CONDITIONAL KNOCKDOWN OF TUBEDOWN-1 IN ENDOTHELIUM RESULTS IN NEOVASCULAR RETINOPATHY

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Conditional Knockdown of Tubedown-1 in Endothelium Results in Neovascular Retinopathy

By

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ABSTRACT

Abnormal blood vessel proliferation in the retina is the physical manifestation promoting blindness in neovascular retinopathies. Neovascular retinopathies include diabetic retinopathy, retinopathy of prematurity, and the 'wet form' of macular degeneration, and constitute the foremost cause of vision loss in developed nations. Uncontrolled angiogenesis is responsible for the aberrant neovascular phenotype. Vascular beds become chaotic and extend to form pre-retinal membranes covering the retinal tissue, and consequently structurally and functionally hinder vision. Elucidating the molecular biological events leading to the unregulated angiogenic surge will help in developing molecular treatments targeting the neovascular phenotype.

Tubedown-1 (Tbdn-1) is a novel protein expressed highly in adult ocular blood vessels. Tbdn-1 is homologous to the N-terminus of yeast Nat1 and co-purifies with an acetyltransferase activity. Previous data implicates Tbdn-1 as a negative regulatory modulator of capillary formation in adults. Furthermore, Tbdn-1 protein expression is down-regulated in retinal blood vessels of patients with proliferative diabetic retinopathy compared to its high expression in normal retinal blood vessels. The objective of this thesis was to further investigate the role of Tbdn-1 in retinal vasculature by studying a mouse model enabling conditional knockdown of Tbdn-1 specifically in endothelial cells. Based on previous results, the hypothesis was suppression of Tbdn-1 in endothelium would result in an increase in retinal angiogenesis. Histological analysis of retinas from mice demonstrating suppression of the Tbdn-1 protein revealed the presence of pre-retinal membranes coinciding with increased retinal neovascularization. The pre-retinal

membranes also displayed the proliferation of glial cells and myofibrobasts, with a decreased expression of basement membrane constituent heparan sulfate proteoglycan. Retinas exhibiting a prolonged down-regulation of Tbdn-1 showed an increase in the size of pre-retinal membranes. Furthermore, the neovascular phenotype resulting from Tbdn-1 down-regulation appeared to be specific to the delicate retinal tissue, as it was not observed in other tissues. These results reinforce the hypothesis that Tbdn-1 functions to dampen capillary formation *in vivo*. Loss of Tbdn-1 expression in the retina may alleviate a constraining force on endothelial cell remodeling allowing the outgrowth of endothelial cells to form neovascular networks.

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ABBREVIATIONS

Ang	Angiopoietin
ASMA	Alpha Smooth Muscle Actin
ASTBDN-1	Antisense Tubedown-1
bFGF	Basic Fibroblast Growth Factor
cBSA	Cationizied Bovine Serum Albumin
Dox	Doxycycline
ECM	Extracellular Matrix
Enh	Enhancer
GCL	Ganglion Cell layer
GFAP	Glial Fibrillary Acidic Protein
H&E	Hematoxylin & Eosin
HGF-1	Hepatocyte Growth Factor-1
HIF-1	Hypoxia Inducible Factor-1
HSPG	Heparan Sulfate Proteoglycan
IEM	Embryonic Endothelial Cells
IGF-1	Insulin-Like Growth Factor-1
INL	Inner Nuclear Layer
IPL	Inner Plexiform Layer
LIF	Leukemia Inhibitory Factor
ONL	Outer Nuclear Layer
OPL	Outer Plexiform Layer

PDR	Proliferative Diabetic Retinopathy
PEGF	Pigment Epithelial Growth Factor
PFV	Persistent Fetal Vasculature
PHR	Photoreceptors
PKC βII	Protein Kinase C βII
ROP	Retinopathy of Prematurity
RPE	Retinal Pigment Epithelium
RtTA	Reverse Tet Transactivator
Tbdn-1	Tubedown-1
TGF-β	Transforming Growth Factor- β
TRE	Tetracycline Response Element
VEGF	Vascular Endothelial Growth Factor
VEGFR2	Vascular Endothelial Growth Factor Receptor 2
VWF	Von Willebrand Factor

1 INTRODUCTION

1.1 The Retina

The retina is the principal ocular tissue responsible for visual perception. Retinal tissue lines the back two-thirds of the eyeball and is structurally divided into seven distinct layers responsible for the initial construction of an image (see Fig. 1-1). These layers are distinguished by the presence of neuronal cell bodies or bundles of synaptic connections establishing intraretinal communication. Light enters the eye through the anterior eye segment and is focused upon the retina via the cornea (the transparent membrane covering the anterior eye) and the lens. Light stimuli must transverse the entire layered retinal tissue before the photons are detected and converted into electrical impulses at the sensory photoreceptor cells located at the farthest outer retinal layer. Intricate neural circuits spanning the retinal tissue allow the vertical propagation of electrical and chemical messages from the photoreceptor cells to the inner nuclear and ganglion cell layers. The signal is then propagated laterally until reaching the optic nerve, the point at which the signal leaves the eye and travels to the brain for further visual processing.

The retina may be divided into the vascular retinal fraction and the avascular retinal fraction. The innermost layers of the retina (the retinal fraction directly adjacent to the gel-like vitreous) comprise small retinal blood vessels that receive its blood supply from the central retinal artery. Adjacent endothelial cells lining these blood vessels adjoin to form seals or tight junctions, the first component constituting the blood-retinal

barrier. To ensure sharp visual acuity the outer photoreceptor fraction is completely avascular and acquires its metabolic needs from the underlying tissue, the highly vascular

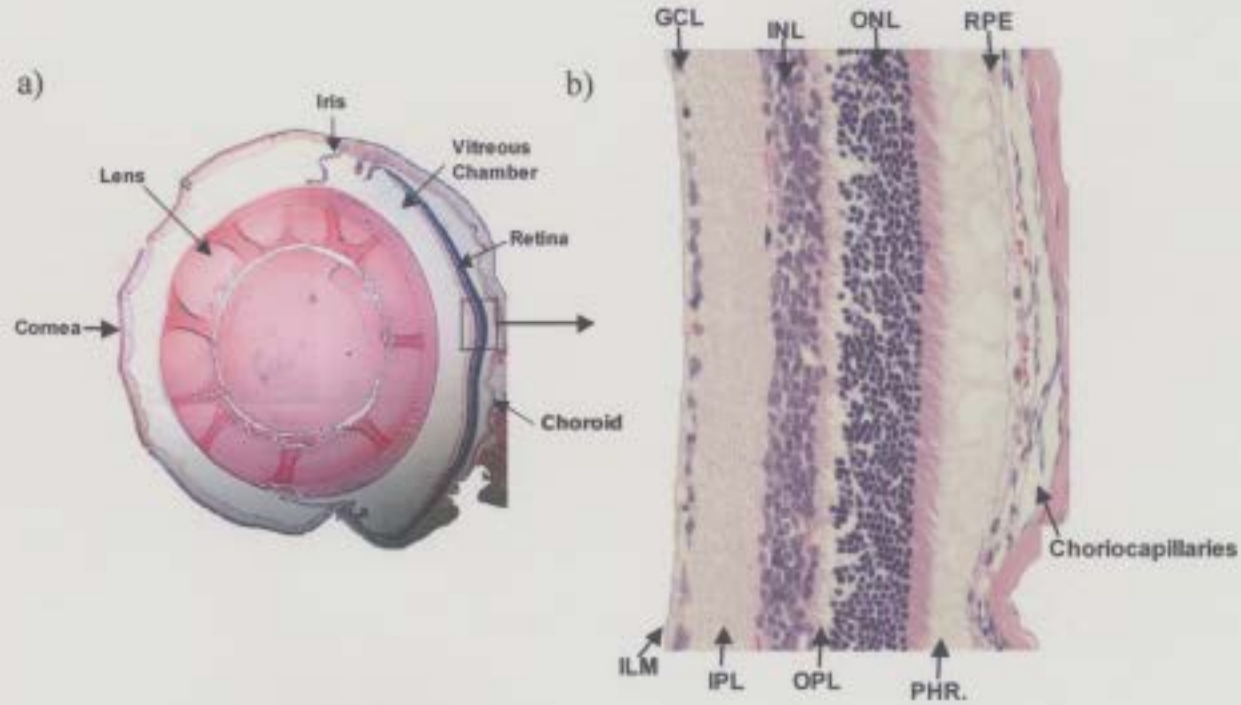

Figure 1-1: The structure of the mouse eye and retina.
1a: A H&E stained sagittal section through a mouse eye illustrates the major anatomical parts of the eye. The anterior eye segment consists of the cornea (the transparent membrane covering the eye which focuses light onto the retina) and the iris (which controls the amount of light entering the eye). In mice, the lens occupies most of the vitreous chamber while in humans the lens is smaller leaving most of the vitreous space unoccupied. The vitreous is an acellular, relatively inert, viscous gel-like material located directly adjacent to the retina. Although the vitreous chamber appears vacant, it consists of hyaluronic acid and highly dispersed collagen. The retina lines the back (posterior segment) of the eyeball and functions as a peripheral sense organ. It is attached to the underlying highly vascular choroid, which serves the metabolic demands of the outer retina. (The detachment of the retina from the choroid seen in this picture is merely an artifact of tissue processing.)
1b: Figure 1b is a magnified view of the boxed region of the retina in figure 1a. The inner limiting membrane (ILM) of the retina forms a diffusion barrier separating the retina from the vitreous. The retina consists of 7 layers: the ganglion cell layer (GCL), the inner plexiform layer (IPL), the inner nuclear layer (INL), the outer plexiform layer (OPL), the outer nuclear layer (ONL), the photoreceptors (PHR) and the retinal pigment epithelium (RPE). The IPL and OPL contain cell axons establishing synaptic connections while the INL and GCL contain cellular nuclei responsible for propagation and modification of the stimuli. The ONL contains the cell bodies of the photoreceptor cells. The choriocapillaries nourish the avascular regions of the outer retina.

choriocapillaries. These capillaries are fenestrated and characteristically leaky to facilitate the transport of essential nutrients from the choroidal blood supply to the retinal pigment epithelium (RPE). Fluid from the choriocapillaries pools beneath the RPE (a thin layer of cubical shaped cells lying directly below the photoreceptors) and is transported to the outer retina by specific transport mechanisms designed to remove waste from the sensory retinal cells while also providing the cells with essential nutrients. RPE cells also adjoin to form tight junctions constituting the second component of the blood retinal barrier (Campochiaro 2000).

The vascularity of the normal adult retina is quiescent. Adult retinal endothelial cells (the cells that line blood vessels) are among the most stable cells in the body with a turnover rate of approximately 25 years (Paques *et al.* 1997). Maturation of retinal blood vessels is marked by the co-localization of the vascular network with supporting cells called pericytes. Pericytes are a class of smooth muscle cells with contractile properties which function to constrain endothelial cell remodeling while promoting endothelial cell survival. Pericytes co-localized with endothelial cells secrete the growth factor Vascular Endothelial Growth Factor (VEGF), which under a normal physiological concentration enhances endothelial cell survival through the inhibition of apoptosis (Wenbiao *et al.* 2002). Pericytes in contact with endothelial cells also activate Transforming Growth Factor-β (TGF-β), a multifunctional growth factor implicated in the inhibition of endothelial cell proliferation (D'Amore 1994). Loss of the intimate association of pericytes with the vascular bed is thought to render endothelial cells prone to fluctuations in the local microenvironment. Vision obscuring diseases of the retina are often initially

characterized by 'pericyte dropout', rendering the endothelial cells susceptible to pathologic disease progression.

