THE ROLE OF TUBEDOWN ON THE PERMEABILITY OF RETINAL ENDOTHELIAL CELLS

THASIN JACKIE ISLAM

THE ROLE OF TUBEDOWN ON THE PERMEABILITY OF RETINAL ENDOTHELIAL CELLS

By

©Thasin Jackie Islam

A thesis submitted to the School of Graduate Studies in partial fulfillment of

the requirements for the degree of

MASTER OF SCIENCE

Division of BioMedical Sciences

Faculty of Medicine

Memorial University

August 2008

St. John's Newfoundland & Labrador

ABSTRACT

Tubedown (Tbdn) is a mammalian homologue of the N-terminal acetyltransferase subunit NAT1 of *S. cerevisiae* and copurifies with an acetyltransferase activity. Previous studies have demonstrated a suppression of Tbdn in the retinal blood vessels of patients with neovascular retinopathy including proliferative diabetic retinopathy (PDR) and retinopathy of prematurity (ROP). Moreover, conditional endothelial knockdown of Tbdn in the mouse blood vessel leads to retinal lesions similar to neovascular retinopathy, characterized by abnormal retinal vascular structures, an increase in numbers of retinal blood vessels, and thickening of the retinal tissues. These results indicate that Tbdn is required for retinal homeostasis. Means to restore Tbdn expression and/or activity may be useful for treating neovascular retinopathy. Recent evidence from our lab suggests that Tbdn binds to the actin binding protein cortactin. Tbdn also has been shown to co-localize with the actin cytoskeleton. The actin cytoskeleton is involved in regulating cell permeability and tight junctions. A change in the permeability of endothelial cells has been shown to occur in neovascular PDR. In addition, tight junctions that are important for the blood-retinal barrier are also disrupted during the process of neovascularization. Therefore, this study investigated whether a decreased level of Tbdn increases the permeability of retinal endothelial cell layers. Our results indicate that knockdown of Tbdn expression in endothelial cells leads to a significant increase in cellular permeability measured by transit of FITC-albumin across a monolayer of retinal endothelial cells. Similarly, under in vivo conditions the extravasation of albumin was seen in retinal blood vessels of mice wherein Tbdn was conditionally knocked down in

the endothelium. Based on our findings we are proposing a model in which Tbdn act in concert with cortactin to regulate the permeability of FITC-albumin in the retinal endothelial cells via its conserved domains. Our results suggest that loss of Tbdn could have clinically relevant significance and Tbdn may be used as a future drug target.

ACKNOWLEDGEMENTS

I would like to express profound gratitude to my supervisors Drs. Robert Gendron and Hélène Paradis for their continuous support, guidance and invaluable advices during the course of this project. Without them this project would not have been at this stage. I would also like to extend thanks to the other members of my supervisory committee, Dr. Ken Hirasawa and Dr. Kenneth Kao for their helpful suggestions and important insights during the initial stages of this project and for the evaluation of this thesis.

I would like to thank the School of Graduate Studies for financial assistance and for awarding me the title "Fellow of the School of Graduate Studies 2007-2008" and Research and Graduate Studies at the Faculty of Medicine for honouring me with the Alfred Burness Graduate Student Award.

Many thanks to my fellow graduate students Adam Green and Darryl Martin for their helpful suggestions during the period of my study. Thank you to our research assistants Dr. Ewa Miskeiwicz and Stephanie Tucker for always being there when I needed them. Thanks to Jackie Walker for helping with all the histological work.

I am grateful to Dr. Daniel Macphee for his expert suggestions on the use of fluorescence microscopy.

Thank you to all my family and friends whom I love dearly back home in Bangladesh and here in Canada. My appreciation also goes to my parent-in-laws, bothers-in laws and sisters-in laws for their guidance and well wishes.

Finally, I want express my deepest gratitude to my beloved parents for their unconditional love and support. I will be forever indebted to you mom and dad for everything you have done for me! I want to thank my brothers Jilan and Jumar.

I want to express heartfelt thanks to my husband Shahid for being a constant source of encouragements and inspirations. Thank You!


DEDICATED TO MY BELOVED PARENTS
MR. RAFIQUL ISLAM
&
MRS. BULBUL AKTHER
AND
MY WONDERFUL HUSBAND
DR. SHAHIDUL ISLAM

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

AMD	Age-related macular degeneration
ARD1	Arrest Defective Protein 1
ASTBDN	Antisense Tubedown
BBB	Blood-Brain Barrier
BRB	Blood-Retinal Barrier
CBV	Choroidal Blood Vessel
DAPI	4',6-Diamidino-2-Phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DOX	Doxycycline
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate-Conjugated Albumin
GCL	Ganglion Cell Layer
INL	Inner Nuclear Layer
IPL	Inner Plexiform Layer
NAT-1	N-terminal Acetyltransferase

NFL	Nuclear Fiber Layer
ONL	Outer Nuclear Layer
OPL	Outer Plexiform Layer
PBS	Phosphate Buffered Saline
PDR	Proliferative Diabetic Retinopathy
RBV	Retinal Blood Vessel
ROP	Retinopathy of Prematurity
RPE	Retinal Pigment Epithelium
TBDN	Tubedown
TRITC	Tetramethyl Rhodamine Isothiocyanate
V	Vitreous Body
VEGF	Vascular Endothelial Growth Factor
ZO-1	Zonula Occludens

List of Publications from this Thesis

1. Paradis H, **Islam T**, Tucker S, Tao L, Koubi S, Gendron RL. Tubedown associates with cortactin and controls permeability of retinal endothelial cells to albumin. *J Cell Sci.* 2008 Jun 15; **121**(Pt 12): 1965-72.

Co-authorship Statement

This research project was completed under the supervision of Drs. Paradis and Gendron. Dr. Paradis and Thasin Islam are jointly responsible for generating the data for Figure 3-1. Thasin Islam is responsible for generating the data for Figures 3-2, 3-3, 3-4, 3-5 and 3-6. Dr. Ewa Miskeiwicz and Thasin Islam assisted Dr. Gendron experimentally for generating the data for Figure 3-7. The following Figures from this thesis were published in the above reference: Figures 3-1, 3-2, 3-3, 3-4, 3-6 and 3-7. Some texts from the above manuscript may be found in this thesis which was jointly written by Dr.Paradis, Dr. Gendron and Thasin Islam.

CHAPTER 1

INTRODUCTION

1.1 Retina and Neovascular pathology/diseases

1.1.1 Anatomy of the retina

The retina, being a highly differentiated organ, plays a major role in the visual system. It is located between the vitreous body (V) and the retinal pigmented epithelium (RPE) and is composed of seven layers; the nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), and finally the outer segments of rods and cones (Fig. 1-1). The retina is supplied by two major vascular structures; the choroidal circulation (CBV) and the retinal vasculature (RBV) (Fig.1-1). The retina also contains a blood-retinal barrier (BRB), which is similar to the blood-brain barrier (BBB) to maintain a neutral environment and to protect the neural retina from the circulating blood (Hosoya and Tomi, 2005). BRB can be further subdivided into two parts forming complex tight junctions of retinal capillary endothelial cells (forming inner BRB) and retinal pigment epithelial cells (forming outer BRB). BRB is responsible for regulating osmotic balance, ionic concentration and transport of nutrients, including sugars, lipids, and amino acids (Erickson, 2007). Similar to the BBB, the adjacent endothelial cells lining the BRB form

a tight barrier junction to limit the permeability of fluids and solutes to the minimum (Harhaj and Antonetti, 2004). Breakdown of the BRB is seen in a number of diseases including retinal diseases with neovascularization (Harhaj and Antonetti, 2004; Erickson et al., 2007), which are usually associated with increased tissue permeability (Harhaj and Antonetti, 2004). Neovascularization involves angiogenesis or formation of new blood vessels from pre-existing vessels. This is a complex process that requires multiple steps. Briefly, during the initial phase of angiogenesis activated blood vessels are vasodilated with increased vascular permeability; surrounding matrix is then degraded leading to endothelial cell proliferation and subsequent migration (Erickson et al., 2007).

