care was taken to minimize handling of the pups to prevent stress, which is known to alter retinal neovascularization (NV).

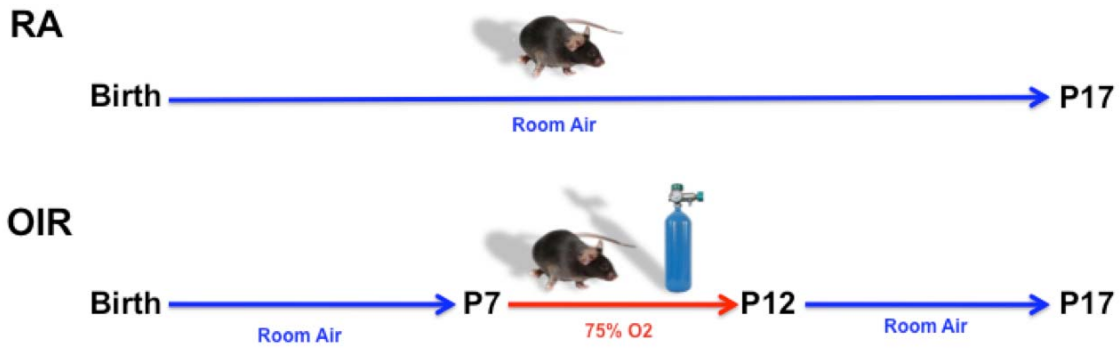

Figure 11. OIR

At the end of the study period, the mice were euthanized. One eye was enucleated for retinal flat mounting or retinal sectioning, and the contralateral retina was collected for mRNA and protein measurements. For enucleation, the eyelids were manually pulled apart to protrude the eyeball. Then with a curved, non-toothed, sharp tip forceps optic nerve was grabbed and the eyeball was pulled out. The eyeball was then immersed in 4% paraformaldehyde for 24 hours for subsequent morphological studies. For retina sample collection, after opening the eyelids and protruding the eye, the eyeball was gently grabbed with a curved toothed forceps behind the equator near the optic nerve and with a No. 11 blade the cornea was cut from limbus to limbus. The eyeball was then gently squeezed with the forceps. The extruded lens and vitreous were discarded and the extruded retina was put in 1.5 ml conical screw cap tube and immediately frozen in liquid nitrogen and subsequently transferred to -80°C.

RT-PCR assay on retinal samples

RNA 4PCR kit (Applied Biosystems, Austin, TX) was used to isolate total RNA. Lysis/binding solution was added to the retina in a 1.5 ml RNAse free tube and homogenized using a homogenizing pestle. The lysate was clarified by centrifuging at top speed with the temperature set at 5°C for 2.5 minutes. The supernatant was transferred to a new tube and an equal volume of 64% ethanol was added and mixed gently by pipetting. The solution was transferred to a filter tube and centrifuged at room temperature for 30 seconds. The flow-through was discarded and wash solution #1 was added to the filter tube and centrifuged; the same was repeated twice with wash solution #2/3. The flow-through was discarded and the cartridge was moved to a new tube and preheated elution solution (75-80°C) was placed on the filter and centrifuged. Next, the filter was discarded and the solution mixed with 10X DNase buffer and DNase, and incubated at 37°C for 30 minutes. Then, DNase inactivation reagent was added and incubated at room temperature for 2.5 minutes. Finally, the tube was centrifuged for 55 seconds and the supernatant was transferred to a new tube and RNA was quantified using NanoDrop ND-1000 Spectrophotometer. RNA samples were diluted to 45 ng/µl. To make cDNA by reverse transcription, preheated RNA samples were incubated with random primer and M-MLV RT enzyme (Invitrogen) at 37°C for two hours. cDNA templates and a mixture of forward and reverse primers along with the Power SYBR Green were added to a 96-well PCR plate, with a total volume of 10 µl in each well. StepOne PCR system (Applied Biosystems, Austin, TX) with the following PCR program was used: step 1, 2 minutes at 94°C; step 2, 30 seconds at 94°C; step 3, 30 seconds at 62°C; step 4, 15 seconds at 72°C; step 5, 40 repetitions of steps 2 to 4; step 6, 7 minutes at 72°C; step 7, hold at 4°C. The fold difference in various transcripts was calculated by the ΔΔCT method using Hprt as an internal control and normalized to RA control. Subsequently, a melting curve, constructed in the range of 60°C to 95°C, was

used to evaluate the specificity of the amplification products. Primer sequences for mouse transcripts were as follows: Hprt For-5'-GAA AGA CTT GCT CGA GAT GTC ATG-3'; Hprt Rev-5'-CAC ACA GAG GGC CAC AAT GT-3'; Fn14 For-5'-CTG GTT TTG GCG CTG GTT-3'; Fn14 Rev-5'-TCT CTC CGG CGG CAT CT-3'; TWEAK For-5'-TGG GAA GAG ACC AAA ATC AAC A-3'; TWEAK-Rev-5'-CCC AAT CTG GCG GTC GTA-3'.

Immunostaining of retinal sections

After fixing in 4% paraformaldehyde for approximately 24 hours, the eyeball was transferred to PBS solution. The retina was then dissected from the choroid and sclera under direct visualization with a Stereomaster Zoom Microscope (Fisher scientific). The retina was equilibrated in 30% sucrose and embedded in optimal cutting temperature compound (Tissue Tek; Sakura Finetek, CA). It was then frozen in liquid nitrogen and cut into 10 μm sections. Three sections were placed on each glass slide and stored at -80°C. At the time of staining, the glass slide was transferred to room temperature and left for 10 minutes to defrost. The slide was washed twice with PBS. The retinal sections were permeabilized with PBS-1% TritonX-100 for 30 minutes at room temperature before blocking with PBS-10% NGS for 1 hour. The sections were incubated overnight at 4°C with Alex 594-labeled isolectin B4 (Griffonia simplicifolia) (1:200, Invitrogen) and primary antibody against Fn14 (1:1000; abcam, MA) or Tweak (1:1000; Novus, CA) in PBS-3% NGS. Next, the sections were washed three times with PBS and were incubated with Alexa Fluor 488-labeled goat anti rabbit secondary Antibody (1:400; Inivitrogen, Carlsbad, CA) and left at room temperature for 1 hour. Then, they were washed three times with PBS and were covered with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA) with an overlying coverslip. Slides were examined by fluorescence microscope (Olympus 1X71)

Isolation of retinal vessels

Retinal vessels were isolated from other components of the retina using a procedure previously described [82, 83]. Briefly, the retina was dissected and incubated in 4°C sterile water for 1 hour. It was then incubated in 4 mL distilled water containing 500 U DNase I (Worthington Biochemical Corp.) in a 60-mm dish for 10 minutes. During the incubation, the solution was gently and repeatedly pipetted on the retinal tissue until the preparation became transparent. The tissue was then transferred back to water and the vessels were separated from debris. The purity of the isolated vessels was assessed by microscopic examination after staining with periodic acid schiff and hematoxylin.

Intravitreal injection

Mice were anesthetized by intraperitoneal injection of a mixture of ketamine hydrochloride (90 mg/kg) and xylzine hydrochloride (7.5-16 mg/kg). Intravitreal injections were performed at P12, using a 36-gauge needle mounted on a 10-µl Hamilton syringe (Hamilton, Reno, NV). The procedures were performed in sterile conditions and under direct visualization using a Leica Wild M650 dissecting microscope (Leica, Bannockburn, IL). A total of 0.5 µl PBS, containing either 2.5 µg soluble Fn14-Fc decoy receptor (R&D systems, Minneapolis, MN) or vehicle (PBS only), was slowly injected into the vitreous cavity through the dorsal limbus.

Immunostaining of whole-mount retina

After fixing the eyeball in 4% paraformaldehyde for approximately 24 hours, it was transferred to a phosphate buffered saline (PBS) solution. The retina was then dissected from the choroid and sclera under direct visualization with a Stereomaster

Zoom Microscope (Fisher scientific). Four radial cuts were made with Vannas micro scissors starting from the periphery and extending posteriorly to halfway between the optic disc and the equator to allow flattening of retina during flat mounting. The retina was then placed in a well of a 96-well plate, washed with PBS and was blocked and permeabilized by incubating in PBS solution containing 10% normal goat serum (NGS) and 1% Triton-X-100 for 30 minutes at room temparature. It was then incubated with Alex 594-labeled isolectin B4 (Griffonia simplicifolia; 1:200, Invitrogen, Carlsbad, CA) in PBS solution containing 3% NGS and 0.3% Triton-X-100 overnight at 4°C. The retina was washed twice with PBS and gentle rocking at room temperature. Next, the retina was flat-mounted on a microscope glass slide, using Vectashield mounting medium (Vector Laboratories, Burlingame, CA) with overlying coverslip, and examined with a fluorescence microscope (Olympus 1X71).

Assessment of retinal vaso-obliteration and neovascularization areas

Digital images of each retina were taken with a fluorescence microscope (Olympus 1X71) using NIS-Elements BR 3.10 software. Magnification of 4X objective was used. Approximately, 9-12 images were required to cover the entire retina. Images of each retina were placed in a separate folder and the folders were renamed by one of my colleagues so that I was completely masked during the entire measurements. The images of each file were stitched together using the photomerge function in Adobe Photoshop Elements 11 to create a single image of the entire retina. In photoshop, the polygonal lasso tool was used to trace the entire retina, and the total area was measured in pixels using the histogram function. Next, the free hand lasso tool was used to trace the avascular (vaso-obliteration) area of the retina and it was measured in pixels using the histogram function. The avascular area was then filled with white color and the image was saved as a separate file[84] (Fig. 12).

Areas of retinal neovascularization were quantified using NIH image J free software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html; 5/16/13) [85] SWIFT_NV macros were downloaded and installed to the Image J. The protocol of the creators of the software was followed [86]. First the image of the entire retina was opened in image J equipped with SWIFT_NV macros. The software automatically divides the retina in 4 quadrants. The threshold was adjusted for each quadrant using 'Adjust Threshold' function to highlight NV (Fig. 12). The measurements, recorded in pixels, were extracted from the 'result" window.

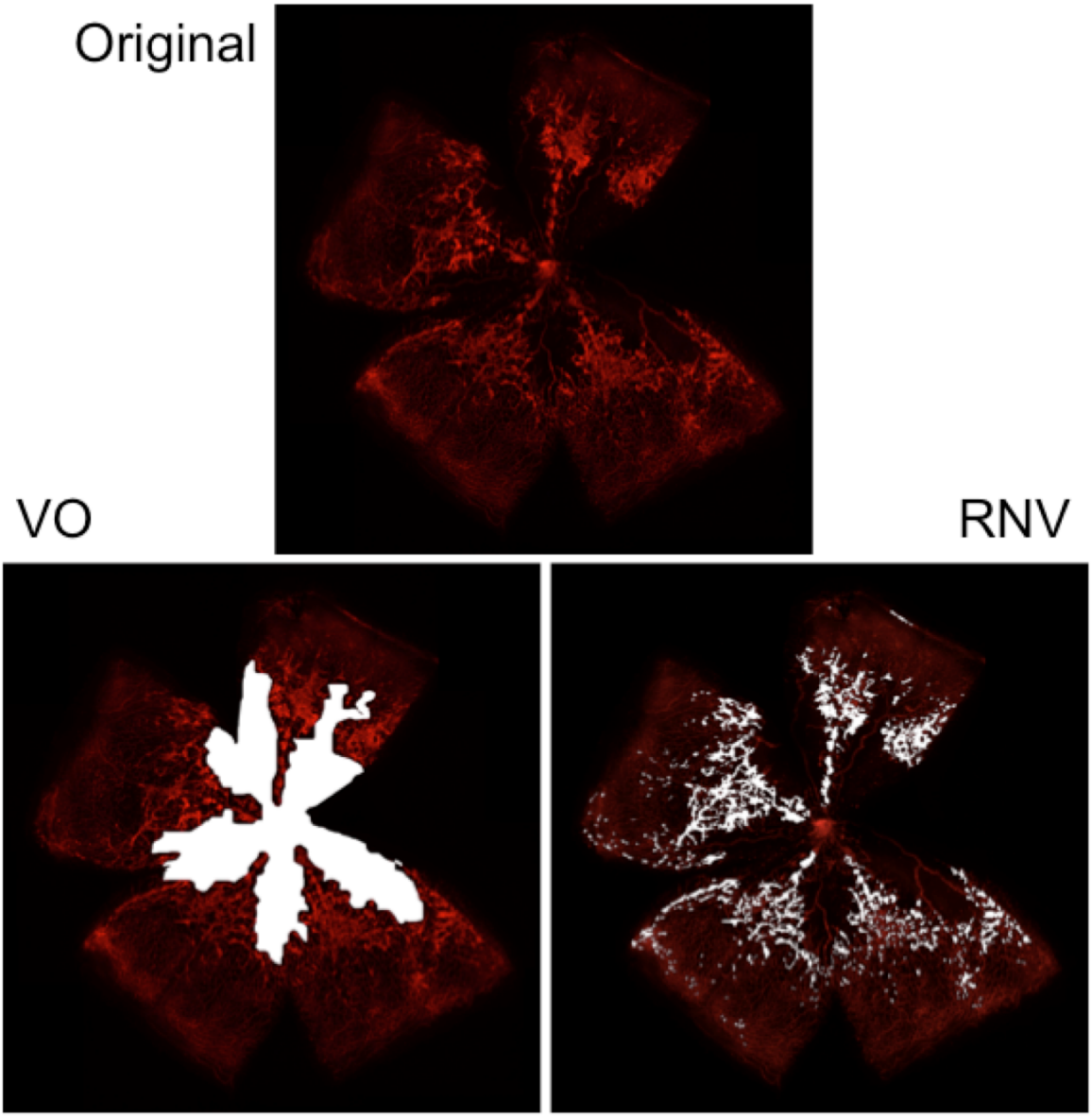

Figure 12. Measuring vaso-obliteration and retinal neovascularization areas

Top: original image of retinal vasculature taken by fluorescence microscopoe after staining retinal flat mount with isolectin B4. Bottom Left: area of vaso-obliteration (VO) selected and filled with white color using Photoshop Elements 11; this area is measured in pixels by photoshop. Bottom Right: areas of retinal neovascularization (NV) selected and filled with white color using NIH Image J loaded with SWIFT_NV macros; these areas are measured in pixels by Image J.

Statistical Analysis

Data are presented as mean + standard error of mean. Two-tailed Student's t-test and one-way ANOVA followed by post hoc Student's t-test were used for comparison between data sets. $P<0.05$ was considered statistically significant.

RESULTS

Localization of TWEAK and Fn14 in the normal mouse retina

For a ligand/receptor to be involved in any process in a given tissue, at least the receptor is expected to be present in that tissue. Localization of TWEAK and Fn14 in the retina could help to identify potential cells involved in TWEAK/Fn14 mediated processes. To address this, immunohistochemistry was performed on the retina of wild type mice, kept in room air at all times, at P17. In addition to TWEAK or Fn14, the triple staining of retinal sections included isolectin B4 and DAPI to facilitate localization by identifing vessels and nuclear layers. As figure 13 displays, TWEAK is diffusely distributed throughout the retina; however, the ganglion cell layer shows the highest expression. There is almost lack of expression of TWEAK in the retinal vessels.