1.2 Neovascular Retinopathies

Blinding proliferative retinopathies, such as proliferative diabetic retinopathy (PDR), branch vein retinal occlusion, and retinopathy of prematurity (ROP), are leading causes of vision loss in the industrialized world and are primarily characterized by aberrant retinal neovascularization. Neovascularization occurs by a process called angiogenesis, the formation of new blood vessels by recycling differentiated endothelial cells (Conway *et al.* 2001). Angiogenesis is vital to the development of the embryonic vascular network and remains an active process in the adult, though restricted to highly specific conditions and vascular beds such as wound healing and female reproduction. Angiogenesis is also the mechanism responsible for nascent blood vessel formation in pathological settings such as tumor progression as well as the overgrowth of retinal blood vessels (Timar *et al.* 2001). In normal conditions angiogenesis is accomplished through a tightly regulated collaborative mechanism involving degradation of the surrounding extracellular matrix, activation of the newly exposed endothelial cells, migration and proliferation of these cells, followed by their assembly into tubular structures (Conway *et al.* 2001). The quiescence of the retina is derived from a balance between pro-angiogenic and anti-angiogenic modulators (Semenza 2003). However, the diseased retina exhibits a disruption in the molecular profile controlling the angiogenic process enabling a prevalence of pro-angiogenic stimuli. The retina is sensitive to the heightened

concentrations of pro-angiogenic factors, which manifests as abnormal retinal neovascularization (Speicher *et al.* 2003).

Neovascular retinopathies involve an unyielding transformation in retinal microvasculature and its associated extracellular matrix. Cellular proliferation at the interface of the retina and vitreous tissue results in the formation of preretinal membranes or retinal lesions, an occurrence evident in both ROP and PDR (Speicher *et al.* 2003; Smith 2003). Glial cells, myofibroblasts, endothelial cells and extracellular matrix proteins are the common residents constituting these fibrovascular membranes (Bochaton-Piallat *et al.* 2000). The contraction or migration of the glial and myofibroblast cell types promotes extracellular matrix remodeling, which may result in tractional detachment of the retina from the underlying RPE (Bochaton-Piallat *et al.* 2000; Mishima *et al.* 1989; Lewis *et al.* 2003). Retinal detachment is a major source of vision loss in patients with both ROP and PDR Kohner 1993; Good *et al.* 2001). The nascent blood vessels characteristic of preretinal membranes are often functionally impaired and leaky, a consequence of a breakdown of the blood-retinal barrier. Blood cells, proteins and plasma pass through or between the diseased vascular walls with the potential for ocular edema. The collection or building of fluid in the delicate retina and vitreous may manifest as vitreal haemorrhage, also a significant cause of blindness associated with proliferative retinopathies (Cai *et al.* 2003).

Although the initial insult which fuels a molecular cascade promoting the neovascular phenotype differs between proliferative retinopathies, the indirect stimulus driving retinal neovascularization is common among these diseases. Hypoxia is the

major stimulus responsible for the surge of events leading to a pro-angiogenic state in the normally quiescent retinal tissue (Smith 2003; Khan *et al.* 2003; Semenza 2003; Stefansson 1990). Although the stimulatory role of hypoxia is not completely understood, hypoxic conditions are detected by an oxygen sensing cytosolic heme protein which responds to low oxygen tension by generating free radicals (Campochiaro 2000; Cai *et al.* 2004). The free radicals activate transcription factors, including Hypoxia Inducible Factor-1 (HIF-1). The HIF-1α subunit exhibits an increased lifespan in response to a decreased oxygen concentration and forms a dimmer by binding to the constitutively expressed HIF-1β subunit. Together these subunits function as a transcription factor, activating proteins with a hypoxia responsive element (Campochiaro 2000; Semenza 2003). Hypoxic conditions result in an increased expression of the potent endothelial cell mitogen and permeability agent VEGF, an important growth factor implicated in the initiation and progression of proliferative retinal diseases (Khan *et al.* 2003; Aiello *et al.* 2000; Witmer *et al.* 2003; Zachery 2003). Transgenic mice demonstrating VEGF overexpression develop severe retinal neovascularization resembling proliferative retinopathy, which advances to retinal detachment (Ohno-Matsui *et al.* 2002; Okamoto *et al.* 1997). Insulin –Like Growth Factor-1 (IGF-1) is another hypoxia responsive cytokine shown to mediate blood vessel formation and exhibits increased expression in patients with an overgrowth of retinal blood vessels (Khan *et al.* 2003; Cai *et al.* 2002; Boulton *et al.* 1997).

Perturbed expression of basement membrane constituents are also documented in neovascular retinopathy patients (Ljubimov *et al.* 1996). Vascular basement membrane

proteins include laminin, collagen I, II, III and IV, heparan sulfate proteoglycans (HSPG), and fibronectin. These proteins form a structural scaffold to support blood vessel integrity while also providing regulatory molecular signals for endothelial cells. Altered expression of such constituents renders endothelial cells exposed and thus vulnerable to the pro-angiogenic microenvironment. Also, mitogen-promoting growth factors, such as basic Fibroblast Growth Factor (bFGF), are sequestered in extracellular matrices (ECM) with matrix degradation releasing the growth factors thereby potentiating neoangiogenic activity (Rusnati 1996). Deviations in the expression of many other growth factors (such as PDGF, HGF, Ang-1, Ang-2) integrins (the receptors through which ECM proteins signal), and matrix metalloproteinases (proteins which regulate ECM degradation) are also implicated in the pathogenesis of neovascular retinopathies (Khan *et al.* 2003; Takagi 2003; Ljubimov *et al.* 1996; Friedlander *et al.* 1996; Au *et al.* 2002).

1.3 Treatment for Neovascular Retinopathies

Pan-retinal or scatter laser photocoagulation is the first-line therapeutic option for patients with proliferative retinopathies. A patient receiving laser treatment is subjected to hundreds or even thousands of burns throughout the peripheral retina while preserving the centrally located macula, the area of the retina accounting for 80% of vision in humans. Laser-mediated destruction of the photoreceptors, the highest oxygen consumers in the retina, results in their replacement with glial cell scar tissue. Ultimately, the destruction of the photoreceptors facilitates the diffusion of oxygen from the choroid to

the ischemic regions of the retina (Wilson *et al.* 2003). Thus, the therapeutic effect of laser treatment arises from relieving retinal hypoxia and consequently destroying the source of the chaotic angiogenic growth factor expression.

Laser treatment contains a window of opportunity for a beneficial outcome with disease progression negatively correlating with a favorable response (Speicher *et al.* 2003). If administered early in disease progression, laser therapy can prevent severe vision loss by 50 to 60% (ETDRS 1985). Unfortunately, timely treatment is often difficult due to the late presentation of symptoms. Also, laser surgery cannot rescue visual impairment previously incurred by neovascular retinopathy (Speicher *et al.* 2003). This treatment is ablative and mechanistically involves the destruction of retinal tissue with treatment itself responsible for some degree of permanent vision loss. Sight-threatening side effects such as loss of peripheral vision, reduced night vision, and decreased visual acuity are also inherent in the destruction of the peripheral retina (Aiello 1997).

Recent research has focused on designing therapies to target the molecules involved in both the onset and progression of retinal disease, in hope of complimenting or replacing laser surgery. VEGF is an appealing target for molecular therapy due to its major proliferative action on endothelial cells and increased expression early in disease development. Models countering VEGF expression in animal models of proliferative retinopathy include targeting its expression through the use of small interfering RNA (Reich *et al.* 2003), gene transfer of soluble VEGF receptors (Bainbridge *et al.* 2002), anti-sense oligonucleotides targeting VEGFR2 (the receptor which relays the mitogenic

properties of VEGF), and VEGF specific monoclonal antibodies (Mechoulam *et al.* 2003). These models have been successful in preventing retinal neovascularization. However, they do not completely or consistently rescue pre-existing pathology. Currently, there are only two molecular-based drugs in clinical trails to combat diabetic proliferative retinopathy. The first is a selective inhibitor of PKC βII, a protein involved in intracellular signaling in response to both VEGF and IGF-1. The second drug, octreotide acetate, combats IGF-1 mediated signaling. Octreotide acetate is a somatostatin analogue which inhibits the phosphorylation of the IGF-1 receptor tyrosine kinase, therefore inhibiting signaling through this pathway. Both of these drugs are designed for the prevention and/or treatment of very early stage proliferative diabetic retinopathy (Speicher *et al.* 2003). Identification of novel proteins, interactions and pathways involved in the pathogenesis of retinal disease will undoubtedly facilitate the breakthrough of molecular based treatments to both prevent and cure neovascular retinopathy.

1.4 Tubedown-1

Tubedown-1 (Tbdn-1) is a recently identified protein isolated from an embryonic endothelial cell line (IEM) (Gendron *et al.* 2000). IEM cells are an attractive model for studying molecular characteristics of endothelial cells because of their ability to form capillary-like structures *in vitro* and emulate normal blood vessel formation in embryo chimeras *in vivo* (Gendron *et al.* 1996). Tbdn-1 is highly expressed in quiescent IEM cell cultures. Upon stimulation with endothelial cell stimulants bFGF and Leukemia Inhibitory Factor (LIF), the IEM cells remodel to form capillary-like structures

coinciding with a decreased protein expression of Tbdn-1 (Gendron *et al.* 2000). The Tbdn-1 protein is also down-regulated during the out-growth of rheus RF/6A retinal-choroidal endothelial cells to form tubular structures compared to their quiescent state (Gendron *et al.* 2001). Furthermore, targeted suppression of Tbdn-1 levels in RF/6A cells using an antisense cDNA construct specific for Tbdn-1 (ASTBDN-1) results in a significant increase in capillary number compared to control cells expressing normal levels of Tbdn-1 (Paradis *et al.* 2002). Together, this data suggests that Tbdn-1 may function to dampen capillary formation *in vitro*.