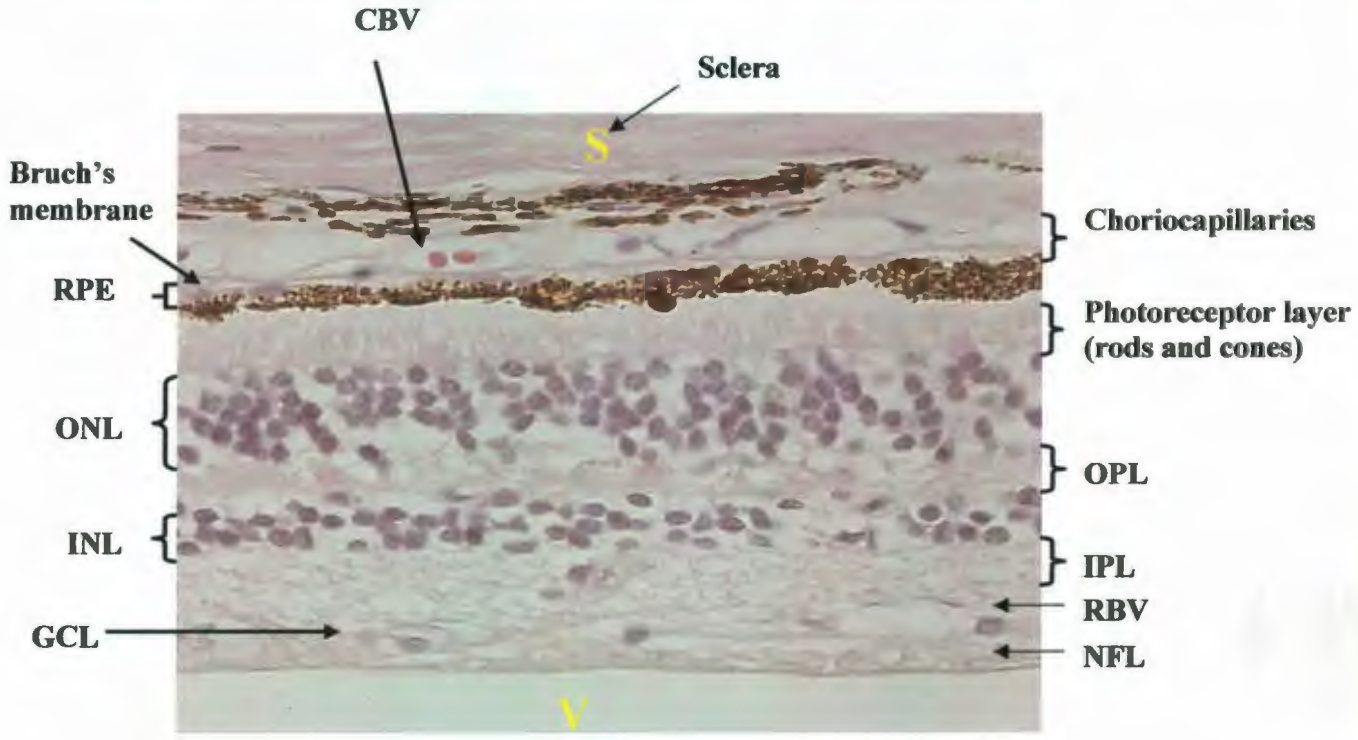

Figure 1-1. Structural features of the human eye. Hematoxylin and Eosin staining at 400X showing different cell layers that comprise the retina (See text above for details).

1.1.2 Ocular Neovascularization

A number of ocular diseases share neovascularization as a common underlying pathology leading to vision loss. The most common among these are the proliferative diabetic retinopathy (PDR), age-related macular degeneration (AMD) and retinopathy of prematurity (ROP). Together these three diseases are the leading cause of blindness in North America (Bradley et al., 2007; Dorrell M et al., 2007) affecting people of all ages. PDR affects mostly adults of working age and is a complication resulting from diabetes mellitus. In the early and non-proliferative stage of the disease there is vasodilation, increased blood flow, tissue (macular) edema, and vascular permeability occurring (Gardner et al., 2002). However, as the disease progresses to a more advanced phase there is growth of new vessels which again have increased leakiness and leads to blurred vision and edema (Erickson et al., 2007). In the final stages of the disease, detachment of the retina may occur causing severe sight-loss. In adults of >65years, AMD is the prevalent cause of blindness (Neely, 1998). AMD can occur in two forms: "wet" (neovascular) and "dry" (non-neovascular) AMD. In the case of the neovascular AMD abnormal choroidal blood vessels begin to grow under the centre of the retina or the macula, responsible for detailed central vision. Like in the PDR these newly formed vessels are fragile and leaky (more permeable) in nature. There is accumulation of fluid or edema in the macula causing damage to the macula and problems with vision follow. Approximately 85-90% of the patients who have AMD fall into the "dry" form category (American Macular Degeneration Foundation, http://www.macular.org/). Dry AMD usually occurs before the development of wet AMD and it tends to progress more slowly

than the "wet" type. During the "dry" form of AMD, the retinal pigment epithelial cells (RPE) located in the macula breaks down due to the deposition of discrete regions of yellow pigmentation also known as drusen in the macula. This process causes thinning and drying of the macula, making it unable to function properly. Patients with this condition also have problems with their central vision, however this is not as severe as in the case of the "wet" AMD. Retinopathy of prematurity is a disease found in infants born prematurely. In humans, the development of retinal blood vessels takes place during the fourth month of pregnancy and reaches the retinal periphery just before birth (Chen and Smith, 2007). However, in premature babies retinal vasculature is poorly developed with a peripheral avascular region. As the infant matures, newly formed vessels grow into the avascular region of the retina. Over time, in severe and untreated cases, neovessels proliferate into the vitreous region leading to retinal detachment and blindness. There are many risk factors for developing ROP including, prematurity, hyperoxia, hypoxia, hypotension, acidosis, blood transfusions, sepsis, antioxidant deficiency, patent ductus, arteriosus and apnea (Chawla et al., 2008).

1.1.3 Molecular basis of neovascular retinopathy

The exact cause of neovascular retinopathies remains to be explored. There are however speculations that genetic factors may be involved for the onset of ROP; since skin pigmentation plays an important role in the development of ROP with Caucasian infants more likely to develop the disease compared to African American infants, as well twins that have a higher concordance rate for acquiring the disease (Good and Gendron,

2007). Similarly, there is a growing amount of evidence linking genetic variants to the development of AMD (Swaroop et al., 2007) and polymorphisms of the human vascular endothelial growth factor (VEGF) gene have been shown to increase the development of PDR in diabetic patients (Szaflik et al., 2007). In particular, in the case of AMD there is strong evidence associating complement factor H (CFH) and locus *LOC387715/HTRA1* to the disease risk of AMD (Swaroop et al., 2007; Scholl, 2007). CFH is an important protein for regulating the complement system and is involved in the immune-inflammatory processes. This is consistent with the fact that AMD is characterized by choroidal neovascularization which occurs due to a chronic inflammatory response within the Bruch membrane (inner most layer of the choroid) and the choroid layer, the vascular layer found between the sclera and the retina (Montezuma et al., 2007).

Currently, some of the key proteins that are thought to contribute to retinal pathogenesis include vascular endothelial growth factor (VEGF), erythropoietin, insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), angiopoietin, platelet-derived growth factor (PDGF) (Kvanta, 2006) and fibroblast growth factor (FGF) (Neely, 1998). VEGF in particularly is a potent inducer of permeability and has been found to be a key contributor of angiogenesis (Bhisitkul, 2006; Andreoli and Miller, 2007; Erickson et al., 2007, Shukla et al. 2007). It is part of a family of proteins that consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PlGF and act through the tyrosine kinase receptors VEGFR-1, VEGFR-2, and VEGFR-3 (Andreoli and Miller, 2007). It is the proper balance of the pro- and anti-angiogenic regulators, which is crucial for maintaining homeostasis of the blood vessels. Since, angiogenesis occurs in multiple steps involving a

wide range of factors and pathways, it will be ineffective to target just one regulator affecting this process. Plus, current treatments are only focusing on growth factors mentioned above and fail to take into account other important homeostatic regulators, which may be implicated in these diseases. In order to develop treatment that will be useful against the nature and complexity of the neovascularization, it is not only necessary but also essential to find other targets, which will have a more global effect on the disease state. One such homeostatic protein has been isolated in our lab and is named Tubedown (Tbdn).

1.1.4 Currently available treatments for retinal diseases

The need for better treatment for neovascular retinopathies is crucial since current therapeutic strategies targeting these diseases are either limited or non-existent. Laser photocoagulation therapy uses a thermal (heat) laser to destroy the newly formed vessels caused by AMD. This form of therapy is only partially effective against reducing blindness and may actually damage the retina since laser has to reach the blood vessels that are embedded underneath the retina. Patients often develop complications associated with the treatment and poor visual acuity remains (Chen and Smith, 2007). Photodynamic therapy was developed to prevent the damages resulting from the laser photocoagulation therapy. However, this procedure requires multiple treatments administered every 3 months (Bradley et al. 2007) and hence can be considered a nuisance for patients and their family. Even with treatments vision improvements rarely occur (Bradley et al. 2007). There is currently no cure for PDR and ROP.

Anti-vascular endothelial growth factor (Anti-VEGF) therapy is an attractive and emerging therapy for neovascular ocular diseases. However, due to the limited number of trials with the anti-VEGF therapy the long-term benefits and safety of this type of therapy remain to be assessed. To date only Macugen (Pegaptanib sodium injection) and Lucentis (Ranibizumab injection) are the only two Foods and Drug Administration (FDA) approved drugs available for the treatment of AMD (Andreoli and Miller, 2007). Ranibizumab is a 48-kDa recombinant, humanized monoclonal anti-VEGF binding fragment (Kaiser et al; 2007). It binds to and inhibits the biologic activity of all isoforms of human VEGF-A by inhibiting the interaction between VEGF-A and its receptors, which is responsible for processes such as endothelial cell proliferation, migration, and survival of cell receptors (Kourlas and Abrams, 2007). A few large, randomized trials have shown the efficacy and safety of the Ranibizumab for clinical use in AMD (la Cour, 2007; Kourlas and Abrams 2007). Another drug named Avastin (Bevacizumab) was approved by FDA in 2004 for the treatment of colorectal cancer (Hussain, 2007).

Bevacizumab is a full-length, humanized monoclonal antibody, which is also directed against all the biologically active isoforms of VEGF-A. The clinical results for AMD with Bevacizumab are similar to the ones with Ranibizumab (Packer and Jampol, 2008). Majority of the published reports regarding Bevacizumab are however, uncontrolled, non-randomized or small case reports (Nagpal, 2007; Packer and Jampol, 2008). Due to this lack of large randomized trials, Bevacizumab has not been approved by the FDA for intraocular use (Andreoli and Miller, 2007). On the other hand, it is widely used as an off-label drug for various ophthalmic diseases which is the main factor

for its cost being very low compared to other FDA approved drugs for eye diseases (Nagpal, 2007). The off-label status of Bevacizumab is responsible for the continuous controversy and ethical dilemma regarding the application of this drug for intraocular use.