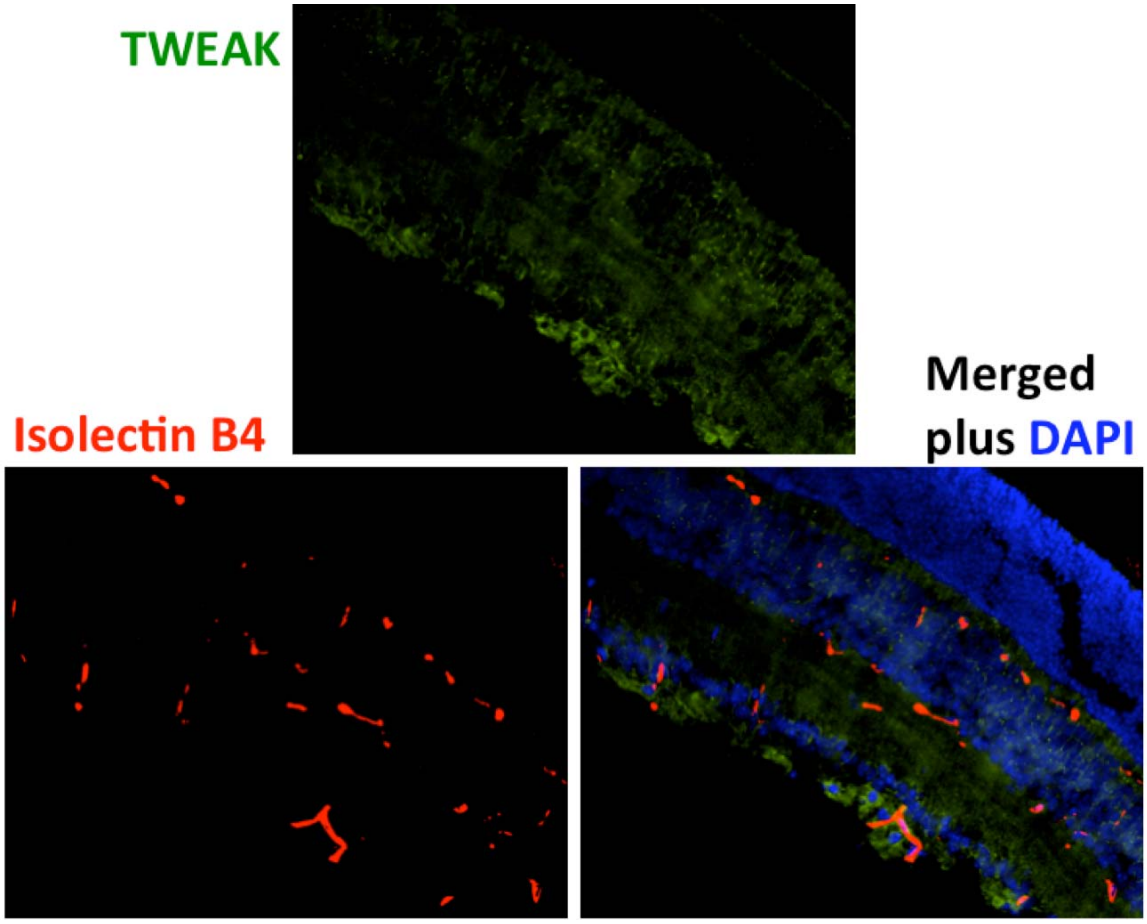

Figure 13. TWEAK immunoreactivity in the normal retina

Retinal sections were triple stained with TWEAK antibody, isolectin B4 and DAPI. Top: TWEAK staining shows diffuse expression throughout the retina, maximum in the ganglion cell layer. Bottom left: isolectin B4 staining shows normal retinal vasculature in three layers. Bottom right: Merged image shows lack of co-localization of TWEAK and isolectin B4 staining in retinal vessels.

In order to rule out non-specific staining of the retina with the secondary antibody, immunostaining was performed on the retinal sections with secondary antibody without adding primary antibody against TWEAK and identical image setting was used for taking pictures. Figure 14 shows lack of retinal staining, confirming the specificity of the TWEAK staining.

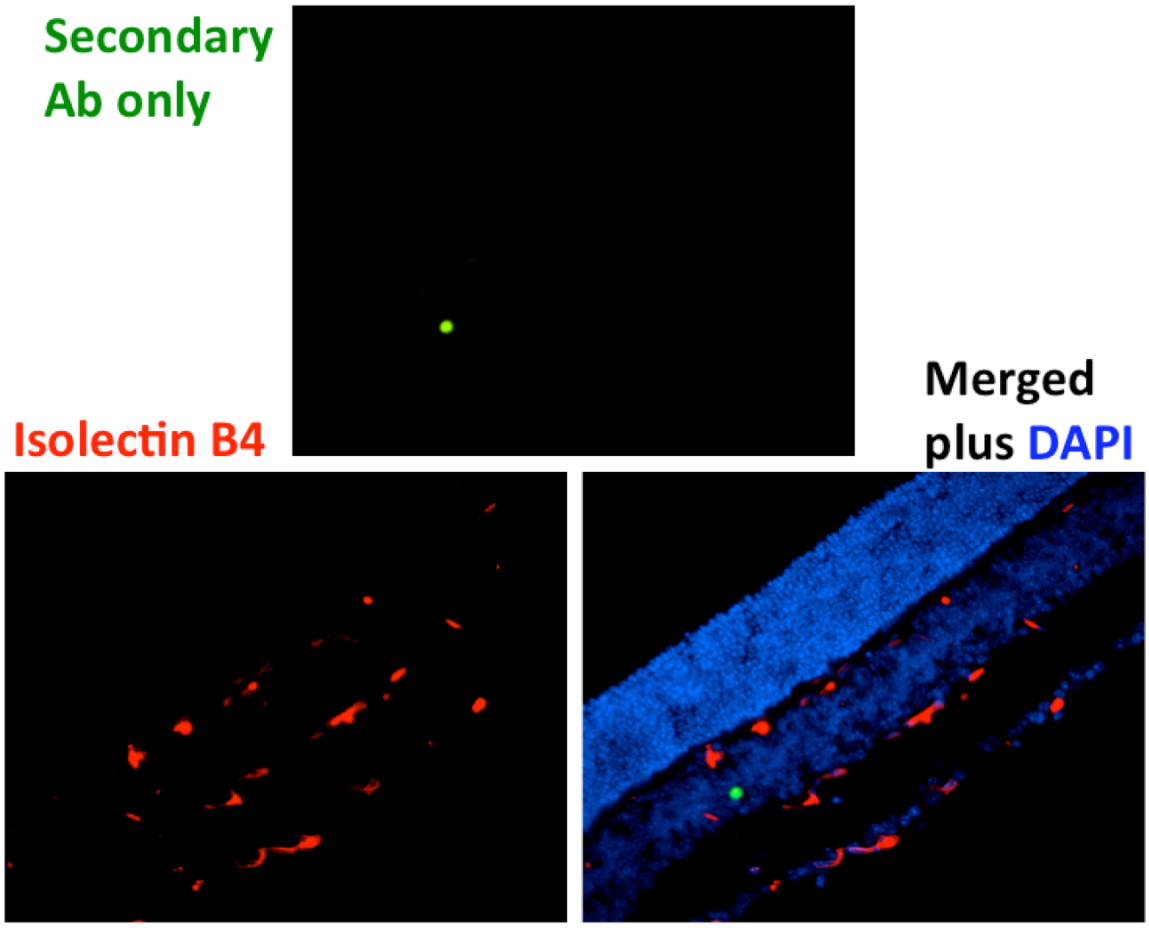

Figure 14. Secondary antibody staining of the normal retina without TWEAK antibody

Retinal sections were double stained with isolectin B4 and DAPI. In addition, secondary antibody for TWEAK staining was used without primary antibody. Top: Staining with secondary antibody alone shows lack of retinal staining. Bottom left: isolectin B4 staining shows normal retinal vasculature in three layers. Bottom right: Merged image shows lack of co-localization of secondary antibody staining with isolectin B4 or DAPI.

Similar to TWEAK, Fn14 showed predominant expression in the neuronal layers. As is seen in figure 15, the highest expression of Fn14 is in the ganglion cell layer and inner nuclear layer. Vessels show minimal expression of Fn14. Fn14 expression appears as intracellular clumps overlapping with the nucleus. Expression of Fn14, a receptor, in the neuronal layers implies that TWEAK/Fn14 may be involved in other processes such as neuronal growth and development.

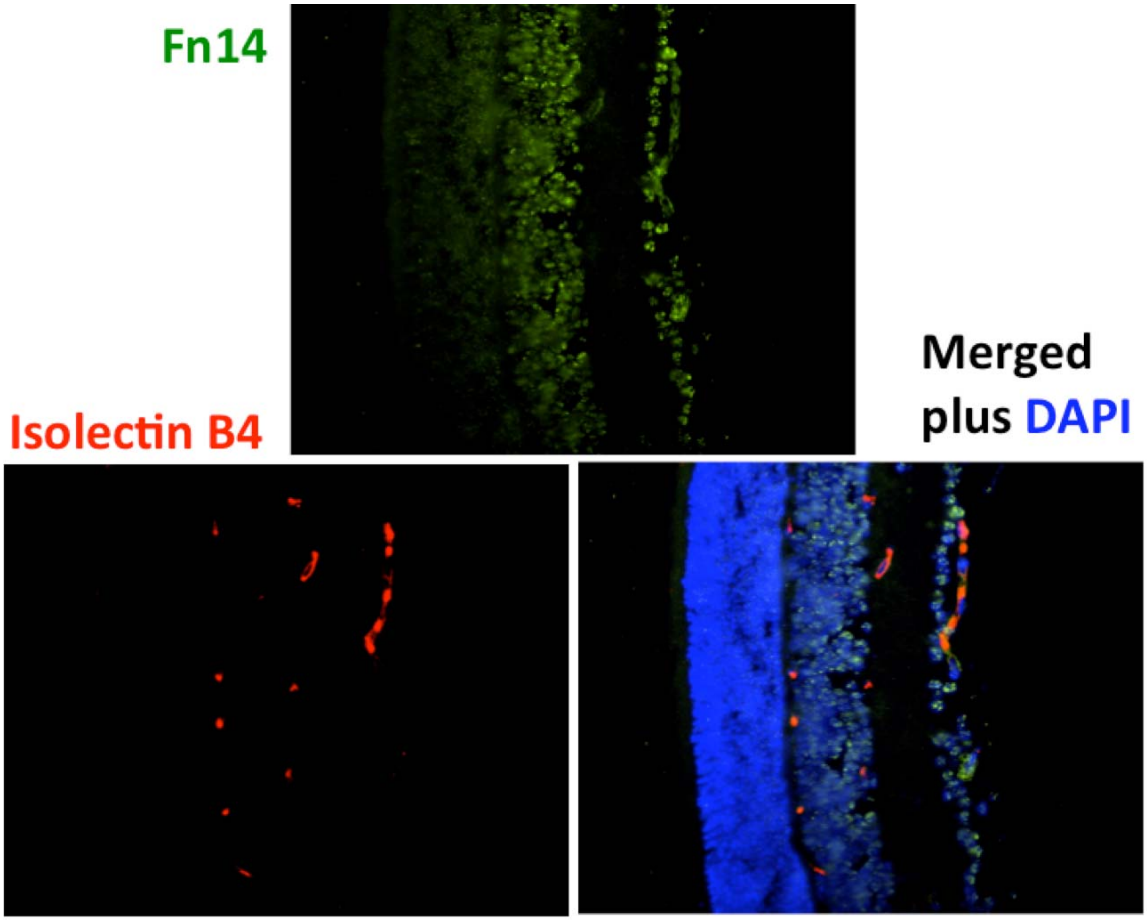

Figure 15. Fn14 immunoreactivity in the normal retina

Retinal sections were triple stained with Fn14 antibody, isolectin B4 and DAPI. Top: Fn14 staining showing predominant expression in the inner nuclear layer and the ganglion cell layer. Bottom left: isolectin B4 staining shows normal retinal vasculature in three layers. Bottom right: Merged image shows lack co-localization of Fn14 and DAPI in the inner nuclear layer and the ganglion cell layer; also note co-localization of Fn14 and isolectin B4 staining in the retinal vessels, although Fn14 staining of vessels is considerably less than that of neuronal layers.

In order to rule out non-specific staining of the retina with the secondary antibody, immunostaining was performed on the retinal sections with secondary antibody without adding primary antibody against Fn14 and identical image setting was used for taking pictures. Figure 16 shows lack of retinal staining, confirming the specificity of the Fn14 staining.

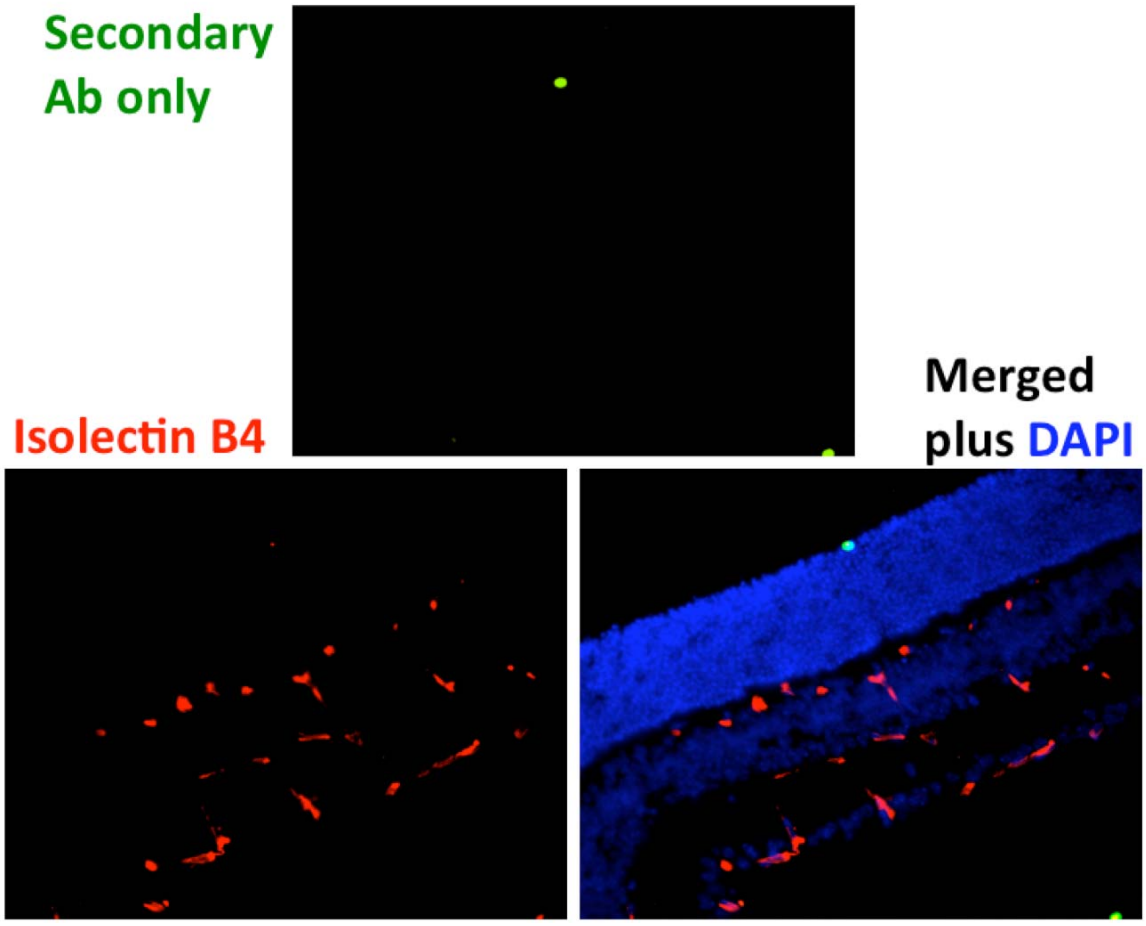

Figure 16. Secondary antibody staining of the normal retina without Fn14 antibody

Retinal sections were double stained with isolectin B4 and DAPI. In addition, secondary antibody for Fn14 staining was used without primary antibody. Top: Staining with secondary antibody alone shows lack of retinal staining. Bottom left: isolectin B4 staining shows normal retinal vasculature in three layers. Bottom right: Merged image shows lack of co-localization of secondary antibody staining with isolectin B4 or DAPI.