Tbdn-1 is expressed highly in developing vasculature during embryogenesis; however, Tbdn-1 expression in the adult is restricted to ocular endothelium, endocardium, bone marrow capillaries, and blood vessels of regressing ovarian follicles (Gendron *et al.* 2000). *In vivo,* Tbdn-1 expression is specifically suppressed in retinal blood vessels of human patients with PDR compared to its high expression in normal retinal blood vessels (Gendron *et al.* 2001). During embryogenesis, the developing posterior ocular tissue is nourished by the transient hyaloid vascular network. The hyaloid artery and branches (vasa hyaloidea propria) nourish the developing posterior eye while the anterior and posterior tunica vasculosa lentis nourish the developing lens (Ito 1999). The hyaloid vascular network regresses late in embryonic life and in mice the involution of hyaloid vascular endothelial cells is complete by post-natal day 30 (Smith 2002). In humans, failure of the hyaloid vascular network to regress results in persistent fetal vasculature (PFV), a potentially blinding disease. TGF-β2 null embryos exhibit a persistent hyaloid mass within the vitreal chamber resembling PFV in humans. This

pathological hyaloid mass also shows suppression of the Tbdn-1 protein. Immunohistochemical analysis has shown that Tbdn-1 is expressed in the hyaloid vessels and tunica vasculosa lentis (the layer of blood vessels nourishing the lens of a fetus) of a human eye at 14 weeks of gestation (Paradis *et al.* 2002), prior to the remodeling events leading to the regression of this vascular bed. Together, this implicates Tbdn-1 as a necessary caveat in vitreal blood vessel regression during development (Paradis *et al.* 2002). Collectively, these results suggest that Tbdn-1 functions in dampening capillary formation *in vivo.*

Although the mechanistic action of Tbdn-1 remains largely unknown, its activity may be regulated through acetylation. Tbdn-1 is homologous to the N-terminus acetyltransferase subunit of yeast NAT1 (Gendron *et al.* 2000). Immunoprecipitates prepared using an anti-Tbdn-1 specific antibody demonstrate an acetyltransferase activity, which may represent an autoacetylation reaction of Tbdn-1 (Gendron *et al.* 2000). The autoacetylation of Tbdn-1 may likely regulate its activity by potentially modifying DNA binding and/or its interaction with other proteins. Tbdn-1 also contains a sequence motif homologous to the tetracotripeptide repeat, a motif known to mediate protein-protein interactions (Gendron *et al.* 2000). Tbdn-1 may function to restrain endothelial cell remodeling through these mechanisms.

1.5 Rationale for Current Study

Angiogenesis is a multi-factorial process. Its proper execution is accomplished by the tight regulation of a host of factors including growth factors, receptors, integrins, and

extracellular matrix elements. Unregulated and unrelenting angiogenesis is witnessed in several disease processes including abnormal retinal neovascularization, where blood vessel proliferation compromises vision. Treatments targeted at defusing angiogenesis may alleviate blindness in diseases caused by an uncontrolled growth of retinal blood vessels, such as in PDR, ROP and the 'wet form' of macular degeneration. Because angiogenesis is regulated by such a wide host of factors, it seems logical that an effective treatment will target more than one facet of the angiogenic process. A thorough understanding of the molecules and molecular cascades characteristic of normal and diseased retinal biology will facilitate the development of anti-angiogenic treatments. Tbdn-1 was isolated from retinal endothelial cells in an attempt to identify and characterize novel regulatory molecules involved in capillary formation (Gendron *et al.* 2000). Preliminary data suggests that Tbdn-1 is a necessary contributor to the maintenance of normal vascular homeostasis (Gendron e*t al.* 2000; Gendron e*t al.* 2001; Paradis *et al.* 2002). The objective of this study was to further characterize the regulatory function of Tbdn-1 in retinal capillaries to further uncover of molecular mechanisms in vascular retinal biology.

1.6 Overview of Methodology for Current Study

Characterization of the role of Tbdn-1 in capillary formation is analyzed in this study using a bitransgenic mouse model enabling conditional knockdown of endothelial-derived Tbdn-1 (see Fig 1-2). The mouse system utilizes two separate gene constructs driven by two distinct promoters: the tie-2 promoter and the tetracycline response

element (pTRE) (for review see Zhu *et al.* 2002). Tie-2 is an endothelial cell specific promoter and controls the expression of the reverse tet transactivator protein (rtTA). The pTRE drives the expression of a specific ASTBDN-1 cDNA. The introduction of an exogenous agent, doxycycline (Dox), to the binary system turns the system 'on' and instigates a decrease in Tbdn-1 protein expression. Specifically, Dox will bind to the rtTA protein resulting in a conformational change. The newly conformed rtTA can then bind to its specific binding site within the TRE promoter allowing the expression of an ASTBDN-1 cDNA, decreasing endogenous Tbdn-1 protein expression. In the absence of Dox, the rtTA will not bind to the TRE promoter and consequently there will not be a decrease in Tbdn-1 protein expression. Analysis of the mouse model was accomplished using histological and immunohistochemical techniques.

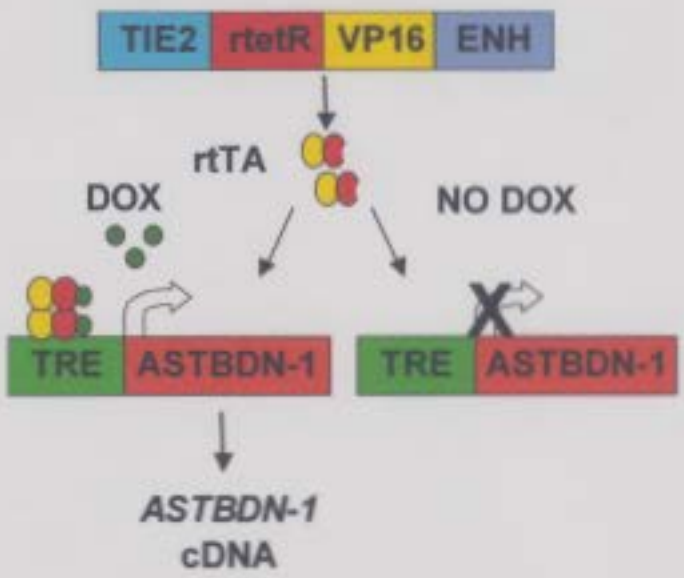

Figure 1-2: Schematic of transgenic system allowing inducible endothelial specific knockdown of Tbdn-1 protein expression.
The tie2 endothelial specific promoter directs the endothelial specific expression of the rtTA protein. Administration of Dox will allow the rtTA protein to activate the pTRE driving the expression of an ASTBDN-1 cDNA and decreasing Tbdn-1 protein expression. In the absence of Dox, the rtTA will not activate the pTRE therefore there will not be expression of the ASTBDN-1 cDNA.

2 MANUSCRIPT

Conditional knockdown of Tubedown-1 in endothelial cells leads to neovascular retinopathy

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2.1 CO-AUTHORSHIP STATEMENT

This project and manuscript was completed under the supervision of Drs. Gendron and Paradis. The TIE2/rtTA/Enh, TRE/ASTBDN-1, and TIE2/rtTA/Enh-TRE/ASTBDN-1 transgenic mouse lines was derived by Dr. Paradis. Mouse work (changing cages, feeding etc.) was performed and supervised by Dr. Paradis with the help of Dr. Gendron, Mandy Woodland , Dr. Ewa Miskeiwicz, and Karina Leblanc. The mouse anti-Tbdn-1 polyclonal serum MS.C10-2OTD was derived by Drs Paradis and Gendron. Characterization of the mouse anti-Tbdn-1 polyclonal serum MS.C10-2OTD was performed by Dana Wall and Karina Leblanc. Mouse genotyping was performed by Dr. Paradis, Mandy Woodland, and Dr. Ewa Miskeiwicz. Dana Wall is responsible for generating the data for Figures 2-1C-2H, 2-3, 2-4, 2-5 and 2-6. Dr. Gendron generated the data for Figure 2A and 2B. Dr. Ewa Miskeiwicz generated data for Figure 2-7 as well as blood vessel density of liver tissue and morphological study of testes and brain tissues, and wrote the sections of the manuscript corresponding to this data. Dana Wall, Dr. Paradis and Dr. Gendron are jointly responsible for writing the text of the manuscript and preparing Figures 2-1 to 2-6. Dr. Good contributed to the manuscript by providing valuable feedback and insight.

2.2 ABSTRACT

Purpose. Identification of novel proteins involved in retinal neovascularization may facilitate new and more effective molecular based treatments for proliferative retinopathy. Tubedown-1 (Tbdn-1) is a novel protein which shows homology to the yeast acetyltransferase subunit NAT1 and co-purifies with an acetyltransferase activity. Tbdn-1 is expressed in normal retinal endothelium but is specifically suppressed in retinal endothelial cells from patients with proliferative diabetic retinopathy. The purpose of this study was to investigate the importance of Tbdn-1 expression in retinal blood vessels *in vivo*.

Methods. A bitransgenic mouse model enabling conditional knockdown of Tbdn-1 specifically in endothelial cells was produced and studied using histological and immunohistochemical techniques.

Results. Tbdn-1 suppressed mice developed retinal neovascularization with intra and preretinal fibrovascular lesions similar to human proliferative retinopathies. Retinal lesions observed in Tbdn-1 suppressed mice increased in severity with prolonged suppression of Tbdn-1. In comparison to normal retina, the retinal lesions displayed alterations in the basement membrane of blood vessels and in the distribution of glial and myofibroblastic cells. Moreover, the pathological consequences of Tbdn-1 knockdown in endothelium were restricted to the retina.

Conclusions. These results indicate that the maintenance of Tbdn-1 expression is important for retinal blood vessel homeostasis and for controlling retinal

neovascularization in adults. Restoration of Tbdn-1 protein expression and/or activity may provide a novel approach for treating proliferative retinopathies.

2.3 INTRODUCTION

In blinding ocular diseases such as proliferative diabetic retinopathy (PDR) and retinopathy of prematurity (ROP), retinal ischemia progresses to a proliferative stage involving both neovascularization and fibrosis of the retina resulting in the formation of preretinal membranes (1- 8). Membranous traction may result in retinal detachment, a significant cause of blindness associated with both PDR and ROP (1-8). Laser photocoagulation administered to peripheral regions of the retina may reduce blindness in patients with neovascular retinopathies (1-8). However, this treatment is ablative and may not prevent sight-impairing complications associated with neovascular retinopathies (1-8). Elucidating the molecular processes of proliferative retinal disease and designing therapies to target molecular abnormalities may offer a means to replace laser surgery and prevent associated complications.

The molecular events leading to the angiogenic phenotype in PDR and ROP is mediated by increased expression of pro-angiogenic growth factors (vascular endothelial growth factor [VEGF], basic fibroblast growth factor [bFGF] and insulin-like growth factor [IGF-1]) (5, 9-11). Integrins, extracellular matrix (ECM) components and glial cells also contribute to pathologic neovascularization in PDR (5, 10-13). Increased production of VEGF in the retina is likely the determining factor in initiating the proliferative phase evident in later stages of diabetic retinopathy (DR [9-10, 14-15]). Treatments specifically targeting either VEGF and its receptors, or specific integrins have

been effective in preventing or reducing retinal neovascularization in animal models (9). However, these treatments do not completely or consistently rescue pre-existing pathology. Since a range of angiogenic factors are present in the microenvironment promoting retinal blood vessel proliferation, targeting of one type of factor for anti-angiogenic therapy may not completely counter the neovascularization evident in proliferative retinopathies. The characterization of common regulators acting in diverse angiogenic pathways will be key in identifying targets which could have a more global effect on controlling retinal neovascularization.