Moreover, predicted statistics on the development of ocular diseases with neovascularization is staggering. For instance, it is estimated that by 2025 the incidence of diabetes worldwide will be in ~ 334 million people and 30.3 million people in the United States of which 25% will display PDR and hence will require effective treatment (Bradley et al. 2007). However, effective and successful development of pharmacological treatments will require a better knowledge of the molecular mechanism or factors responsible for causing ocular neovascularization. We investigate here the role of such an important factor known as Tubedown, in order to gain a better understanding of retinal diseases with neovascularization.

1.2 Tubedown

1.2.1 What is Tubedown?

Tubedown (Tbdn), also referred to as mNat1, NATH and Narg1 is a 100-kDa novel acetyltransferase protein originally identified from an embryonic endothelial cell line (IEM) (Gendron et al., 2000). It displays homology to Nat1 of yeast *S. cerevisae,* which forms a complex with the acetyltransferase Ard1 to form the essential subunit of the yeast N-terminal acetyltransferase NatA (Park and Szostak, 1992; Gendron et al., 2000). In the

mammalian system, Tbdn shows a great extent of homology to mNat2 (70% identity) and Ard1 has been reported to show 81% identity with Ard2 (Sugiura et al., 2003; Arnesen et al., 2006). In yeast, NatA likely mediates co-translational acetylation of nascent polypeptides at specific residues in the second position of the N-terminus upon cleavage of the initial methionine by methionine aminopeptidases (Gautschi et al. 2003; Polevoda et al., 2003). Acetylation of lysine residues has also been suggested as a function of Ard1 and a wide variety of potential substrates for NatA have been reported (Polevoda et al., 2003; Kimura et al., 2003; Wang et al., 2004; Geissenhoner et al., 2004; Lim et al., 2006). Recently, another protein found in association with the NatA complex is the putative acetyltransferase Nat5 (also called San in *D. melanogaster*) which interacts with Nat1 in the yeast ribosome (Gautschi et al. 2003; Williams et al., 2003; Arnesen et al., 2005; Arnesen et al., 2006c; Hou et al, 2007). Both in the yeast and in mammalian cell lines, Tbdn acts in a complex with the acetyltransferase Ard1 and is involved in the regulation of a wide range of cellular processes including cell growth and differentiation (Park and Szostak, 1992; Paradis et al., 2002; Willis et al., 2002; Sugiura et al., 2003; Gautschi et al., 2003; Kimura et al., 2003; Wang et al., 2004; Asaumi et al., 2005; Arnesen et al., 2005; Arnesen et al., 2006b). In 2000, Gendron et al. tested the acetylation activity of Tbdn in an embryonic endothelial cell line and found that immunoprecipitates prepared with anti-Tbdn antibody display an acetyltransferase activity, confirming the presence of acetylation activity in the mammalian cell line. Acetylation of transcription factors is in turn one of the most significant processes regulating gene expression (Gregory et al., 2001). Acetylation has been shown to play a wide variety of role in

nuclear import, protein-protein interaction and protein stability (Gregory et al., 2001). Further, lack of acetyltransferase due to a defect in either the *nat1* or *ard1* gene leads to similar phenotypes resulting in abnormal cell cycle control and lack in sporulation (Whiteway and Szostak, 1985; Whiteway et al., 1987; Mullen et al., 1989) indicating that both gene products perform a common function in yeast (Mullen et al., 1989) and in the mammalian system (Asaumi et al., 2005; Arnesen et al., 2005).

1.2.2 Tubedown expression

The expression of Tbdn has been studied in a number of embryonic and adult tissues (Gendron et al., 2000; Gendron et al., 2001; Paradis et al., 2002) at both RNA and protein level. The results indicate that the expression of Tbdn is developmentally regulated during embryogenesis while during adulthood high levels appear to be limited to only few tissues including the ocular endothelium, blood vessels of regressing ovarian follicles and the choroid plexus endothelium (Gendron et al., 2000; Gendron et al., 2001; Paradis et al., 2002; H. Paradis, personnel communication). Tbdn is also transiently expressed during embryogenesis in the developing vasculature and neuronal tissues where its regulation is associated with differentiation (Gendron et al, 2000; Sugiura et al., 2003; Arnesen et al., 2006; Martin et al, 2007). The high level of Tbdn expression in these tissues may suggest a unique role for Tbdn in these particular areas and more importantly in the retinal blood vessels.

1.2.3 Function of Tubedown

The functional role of Tbdn is still being investigated. Although some information now exists on its role in growth and differentiation as well as its importance in maintaining the ocular homeostasis, the full mechanistic pathways to explain these processes still remain to be explored. Earlier studies looking at the expression level of Tbdn in healthy and diseased retinal blood vessels may help shed some light on its role in vascular remodeling and angiogenesis (Fluge et al., 2002; Paradis et al., 2002; Wall et al., 2004; Gendron et al., 2006). In one study, *in vitro* capillary formation in embryonic endothelial cells as well as in retinal endothelial cells was investigated where Tbdn showed significant downregulation during capillary growth suggesting it may play a role in vascular remodeling (Gendron et al., 2000). In addition, clones of the RF/6A fetal choroid-retina endothelial cell line showing suppression of Tbdn levels after overexpression of an antisense *TBDN* cDNA display a significant increase in the formation of capillary-like structures *in vitro* compared with controls (Paradis et al., 2002). Further, Wall et al. (2004) generated a bitransgenic mouse model that enables conditional knockdown of Tbdn specifically in endothelial cells. Their results indicated that mice with suppression of Tbdn displayed retinal and choroidal neovascularization with intra- and preretinal fibrovascular lesions similar to human proliferative retinopathies. Retinal lesions observed in Tbdn suppressed mice increased in severity with prolonged suppression of Tbdn. 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. When Tbdn expression was studied in diabetic adult human eye specimens in parallel with the normal samples, it was found that there is a significant suppression of Tbdn in retinal blood vessels of human specimens of neovascularization PDR (Gendron et al., 2001). Likewise, the expression of Tbdn was characterized in neonatal retinal neovascularization using a hyperoxia-induced retinopathy mouse model and human specimen of stage 3 ROP (Gendron et al., 2006). Again, the authors observed downregulation of Tbdn expression in the mouse model, which was accompanied by increased neovascularization and retinal thickness. The human sample also showed a lower level of Tbdn staining compared to the age-matched control. Loss of Tbdn expression is again seen in human specimens of AMD compared to age-matched controls (H. Paradis, personnel communication). Taken together, these studies clearly indicate that suppressed levels of Tbdn is associated with angiogenesis and pathological neovascular retinopathies (Gendron et al., 2001; Paradis et al., 2002; Wall et al., 2004; Gendron et al., 2006) as well, highlights the importance of Tbdn in maintaining the homeostasis of the retinal blood vessels.

Recently, Asaumi et al. (2005) investigated the role of ARD1 and Tbdn in the endocytosis of beta-amyloid precursor protein and its effect on amyloid beta protein secretion. They reported that coexpression of the human homologues of the yeast Ard1 and Tbdn in cells suppressed amyloid beta secretion and inhibition of endocytosis of cell-surface amyloid precursor protein was also seen. This suppression correlated with the enzyme activity of Tbdn and Ard1. This is a significant piece of evidence indicating a possible functional role for Tbdn in membrane trafficking. Moreover, Paradis et al.

(2008) showed that Tbdn forms a complex with the actin binding protein cortactin. Cortactin is known to be important for regulating the actin cytoskeleton dynamics (Weed and Parsons, 2001; Daly 2004) and by doing so it is involved in processes requiring plasma membrane remodeling such as cell migration, endocytosis and in intracellular movement of vesicles (Weed and Parsons, 2001; Daly, 2004; Kessels and Qualmann, 2005; Kowalski et al., 2005; Mehta and Malik, 2006). Since, cortactin is also implicated in endothelial permeability and migration (Daly, 2004; Kowalski et al., 2005; Mehta and Malik, 2006), it is tempting to ask the question "whether there is a possible role for Tbdn in the vesicle transport mechanism including endothelial permeability?" To be able to answer this question it is necessary to closely look at the current transport mechanisms.

1.3 Vascular Permeability/ Rationale for current study

The vascular system of our body originates from and is largely mediated by the endothelial cells, which plays a major role in regulating various homeostatic functions including the control of vasomotor tone, the trafficking of cells and nutrients, the maintenance of blood fluidity, the growth of new blood vessels (Cines et al., 1998) as well as transport of plasma molecules, employ bidirectional receptor-mediated and receptor-independent transcytosis and endocytosis (Simionescu and Antohe, 2006). When endothelial cells fail to maintain the homeostasis, it leads to a number of patho-physiological conditions such as inflammation, cardiovascular diseases (Esper et al., 2006 and Trepels et al., 2006) and angiogenesis (Polverini, 1995; Lee et al., 1998; Lamalice et al., 2007). As mentioned above, ocular diseases such as ROP, PDR, and

AMD are responsible for majority of the vision loss in the general population (Erickson et al., 2007). The blood-retinal barrier is greatly compromised in these diseases as an imbalance in nutrients and oxygen demand/supply occur which leads to angiogenesis (Erickson et al., 2007). In order to understand the molecular events leading to angiogenesis and increased vascular permeability, it is important to understand permeability pathways.