Upregulation of Fn14 in OIR in the absence of significant change in TWEAK

In OIR, exposure to 75% oxygen from P7 to P12 causes obliteration of immature retinal vessels in the central retinal area. Upon return to room air at P12, relative hypoxia results in upregulation of many angiogenic and inflammatory genes, which leads to pathological retinal neovascularization in the mid-periphery, best evident at P17 [87, 88]. To investigate the potential role of TWEAK/Fn14 in NV, retinal mRNA levels were measured at P17. Figure 17 shows approximately five fold upregulation of Fn14 mRNA in OIR compared to RA, the age-matched control mice which were kept at room air at all times.

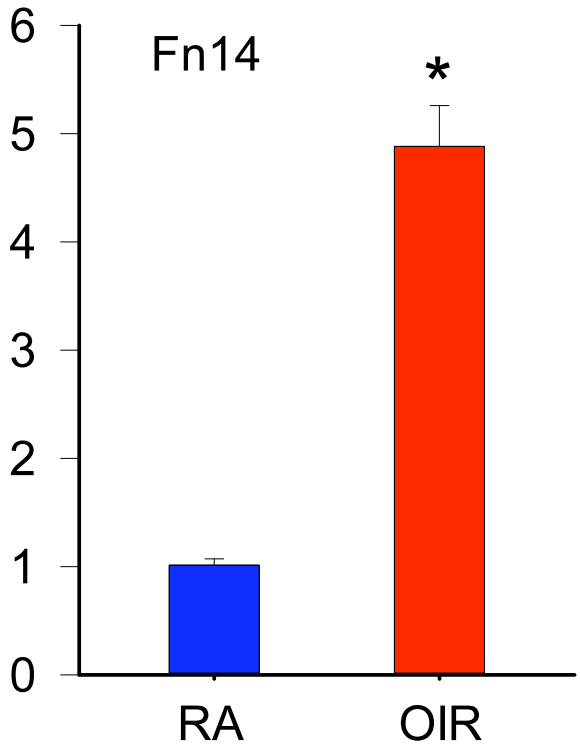

Figure 17. Fn14 mRNA is increased in OIR.

Retinas were collected from OIR and RA mice at P17. Fn14 mRNA levels were quantified by qPCR and normalized to RA. n=6, *P<0.05.

Unlike Fn14 mRNA, which is significantly upregulated in OIR at P17, TWEAK mRNA remains relatively unchanged (Fig. 18).

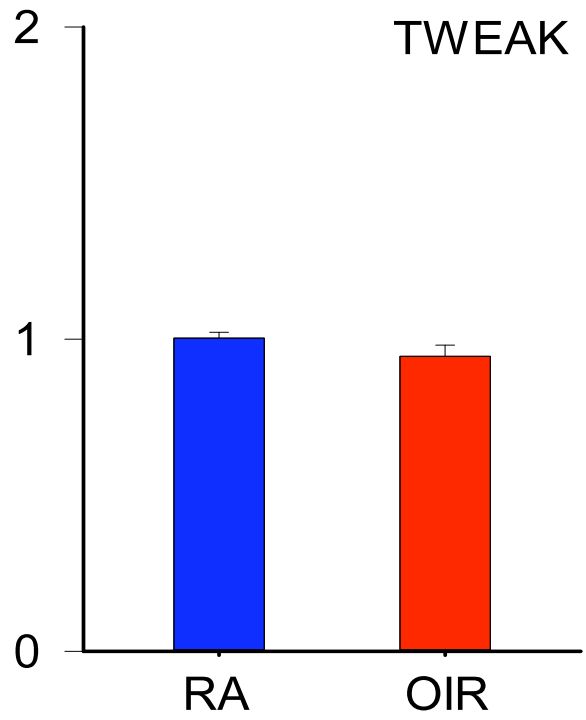

Figure 18. TWEAK mRNA remains unchanged in OIR.

Retinas were collected from OIR and RA mice at P17. TWEAK mRNA levels were quantified by qPCR and normalized to RA. There is no statistically significant difference between the two groups. n=6.

Localization of Fn14 and TWEAK in neovascular tufts in OIR

As I showed earlier, both TWEAK and Fn14 are constitutively present in the normal retina. The next question was that, given the changes in Fn14 mRNA expression in OIR, would there be any changes in retinal expression of TWEAK and Fn14 in OIR? To answer this question, retinal sections of OIR mice were immunostained for TWEAK or Fn14 in addition to isolectin B4 and DAPI. As seen in figure 19, the OIR retina shows

predominant expression of Fn14 in neovascular tufts and less obvious expression in neuronal layers. This is in contrast to the normal retina, obtained from RA mice (Fig. 15), which shows expression of Fn14 in neuronal layers with minimal expression in normal vessels

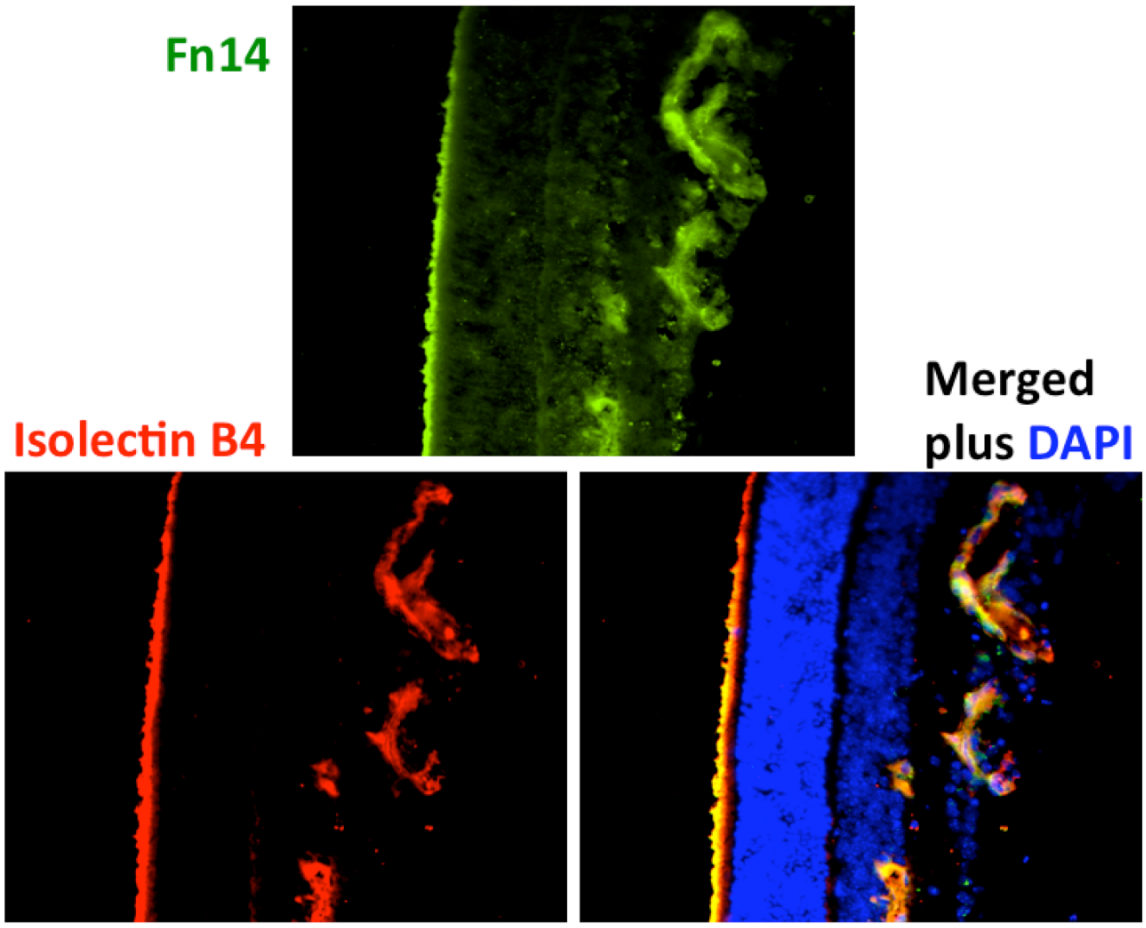

Figure 19. Fn14 localization in the OIR retina

Retinal sections were triple stained with Fn14 antibody, isolectin B4 and DAPI. Top: Fn14 staining shows predominant expression in neovascular tufts. Bottom left: isolectin B4 staining shows pathological neovascularization. Bottom right: Merged image shows co-localization of Fn14 and isolectin B4 staining in neovascular tufts.

In order to rule out non-specific staining of the OIR retina with the secondary antibody, immunostaining was performed on the retinal sections with secondary antibody without adding primary antibody against Fn14 and identical image setting was used for taking pictures. Figure 20 shows lack of retinal staining, confirming the specificity of the Fn14 staining in OIR.

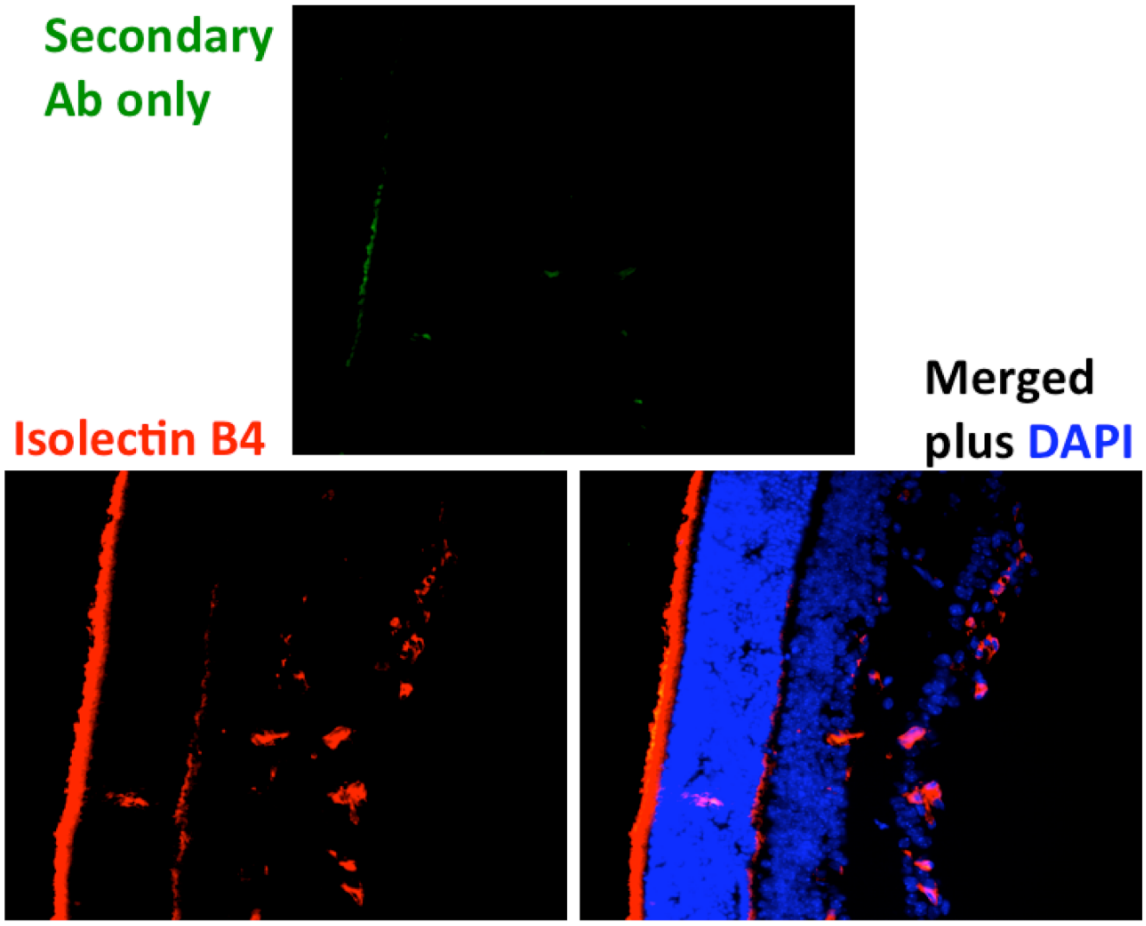

Figure 20. Secondary antibody staining of the OIR retina without Fn14 antibody

Retinal sections were double stained with isolectin B4 and DAPI. In addition, secondary antibody for Fn14 staining was used without primary antibody. Top: Staining with secondary antibody alone shows lack of retinal staining. Bottom left: isolectin B4 staining shows pathological neovascularization. Bottom right: Merged image shows lack of co-localization of secondary antibody staining with isolectin B4 or DAPI.

Interestingly, similar to Fn14, TWEAK also shows predominant expression in neovascular tufts in OIR (Fig. 21). This is despite lack of change in TWEAK mRNA in OIR compared to RA (Fig. 18). Compared to RA control (Fig. 13) there is less expression of TWEAK in other retinal layers in OIR.

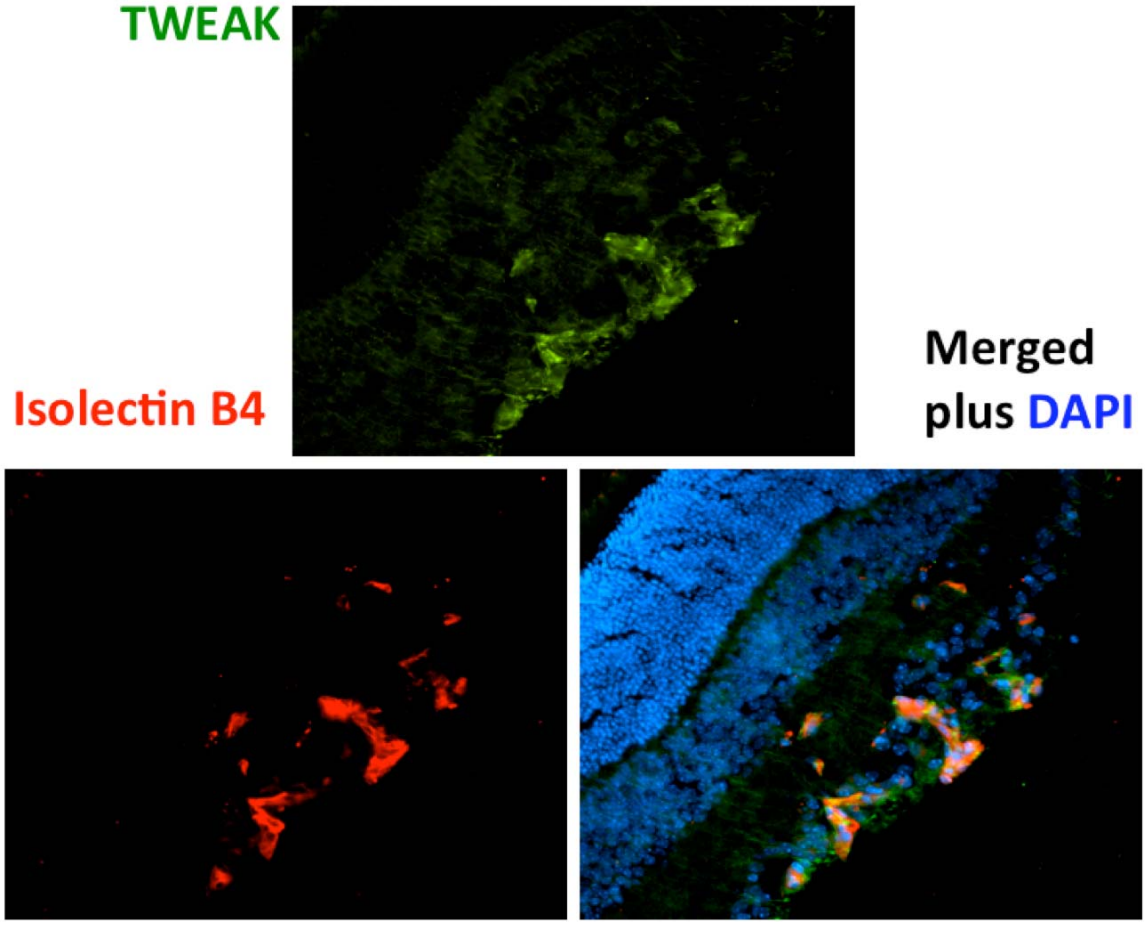

Figure 21. TWEAK localization in the OIR retina

Retinal sections were triple stained with TWEAK antibody, isolectin B4 and DAPI. Top: TWEAK staining shows predominant expression in neovascular tufts. Bottom left: isolectin B4 staining shows pathological neovascularization. Bottom right: Merged image shows co-localization of TWEAK and isolectin B4 staining in neovascular tufts.