Tubedown-1 (Tbdn-1) is a mammalian homologue of the yeast acetyltransferase subunit NAT1 (16). Tbdn-1 expression is regulated during development and becomes restricted to normal ocular endothelial cells, atrial endocardium, blood vessels of regressing ovarian follicles and bone marrow capillaries in adults (16-17). We have found that Tbdn-1 expression is suppressed in retinal blood vessels of neovascular areas in patients with PDR (17). Both *in vitro* experiments and *in vivo* observations have suggested that Tbdn-1 suppression in retinal endothelial cells leads to increased angiogenesis (16-18). Collectively, these data have led us to hypothesize that Tbdn-1 in the adult functions to suppress retinal neovascularization *in vivo*. In order to directly examine if Tbdn-1 is an important regulator of retinal neovascularization *in vivo*, we have generated a binary antisense *TBDN-1* (*ASTBDN-1*) transgenic mouse model driven by the *TIE2* pan-endothelial promoter to enable conditional knockdown of Tbdn-1 expression in endothelial cells. Here we present *in vivo*

evidence for the requirement of Tbdn-1 expression for the maintenance of normal adult retinal vascular homeostasis.

2.4 METHODS

Endothelial specific conditional knockdown of Tbdn-1 in a bitransgenic mouse model

To facilitate conditional knockdown of Tbdn-1 in endothelial cells in mice, two different lines of transgenic mice were derived. For the construction of the first line, the BamHI/PvuII fragment from pcDNA3.1/Zeo-*ASTBDN-1* (18) containing the antisense cDNA fragment sequence 1-1413 of *TBDN-1* was inserted into the pTRE vector (Clontech, Palo Alto California) at the BamHI and PvuII sites. This antisense *TBDN-1* fragment 1413-1 does not produce aberrant proteins that could confer toxic and non-specific effects. The resulting construct pTRE/*ASTBDN-1* was digested with AatII and PvuII. The fragment containing the TRE promoter followed by *ASTBDN-1* (*TRE/ASTBDN-1*) was used to create FVB transgenic mouse lines (Transgenic Core Facility at Children's Hospital Research Foundation [Cincinnati, Ohio]).

Construction of the second line of transgenic mice was as follows: A DNA fragment encompassing coding sequences for *rtTA* followed by the SV40 poly A site were amplified by PCR from the pTet-On vector (Clontech, Palo Alto California) using the following primers: 5'-CGGCCCCGAATTAATATGGCTAGATTA-3' and 5'-TCCATTTTAGCGGCCGCAGCTCCTGAA-3'. The purified amplicon was digested with HindIII and NotI, and inserted into the pHHSDKXK construct (19) previously digested with HindIII and NotI removing the HH (HindIII to HindIII) portion of the

promoter and the coding sequences for *LacZ* (SDK). The resulting construct, pH*rtTA*XK, was digested with ApaI and ligated with the HH portion of the promoter previously amplified from the pHHSDKXK construct (using primers: 5'-TGGTACCGGGCCCCCCCT-3' and 5'-TCATCGCGGGCCCTGGTGGCCCT-3') and digested with ApaI. Orientation of the *TIE2* promoter in the construct was verified to be as previously described for endothelial specificity (19). The resulting construct pHH*rtTA*XK was digested with KpnI and the fragment harboring the *TIE2* promoter, *rtTA* coding sequence, SV40 poly A site and *TIE2* enhancer (*TIE2/rtTA/Enh*) was used to create FVB transgenic mouse lines (Transgenic Core Facility at Children's Hospital Research Foundation).

Genotyping was performed by Southern blot analysis using either a probe encompassing the *TRE* promoter for the *TRE/ASTBDN-1* transgenic lines or a probe encompassing the coding sequence for *rtTA* for *TIE2/rtTA/Enh* lines. Lines positive for HH*rtTA*XK transgene integration were further tested for rtTA protein expression by western blot analysis (18) of lysates of tail specimens and by immunohistochemistry of the vasculature using an affinity purified rabbit polyclonal antibody that recognizes the VP-16 sequence present in the rtTA protein (Clontech, Palo Alto California). FVB transgenic lines were backcrossed into the C57BL/6 background. Bitransgenic *TIE2/rtTA/Enh-TRE/ASTBDN-1* mice were derived by mating 4 different *TRE/ASTBDN-1* positive lines to 5 different *TIE2/rtTA/Enh* positive lines.

For conditional knockdown of Tbdn-1, induction with doxycycline (Dox) was performed by feeding adult animals with commercially prepared mouse chow containing

Dox (600 mg/kg [Bio-Serv, New Jersey]). Dox fed mice were sacrificed after various time points and tissues were analyzed grossly, histologically and immunohistochemically. The care and use of animals in this study followed the guidelines set forward by the Canadian Council on Animal Care and were approved by the Institutional Animal Care Committee of Memorial University of Newfoundland. All experiments involving animals adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Mouse anti-Tbdn-1 serum

To generate a mouse anti-Tbdn-1 serum (MS.C10-20TD), mice were immunized with a peptide corresponding to the amino acid sequence 10 to 20 of mouse Tbdn-1 (16) coupled to the carrier protein cationizied BSA (cBSA) (Pierce, Rockford IL). The specificity of the mouse anti-Tbdn-1 antiserum was validated by confirming that the recombinant Tbdn-1 protein was immunoprecipitated by MS.C10-20TD antiserum. Briefly, in vitro translation of Tbdn-1 was carried out with TNT T7 Quick Coupled Transcription/Translation System (Promega, Madison WI), using [^{35}S]-methionine/cysteine and pcDNA3.1-*TBDN-1* construct (16). Analysis of immunoprecipitations of in vitro translated ^{35}S-labeled Tbdn-1 with the mouse anti-Tbdn-1 serum MS.C10-20TD was as previously described (18).

Histology and immunohistochemistry of Tbdn-1 suppressed mice

Mouse tissues were fixed in 4% paraformaldehyde, embedded in paraffin or methacrylate resin and processed for histology and immunohistochemistry as previously described (18,20). Sections were stained with hematoxylin and eosin (H&E), toluidine blue or with

different antibodies. Antibodies used for immunohistochemistry included chicken anti-Tbdn-1 Ab1272 antibody (16), mouse anti-Tbdn-1 serum MS.C10-20TD, rabbit anti-VP-16 (Clontech, Palo Alto, California), rabbit anti-Von Willebrand Factor (vWF [DAKO, Glostrup Denmark]), rabbit anti-laminin (DAKO, Glostrup Denmark), mouse anti-glial fibrillary acidic protein (GFAP) and mouse anti-alpha smooth muscle actin (ASMA [Sigma, St. Louis, MO]). Mouse anti-basement membrane heparan sulfate proteoglycan (HSPG) antibodies C17 (21) and 33 (22) were obtained from the Developmental Studies Hybridoma Bank (University of Iowa). Immunohistochemical reactions were developed using species appropriate secondary antibody (Promega, Madison WI) conjugated to alkaline phosphatase or horseradish peroxidase. Sections destined for peroxidase development were previously incubated in 0.3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. Prior to incubation with anti-laminin, tissues were digested with 0.1% trypsin at 35°C for 20 minutes. Mouse antibodies were applied following a 30 minute incubation in rabbit anti-mouse blocking preparation.
The sections were counter-stained with methyl green and mounted in Permount.

Blood vessel counts

Blood vessels in mouse liver, kidney cortex, brain ventricle and testes were identified by immunostaining with vWF and/or laminin specific antibodies as described above. Pictures of ten random non-overlapping microscopic fields (250x magnification, 35200μm^2) within at least two independent sections for each treatment (two specimens per treatment) were captured and the number of blood vessels counted in each field. The total number of blood vessels for each tissue per specimen was calculated and compared

between transgenic specimens. Blood vessel counts in mouse retina and choroid were based on photographed H&E and Trichrome stained sections. Blood vessels from approximately ten microscopic pictures (400x) spanning each retina and choroid from control mice (n=7; Dox-induced *TIE2/rtTA/Enh* and wild-type mice) and pathological retinal lesions regions of Dox-induced *TIE2/rtTA/Enh-TRE/ASTBDN*-1 mice (n=5) and were counted for each retinal layer and standardized by dividing the blood vessel count by both the length and area of the corresponding retinal layer. Damaged pictures were removed from the analysis. Pictures were taken using a QImaging CCD camera and analyzed with Openlab software (Improvision, Guelph, ON).

2.5 RESULTS

Conditional knockdown of Tbdn-1 in endothelium of TIE2/rtTA/Enh-TRE/ASTBDN-1 bitransgenic mice

To explore the role of Tbdn-1 in endothelium *in vivo*, a binary antisense *TBDN-1* (*ASTBDN-1*) transgenic mouse model (*TRE/ASTBDN-1* and *TIE2/rtTA/Enh*) driven by the tetracycline responsive element (*TRE*) and the *rtTA* cDNA under the control of the *TIE2* endothelial promoter was generated to conditionally suppress Tbdn-1 expression upon doxycycline (Dox) treatment. The *TIE2* promoter confers transgene expression in all endothelial cells, including retinal endothelium *in vivo* (19). Expression of the rtTA protein in the vasculature of mouse lines identified to contain the *TIE2/rtTA/Enh* sequences was confirmed by western blot and immunohistochemical analysis of tissues such as the endocardium and bone marrow blood vessels compared to non-transgenic control mice (Fig. 2-1*a*, 2-1*b* and not shown). Mice harboring both *TIE2/rtTA/Enh* and

TRE/ASTBDN-1 transgenes were treated with Dox and analyzed for the efficacy to specifically suppress Tbdn-1 levels in endothelium. Dox-induced *TIE2/rtTA/Enh-TRE/ASTBDN-1* mice showed suppression of Tbdn-1 protein expression in both retinal blood vessels and bone marrow blood vessels compared to double transgenic non-induced animals or single transgene Dox-induced controls (Fig. 2-1*c-f* and not shown). To confirm that the knockdown of Tbdn-1 expression in the retinas of double induced transgenic mice does not result from the loss of retinal blood vessels, basal lamina of retinal blood vessels of either non-induced or Dox-induced *TIE2/rtTA/Enh-TRE/ASTBDN-1* were stained for laminin. Laminin staining of sections adjacent to those showing Tbdn-1 suppression revealed the presence of complex vascular networks, confirming specific Tbdn-1 suppression rather than the loss of retinal blood vessels in Dox-induced *TIE2/rtTA/Enh-TRE/ASTBDN-1* mice (Fig. 2-1*g* and 2-1*h*). Moreover, laminin staining of the basal lamina of retinal blood vessels indicated a focal increase in blood vessel density in Tbdn-1 suppressed retinas compared to control specimens (Fig. 2-1*g*, 2-1*h*, 2-5*a* and 2-5*b*).