Permeability is defined as the transport of solutes and water across endothelial and epithelial layers and arranged via 2 distinct pathways (Fig. 1-2). The paracellular pathway is mediated by tight junctions and here solutes pass through the lateral spaces between the contacting cells. On the other hand, via the transcellular route molecules pass from apical to basal cell membranes and are mostly regulated by channels, carriers, pumps and vesicles. Simionescu (1979) originally coined the term transcytosis, which involves the transport of large molecules by membrane-bound vesicles and require both endocytosis and exocytosis (Predescu et al., 2007). More specifically transcellular mechanism consists of the following steps; invagination of the plasma membrane, formation of a sealed vesicle by membrane fission, intracellular movement of the vesicle across the cell, followed by fusion of vesicle membrane with the plasmalemma on the opposite front of the EC, and discharge of vesicular content into the extracellular space (Predescu et al., 2007) (Fig. 1-2). Transcytosis is not limited to one particular cell type as previous reports show this process to occur in epithelial cells, osteoclasts, neurons (Predescu et al., 2007) and endothelial cells (Mehta and Malik, 2006).

Transcytosis of albumin is a well-documented system and is of particular interest

to us because transcytosis of albumin has been shown to occur in the eye (Vinores et al. 1998). Albumin is the most abundant protein in human plasma making up for 60% of the total protein content (Mehta and Malik, 2006). Plasma albumin acts as a carrier protein for a variety of hydrophobic molecules, enzymes and hormones across the endothelium (Mehta and Malik, 2006). Further evidences on the function of albumin indicate that it is implicated in controlling the endothelial barrier stability as well as plays an important role in maintaining the oncotic pressure across the capillaries (Mehta and Malik, 2006).

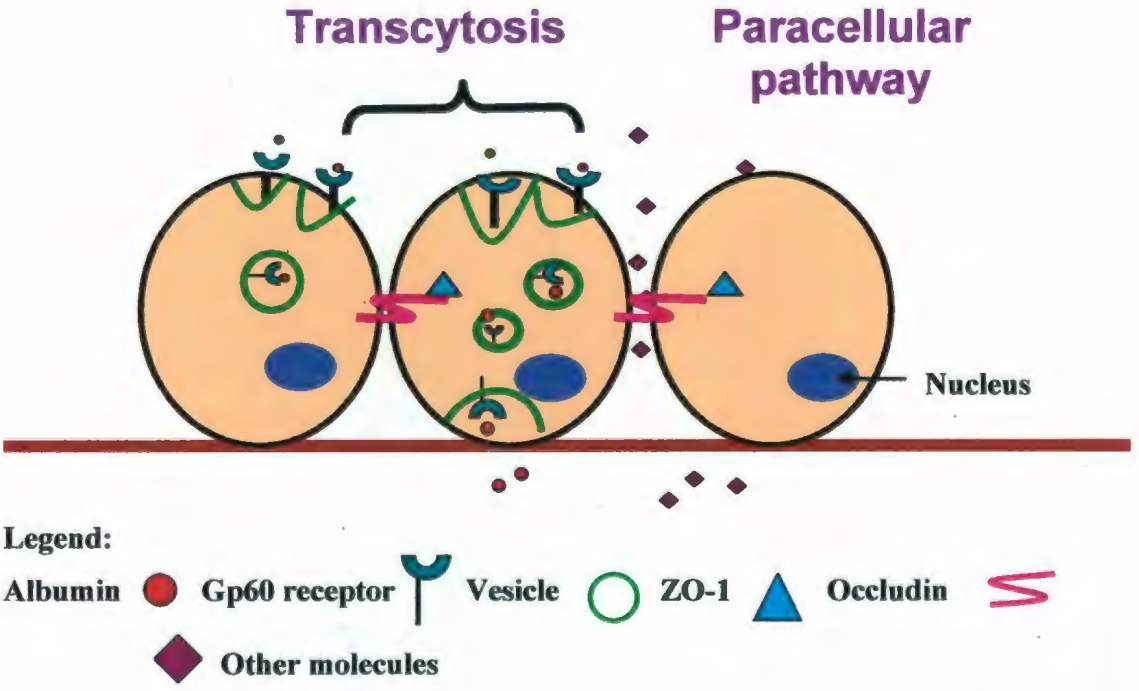

Figure 1-2. Permeability Pathways. Illustration shows transcytosis of albumin where, transcellular permeability is regulated by specific receptors found on the cell-surface. After ligand (albumin) binds to receptor (gp60), it is internalized (vesicle), shuttled across the cell and released on the other side of the cell membrane. In the paracellular permeability passive movement of molecules occur through the junctions (mediated by

ZO-1 and Occludin) found between adjacent cells.

In contrast, tight junctions also known as Zonula Occludens found on most apical component of intercellular junctional complex are associated with paracellular permeability. They play a pivotal role in membrane compartmentalization and serve as barriers in epithelial and endothelial cells. For instances, the blood–brain barrier (BBB) and the blood–retinal barrier (BRB) are mainly composed of tight junction structures (Harhaj and Antonetti, 2004). Both of these barriers are strictly regulated and allow minimal vesicular transport, characterized by transcellular flux across the retinal barrier, to occur (Ishibashi et al., 1980). Tight junctions consist of proteins such as ZO-1, occludin, claudins, JAM-1 and others. These and other proteins associated with tight junctions are connected to components of the cytoskeleton and hence serve as essential link to other cell-cell and cell-substratum adhesion sites (Schneeberger and Lynch, 2004). Moreover, a wide range of cytoplasmic molecules which are a part of these junctions modulate a variety of cellular processes such as transcription, cell proliferation, cell polarity and the formations of a regulated diffusion barrier (Schneeberger and Lynch, 2004). The paracellular pathway may be regulated directly through modification of tight junction proteins, or indirectly through effects on the cytoskeleton (Harhaj and Antonetti, 2004). In 2005, Yu et al. investigated the role of the cytoskeleton on the pathogenesis of diabetic retinopathy using the streptozotocin-induced rat model. The authors observed changes in the F-actin cytoskeleton, which are filamentous polymers and are essential part of the actin cytoskeleton component to occur early in rats with diabetic retinopathy

and this alteration has been associated with microvascular leakage in the retinal capillaries.

The present study, looking at the role of Tbdn in the permeability of endothelial cells was carried out with the hope to further understand and characterize the function and the mechanism of action of our protein of interest in health and disease. The cellular expression of Tbdn, at the leading edge of cell membranes and also in the perinuclear regions indicate its interaction with proteins involved in regulating the actin cytoskeleton. Recent data from our lab also show Tbdn acting in complex with cortactin, an actin binding protein (Paradis et al., 2008). Since originally being described as a filamentous actin binding protein and substrate of the tyrosine kinase $pp60^{src}$ in primary embryo fibroblasts, smooth muscle cells and endothelial cells (Wu and Parsons, 1993), the functional role of cortactin in regulating the dynamics of the actin cytoskeleton assembly has become well established (Weed and Parsons, 2001; Daly 2004). By regulating the actin cytoskeleton, cortactin is involved in processes requiring plasma membrane remodeling such as cell migration and endocytosis and in intracellular movement of vesicles (Weed and Parsons, 2001; Daly, 2004; Kessels and Qualmann, 2005; Kowalski et al., 2005; Mehta and Malik, 2006). Cortactin function is mediated through its interaction with Arp2/3 complex, N-WASP and diverse proteins associating with its SH3 domain such as dynamin (Weed and Parsons, 2001; Daly, 2004; Kessels and Qualmann, 2005, Mehta and Malik, 2006). The immunofluorescence staining analyses revealed that Tbdn co-localizes with cortactin in the cytoplasm and that the co-localization is most intense at the cortex and in perinuclear regions of cultured retinal endothelial cells

(Paradis et al. 2008). The co-localization of Tbdn and cortactin at perinuclear regions indicates that the complex might play a role in organelles surrounding the nucleus such as the endoplasmic reticulum and Golgi apparatus. In agreement with this hypothesis, the yeast NatA complex has been shown to be involved in ribosome biogenesis (Gautschi et al., 2003; Polevoda et al, 2007). In addition, Tbdn co-localizes with the F-actin cytoskeleton in the cytoplasm. The actin cytoskeleton is in turn involved in regulating cell permeability and tight junctions (Clements et al., 2005; Yu et al., 2005; Clark et al., 2007; Tyagi et al., 2008, Madara et al., 1986). Since cortactin is also known to regulate cellular permeability through its interaction with the actin cytoskeleton, which is critical for endothelial cell homeostasis, the role of Tubedown on endothelial cell permeability was examined. In addition, since previous studies have shown Tbdn suppression leading to many ocular diseases (Wall et al., 2004; Gendron *et al.*, 2001; Gendron et al., 2006), we hypothesize that the expression level of Tbdn is an important factor regulating permeability either through the paracellular pathway or the transcellular pathway and hence is a required component for maintaining ocular homeostasis. Studying Tbdn and its role on the permeability of the endothelial cells may help us to better understand pathologies of the ocular diseases and subsequently will allow development of new pharmacological strategies to treat ocular retinopathies.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell culture

RF/6A rhesus macaque choroid-retina endothelial cells, an immortalized cell line were obtained from AmericanType Culture Collection (Massas, VA). Cells were grown in Dulbecco's Modified Eagle Media (DMEM) (Invitrogen) supplemented with 4 mM glutamine plus 10% fetal bovine serum (FBS) and 50 μM of nonessential amino acids. Cells were maintained at 37°C in 10% CO_2 atmosphere. The antisense clones with suppressed level of Tbdn were derived in our lab by stably transfecting RF/6A cells with a vector harboring a *TBDN* cDNA fragment in an antisense fashion (*ASTBDN*) (Paradis et al., 2002).