In order to rule out non-specific staining of the OIR retina with the secondary antibody, immunostaining was performed on the retinal sections with secondary antibody without adding primary antibody against TWEAK and identical image setting was used for taking pictures. Figure 22 shows lack of retinal staining, confirming the specificity of TWEAK staining in OIR.

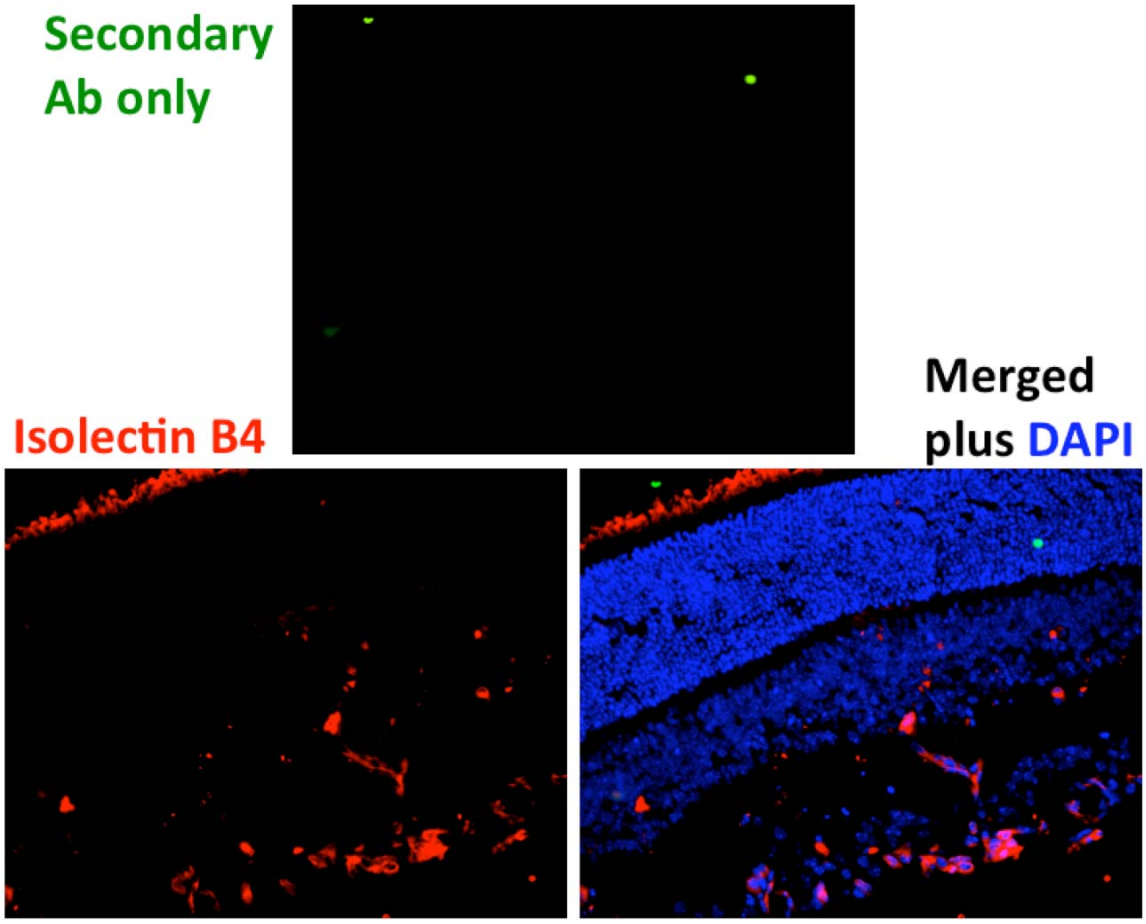

Figure 22. Secondary antibody staining of the OIR retina without TWEAK antibody

Retinal sections were double stained with isolectin B4 and DAPI. In addition, secondary antibody for TWEAK staining was used without primary antibody. Top: Staining with secondary antibody alone shows lack of retinal staining. Bottom left: isolectin B4 staining shows pathological neovascularization. Bottom right: Merged image shows lack of co-localization of secondary antibody staining with isolectin B4 or DAPI.

Dramatic upregulation of Fn14 and significant downregulation of TWEAK in the retinal vasculature in OIR

Given that the endothelial cells making up the neovascular tufts in OIR are newly proliferated cells, it was hypothesized that florid expression of Fn14 in neovascular tufts is probably due to increased transcription of Fn14. To test this hypothesis, retinal vasculature were isolated and mRNA levels were measured by qPCR. Figure 23 shows approximately 6.5-fold upregulation of Fn14 mRNA in OIR compared RA. Higher rate of upregulation of Fn14 mRNA in isolated retinal vasculature compared to the whole retina suggests that perhaps most of the changes seen in the whole retina mRNA may emanate from the changes in the vasculature mRNA.

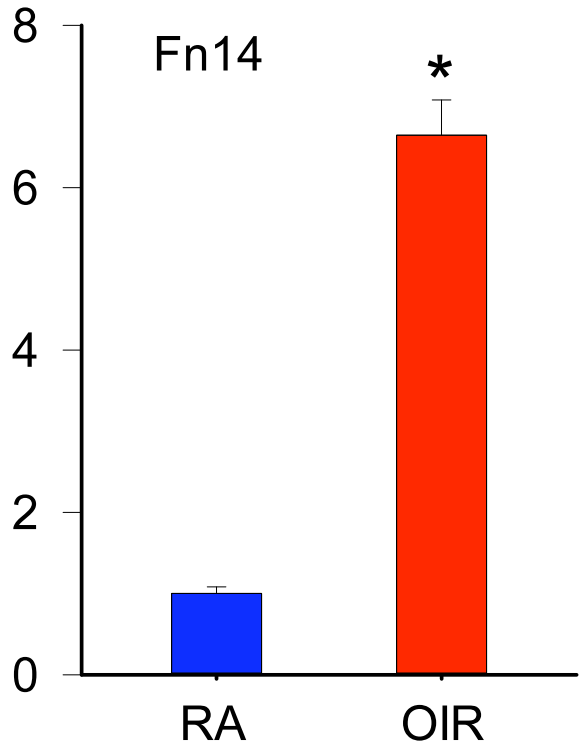

Figure 23. Fn14 mRNA is dramatically increased in the retinal vasculature in OIR

Retinal vessels were isolated and Fn14 and TWEAK mRNA were determined by qPCR. n=3 (each n represents a pool of 6 retinas), *P<0.05.

Because TWEAK is a ligand, its increased expression in neovascular tufts in OIR could be either due to increased transcription or secondary to displacement from other cells to its receptor, Fn14, highly expressed on newly formed endothelial cells. As shown in figure 24, TWEAK mRNA not only does not increase in the retinal vasculature in OIR, it even shows an approximately 50% reduction. This is suggestive that increased immunoexpression of TWEAK in neovascular tufts is secondary to its displacement from other retinal layers to the endothelial cells.

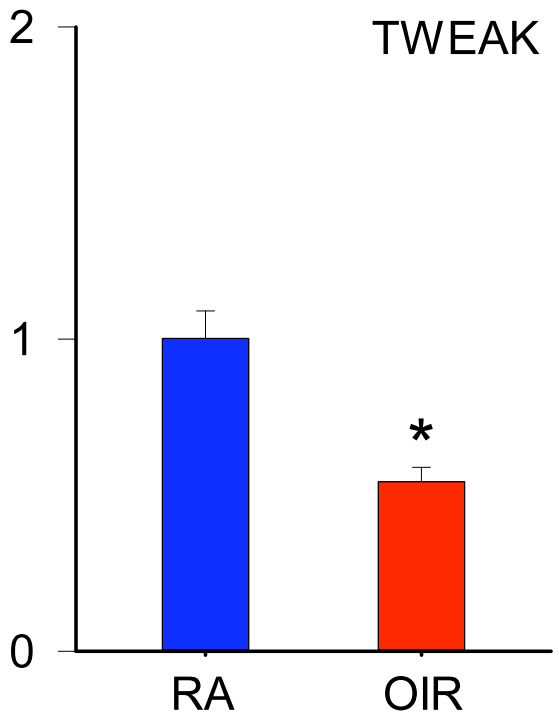

Figure 24. TWEAK mRNA is significantly decreased in the retinal vasculature in OIR

Retinal vessels were isolated and Fn14 and TWEAK mRNA were determined by qPCR. n=3 (each n represents a pool of 6 retinas),

The TWEAK/Fn14 pathway contributes to retinal neovascularization in OIR

The above-mentioned experiments showed that TWEAK and Fn14 are dysregulated in OIR, but further experiments were required to show involvement of the TWEAK/Fn14 pathway in creation of NV. To evaluate whether the TWEAK/Fn14 pathway is required for ischemia-induced pathological retinal neovascularization, the interaction between TWEAK and Fn14 was blocked by Fn14-Fc decoy receptor at P12, the beginning of the relative hypoxia period. Fn14-Fc decoy receptor was injected into the vitreous cavity of the OIR mice at P12 and the extent of NV was compared to that of the OIR control group that received intravitreal injection of vehicle. As displayed in figure 25, blockade of the TWEAK/Fn14 pathway is associated with a significant reduction in the extent of NV. Also, the areas of retinal vaso-obliteration (VO) was measured and compared between the two groups. As the figure shows, there is a slight, but not statistically significant, reduction in the extent of retinal VO in the group that received intravitreal Fn14-Fc decoy.

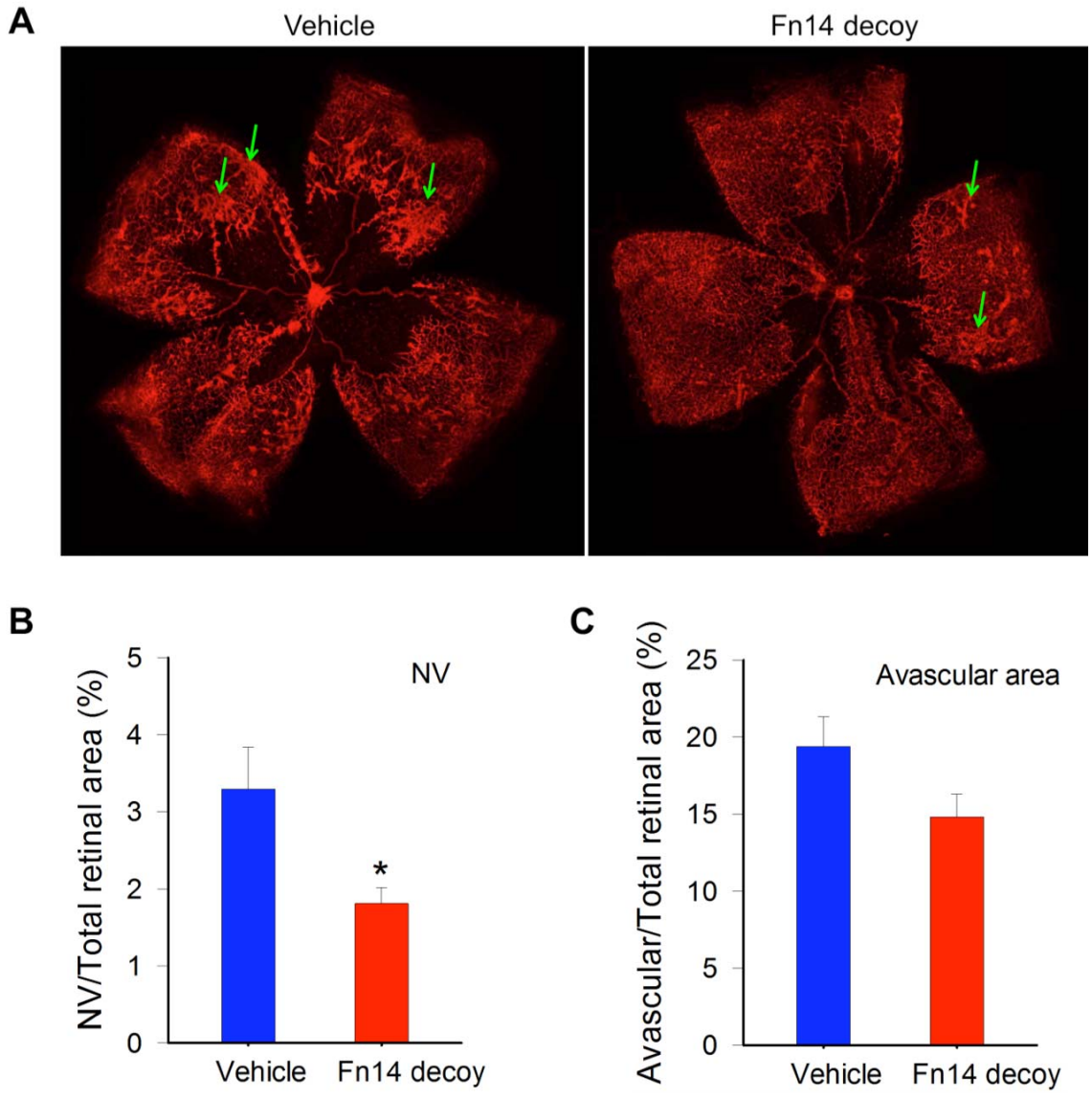

Figure 25. Blockade of the TWEAK/Fn14 pathway reduces NV in OIR

OIR mice received intravitreal injection of a soluble Fn14-Fc decoy receptor or vehicle (PBS) at P12. Representative images of the retinal flat mounts stained with isolection B4 demonstrate reduced retinal neovascularization (green arrows) in the Fn14-Fc group at P17 (A). Quantification of the extent of neovascularization using NIH Image J loaded with SWIFT_NV macros shows a significant reduction in the treatment group (B). Quantification of the extent of vaso-obliteration (VO) using Photoshop shows a slight reduction in the treatment group (C). n=6, *P<0.05.