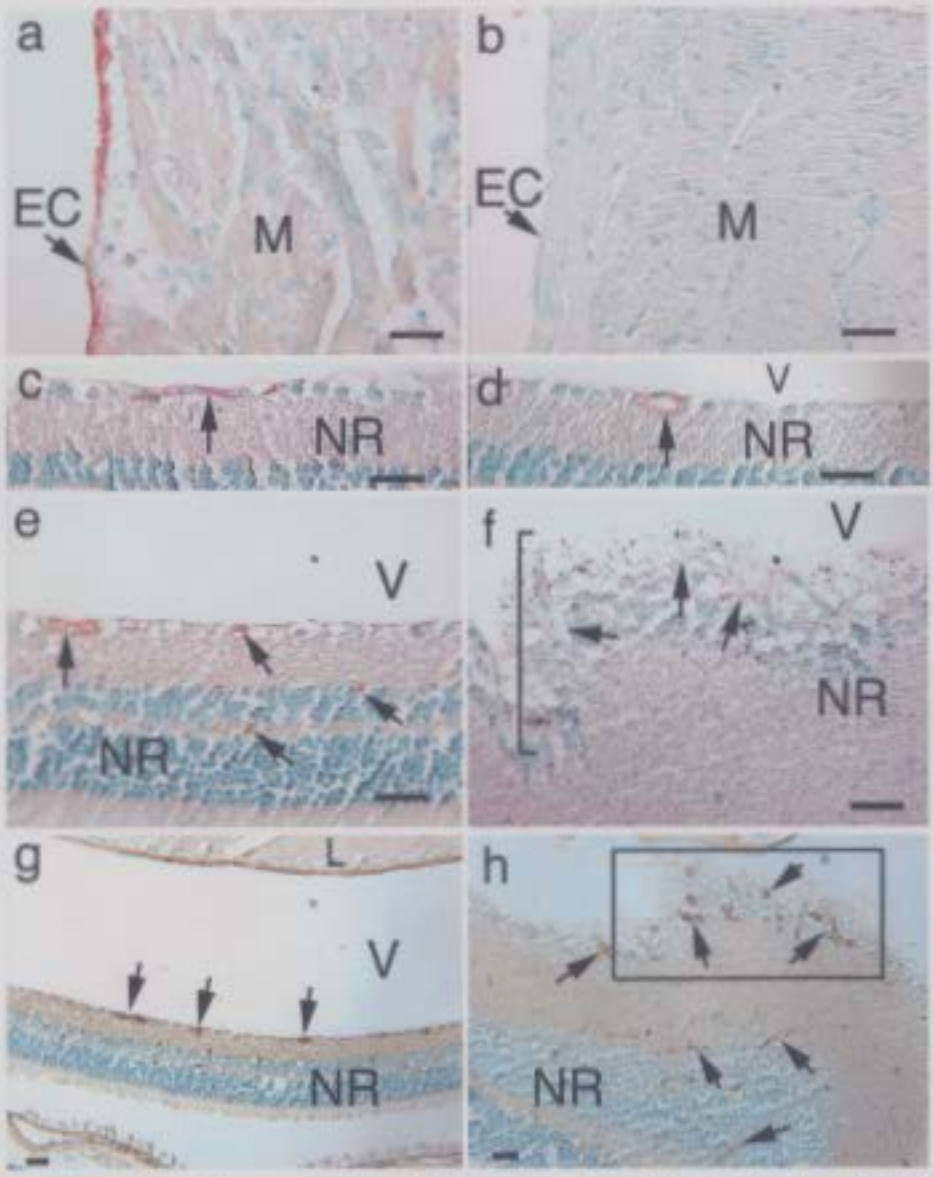

Figure 2-1: Conditional knockdown of Tbdn-1 in blood vessels of TIE2/rtTA/Enh-TRE/ASTBDN-1 bitransgenic mice

a and b, Immunohistochemical analysis of rtTA protein expression (positive red staining) in heart tissue from mice harboring the TIE2/rtTA/Enh construct (a) compared to non-transgenic mice (b) illustrate the successful incorporation of the rtTA transgene. Arrows point to endocardium (EC) and myocardium (M). c-f, Tbdn-1 immunohistochemical analysis (red staining) of retinal vessels in sections of 6 week Dox-induced TIE2/rtTA/Enh (c), 6 week Dox-induced TRE/ASTBDN-1 (d), non-induced TIE2/rtTA/Enh-TRE/ASTBDN-1 (e), and 6 week Dox-induced TIE2/rtTA/Enh-TRE/ASTBDN-1 (f) transgenics show a downregulation of the Tbdn-1 protein in double induced transgenics compared to control mice. Arrows in c-h point to retinal blood vessels and the bracket indicates a preretinal membrane, (V) vitreous, (NR) neural retina, (L) lens. g and h, Laminin staining (brown staining) of the basal lamina of retinal vessels of an adjacent section of the non-induced TIE2/rtTA/Enh-TRE/ASTBDN-1 specimen shown in e (g) and of an adjacent section of the 6 week Dox-induced TIE2/rtTA/Enh-TRE/ASTBDN-1 specimen shown in f (h) reveals the vascular network verifying the specific loss of Tbdn-1 expression as opposed to the absence of blood vessels. Arrows point to brown (horseradish peroxidase) laminin positive vessels. The boxed area in panel h is a low magnification view of a section adjacent of the pathology seen in f. Magnification: a, b, c, and d: (250X); e and f (100X). Counterstain in all panels is methyl green. Scale bars equal 25 μm.

Conditional knockdown of Tbdn-1 in endothelium results in preretinal fibrovascular lesions and retina-lens adhesions

Morphological assessment of retinas in Dox-induced *TIE2/rtTA/Enh-TRE/ASTBDN-1* mice exhibiting Tbdn-1 suppression in endothelial cells (n=15) revealed a phenotype characterized by the focal or localized formation of preretinal membranes displaying retinal neovascularization, fibrovascular growth and retina-lens adhesions (Fig. 2-1c-h, 2-6). Knockdown of Tbdn-1 in retinal blood vessels resulted in abnormal retinal blood vessel structures and patterns (Fig. 2-1h, 2-2c-f, 2-4, and 2-5b). The inner layers of the retina (GCL/IPL/INL; see Fig. 1-1) in Tbdn-1 suppressed mice showed a statistically significant ($p<0.05$) 3.0 fold increase in the number of retinal capillaries per length of retina compared to control retinas (Fig. 2-3). However, the number of retinal blood vessels per area of each retinal layer is not increased in mice exhibiting Tbdn-1 suppression in comparison with control mice due to retinal thickening corresponding with the retinal neovascularization. There was no significant difference in the number of blood vessels in the outer retina layers (OPL/ONL/PHR; see Fig 1-1) or the choriocapillaries in Tbdn-1 suppressed mice compared to control mice (Fig 2-3). Retinal and vitreal tissues of Tbdn-1 suppressed mice contained tortuous, highly branched vasculature enlaced in a fibrovascular material extending beyond the vitreo-retinal boarder (Fig. 2-1h, 2-2c-f, 2-4, and 2-5b). Some capillaries within the retinal lesions appeared abnormally elongated and distended. In contrast, both Dox-induced TRE/ASTBDN-1 and Dox-induced TIE2/rtTA/Enh control mice (n=25), as well as non-induced TIE2/rtTA/Enh-

TRE/ASTBDN-1 mice (n=6) did not show Tbdn-1 suppression or any ocular pathology (Fig. 2-1c-e, 2-1g, 2-2a, 2-2b, 2-4a, 4c,2- 4e, 2-5c, and 2-5d).

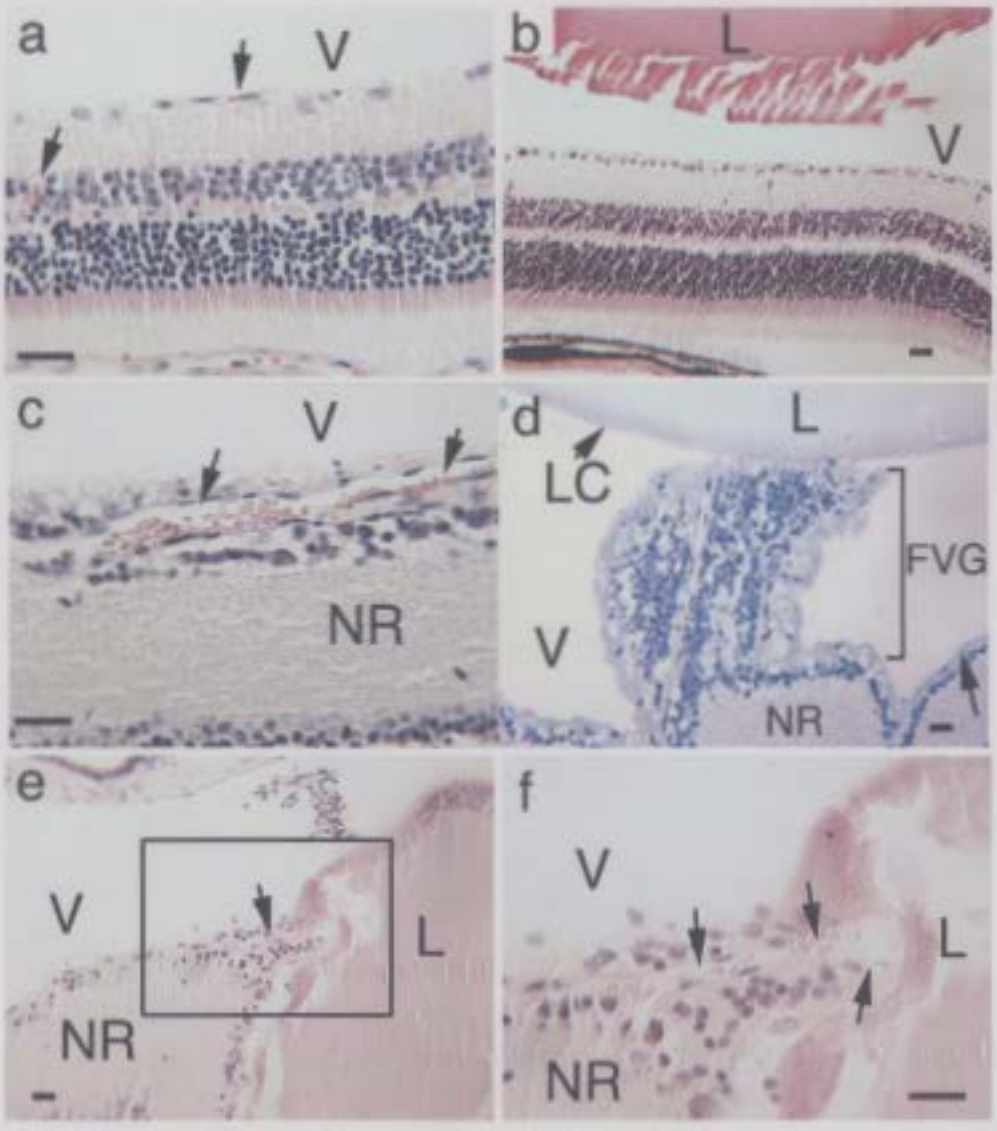

Figure 2-2: Endothelial suppression of Tbdn-1 expression results in retinal neovascularization, preretinal fibrovascular lesions and retina-lens adhesions

a and b, Control 6 week Dox-induced TRE/ASTBDN-1 transgenic (a) and non-induced *TIE2/rtTA/Enh-TRE/ASTBDN-1* bitransgenic (*b*) do not show any retinal pathology. The arrows point to normal retinal blood vessels from a control mouse. ***c***, In striking contrast, Dox-induced (12 week) *TIE2/rtTA/Enh-TRE/ASTBDN-1* mice present large abnormal distended vessels extending along the retina (arrows in panel *c*). ***d***, Dox-induced (10 week) *TIE2/rtTA/Enh-TRE/ASTBDN-1* retinas also exhibit fibrovascular growth extending from the inner limiting membrane (arrow in lower right corner) and adhering to the lens capsule. ***e*** and ***f***, Tbdn-1 suppressed retinal vessels (arrows) from Dox-induced (6 week) mice also invade through the lens capsule. ***f***, Magnified view of the boxed area in *e* showing the blood vessel (arrows) invading through the lens capsule and extending into the lens. Magnification: *a*, *c*, and *f* (250X); *b*, *d*, and *e*: (100X). H & E staining (*a*, *b*, *c*, *e* and *f*); Toluidine blue staining (*d*). (L), lens; (LC), lens capsule; (V), vitreous; (NR), neural retina; (FVG), fibrovascular growth. Scale bars equal 25 μm.