2.2 Permeability Assay

To determine the effect of the antisense *TBDN* construct knockdown for Tbdn on RF/6A permeability, an in vitro permeability assay using FITC-labeled albumin was performed. RF/6A cells (parental cells, stable AS*TBDN* clones and control clones) were seeded onto 1%-gelatin-coated polystyrene filter inserts (Costar Transwell, no. 3470, 6.5 mm diameter, 0.4 μm pore size, Sigma, St. Louis, MO) at a density of 30,000 cells/insert (0.3×10^6 cells/ml) (Fig. 2-1). The cells were grown to confluence on the Transwell

inserts over 24 hours. The integrity of the cellular monolayer was evaluated for confluence by phase contrast light microscopy and photographed. Cells were then washed 3 times in serum-free DMEM and the inserts were transferred into new 24-well plates containing serum-free DMEM and incubated for 2 hours. FITC-labeled albumin (Sigma A9771, Sigma, St. Louis, MO) suspended in serum-free DMEM was added to the endothelial cell monolayers to achieve a final FITC-albumin concentration of 100 μM (Irwin et al., 2005). Transit rate of FITC-albumin across the monolayer was assessed by measuring the increase in FITC-albumin in the lower well at different time points from 0 to 60 min (Irwin et al., 2005). FITC-albumin was quantified against a standard curve of FITC-albumin using a FLUOstar Optima spectrofluorometer (BMG Labtechnologies) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

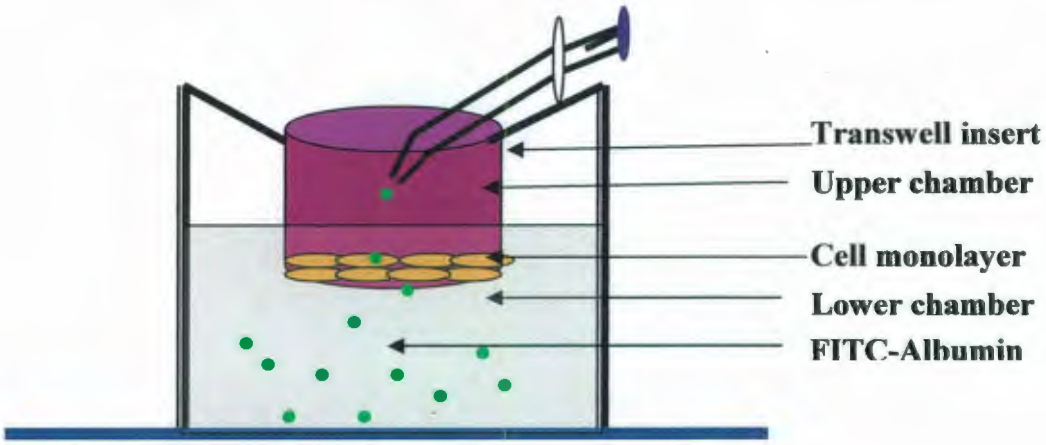

Figure 2-1. Transwell system used for permeability assay. RF/6A cells (parental cells, stable AS*TBDN* clones and control clones) were plated in the top chamber overnight. FITC-albumin at a final concentration of 100 μM was added the next day and the intensity of the tracer was measured by spectrofluorometer.

2.3 Immunofluorescence Staining

To follow FITC- albumin distribution intracellularly, RF/6A cells were cultured overnight at 0.3 x 10^6 cells/ml on 18 mm coverslips. Next day cell layers were washed three times with serum-free DMEM and incubated 2 hrs at 37°C. 100 µM FITC-albumin (Sigma) was added to the cell layer. Cells were incubated for T= 60 min then washed 3 times with PBS. Cells were then fixed with 4% paraformaldehyde for 15 min after which they were incubated with PBS containing 0.1 % TritonX-100 for 15 min and stained with 2.5 µM Phalloidin-TRITC (Sigma). After 40 min of incubation, cells were washed again 3 times with PBS and mounted in Vectashield (Vector laboratories, CA) and sealed with nail polish. All steps were performed at room temperature unless otherwise indicated. For double-labeling experiments, RF/6A parental cells were plated at 0.75 x 10^6 cells/ml. Coverslips were incubated overnight with either a mouse monoclonal OE5 antibody (Martin et al. 2007) against Tbdn at 15 µg/ml or a negative control isotype-matched IgG2a antibody at 15 µg/ml (DAKO, Mississauga Ontario, Canada). Fluorescence was visualized with a Leica DMIRE2 microscope (Germany) connected to a Qimaging RETIGA Exi camera. Images were viewed with a 40X objective and image data processed with the Openlab software (Version 3.1.3, Improvision; Lexington, MA, USA).

2.4 Western blot Analysis

Protein extraction was performed as described previously (Gendron et al., 2000). Cell extracts were prepared using TNB 0.5% lysis buffer (50 mM Tris [pH 7.6], 150 mM NaCl, 0.5% Brij 96) supplemented with protease inhibitors (1mM phenylmethylsulfonyl

fluoride [PMSF], 10 μg/ml aprotinin and 10 μg/ml leupeptin) and phosphatase inhibitors (2 mM sodium orthovanadate, 50 mM sodium fluoride, and 25 mM ß-glycerophosphate). Lysates were clarified by centrifugation, protein concentration of supernatant was quantified and samples separated by SDS-PAGE. Western blot analysis was performed by standard procedures using chemiluminescence detection (ECL Plus reagent; Amersham Pharmacia Biotech, Piscataway, NJ). To ensure loading equivalency, Western blots were stripped and reprobed with ERK-1 rabbit polyclonal antibody (Santa Cruz Biotech, CA) and α-tubulin mouse monoclonal antibody (Sigma, St. Louis, MO). ZO-1 antibody was used at 1:500 (Santa Cruz Biotech, CA, Cat.# Sc-33725). For detection of Tbdn an affinity-purified rabbit anti-Tbdn C10-20 antibody (Gendron et al. 2000, Paradis H. et al. 2008) was used and for detecting ARD1 band affinity-purified goat anti-Ard1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Densitometry analyses were done using the Kodak Gel Logic 200 Imaging System (Eastman Kodak Company, Rochester, NY) and intensities of the expressed bands were analyzed using Kodak Molecular Imaging Software (Version 4.0, Eastman Kodak Company, Rochester, NY).

2.5 Immunostaining of eye sections with anti-albumin antibody

Paraffin sections of eyes from doxycycline-fed endothelial-specific Tbdn knockdown mice and control mice sacrificed at 10 months were processed for immunostaining as described previously (Wall et al., 2004) and analyzed by albumin immunohistochemistry to assess retinal albumin expression/localization. Mouse tissues were fixed in 4% paraformaldehyde, embedded in paraffin and processed for

immunohistochemistry, as previously described (Paradis et al., 2002). Briefly, sections were dewaxed in xylene and rehydrated in graded alcohols. Endogenous peroxidases were blocked in 0.3% H_2O_2 for 10 min followed by post-fixation with 4% paraformaldehyde for 10 min. Sections were incubated for 1 hour with 6% fat-free skim powdered milk in 10 mM Tris HCl pH 7.6, 150 mM NaCl, 0.05% Tween 20 (TBST) for blocking nonspecific binding sites followed by incubation with goat anti-albumin horseradish peroxidase-conjugated antibody (GeneTex Inc., San Antonio, TX) in 3% powdered milk/TBST overnight at room temperature. The peroxidase activity was detected using NovaRed substrate kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Negative control for the albumin staining was goat-anti-rabbit horseradish peroxidase-conjugated antibody (Promega). Sections were then air-dried and mounted in Permount (Fisher Scientific, Pittsburg, PA). Adjacent sections were stained with hematoxylin and eosin to assess tissue integrity and pathology. The care and use of animals in this study followed the guidelines set by the Canadian Council on Animal Care and were approved by the Institutional Animal Care Committee of Memorial University of Newfoundland.

2.6 FITC-Albumin uptake experiment

Intracellular FITC-albumin content was measured using a procedure adapted from Takano *et al.*, 2002. RF/6A cells were plated at 0.75 x 10^6 cells/ml on 35 mm culture dishes. The cells were grown to confluence over 24 hrs. Cells were then washed three times in DMEM and incubated for 2 hours. 10 μM FITC-albumin was added to the plates

and cells were incubated for 0 to 120 min at 37°C. After each incubation period, the dishes were rinsed rapidly three times with 1 mL of ice-cold PBS. The cells were then scraped in ice-cold PBS and centrifuged at 4°C for 5min. Cells were lysed with PBS containing 0.1% Triton X-100 and the fluorescence of the homogenates was measured as described above. Protein quantitation was done by using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA).

2.7 Statistical Analysis

Differences between experimental groups were analyzed for statistical significance using the two-tailed Student's *t* test by the Microsoft Excel program. Values were expressed as the mean percentage of the control ± standard error of the mean (s.e.m.) except for the FITC-albumin uptake experiments where values are expressed as mean of pmol /μg of protein ± s.e.m. Differences were considered significant at $P<0.05$.