DISCUSSION

The underlying mechanisms of vision loss in retinal vascular disease are vasopermeability and vasoproliferation. While vasopermeability could result it macular edema and mild to moderate, often reversible, vision loss, vasoproliferation may lead to neovascularization of the retina or iris and subsequently profound irreversible vision loss. Laser photocoagulation is the standard of care for both vasopermeability and vasoproliferative conditions, although the paradigm for the treatment of macular edema is gradually shifting more toward pharmacological treatment. Intraocular injection of steroids has been effective in reducing macular edema but has been associated with high rate of glaucoma and catarat formation [33, 89, 90]. In recent years, intravitreal anti-VEGF medications, originally developed for the treatment of neovascular age related macular degeneration, have been frequently used to treat macular edema of various causes such as diabetic retinopathy and retinal vein occlusion with relative success [14-16, 29, 91]. However, in vasoproliferative conditions, anti-VEGF drugs have been mainly used preoperatively to reduce the risk of intraocular hemorrhage, or as an adjunct to retinal laser photocoagulation [92-97]. In either case, the effects of anti-VEGF drugs are short-lived and they require repeated intraocular injections, which could increase the risk of complications. Moreover, we have previously demonstrated that in a rabbit model of retinal neovascularization, intravitreal injection of bevacizumab, an anti-VEGF drug, may result in closure of retinal capillaries and exacerbate retinal ischemia [68].

Here, I provide the first evidence that TWEAK/Fn14 pathway could be a novel target for the prevention and treatment of vasoproliferative retinal vascular disease. I used OIR mouse model, which has been widely used for studying mechanisms of pathological NV and for evaluating efficacy of various therapeutic approaches [64, 98-104]. I found that both TWEAK and Fn14 are constitutively present in the retina and are predominantly

expressed in neuronal layers. Although both TWEAK and Fn14 are immunoexpressed in multiple layers of the retina, their pattern of expression is different.

Fn14 expression is predominantly in inner nuclear layer and ganglion cell layer; there is also minimal expression of Fn14 in retinal vasculature. I have chosen P17 for my studies because of my goal of studying NV. Although physiological retinal vascularization is already complete, retinal neuronal maturation is not complete at this point. After opening of the eyes in mice at P12-13, neuronal maturation continues beyond P17 and reaches a peak level at P21 [105]. Why Fn14 is predominantly expressed in ganglion cell layer and neuronal layer at P17 is not clear but it may be related to neuronal maturation and potential role of TWEAK/Fn14 in this process. At cellular level, in neuronal layers, Fn14 displays intracellular speckled staining, which appears to be both intracytoplasmic and intranuclear. Given that Fn14 is believed to be a transmembrane receptor, this pattern of staining is rather unexpected and further studies are required to explore its significance. Receptors could be transferred to other cells, for example, platelet-derived microparticles harbor functional glycoprotein IIb/IIIa receptors that could be transferred to neutrophils and participate in pro-inflammatory signaling [106]. Whether intracellular Fn14 has intracytoplasmic or intranuclear functions, or acts as a reservoir for neuronal cells or even other cells remains to be known.

TWEAK expression is predominantly in the ganglion cell layer and overall is more diffuse than Fn14. Unlike Fn14 which is minimally expressed in normal vasculature, TWEAK is not expressed in normal vessels. At cellular level, TWEAK immunoreactivity appears to be intracytoplasmic and maybe even extracellular but not intranuclear. Since TWEAK is a ligand and becomes soluble when cleaved, its diffuse expression throughout retina may indicate its abundant presence in the extracellular space. Its higher expression in the ganglion cell layer may point to ganglion cells as a potential source of TWEAK production. Its expression in neurons has been reported before [58]. Lack of expression of TWEAK in retinal vessels is not unexpected; it is

plausible that TWEAK is released from other cells such as ganglion cells and acts on target tissue via binding to Fn14.

I have demonstrated that Fn14 mRNA is upregulated in the OIR retina. In the retinal vasculature, Fn14 upregulation is even more dramatic. In OIR, higher rate of upregulation of Fn14 mRNA in isolated retinal vasculature compared to the whole retina suggests that perhaps most of the global changes may stem from the changes in the vasculature. Worth mentioning that the isolated retinal vasculature used in this study includes all the vasculature and neovasular tufts constitute only a portion of the whole vasculature. One can postulate that Fn14 mRNA upregulation in neovascular tufts may be even higher than what measured in the retinal vasculature.

In contrast to Fn14, TWEAK mRNA is downregulated in the retinal vasculature in OIR, and when measured globally in the whole retina, it remains relatively unchanged. This is a rather unexpected result. One would expect to see upregulation of TWEAK in OIR, at least in non-vascular components of the retina. In he case of retinal vasculature, perhaps there are regulatory mechanisms that culminate in downregulation of TWEAK in retinal vasculature. When the scale of dysregulation of FN14 and TWEAK are compared, the magnitude of Fn14 upregulation is higher than the magnitude of TWEAK downregulation; in other words, the ratio of Fn14 to TWEAK increases in OIR. This phenomenon of high receptor/ligand ratio as an activation mechanism has been previously described; for example, it has been shown that high ratio of Eph receptor to its ligand, ephrin, facilitates tumorogenesis [107, 108]. Since the TWEAK/Fn14 pathway is involved in different processes in various tissues, it is possible that activation of TWEAK/Fn14 in each group of cells in the retina results in activation of a process specific to that group of cells. Given constitutive expression of Fn14 in neuronal layers, activation of the TWEAK/Fn14 pathway in these cells will likely have an effect, which is different from angiogenesis. One can postulate that a higher Fn14/TWEAK ratio in the

endothelial cells may offer selective activation of these cells resulting in NV, without activating other processes in neuronal layers.

When compared to normal retina collected from RA control, immunoreactivity of both TWEAK and Fn14 changes in the OIR retina. In OIR, expression of both TWEAK and Fn14 is predominantly seen in neovascular tufts; and compared to RA control their expression in neuronal layers decreases. An interesting phenomenon is increased immunoreactivity of TWEAK in neovascular tufts despite downregulation of the TWEAK mRNA. Since Fn14 is highly expressed in neovascular tufts, increased TWEAK expression is likely due to its displacement from other cells to its receptor, Fn14, on newly formed endothelial cells.

In the mouse OIR model, when the animal is maintained in a high oxygen condition, capillaries become obliterated in the central retina, and at P12 a large area of the central retina becomes avascular. At P15, three days after returning to room air, pathological retinal neovascularization starts and becomes fully evident at P17. I showed that blockade of the TWEAK/Fn14 pathway by intravitreal injection of Fn14-Fc decoy receptor at P12 results in a significant reduction in the extent of NV. The extent of vaso-obliteration is also less when the TWEAK/Fn14 pathway is blocked but it is not statistically significant. This indicates that the TWEAK/Fn14 pathway contributes to pathological neovascularization. The finding that one intravitreal injection of Fn14-Fc decoy does not fully prevent NV, may be due to one or more of the following possibilities: 1) Given that retinal neovascularization generally starts three days after the time of injection and continues to grow for several days afterwards, one intravitreal injection at P12 may not be enough to fully block the pathway. Future studies with repeated intravitreal injections at short intervals or continuous slow release of Fn14-Fc decoy may help to constantly block the pathway. However, these invasive experiments may be associated with other problems that may affect the final outcome. 2) There are many angiogenic factors and the role of VEGF in pathological angiogenesis is well

known [20, 21]. If we assume that one intravitreal injection of Fn14-Fc decoy is enough to fully block the TWEAK/Fn14 pathway, presence of some neovascularization despite intravitreal injection means that either the TWEAK/Fn14 pathway is independent of VEGF signaling or it only partially contributes to VEGF-mediated angiogenesis. 3) Another possibility is TWEAK-independent activation of the Fn14 signaling. There are in vitro studies that have reported activation of NF-kB signaling pathway, neuronal growth, and cell migration by ectopic expression of Fn14 [109-111]. In most reported in vivo studies, TWEAK is upregulated along with Fn14; e.g. in ischemic brain tissue, kidney injury, and skeletal muscle [58, 112-114]. However, in brain tumors, Fn14 upregulation has been observed in the absence of change in TWEAK level [80, 115]. Although TWEAK independent activation of Fn14 signaling is a possibility, the fact that there is a reduction in NV after blockade of the TWEAK/Fn14 pathway by intravitreal injection of Fn14-Fc decoy indicates that at least to some extent Fn14 signaling in retinal endothelial cells is TWEAK dependent. This is further supported by the finding that in the OIR retina, immunoreactivity of TWEAK in neovascular tufts parallels that of Fn14, while Fn14 mRNA is reduced in the retinal vasculature. Displacement of TWEAK to endothelial cells is likely due to its binding to Fn14, suggesting that it may be required for Fn14 signaling activation.

In summary, upregulation of Fn14 in the retinal vasculature and a shift in the inmmunoexpression of TWEAK and Fn14 in the OIR retina to newly formed endothelial cells is suggestive of the involvement of the TWEAK/Fn14 pathway in the formation and maintenance of ischemia-induced pathological retinal angiogenesis. The finding that blockade of the TWEAK/Fn14 pathway significantly reduces the extent of retinal NV supports this possibility. At present, it is unknown how TWEAK/Fn14 is involved in NV. The development of retinal NV is a process that involves multiple factors including upregulation of angiogenic factors, inflammation, and breakdown of internal limiting membrane. TWEAK/Fn14 has been shown to induce expression of many angiogenic

factors such as VEGF, IL-6 and IL-8 [60, 61]. TWEAK/Fn14 pathway also promotes production of matrix metalloproteinase proteins, which may facilitate the breakdown of internal limiting membrane [62, 63]. Further studies are required to explore the mechanism of TWEAK/Fn14 induced NV. The TWEAK/Fn14 pathway is involved in various processes, and has been reported to be a key mediator in kidney injury, neuronal damage, and muscle atrophy [57-59]. Although the angiogenic function of this pathway was recognized soon after TWEAK was identified, its role in physiological or pathological angiogenesis has not been explored [52]. Our novel finding that TWEAK/Fn14 plays an essential role in pathological retinal angiogenesis may encourage further exploration of the role of this pathway in angiogenesis in other diseases such as tumors, limb ischemia, and neovascular age-related macular degeneration.

Chapter 2

TWEAK/Fn14 Pathway is Induced by Hypoxia

INTRODUCTIOIN

In chapter 1 I briefly reviewed common causes of vision loss in retinal vascular disease and described the standard of care for DR, RVO and ROP. As described, NV is a major complication of these conditions and despite advances in ophthalmology, many patients continue to loose vision and there is an urgent need to develop new treatment modalities. TWEAK and its receptor, Fn14 have been implicated in angiogensis but their role in retinal vascular disease has never been explored. In chapter 1 I presented convincing data, from the mouse OIR model, implicating the TWEAK/Fn14 pathway in NV. As discussed, Fn14 is significantly upregulated in the retina of OIR compared to normal retina and blockade of TWEAK/Fn14 decreases the extent of NV. This chapter will explore the mechanism of activation of TWEAK/Fn14 in OIR.

Hypoxia and NV

The mouse OIR model was developed when there was a full understanding of the role of hypoxia in iris or retinal neovascularization. Indeed, the association between oxygen therapy and NV in ROP was clinically discovered more than half a century ago when it was noted that the rate of severe ROP was higher in premature newborns treated with oxygen [116, 117]. Observations that NV and retinal detachment occurred weeks after discontinuation of oxygen led to the hypothesis that hyperoxia causes vaso-obliteration and brings normal retinal angiogenesis to a halt, making a large area of the retina ischemic. Following discontinuation of oxygen therapy, a relative hypoxia state is

created, which triggers NV through release of angiogenic factors. The mouse OIR model was developed based on this understanding and supported the hypothesis. In diabetes mellitus, the association between vaso-obliteration and NV and iris neovascularization has been described for many decades [18, 118]. The same is true about RVO, which has been classified into ischemic and non-ischemic types based on the extent of vaso-obliteration [119, 120]. It is widely known that patients with ischemic RVO are at tremendously higher risk of developing neovascularization of the iris and retina and profound irreversible vision loss.

HIF-1α and NV

Hypoxia inducible factor (HIF)-1 is a transcription factor composed of two subunits: HIF-1α and HIF-1β. While HIF-1β is constitutively expressed and is stable, HIF-1α, made of 826 amino acids, is labile and its expression increases in hypoxia [121]. HIF-1α is constitutively expressed but has a half-life of less than ten minutes and is rapidly degraded under normoxia conditions [122]. Oxygen sensing occurs through various mechanisms detecting hypoxia condition [123, 124]. Hypoxia stabilizes HIF-1α by suppressing its degradation and results in its accumulation in the nucleus [125]. Once in the nucleus, HIF-1α binds to the specific sequences of many target genes and increases their transcription. VEGF and erythropoietin are the most important genes regulated by HIF-1α [126, 127].

HIF-1α has been implicated in both physiological and pathological angiogenesis. In adult rats, after three hours of hypoxia exposure, both the HIF-1α mRNA and the VEGF mRNA increase and reach a maximum at 24 hours [128]. Hypoxia injury in adult rat, produced by laser induced retinal vascular occlusion, is associated with increased expression of HIF-1α in the ganglion cell layer and to less extent in the inner nuclear layer 2-4 days after the hypoxia insult [121]. In mice, HIF-1α protein level increases at the

time of retinal vascular development, reaching a maximum at P4 and decreasing to a baseline as vascularization in complete [129]. At P4, the ganglion cell layer is the main layer of the retina expressing HIF-1α followed by some expression in the inner nuclear layer. In the mouse model of OIR, HIF-1α shows maximum upregulation two hours after the start of the relative hypoxia and decreases afterwards. At this point, HIF-1α expression is predomenently seen in the inner nuclear layer and the ganglion cell layer [129]. VEGF upregulation follows that of HIF-1α by a few hours delay and remains high for several days.

MATERIALS AND METHODS

For creating OIR, performing RT-PCR on retina samples, and statistical analysis refer to Materials and Methods in Chapter 1.

Hyperoxia treatment (HT)

As explained in detail in chapter 1, OIR was produced by maintaining C57BL/6 wild type neonatal mice in 75% oxygen from P7 to P12 and room air from P12 to P17. For hyperoxia treatment (HT), the OIR mice were returned to 75% hyperoxia chamber from P14 to P17 (Fig. 26).

Cell culture

Primary HRE cells, purchased from Cell Systems (Kirkland, WA), were cultured in CSC attachment factor (Cell System Corporation, Wilmington, DE) coated cell culture dish using complete medium made of 50% CSC complete medium (CSC, Wilmington, DE) and 50% EGM (Lonza, Switzerland). When confluent, cells were transferred to collagen coated 12-well plates.