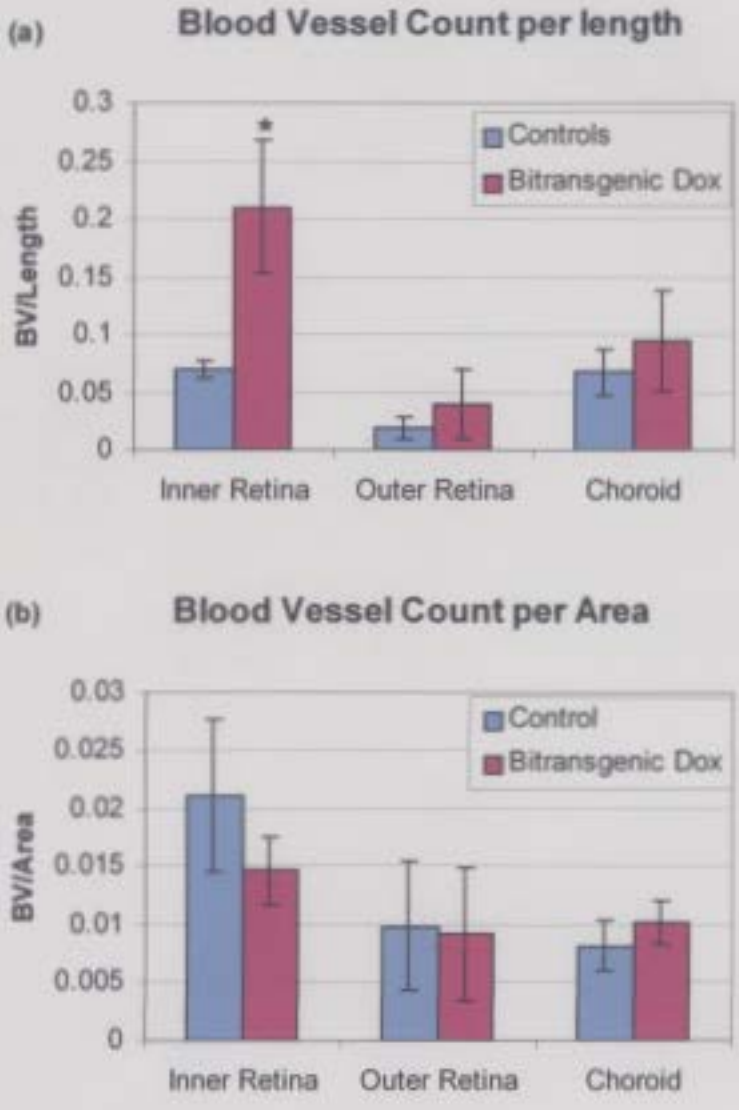

Figure 2-3: The number of blood vessels per length of retina in Tbdn-1 suppressed mice is increased in the inner retina compared to control retinas.

(a) Retinal sections from *TIE2/rtTA/Enh-TRE/ASTBDN- 1*(Bitransgenic) mice (n=5) induced with Dox for 6-8 weeks show a significant increase ($p<0.05$) in the number of blood vessels in the inner retina layers (GCL/IPL/INL) per retinal length compared to Dox-induced single transgenic control retinas and wild-type Dox-induced mice (n=7). There is no significant difference ($p>0.05$) in the number of blood vessels in the outer retina (OPL/ONL/PHR) or choroid in Bitransgenic Dox-induced mice compared to control mice. (b) Bitransgenic mice induced with Dox for 6-8 weeks do not show an increase in the number of retinal or choroidal blood vessels per area compared to control mice. Bars represent standard deviations. Asterisk denotes significance.

The retinal pathology in Dox-induced *TIE2/rtTA/Enh-TRE/ASTBDN-1* mice increased in severity with prolonged suppression of endothelial-derived Tbdn-1 expression (Fig. 2-4), while control mice did not show retinal pathology with prolonged Dox induction. *TIE2/rtTA/Enh-TRE/ASTBDN-1* mice induced with Dox for 2 weeks presented with a focal pathology including thickening of the retina and the initial formation of a preretinal membrane harboring neovascular elements (Fig. 2-4*a* and 2-4*d*). Six weeks after suppression of Tbdn-1 in endothelium, the retinal fibrovascular lesions increased in size (Fig. 2-4*b* and 2-4*e*) with some specimens exhibiting retinal-lens adhesions and penetration of aberrant blood vessels through the lens capsule into the lens (Fig. 2-2*e* and 2-2*f*). At a period of 10 to 12 weeks of Tbdn-1 suppression, the fibrovascular lesions of the retina often filled the vitreal space and adhered to the lens (Fig. 2-2*d*, 2-4*c* and 2-4*f*).

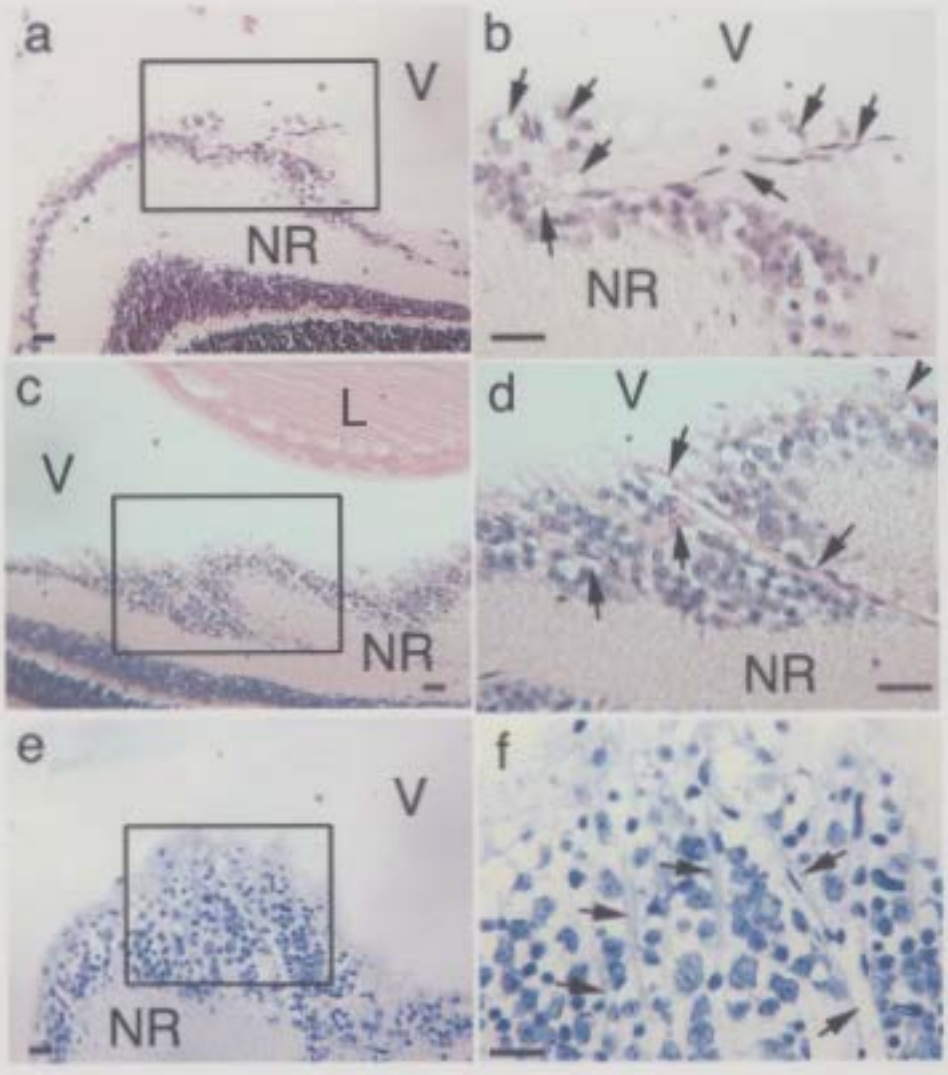

Figure 2-4: Retinal lesions increase in severity with prolonged Tbdn-1 suppression

a-f. Retinas from *TIE2/rtTA/Enh-TRE/ASTBDN-1* mice after two weeks of Dox induction (*a* and *b*) show evidence of nascent blood vessels and fibrovascular proliferation. The severity of pathology of retinal lesions increases with Dox-induction of six weeks (*c* and *d*) and ten weeks (*e* and *f*). The arrows point to neovascularization occurring within the fibrovascular lesions. The boxes in *a*, *c*, and *e* indicate areas of pathology shown at higher magnification in *b*, *d*, and *f*, respectively. Magnification: *a*, *c*, and *e*: (100X); *b*, *d*, and *f*: (250X). H & E staining (*a-d*); Toluidine blue staining (*e* and *f*). (L), lens; (V), vitreous; (NR), neural retina. Scale bars equal 25 μm.

Tbdn-1 suppressed retinal lesions display abnormal distribution of ECM, myofibroblasts and glial cells

To further characterize the fibrovascular lesions associated with endothelial knockdown of Tbdn-1, expression patterns of ECM components were analyzed by

immunohistochemistry in Dox-induced *TIE2/rtTA/Enh-TRE/ASTBDN-1* mice. Laminin expression was utilized as a marker to label the abnormal retinal vascular network in Dox-induced *TIE2/rtTA/Enh-TRE/ASTBDN-1* mice as well as normal blood vessels of control retinas (Fig. 2-5). Sections adjacent to those stained for laminin were analyzed for the expression of basement membrane HSPG using two different anti- basement membrane HSPG monoclonal antibodies (C17 and 33). Results using both anti-HSPG antibodies revealed that the expression of basement membrane HSPG in blood vessels comprising Tbdn-1 suppressed retinal lesions was very low or un-detected compared to HSPG expression in normal retinal blood vessels expressing normal levels of Tbdn-1 (results using antibody C17 shown in Fig. 2-5*c* and 2-5*d*). Furthermore, knockdown of Tbdn-1 expression in endothelium resulted in a global decrease in retinal basement membrane HSPG expression, including retinal blood vessels localized outside the fibrovascular lesions in areas where the retinal morphology appeared normal (Fig. 2-5*e* and 2-5*f*).