CHAPTER 3

RESULTS

3.1 ***Transcellular permeability of RF/6A endothelial cells.*** The role of Tubedown on the permeability of endothelial cells is unknown. However, a recent study by Asuami et al., suggested Tbdn being involved in the regulation of the endocytosis of beta–amyloid precursor transmembrane protein (Asaumi et al., 2005). Since transcellular permeability requires endocytosis, these findings prompted us to study the role of Tbdn expression on the permeability of endothelial cells in vitro and in vivo. As a first step, an in vitro permeability assay was used to measure the transit of FITC-albumin across monolayers of RF/6A endothelial cell clones in which Tbdn expression had been knocked down by stable expression of an antisense *TBDN cDNA* construct AS*TBDN* (Paradis et al., 2002; Wall et al., 2004). As shown in other cellular systems (Arnesen et al., 2006c; Hou et al., 2007; Polevoda et al., 2007), the knockdown of Tbdn protein expression in RF/6A cells is associated with the suppression of Ard1 protein expression (Fig.3-1 A, B). The knockdown of Tbdn in RF/6A endothelial cells resulted in a significant increase in the percentage of FITC-albumin transit across the cellular monolayers compared to the RF/6A parental cell line and negative control RF/6A clones in which Tbdn expression was not suppressed (Fig. 3-2 and 3-3). Differences were considered significant at $P<0.05$.

3.2 Immunofluorescence localization of FITC-albumin in RF/6A cells. As previously reported for endothelial cells (Mehta and Malik, 2006), both fluorescence microscopy of RF/6A cells incubated with FITC-albumin and measurement of intracellular uptake of FITC-albumin by RF/6A cells indicated that the permeability of endothelial cells to FITC-albumin was mediated by a transcytosis mechanism (Fig. 3-4 and Fig. 3-6 respectively). Fluorescence images were consistent with the presence of FITC-albumin vesicles (green) detected inside endothelial cells which appeared to align along the phalloidin-labeled F-actin cytoskeleton (red) (Fig. 3-4). We next examined the co-localization of FITC-albumin with an anti-Tbdn monoclonal antibody (Martin et al., 2007). Figure 3-5 depicts individual staining of FITC-albumin (green), Tbdn (red) concentrated in the perinuclear region, nuclei shown by DAPI (blue) and a merged picture taken after 60 minute of incubation with 10 μM of FITC-albumin. There was no co-localization seen between FITC-albumin and Tbdn staining.

3.3 FITC-albumin uptake in parental and antisense clones. Measurements of intracellular uptake of FITC-albumin by RF/6A endothelial cell monolayers by spectrofluorometry indicated a linear increase from 0 to 120 min (Fig. 3-6). There was no statistically significant difference between RF/6A parental endothelial cells, ASTB#1 and CTR#1. This measurement was specific to albumin as the fluorescence signal was competed by excess unlabeled albumin (data not shown).

3.4 In vivo measurement of FITC-albumin. Barrier function is an important functional property of retinal endothelial cells that usually limits plasma albumin to intravascular areas (blood vessel lumens) in normal disease-free neural retinal tissues (Pino and Thouron, 1983; Liao and Gonzalez-Fernandez, 2004) as shown by albumin immunostaining of sections of control mouse retina (Fig. 3-7A). The effect of Tbdn loss on the permeability of retinal endothelial cells to albumin was next examined in mice in vivo. A previously described conditional endothelial specific transgenic Tbdn-knockdown mouse model (Wall et al., 2004) was used for these experiments. The conditional endothelial knockdown of Tbdn in adult mice is associated with fibrovascular growth and thickening of the retinal tissues (Wall et al., 2004) (Fig. 3-7 B,F). Compared to control age matched mice (Fig. 3-7A), the abnormal blood vessels in retinal neovascular lesions of Tbdn knockdown mice leaked albumin as shown by staining of extravasated albumin (Fig. 3-7B). The leakage was most significant from inner retinal blood vessels which are situated in areas in which we have previously shown increases in tissue thickness and abnormal fibrovascular growth (Wall et al., 2004).

3.5 Expression of ZO-1 protein in RF/6A cells. One of the important molecules for maintaining the tight barrier in inter-endothelial junctions and regulating the paracellular permeability is ZO-1. We examined the protein expression of ZO-1 in our parental, Tbdn knockdown clones and control clones (Fig. 3-8 A, B). There was no correlation seen between the permeability data (Fig. 3-2) and the level of ZO-1 in the parental or antisense clones.

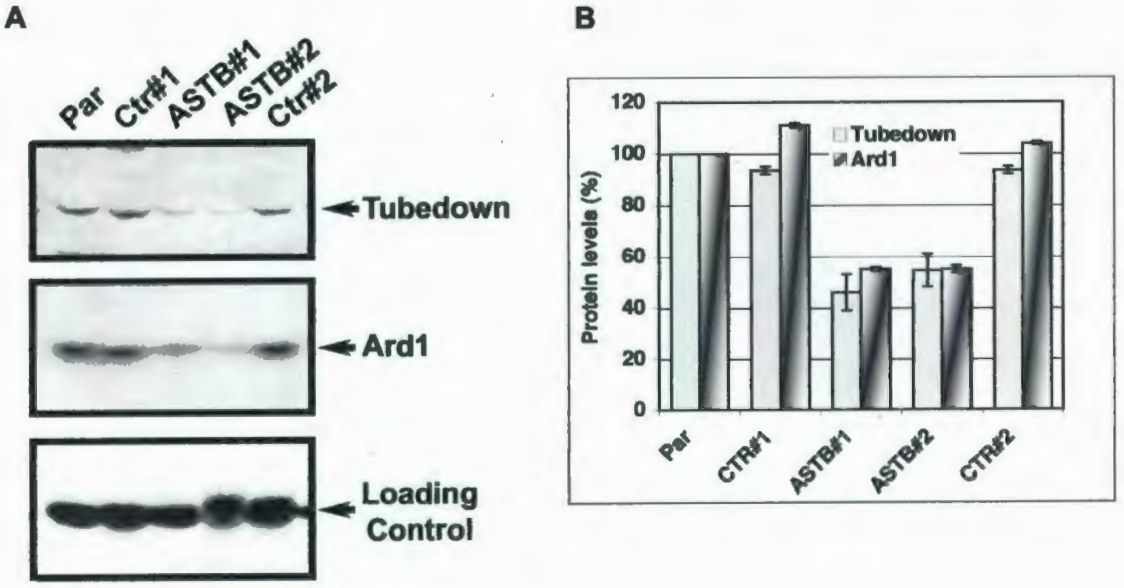

Figure 3-1. Protein expression level of Tubedown/Ard-1. A, RF/6A parental endothelial cells (Par), Tbdn knockdown clones AS-*TBDN* (ASTB#1 and #2) and control clones (CTR#1 and #2) analyzed by Western blot for Tbdn and Ard1 expression (Top and middle panels, respectively). Blots were reprobed and analyzed for Tubulin as loading control (Bottom panel). A representative experiment is shown in **A,** while the average of Tbdn and Ard1 levels ± s.e.m. are shown in **B.**

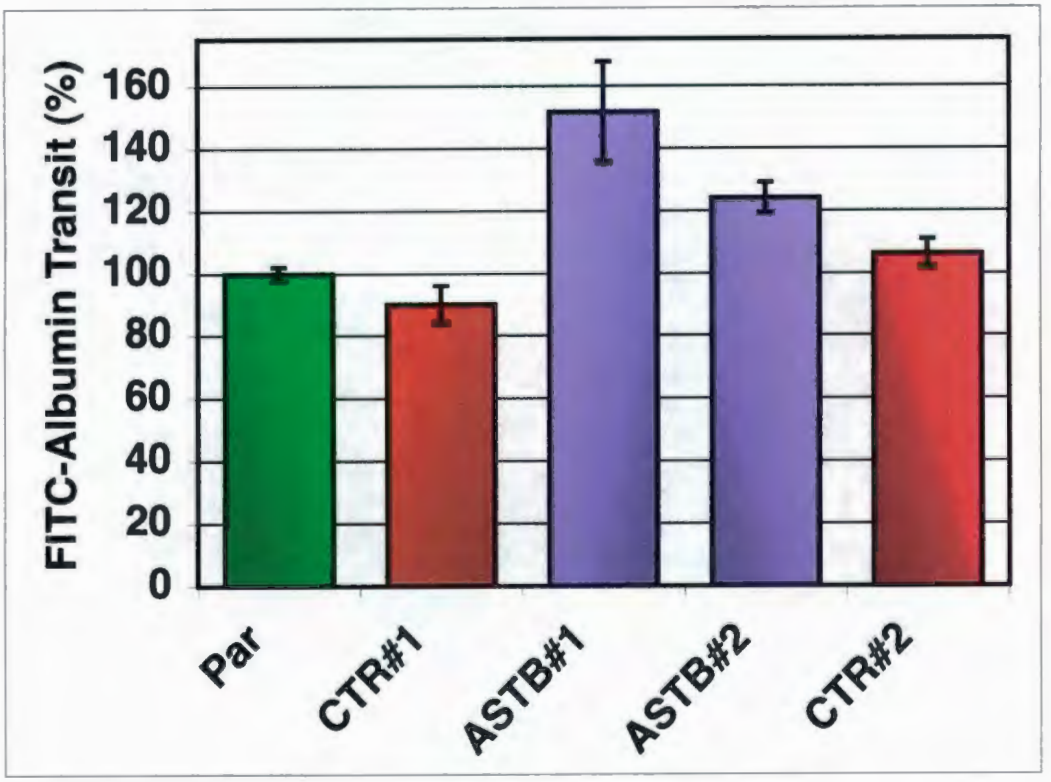

Fig. 3-2. Tbdn/Ard1 knockdown in endothelial cells is associated with increased cellular permeability. FITC-albumin transit across monolayers of RF/6A knocked-down for Tbdn expression (ASTB#1 and #2) or controls (Par, CTR#1 and #2) expressed as percentage of control Par at 30 min. Significantly higher percentages of FITC-albumin transit are observed in two AS-*TBDN* clones (ASTB#1 and #2) as compared to parental cells (Par) and two negative control clones (CTR#1 and #2). Data shown in are expressed as mean +/- s.e.m. of four duplicate experiments in each group.