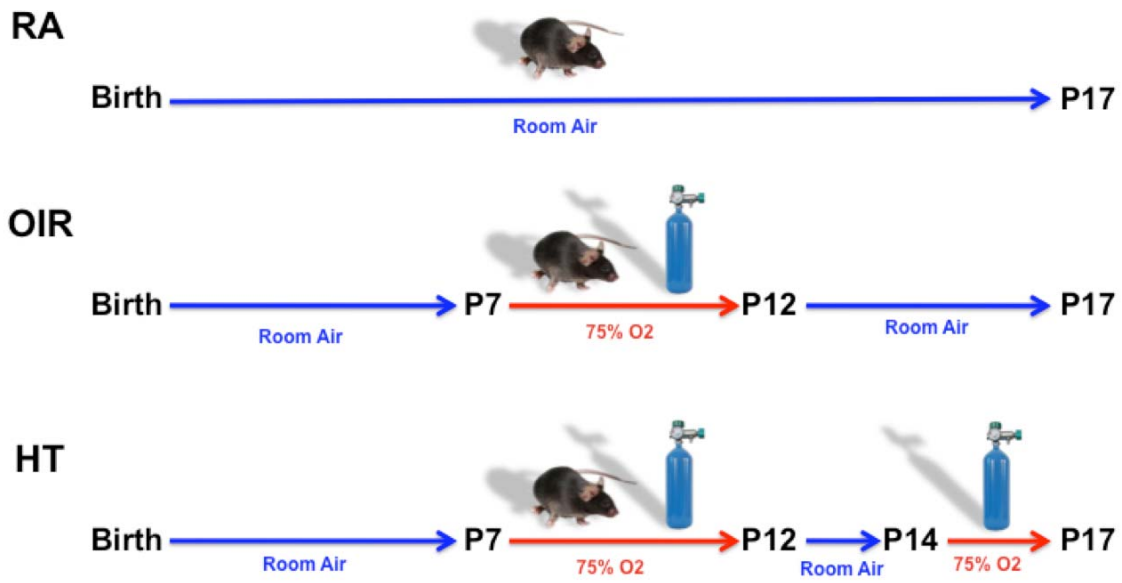

Figure 26. Hyperoxia treatment

Adenovirus infection of HRE cells

HRE cells, grown in 50% CSC complete medium (CSC, Wilmington, DE) -50% EGM (Lonza, Walkersville Inc., Walkersville, MD) until confluent, were infected with adenovirus carrying HIF1A or GFP vectors in opti-MEM (Life Technology) for 6 hours. The following day, cells were starved overnight with serum free EBM (Lonza, Walkersville Inc., Walkersville, MD). Approximately 48 hours from the start of adenovirus infection, cells were stimulated with VEGF (R&D systems, Minneapolis, MN), bFGF (PeproTech, Rocky Hill, NJ) or IL-1β (PeproTech) for two hours and samples were collected for Western blotting or qPCR.

Western blotting on cell culture lysate

At the end of experiments, loading buffer was added to each well of a 12-well plate containing HRE cells and homogenized. The lysate was put on heat block (95-100°C) for 10 minutes. Protein samples were subjected to 10% SDS polyacrylamide gel electrophoresis. The samples and molecular weight marker (Bio-Rad, Hercules, CA) were loaded in separate wells. Proteins were then transferred onto a nitrocellulose membrane. Transfer efficiency was assessed using Ponceau S stain. The membrane was blocked with 10% skim milk-TBST for one hour and washed with TBST. The membrane was then incubated overnight at 4°C with primary antibody against HIF-1α (1:200, rabbit; Santa Cruz biotechnology, TX) or tubulin (1:5,000, mouse; Sigma-Aldrich, St. Louis, MO) in 1% bovine serum albumin (BSA)-TBST. The following day, the membrane was washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibody (1:2000, GE Healthcare, Piscataway, NJ) in 1% BSA-TBST for 1 hour. Immunoreactive proteins were detected with the enhanced chemiluminescence system (GE Healthcare, Piscataway, NJ).

RT-PCR Assay on cell culture lysate

The experiments were performed with cells grown in 12-well plates. At the end of experiments each well was washed with DPBS for cell culture and Trizol (company) was added to each well and transferred to a 1.5 ml RNAse free tube. Chloroform was added and mixed, and the solution was incubated at room temperature for 2.5 minutes and centrifuged at 5°C for 15 minutes. The upper colorless aqueous phase was transferred to a new tube, mixed with equal amount of isopropyl alcohol and incubated at room temperature for 10 minutes before centrifuging for 15 minutes. The supernatant was discarded and 60% ethanol was added to the RNA pellet and gently mixed and centrifuged for 5 minutes. The supernatant was discarded and the RNA pellet was

allowed to dry at 37°C for 5-30 minutes until ethanol completely evaporated. RNAse free biological water was added to the tube and RNA was incubated at 55-60°C for 10 minuts before quantification using NanoDrop ND-1000 Spectrophotometer. RNA samples were diluted to 45 ng/µl. To make cDNA by reverse transcription, preheated RNA samples were incubated with random primer and M-MLV RT enzyme (Invitrogen) at 37°C for two hours. Templates and a mixture of forward and reverse primers along with the Power SYBR Green were added to a 96-well PCR plate, with a total volume of 10 µl in each well. StepOne PCR system (Applied Biosystems, Austin, TX) with the following PCR program was used: step 1, 2 minutes at 94°C; step 2, 30 seconds at 94°C; step 3, 30 seconds at 62°C; step 4, 15 seconds at 72°C; step 5, 40 repetitions of steps 2 to 4; step 6, 7 minutes at 72°C; step 7, hold at 4°C. The fold difference in various transcripts was calculated by the ΔΔCT method using Hprt as an internal control and normalized to RA control. Subsequently, a melting curve, constructed in the range of 60°C to 95°C, was used to evaluate the specificity of the amplification products. Primer sequences for human transcript were as follows: GAPDH For-5'-ACC ACA GTC CAT GCC ATC AC-3'; GAPDH Rev-5'- TCC ACC ACC CTG TTG CTG TA-3'; Fn14 For-5'- GCT CTG AGC CTG ACC TTC GT-3'; Fn14 Rev-5'-CGA CCA CAC AGC GAC TTC TG-3'; TWEAK For-5'- GGA AAA CAC GGG CTC GAA-3'; TWEAK-Rev-5'-CCA GGT CGT GGA TGA ACT TCA-3'.

RESULTS

Upregulation of Fn14 with hypoxia

As it was explained in chapter 1, the mouse OIR model is produced by maintaining pups in hyperoxia condition from P7 to P12 and keeping them at room air

from P12 to P17. This creates a relative hypoxia from P12 onward that results in neovascular formation starting at P15. To evaluate whether Fn14-induced NV is hypoxia driven retinal Fn14 mRNA was measured immediately after the pups were returned to room air at P12 and the following days until P17. The results were compared to RA control. As displayed in figure 27, at P12, which is the end of the hyperoixa period, Fn14 mRNA remains relatively unchanged. However, from P13, Fn14 mRNA increases and remains high through P17. In other words, Fn14 upregulation occurs at the time of relative hypoxia before the appearance of NV (P13-P15) and remains high even at the time of new vessel formation (P15-P17). This indicates association of Fn14 upregulation with both hypoxia and NV.

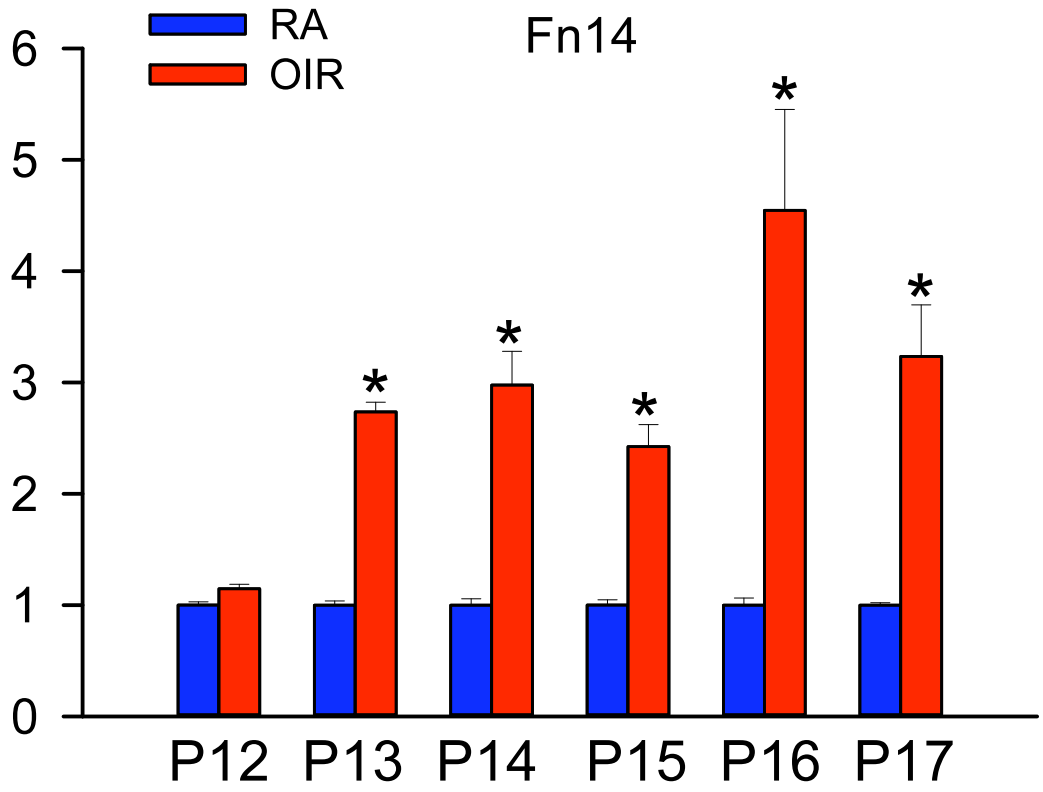

Figure 27. Fn14 mRNA is increased before, during and after NV in OIR

Retinas were collected from OIR and RA mice at P12-P17. Fn14 mRNA levels were quantified by qPCR and normalized to RA at P12. n=3, *P<0.05.

Unlike Fn14, TWEAK mRNA minimally decreases from P13 to P17 in OIR compared to RA (Fig. 28). Although these changes are statistically significant at P14 and P17, the amount of change is less than 15%. In Chapter 1 (Fig. 17) I showed that, with an n=6 in each group, the TWEAK mRNA remained relatively unchanged in OIR compared to RA. Given there is a 50% reduction in the TWEAK mRNA in the retinal vasculature in OIR (Fig. 24), the negligible global reduction in the retina may be secondary to the 50% reduction in the retinal vasculature.

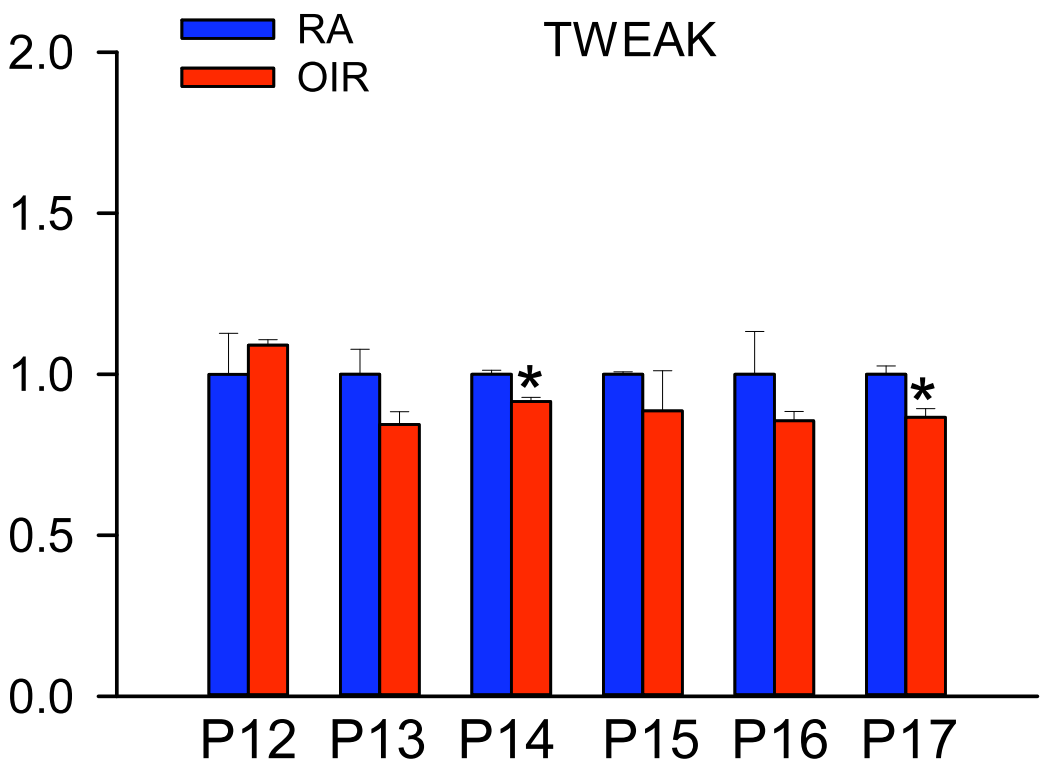

Figure 28. TWEAK mRNA is slightly decreased from P13-P17 in OIR

Retinas were collected from OIR and RA mice at P12-P17. TWEAK mRNA levels were quantified by qPCR and normalized to RA at P12. n=3, *P<0.05.

Normalization of Fn14 and TWEAK with HT

After finding the association between hypoxia and Fn14 upregulation, the question was whether eliminating hypoxia would decrease the amount of Fn14 upregulation. To answer this question the OIR mice were treated with hyperoxia by maintaining at 75% oxygen from P14 to P17 after being in room air for two days from P12 to P14. Figure 29 shows that comparing to untreated OIR, HT is associated with an increase in Fn14 mRNA to RA levels.

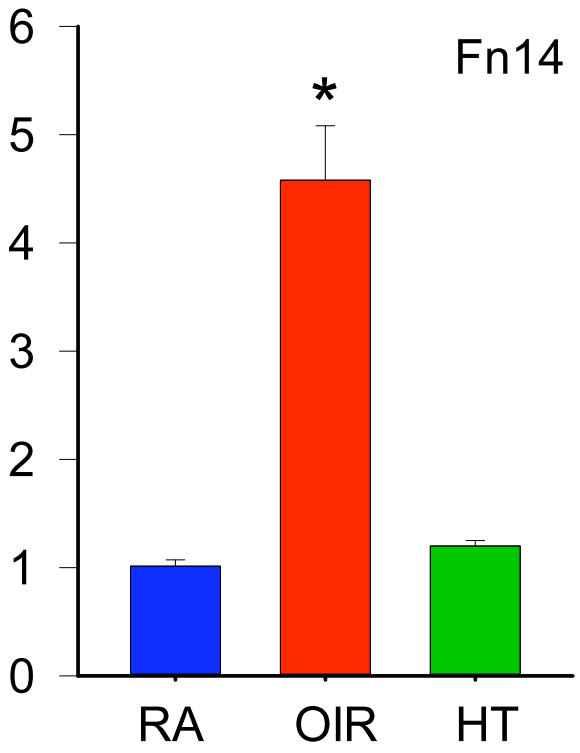

Figure 29. Normalization of Fn14 mRNA with HT

Retinas were collected from RA, OIR and HT mice at P17. Fn14 mRNA levels were quantified by qPCR and normalized to RA. n=6, *P<0.05.

As I presented earlier, the TWEAK mRNA remains relatively unchanged or it may show negligible reduction when measured in the whole retina of OIR (Fig. 17 & 27). Given this, no change was expected to occur with HT. Indeed, as figure 30 displays, TWEAK mRNA, which is reduced by a negligible amount in OIR, returns toward normal level after HT.

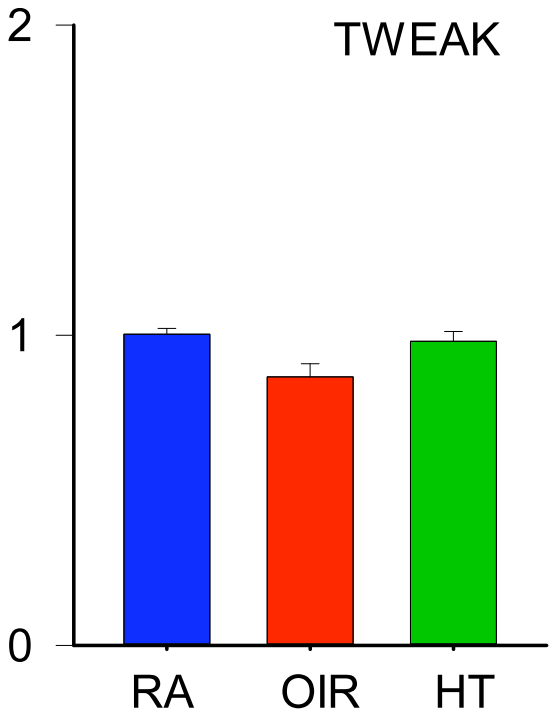

Figure 30. Normalization of Fn14 mRNA with HT

Retinas were collected from RA, OIR and HT mice at P17. TWEAK mRNA levels were quantified by qPCR and normalized to RA. n=6.