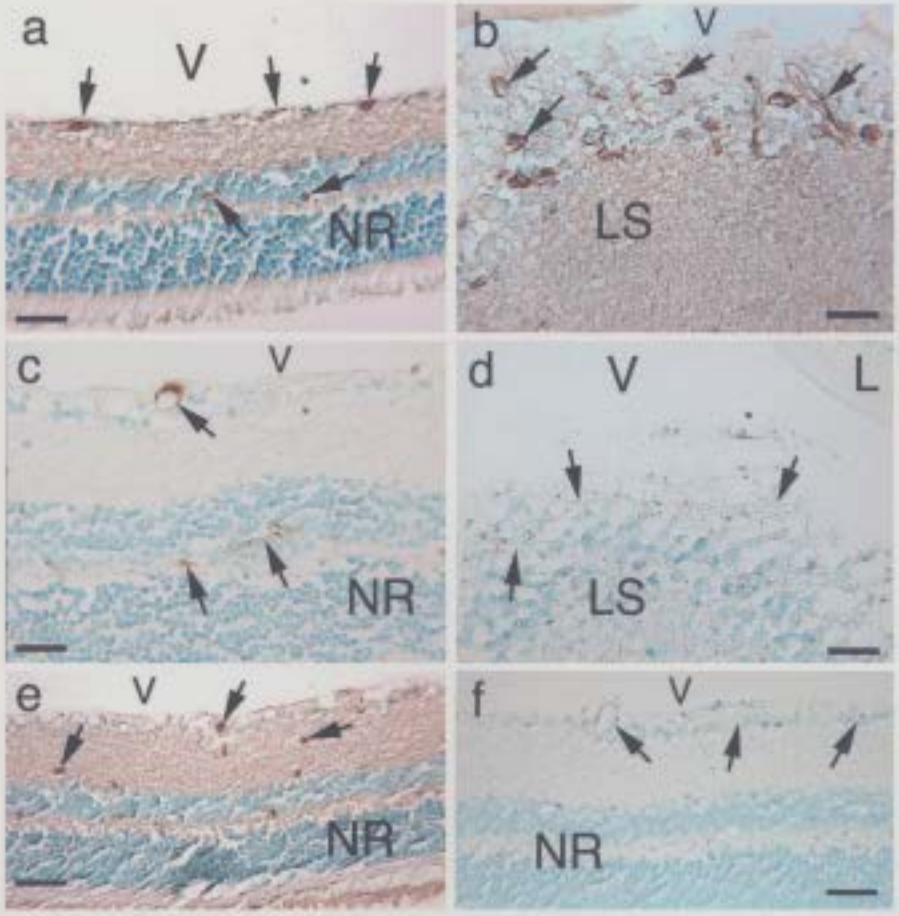

Figure 2-5: Endothelial knockdown of Tbdn-1 results in suppression of HSPG expression in retinal blood vessels

a and ***b***, Laminin staining (brown horseradish peroxidase staining) labels the basal lamina of vessels (arrowed) revealing the vascular networks present in the retina of both non-induced control (*a*) and 6 week Dox-induced *TIE2/rtTA/Enh-TRE/ASTBDN-1* mice (*b*). Laminin expression patterns reveal an increase in retinal blood vessels in Dox-induced *TIE2/rtTA/Enh-TRE/ASTBDN-1* mice showing suppressed Tbdn-1 levels (*b*). ***c*** and ***d***, In contrast to normal vessels in non-induced *TIE2/rtTA/Enh-TRE/ASTBDN-1* retina (*c*), vessels (arrowed) present in the retinal fibrovascular lesions of Tbdn-1 suppressed mice (6 week Dox-induced *TIE2/rtTA/Enh-TRE/ASTBDN-1*) show low or no staining (brown staining) for HSPG (*d*). ***e*** and ***f***, Ocular vessels outside fibrovascular lesions (arrowed) in Dox-induced *TIE2/rtTA/Enh-TRE/ASTBDN-1* mice also show normal levels of laminin staining (*e*) but suppressed levels of HSPG (*f*) compared to control mice expressing normal levels of Tbdn-1 (*a* and *c*, respectively). Magnification: *a-f* (250X). Counterstain in all panels is methyl green. (V), vitreous; (NR), neural retina; (L), Lens; (LS), retinal lesion. Scale bars equal 25 μm.

To further characterize the cell types present in the retinal lesions of Tbdn-1 suppressed mice, retinas of Dox-induced *TIE2/rtTA/Enh-TRE/ASTBDN-1* mice were analyzed for the expression of ASMA, a myofibroblast cell marker, and GFAP, a glial

cell marker. Expression of ASMA was detected in the fibrovascular lesions of Dox-induced *TIE2/rtTA/Enh-TRE/ASTBDN-1* mice (Fig. 2-6*a*). ASMA positive staining in the retinal lesions appeared to be concentrated around larger blood vessels in a continuous pattern, rather than compact staining of small retinal capillaries that would indicate the normal pericytic coverage of vessels. ASMA staining outside the retinal lesions in Tbdn-1 suppressed animals displayed an expression pattern similar to pericyte staining in control retinas (Fig. 2-6*c* and data not shown). Fibrovascular retinal lesions resulting from Tbdn-1 suppression also showed an increase in the number of cells positive for GFAP staining compared to control retinas expressing normal levels of Tbdn-1 (Fig. 2-6*b* and 2-6*d*). However, the increase in GFAP positive cell number in Tbdn-1 suppressed retinas was restricted to the fibrovascular lesions (data not shown).

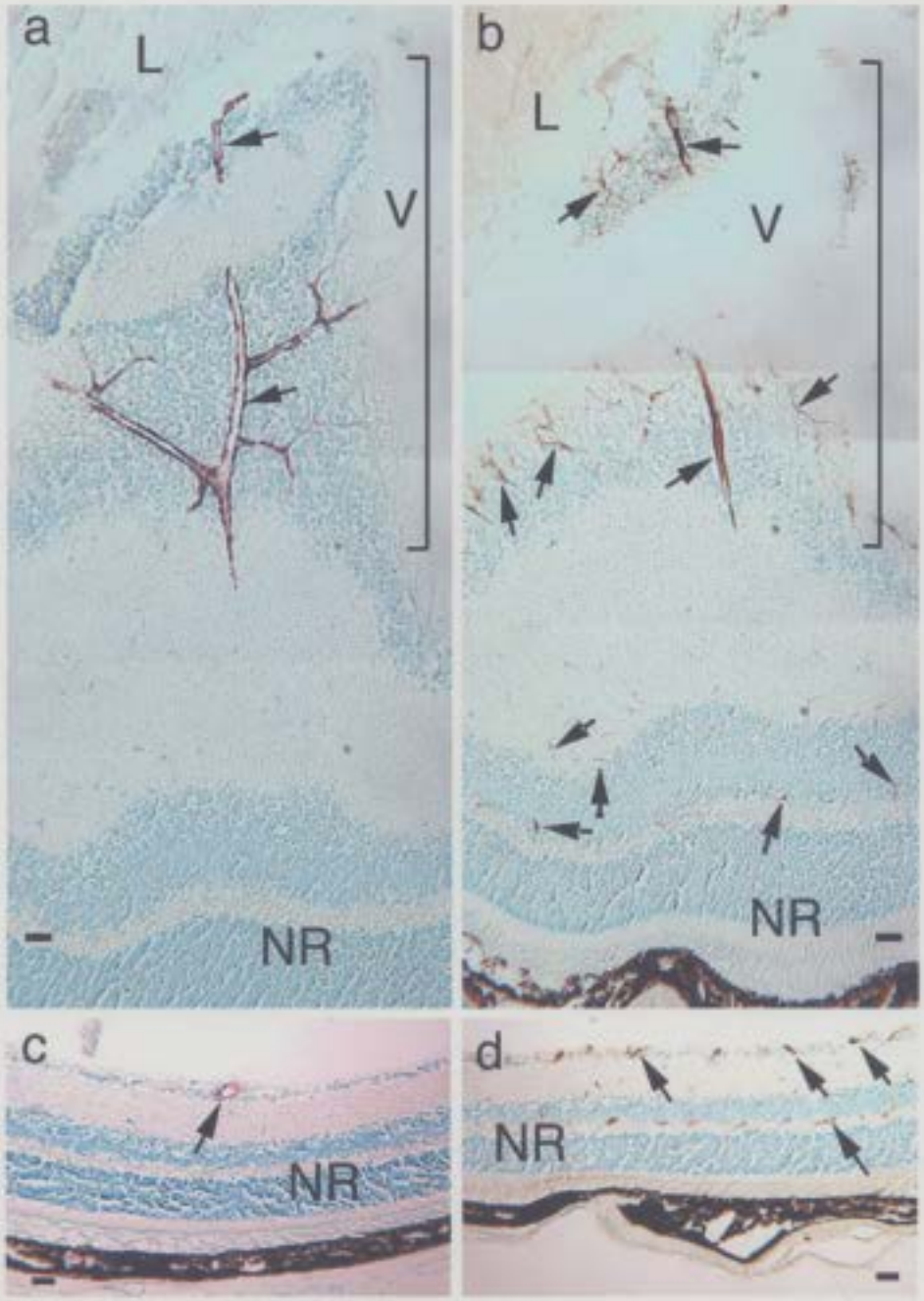

Figure 2-6: Fibrovascular lesions in Tbdn-1 suppressed retinas contain myofibroblasts and glial cells

a and ***c,*** ASMA immunostaining (brown reddish staining, arrowed) of a fibrovascular retinal lesion from a 6 week Dox-induced *TIE2/rtTA/Enh-TRE/ASTBDN-1* mouse (*a*) reveals large abnormal vessels containing myofibroblast cells (bracket) while immunostaining outside the lesion displays a normal pattern (*c*). ***b*** and ***d***, GFAP immunohistochemistry (brown horseradish peroxidase staining) of retinas from 6 week Dox-induced *TIE2/rtTA/Enh-TRE/ASTBDN-1* mice (*b*) reveal an increase in the number of glial cells (arrowed) in fibrovascular tissue (bracket) of retinal lesions compared to control retina expressing normal levels of Tbdn-1 (*d*). Magnification: *a-d* (100X). Counterstain in both panels is methyl green. (V), vitreous; (NR), neural retina; (L), lens. Scale bars equal 25 μm.

Pathology associated with the endothelial knockdown of Tbdn-1 is limited to the retina

Since the *TIE2* pan endothelial promoter was utilized for targeting expression of antisense *TBDN-1* exclusively to all endothelial cells, it is conceivable that the vasculature of tissues other than the retina might be susceptible to the potentially deleterious effects of Tbdn-1 suppression. To address this issue, several adult tissues of Dox-induced *TIE2/rtTA/Enh-TRE/ASTBDN-1* mice were analyzed in comparison to controls for pathology and for blood vessel integrity using endothelial cell and blood vessel markers. All other adult tissues examined (heart, bone marrow, salivary gland, lung, spleen, liver, kidney, brain, and testes) showed no discernable gross or histological pathology of the tissues or blood vessels (not shown). Kidney, liver, brain, and testes from Dox-induced *TIE2/rtTA/Enh-TRE/ASTBDN-1* mice all showed no difference in overall blood vessel density compared to controls (Fig. 2-7 and not shown). Therefore, the neovascularization as well as the pathological changes evident in Dox-induced *TIE2/rtTA/Enh-TRE/ASTBDN-1* mice appear to be specific to the retina.