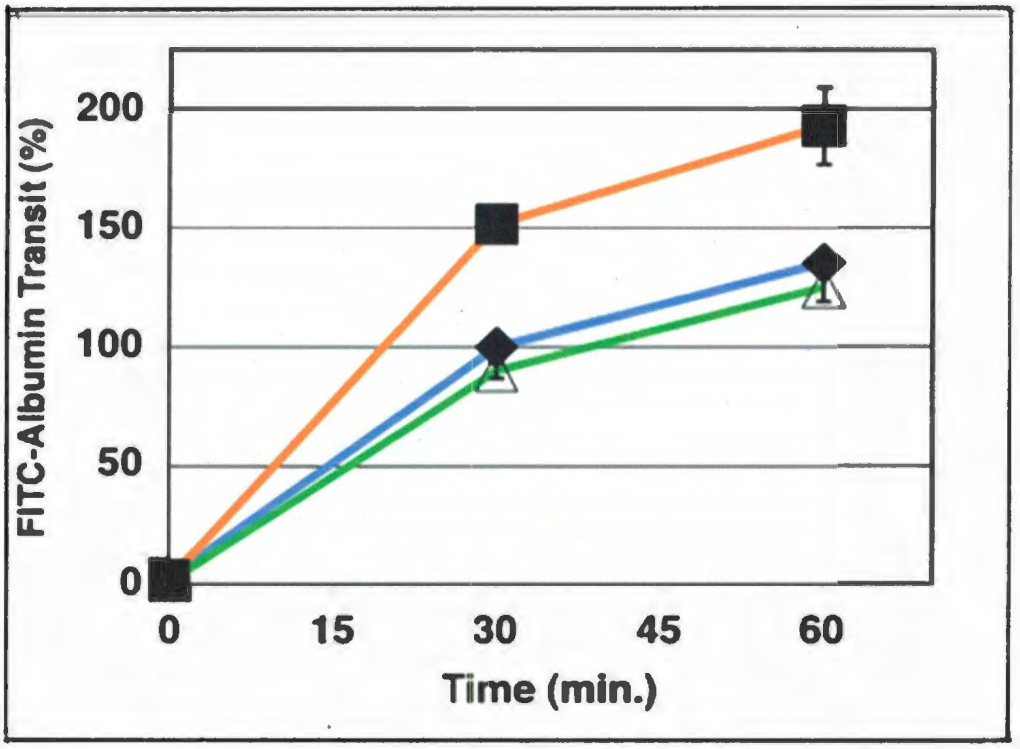

Figure 3-3. FITC-albumin permeability in RF/6A endothelial cells increases with time. Time-course of FITC-albumin transit across cellular monolayers of Tbdn knockdown RF/6A cells (ASTB#1: filled square) as compared to parental cells (filled diamond) and control clone CTR#1 cells (opened triangle) expressed as percentage of arbitrary units. Data shown are expressed as mean ± s.e.m. of four duplicate experiments in each group. Differences were considered significant at $P<0.05$.

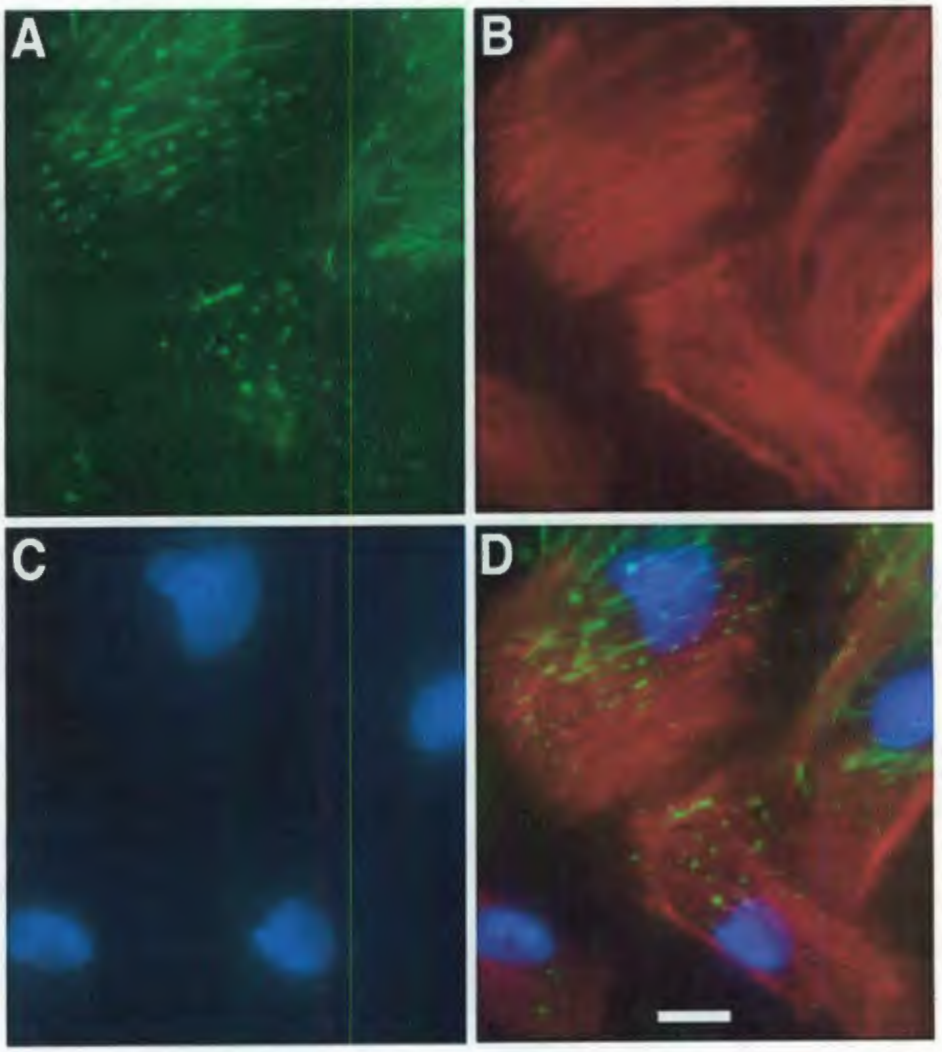

Figure 3-4. FITC-albumin localization in the retinal endothelial cells. A-D, Fluorescence microscopy of FITC-albumin (**A** and **D**, green fluorescence) in RF/6A endothelial cells after 60 min. of incubation in the presence of FITC-albumin on cover slip and washes of excess of FITC-albumin, and staining for F-actin with phalloidin (**B** and **D**, red fluorescence). Cellular nuclei are highlighted by DAPI staining (**C** and **D**, blue fluorescence). The image in **D** represents the merge images of **A**, **B** and **C**. The scale bar shown in D represents 20 μm while the magnification is 400X for A-D.