HIF-1α binding sites in the Fn14 region of genome

The role of HIF-1α in hypoxia-induced angiogenesis is known [130]. To study potential role of HIF-1α in hypoxia-induced Fn14 upregulation, rVISTA software was used to perform a comparative sequence analysis of mouse and human genome. Analysis of Fn14 region and upstream of 6 kilo base pairs (kb) in size, revealed presence of 2,495 transcription factor binding sites (TFBS) in human compared to 1,998 in mouse, and 386 TFBS conserved between human and mouse (Fig. 31 A). Three binding sites for HIF-1

were indentified in the intron and eight binding sites were identified upstream of Fn14 transcriptional starting point (Fig. 31 B).

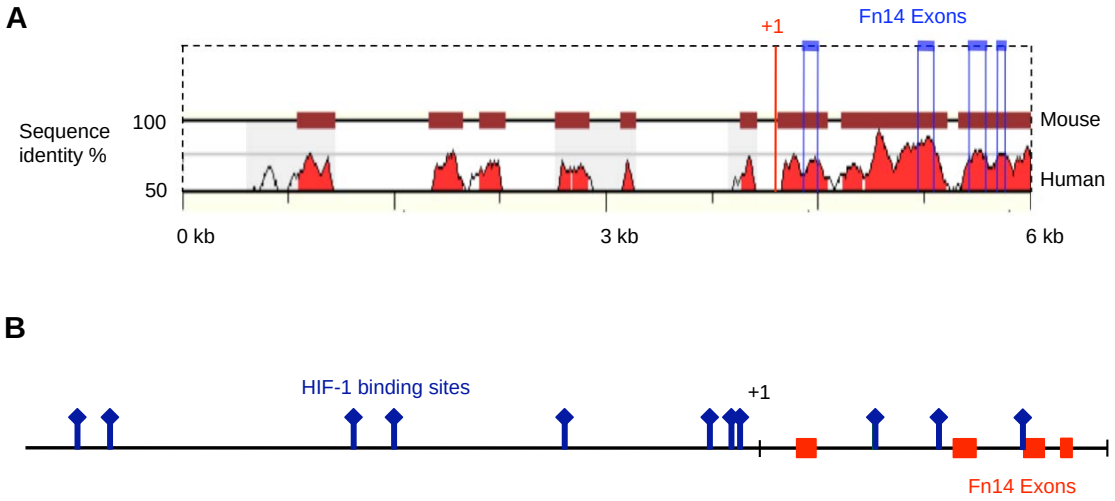

Figure 31. Identification of transcription factor binding sites in Fn14 region

A. rVISTA plot shows regions of homology between mouse and human in the in the region of Fn14 gene and upstream. **B.** Schematic diagram shows presence of 11 binding sites for HIF-1 in the Fn14 region and upstream.

HIF-1α upregulates Fn14

Presence of HIF-1α binding sites on Fn14 genome is suggestive of HIF-1α involvement in hypoxia-induced Fn14 upregulation. To evaluate whether Fn14 mediated neovascularization is induced by HIF-1α, HRE cells were infected with adenovirus containing either HIF1A (AdHIF1A) or GFP as control (AdGFP). Western blot was performed on cell lysate to test efficiency of HIF1A induction. As seen in figure 32 cells infected with AdHIF1A showed a clear overexpression of HIF-1α protein whereas cells infected with control vector did not overexpress HIF-1α.

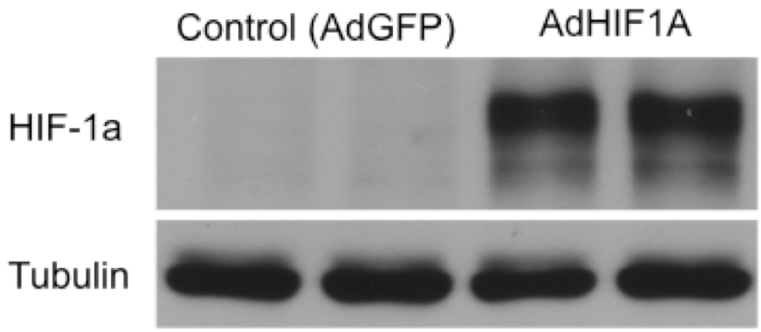

Figure 32. Overexpression of HIF-1α in HRE cells infected with adenovirus containing HIF1A

HRE cells were infected with adenovirus either expressing HIF1A (AdHIF1A) or GFP as control (AdGFP) for 48 hours. Western blot, performed on cell lysate at 48 hours, shows HIF-1α overexpression in cells infected with AdHIF1A but not AdGFP. Two samples in each group is shown. Tubulin, used as an internal control, shows almost equal loading in each well.

Following confirmation of HIF-1α overexpression, Fn14 mRNA levels were determined by qPCR 48 hours after adenovirus infection in both groups. HIF-1α overexpression significantly increases Fn14 mRNA (Fig. 33).

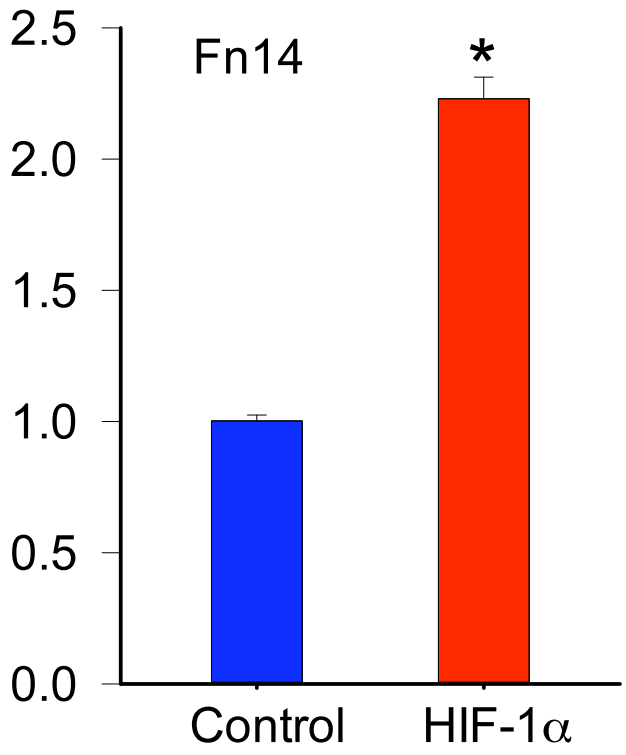

Figure 33. HIF-1α overexpression increases Fn14 mRNA in HRE cells

HRE cells were infected with adenovirus either expressing HIF1A (AdHIF1A) or GFP as control (AdGFP) for 48 hours. Fn14 mRNA levels were quantified by qPCR and normalized to control. n=5, *P<0.05.

Interestingly, HIF-1α overexpression in HRE cells causes a mild increase in TWEAK mRNA (Fig. 34). This is inconsistent with in vivo results I presented in Chapter 1 and earlier in this chapter, in that, TWEAK mRNA significantly decreases in the retinal vasculature in OIR. Therefore, there must be some negative feedback mechanisms causing downregulation of TWEAK in vivo.

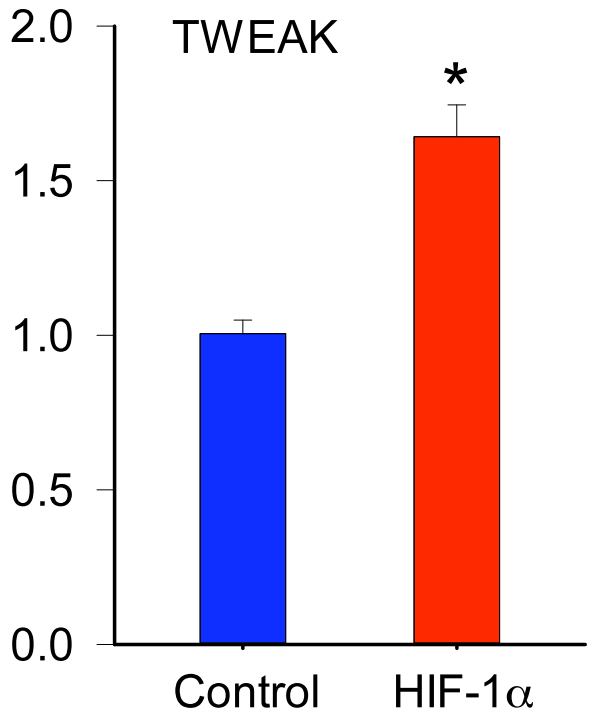

Figure 34. HIF-1α overexpression increases TWEAK mRNA in HRE cells

HRE cells were infected with adenovirus either expressing HIF1A (AdHIF1A) or GFP as control (AdGFP) for 48 hours. TWEAK mRNA levels were quantified by qPCR and normalized to control. n=5, *P<0.05.

Effects of VEGF, bFGF and IL-1β on HIF-1α-induced Fn14 and TWEAK upregulation

To study the effects of proangiogenic and proinflammatory molecules on HIF-1α-induced Fn14 and TWEAK upregulation, HRE cells were stimulated with VEGF, basic fibroblast growth factor (bFGF) or Interleukin (IL)-1β. Stimulations were conducted 48 hours after cells were infected with either AdHIF1A or AdGFP and lasted two hours. The duration of stimulation was chosen because it has previously been shown that Fn14

mRNA upregulation in response to growth factors such as VEGF is highest at two hours [131]. As seen in figure 35, VEGF stimulation of HRE cells slightly upregulates Fn14 and has a minimal additive effect with HIF-1α. However, VEGF stimulation does not change TWEAK mRNA levels in control HRE cells or cells overexpressing HIF-1α (Fig. 36).

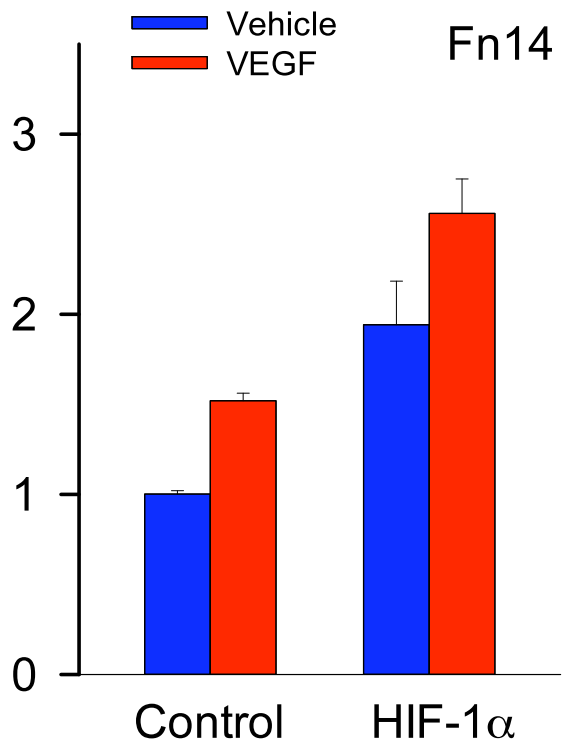

Figure 35. VEGF stimulation increases Fn14 mRNA in both control and HRE cells overexpressing HIF-1α. n=3.

HRE cells were infected with adenovirus either expressing HIF1A (AdHIF1A) or GFP as control (AdGFP) for 48 hours before stimulating with VEGF for two hours. Fn14 mRNA levels were quantified by qPCR and normalized to vehicle control. n=3.

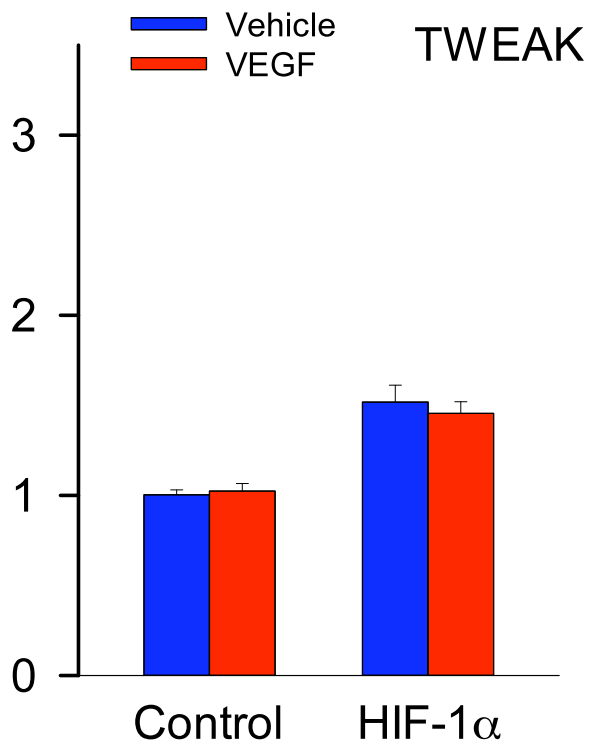

Figure 36. VEGF stimulation does not change TWEAK mRNA in control or HRE cells overexpressing HIF-1α

HRE cells were infected with adenovirus either expressing HIF1A (AdHIF1A) or GFP as control (AdGFP) for 48 hours before stimulating with VEGF for two hours. TWEAK mRNA levels were quantified by qPCR and normalized to vehicle control. n=3.

Stimulation of HRE cells with bFGF does not have significant effect on Fn14 mRNA in control cells or cells overexpressing HIF-1α (Fig. 37). Similarly, bFGF has no effect on TWEAK mRNA levels in each group of cells (Fig. 38).

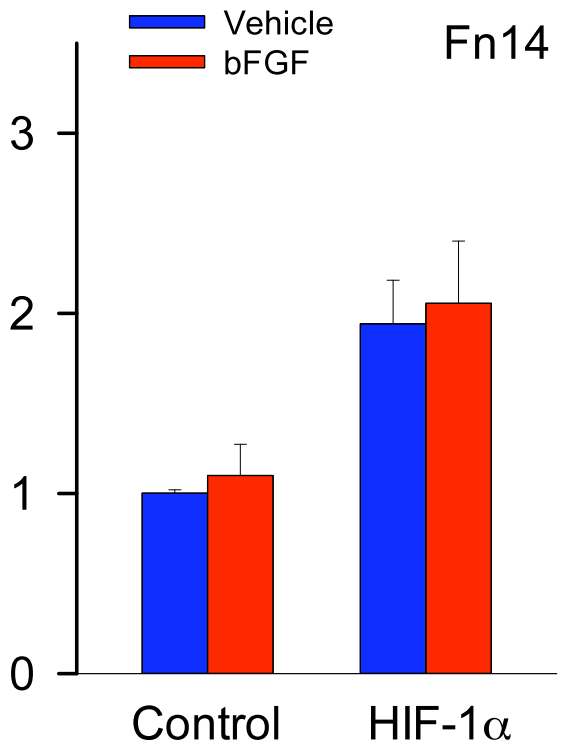

Figure 37. bFGF stimulation does not significantly change Fn14 mRNA in control and HRE cells overexpressing HIF-1α

HRE cells were infected with adenovirus either expressing HIF1A (AdHIF1A) or GFP as control (AdGFP) for 48 hours before stimulating with bFGF for two hours. Fn14 mRNA levels were quantified by qPCR and normalized to vehicle control. n=3.