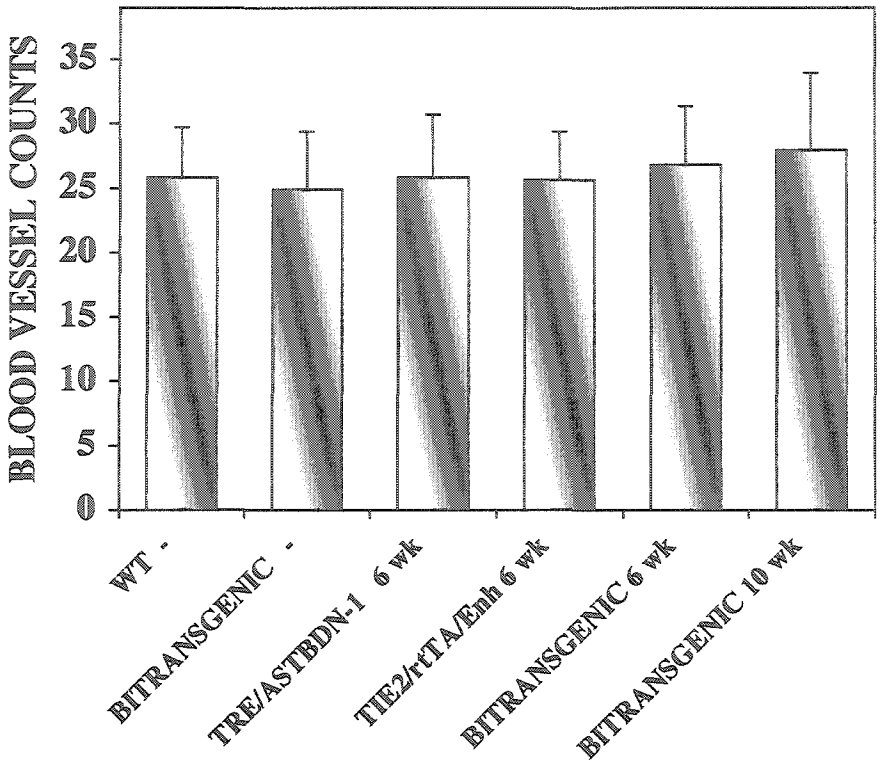

Figure 2-7: Blood vessel density in kidneys of Tbdn-1 suppressed mice is similar to control mice

Kidney sections from Dox-induced *TIE2/rtTA/Enh-TRE/ASTBDN-1* mice at both 6 and 10 weeks of Dox induction (BITRANSGENIC 6 wk and BITRANSGENIC 10 wk, respectively) showed no difference in overall blood vessel density compared to non-induced wild-type (WT -), non-induced *TIE2/rtTA/Enh-TRE/ASTBDN-1* (BITRANSGENIC -), and 6 week Dox-induced single transgenic (TRE/ASTBDN-1 6 wk and TIE2/rtTA/Enh 6 wk) controls as indicated. Bars represent standard deviations.

2.6 DISCUSSION

The suppression of Tbdn-1 expression in mouse endothelium results in several pathological features observed in human proliferative retinopathies including retinal neovascularization, preretinal fibrovascular proliferation and retinal-lens adhesions. Manipulation of specific protein expression has previously been demonstrated to induce retinal neovascularization *in vivo*. Previously described models of neovascular retinopathy include the overexpression of specific growth factors. Transgenic mouse models conferring VEGF overexpression in the retina have proven sufficient to cause retinal neovascularization which progresses to tractional retinal detachment (23-24). Transgenic mice overexpressing PDGF-B in photoreceptors soon after birth develop retinal lesions harboring glial, endothelial and pericyte elements also leading to retinal detachment (25). In contrast to the ocular specific phenotype resulting from Tbdn-1 suppression, the effects of endothelial ablation of PDGF-B and over-expression of VEGF are not restricted to the ocular vascular bed with consequences observed in other vascular networks (26-29). In the adult, high levels of Tbdn-1 expression are restricted to specialized vasculature including ocular blood vessels, bone marrow capillaries, atrial endocardium and blood vessels of regressing ovarian follicles (16-17). The restriction of pathological changes to the retina in Dox-induced *TIE2/rtTA/Enh-TRE/ASTBDN-1* mice indicates that the effects resulting from Tbdn-1 suppression in endothelium is highly specific to retinal blood vessels. The lack of pathology in other adult tissues might be explained either by the absence of Tbdn-1 expression in the corresponding vascular bed (16-17) or by the absence of a critical binding partner or cofactor important for mediating

Tbdn-1 activity in the given tissue. Such possible binding partners might include mammalian orthologues of the yeast ARD1 acetyltransferase (30-31). Possible mechanisms of action of the Tbdn-1 pathway may include affecting the stability and/or activity of proteins involved in regulating angiogenesis through acetylation. Recent studies indicate that the mammalian acetyltransferase ARD1 plays a role in the targeted degradation of HIF-1α (32). HIF-1α regulates VEGF expression during hypoxia (33). Therefore, acetyltransferase complexes involving ARD1 or Tbdn-1 represent new candidates for upstream regulators of cytokines and growth factors which could control retinal neovascularization. Upstream regulators of signaling cascades controlling angiogenesis in the retina could offer new molecular based therapeutic targets for treating neovascular retinopathies.

In the present study, we find that the proliferative retinopathy resulting from suppression of Tbdn-1 expression in endothelium in mice is accompanied by a global decrease of basement membrane HSPG expression in retinal blood vessels. Although the thickening and alteration of the vascular basement membrane are well known features associated with proliferative retinopathies, the mechanisms that regulate these processes are not completely elucidated (12, 34). Basement membrane ECM constituents provide a tissue specific barrier between endothelial cells and underlying connective tissues and participate in controlling angiogenesis (35). Altered expression patterns of ECM constituents could promote the disorganization and breakdown of vascular basement membranes and eventually lead to endothelial cell proliferation (35). The loss of HSPG from basement membranes has been implicated in the breakdown of blood vessel

integrity in diabetic nephropathy (36-38). Moreover, retinas of diabetic rats show a decrease in the synthesis and expression of the HSPG perlecan (39). HSPG can regulate the activity of angiogenic factors (e.g. VEGF, bFGF) by regulating the binding to their respective receptor on endothelial cells (40-41). Our results indicate that Tbdn-1 is an upstream regulator of HSPG expression in blood vessel basement membranes in the retina. Currently, it is not clear if suppression of basement membrane HSPG in retinal blood vessels of Tbdn-1 suppressed mice is the result of a reduction in HSPG synthesis or an increase in HSPG degradation. Future studies will focus on the identification of specific basement membrane HSGPs regulated by Tbdn-1 and the mechanisms of action underlying this process.

Although retinal detachment is common in later stage PDR, in rodents retinal detachment is rare (42). Nevertheless, the retinal pathology in Dox-induced *TIE2/rtTA/Enh-TRE/ASTBDN-1* mice displays several features which could promote tractional retinal detachment. The Tbdn-1 suppressed retinas display retinal-lens adhesions and invasion of the lens capsule and lens by blood vessels from the adjacent fibrovascular lesional tissue. Also, the fibrovascular lesions in Tbdn-1 suppressed retinas show an increase in the number of cells expressing the glial marker GFAP compared to control retinas. In conjunction with an increase in GFAP-positive cells, the myofibroblastic marker ASMA is increased in large caliber blood vessels in the Tbdn-1 suppressed retinal lesions. Myofibroblasts expressing ASMA and glial cells expressing GFAP are thought to exert contractile forces which promote tractional retinal detachment in neovascular retinopathy (43-49). The presence of ASMA expressing myofibroblasts

and GFAP expressing glial cells in Tbdn-1 suppressed lesions supports the view that the exertion of contractile forces and subsequent tractional retinal detachment is possible in the advanced retinal lesions of Tbdn-1 suppressed mice. However, this cannot be confirmed histologically due to potential artifacts inferred by tissue processing.

The conditional knockdown of Tbdn-1 in endothelium in the *TIE2/rtTA/Enh-TRE/ASTBDN-1* bitransgenic model clearly provides evidence for the necessity of Tbdn-1 expression for the maintenance of adult retinal blood vessel homeostasis by suppressing abnormal retinal blood vessel growth. The phenotype associated with Tbdn-1 suppression in endothelium resembles the sight disrupting pathology observed in human proliferative retinopathy. This, together with our previous evidence of Tbdn-1 suppression in the retinal endothelium in patients with PDR (17), supports the hypothesis of a contributing role for loss of Tbdn-1 expression in the progression of proliferative retinopathies. Tbdn-1 may serve as a valuable pathway for developing new therapies aimed at controlling proliferative retinopathies.

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3 SUMMARY

Blinding neovascular ocular diseases are characterized by a disparity in the equilibrium between proangiogenic and antiangiogenic molecular determinants. A molecular shift favoring a pro-angiogenic microenvironment stimulates neovascularization, fibrovascular scar formation and retinal detachment, all hallmarks of several common sight threatening diseases such as PDR, the "wet" form of macular degeneration and ROP. A comprehensive understanding of the molecular mechanisms driving the pathophysiology resulting in these phenotypic responses is necessary in preventing and treating neovascular ocular disease.

Previous *in vitro* and *in vivo* evidence suggest that Tbdn-1 functions as a homeostatic modulator during the maturation and quiescence of retinal vascular networks (Gendron *et al.* 2000; Gendron *et al.* 2001; Paradis *et al.* 2002). Tbdn-1 is suppressed in neovascular areas of retinal endothelium in patients with diabetic retinopathy, the leading cause of blindness in the working age population in developed nations (Gendron *et al.* 2001). For the purpose of investigating the role of Tbdn-1 in retinal endothelium *in vivo*, a mouse model was developed utilizing the Dox controlled tet-on system and taking advantage of the tie2 pan endothelial promoter for targeting expression of an ASTBDN-1 cDNA exclusively to endothelial cells (Sato *et al.* 1993). Confirmation of the efficacy of this system to down-regulate Tbdn-1 levels in endothelium was accomplished using immunohistochemical analysis of rtTA and Tbdn-1 protein expression.

The present study has reinforced our hypothesis that Tbdn-1 regulates retinal blood vessel homeostasis by demonstrating *in vivo* the retinal consequences of specifically

suppressing Tbdn-1 protein expression in endothelium. Knockdown of Tbdn-1 protein expression in endothelial cells results in the formation of pre-retinal membranes, retinal neovascularization, and cellular proliferation of contractile cells such as glial and myofibroblastic cell types. This phenotype potentially promotes vision loss in several ways. The formation of neovascular lesions extending from the inner retina and filling the vitreous chamber may structurally obscure the passage of light photons through the lesioned retina to the sensory photoreceptor cells. The pathological blood vessels constituting the lesions are likely leaky, as the capillaries are distorted and specifically down-regulate basement membrane constituent HSPG, resulting in leakage of fluid from the capillaries into the retina and vitreous promoting vitreal hemorrhage. Also, the presence of the contractile cell types may promote the traction of the retina thereby tearing the retina from the RPE. However, the definitive diagnosis of retinal detachment in mice is difficult due to potential artifacts during tissue processing (Smith 2002). This work implicates Tbdn-1 as an important regulatory protein in retinal vascular biology *in vivo*. Tbdn-1 expression in adult retinal endothelium is a necessary negative modulator of blood vessel remodeling. Loss of the Tbdn-1 protein in retinal capillaries may promote endothelial cell outgrowth to form vascular networks.

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