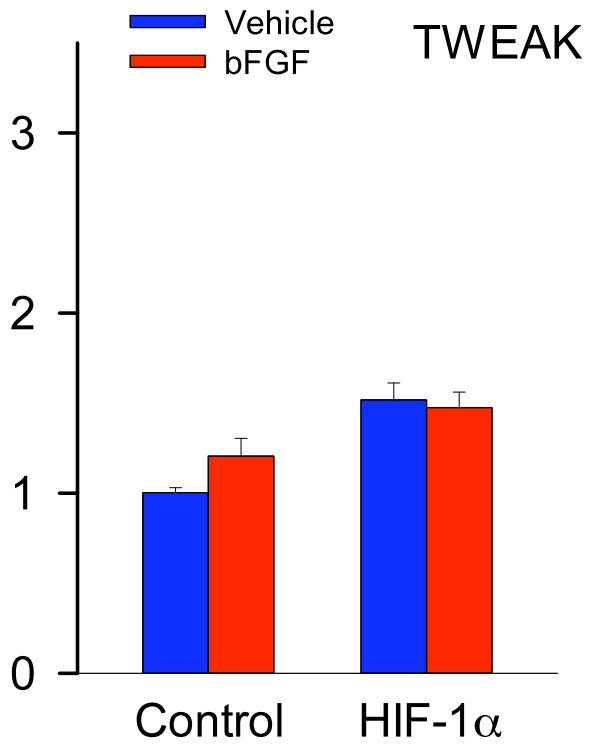

Figure 38. bFGF stimulation does not change TWEAK mRNA in control or HRE cells overexpressing HIF-1α. n=3

HRE cells were infected with adenovirus either expressing HIF1A (AdHIF1A) or GFP as control (AdGFP) for 48 hours before stimulating with bFGF for two hours. TWEAK mRNA levels were quantified by qPCR and normalized to vehicle control. n=3.

Stimulation of HRE cells with IL-1β does not change Fn14 mRNA in control cells but it minimally reduces its level in cells overexpressing HIF-1α, although this reduction is not statistically significant (Fig. 39). IL-1β slightly reduces TWEAK mRNA levels in both groups but they are not statistically significant (Fig. 40).

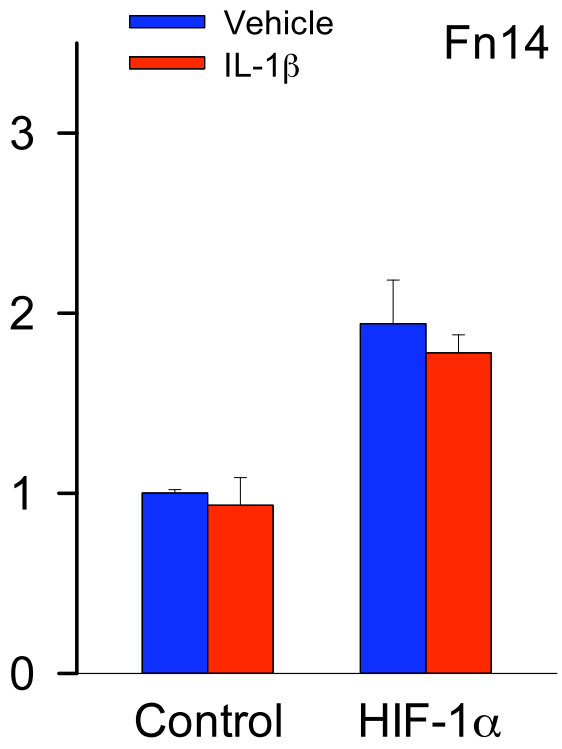

Figure 39. IL-1β stimulation does not change Fn14 mRNA in control but slightly reduces its level in HRE cells overexpressing HIF-1α

HRE cells were infected with adenovirus either expressing HIF1A (AdHIF1A) or GFP as control (AdGFP) for 48 hours before stimulating with IL-1β for two hours. Fn14 mRNA levels were quantified by qPCR and normalized to vehicle control. n=3.

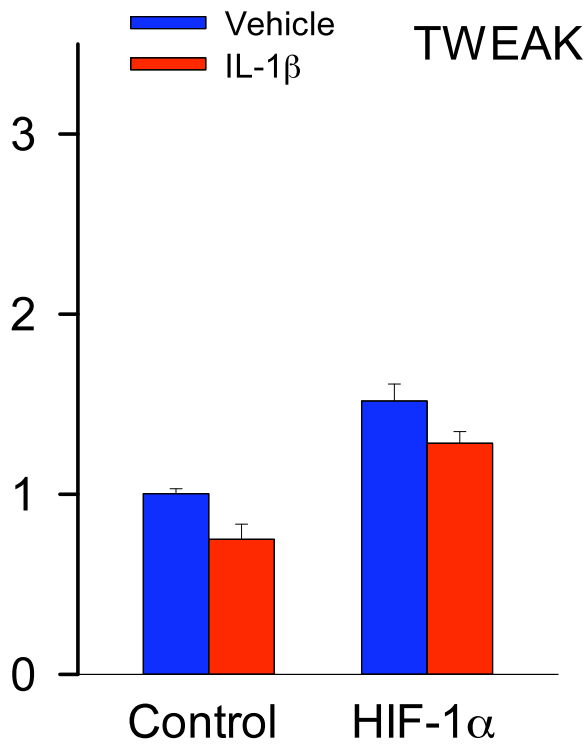

Figure 40. IL-1β stimulation minimally reduces TWEAK mRNA in control and HRE cells overexpressing HIF-1α

HRE cells were infected with adenovirus either expressing HIF1A (AdHIF1A) or GFP as control (AdGFP) for 48 hours before stimulating with IL-1β for two hours. TWEAK mRNA levels were quantified by qPCR and normalized to vehicle control. n=3.

DISCUSSION

There have been significant improvements in the treatment of retinal vascular disease in recent years. However, as it is true in most other conditions, each treatment is effective in a certain group of patients and in a certain time point in the disease. This is because of the complexity of disease processes. The more we understand about

mechanisms of retinal vascular disease the more likely to find new treatments for those who are not responsive to current treatments. In Chapter 1 I presented data implicating the TWEAK/Fn14 pathway in NV, a complication of retinal vascular disease. The data presented in this chapter focused on how the TWEAK/Fn14 pathway is activated. Since ischemia is a known cause of NV in retinal vascular disease and the mouse OIR model, it is reasonable to explore its potential role in activation of the TWEAK/Fn14 pathway.

As it was demonstrated earlier, in OIR, Fn14 is upregulated shortly after the beginning of the relative hypoxia period prior to the appearance of NV tufts. This is suggestive of hypoxia as an inciting mechanism for activation of the TWEAK/Fn14 pathway in OIR. Normalization of Fn14 following elimination of hypoxia by HT further indicates that hypoxia plays a significant role in the activation of the TWEAK/Fn14 pathway in OIR. In addition it further supports involvement of the TWEAK/Fn14 in NV, as it has previously been shown that HT accelerate the process of revascularization and prevents development of NV in OIR [132]. Similar to the findings presented in Chapter 1, I showed earlier in this chapter that the effects of both hypoxia and hyperoxia on TWEAK mRNA are negligible. This indicates that at least in the mouse OIR model, Fn14 is a major regulatory point in the TWEAK/Fn14 pathway.

How does hypoxia activate the TWEAK/Fn14 pathway leading to NV? In the case of VEGF mediated angiogenesis hypoxia causes upregulation of HIF-1α, which in turn increases production of VEGF in retinal cells such as Muller cells and neuronal cells [129, 133-136]. Angiogenesis then occurs because of the effects of high VEGF level on the endothelial cells. Could TWEAK/Fn14 mediated angiogenesis be the same as VEGF mediated angiogenesis? The data I have presented so far suggests that at least in some parts TWEAK/Fn14 mediated angiogenesis may be different from that of VEGF. Given the lack of significant changes in TWEAK levels at the time of hypoxia or during neovascularization in the OIR retina, it is less likely that non-vascular cells such as Muller cells or neuronal cells are involved. Therefore, activation of the TWEAK/Fn14

pathway may occur within the endothelial cells via upregulation of Fn14, without other cells being significantly involved.

The role of HIF-1α in hypoxia is well studied. By means of binding to its binding sites on many genes, HIF-1α increases their transcription. I presented the analysis of Fn14 region and upstream earlier in this chapter and showed that there are at least two binding sites for HIF-1α on Fn14 gene, suggesting that hypoxia mediated upregulation of Fn14 may be through HIF-1α. Subsequently, I showed that overexpression of HIF-1α in HRE cells increases both Fn14 mRNA and TWEAK mRNA levels. These data support the hypothesis that the TWEAK/Fn14 pathway activation is hypoxia driven and is mediated by HIF-1α. To the best of my knowledge this is the first study to link HIF-1α to Fn14.

Since VEGF is a potent angiogenic factor, it is important to explore its interactions with the TWEAK/Fn14 pathway. TWEAK has been reported to have no effects on VEGF dependent proliferation and migration of human umbilical vein endothelial cells (HUVEC) but showing antagonistic effects on the morphogenic response of endothelial cells to VEGF [55]. VEGF Stimulation of HUVEC and human microvascular endothelial cells resulted in upregulation of Fn14 mRNA [131]. TWEAK has been shown to enhance mitogenic activity of VEGF on endothelial cells but have no effects on VEGF induced cell migration [131]. I presented earlier in this chapter that VEGF stimulation increases Fn14 mRNA in HRE cells and may have minimal additive effects to HIF-1α induced Fn14 upregulation. However, VEGF stimulation of endothelial cells has no effects on TWEAK mRNA expression in control HRE cells or on cells overexpressing HIF-1α.

TWEAK has been shown to cooperate with bFGF in inducing proliferation and migration of human endothelial cells and have synergistic effects on bFGF mediated microvessel formation [55]. However, I showed in this chapter that bFGF stimulation does

not significantly change Fn14 mRNA or TWEAK mRNA in control HRE cells or in cells overexpressing HIF-1α.

The TWEAK/Fn14 pathway has been implicated in inflammation and TWEAK has been reported to potentiate the effects of IL-1β on vaginal and cervical epithelial cells in producing IL-8 [137]. TWEAK also enhances IL-8 and CC chemokine ligand 20 (CCL20) production in human gingival fibroblasts stimulated with IL-1β [138, 139]. However, as presented earlier in this chapter, IL-1β stimulation does not significantly change Fn14 mRNA or TWEAK mRNA in control HRE cells or in cells overexpressing HIF-1α; if any effects, it seems to be slightly antagonistic.

In summary, the TWEAK/Fn14 pathway is involved in the initiation, progression and maintenance of NV in OIR. Activation of the TWEAK/Fn14 pathway is induced by hypoxia and is mediated by HIF-1α (Fig. 41).

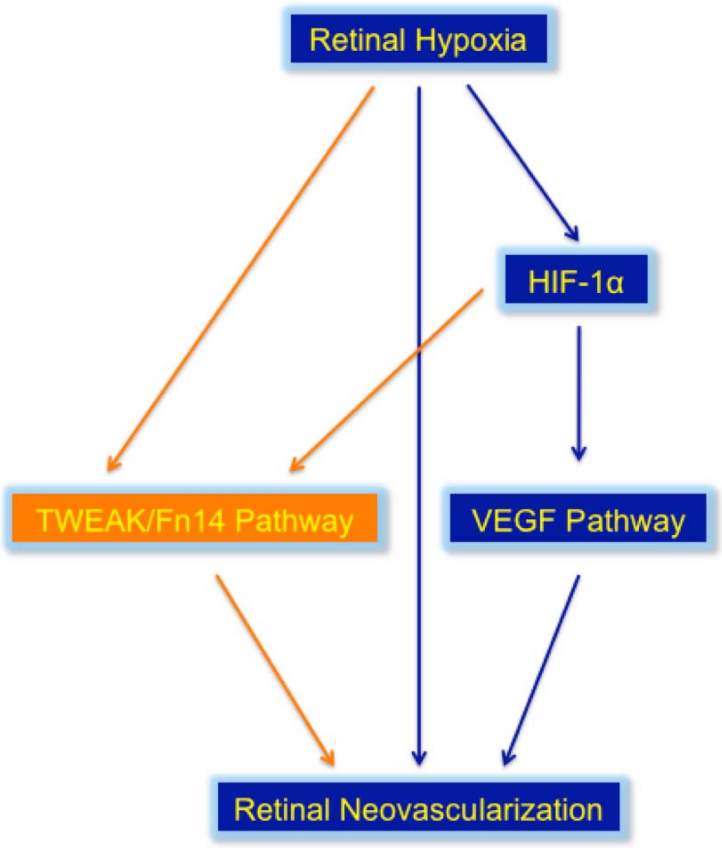

Figure 41. Block diagram of TWEAK/Fn14 involvement in NV

Blue lines represent established pathways. Orange lines show novel findings about the TWEAK/Fn14 involvement in hypoxia induced NV in OIR

Future work

I focused on vasoproliferative complication of retinal vascular disease, however, TWEAK injection into the brain has been shown to increase blood-brain barrier permeability [140]. Given macular edema is a common cause of mild to moderate vision loss in retinal vascular disease and it occurs because of the blood-retina barrier is compromised, further studies are warranted to explore the role the TWEAK/Fn14 pathway in macular edema.

The immunohistochemistry data presented here were performed on 17-day-old mice. Since retinal maturation continues beyond this point, localization of TWEAK and Fn14 may be different in adult mice; younger mice may also display a different localization. For example, in younger age before completion of physiological retinal vascularization, higher Fn14 expression in growing retinal vasculature may indicate TWEAK/Fn14 involvement in physiological angiogenesis. On the other hand, a lower expression of TWEAK or Fn14 in neuronal layers in adult mice may be suggestive of their potential involvement in retinal maturation. Future immunohistochemical studies carried out at different age groups may help to better understand various aspects of the TWEAK/Fn14 involvement in physiological processes.

The results I have presented here show involvement of the TWEAK/Fn14 pathway in pathological neovascularization of the retina and the mechanism of its activation by hypoxia. There may be other mechanisms by which hypoxia activates this pathway and this needs to be explored. More importantly, little is known about the mechanisms by which the TWEAK/Fn14 pathway causes pathological neovascularization and certainly none about the mechanism of neovascularizaiton in the retina. Additional studies, including both in vitro and in vivo experiments, will expand our knowledge in this field and will help to introduce new treatments for retinal vascular diseases. These studies may focus on demonstrating creation of retinal neovascularization by artificially

activating the TWEAK/Fn14 pathway. Given the lack of change or even slight downregulation of TWEAK in the mouse model of retinal neovascularization, blockade of the pathway with Fn14 Fc decoy in other models of retinal neovascularization could help to realize whether this method is sufficient for blocking the pathway or other means that directly act on the receptor or downstream are required for eliminating the effects of TWEAK/Fn14. Human studies on patients with retinal vascular diseases could expand our knowledge about the extent of involvement of TWEAK/Fn14 in these conditions by exploring potential correlation between severity of the disease and the extent of the TWEAK/Fn14 pathway activation.

My studies on the mouse model of retinal neovascularization may imply potential involvement of the TWEAK/Fn14 pathway in neovascular age related macular degeneration but are not directly applicable to this disease since the major component of the age related macular degeneration is choroidal neovascularization. Future studies on animal models of choroidal neovascularization could help to explore potential contribution of the TWEAK/Fn14 pathway in the formation and maintenance of the choroidal neovascular membrane in age related macular degeneration.

Finally, the novel finding that TWEAK/Fn14 plays an essential role in pathological retinal angiogenesis may encourage further exploration of the role of this pathway in angiogenesis in other diseases such as tumors and limb ischemia.

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Vita

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