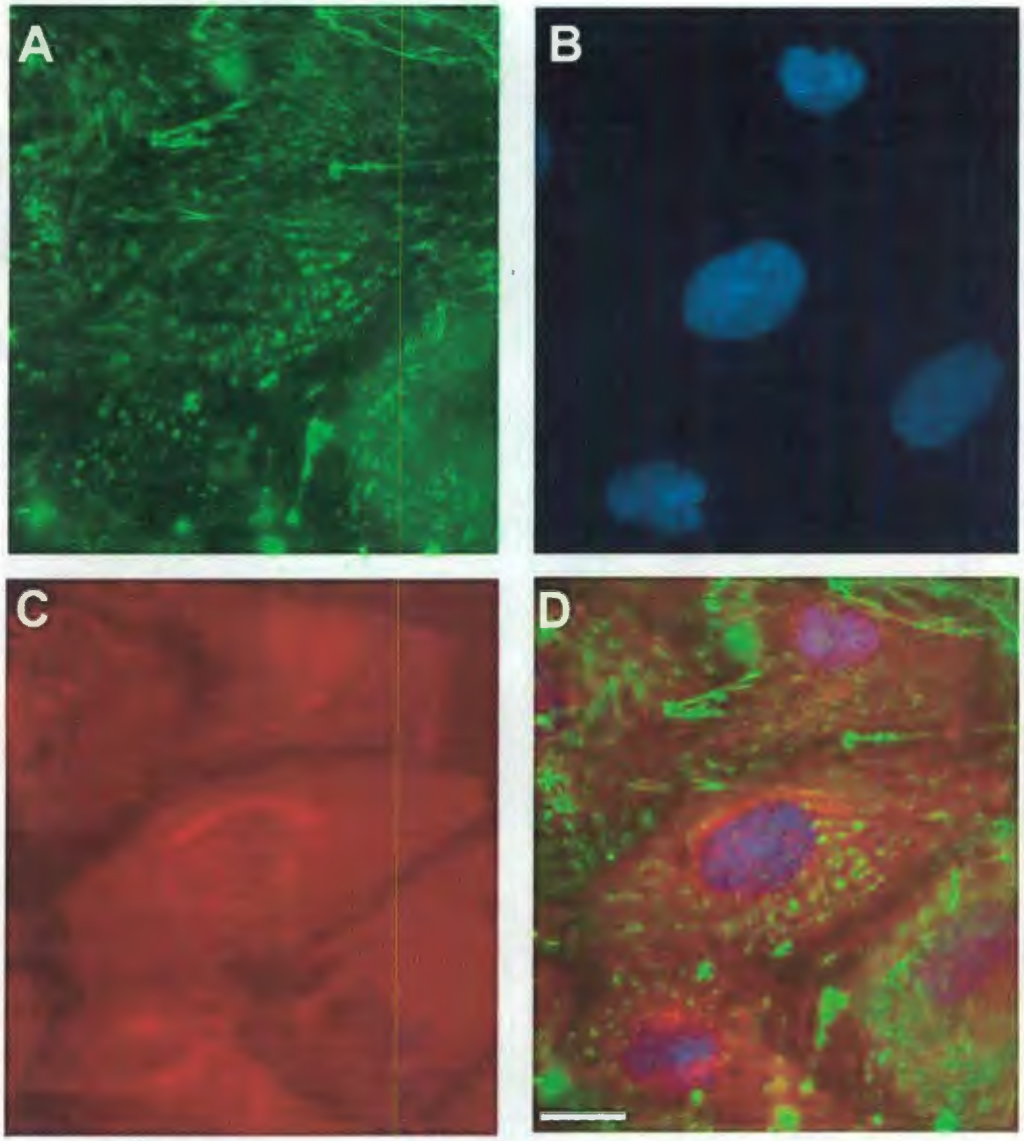

Figure 3-5. Intracellular localization of FITC-albumin and Tubedown in RF/6A cells. RF/6A parental endothelial cells cultured on glass coverslips were incubated with FITC-albumin tracer (**A, green**) and OE5 anti-Tbdn antibody (**C, red**) for overnight at room temperature. Panel **B** shows staining of the nuclei by DAPI (blue). The image in **D** illustrate the merge images of **A,B** and **C.** The scale bar shown in D represents 20 μm while the magnification is 400X for A-D.

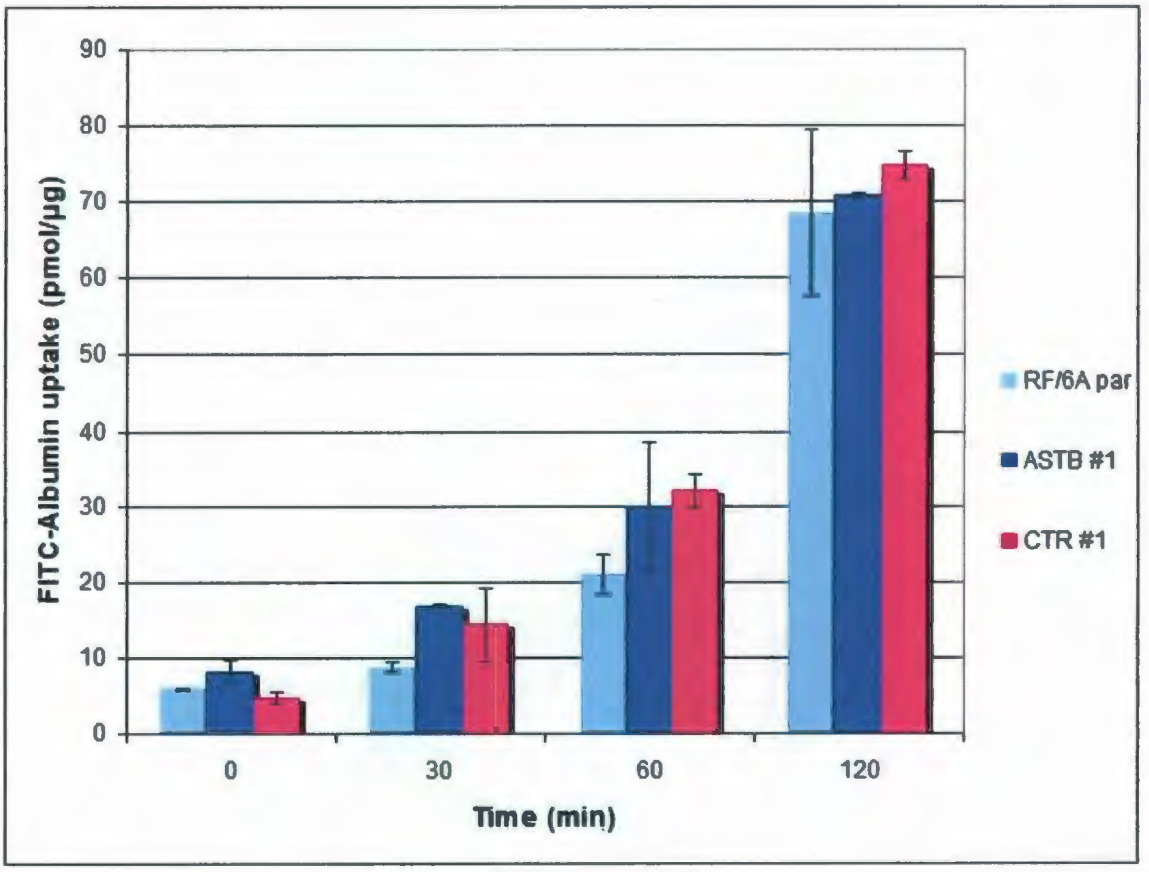

Figure 3-6. Quantitative analysis of FITC-albumin uptake by RF/6A cells as a function of time. Intracellular FITC-albumin uptake increased with time. There was no statistically significant difference between the RF/6A par cells and the ASTB #1 and CTR#1 clones, $p>0.05$, n=3 experiments. Data are expressed as the mean of pmol per µg of protein ± s.e.m.

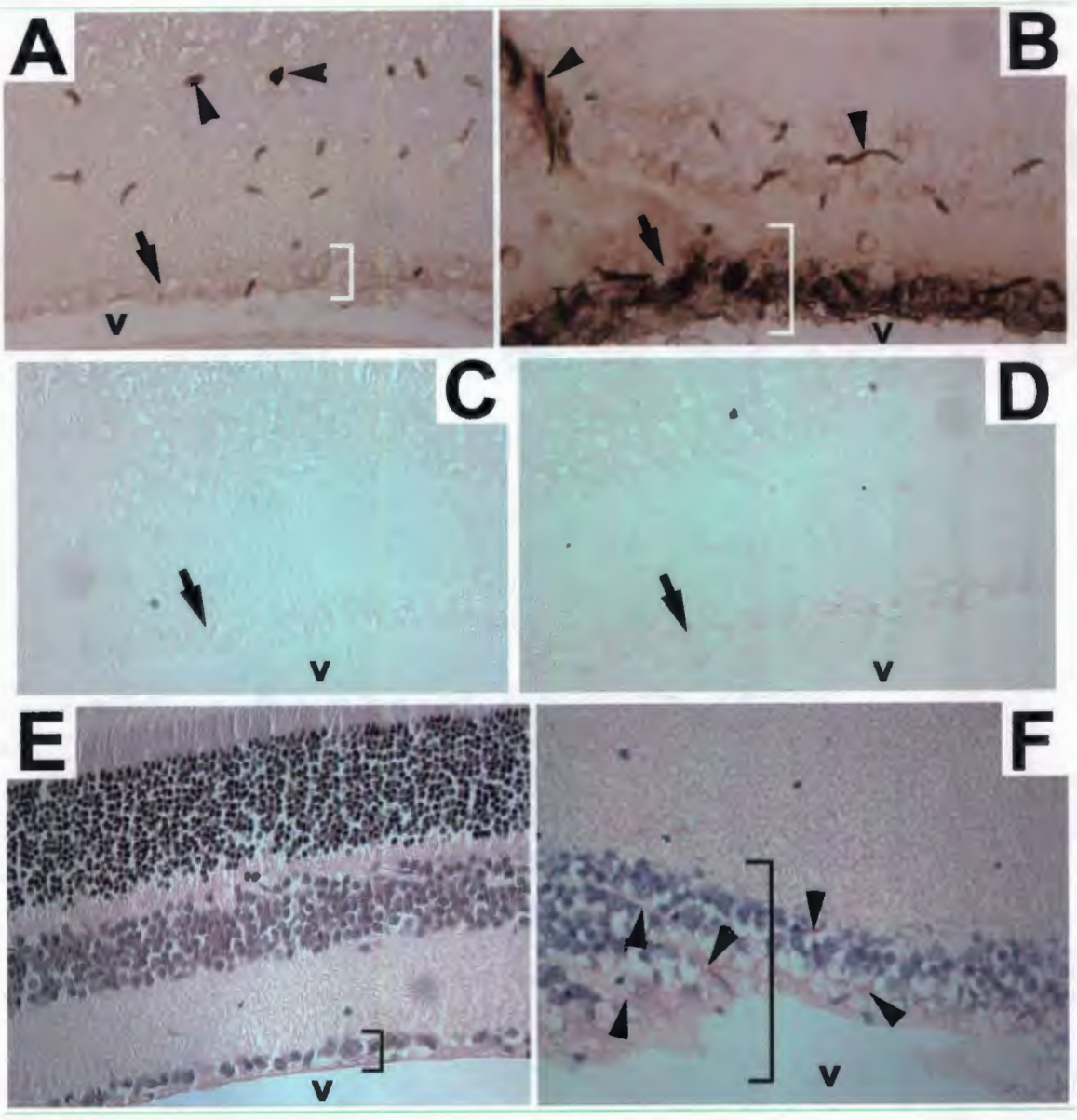

Figure 3-7. Extravasation of serum albumin through the retinal-blood barrier in endothelial-specific-Tbdn-knockdown mice. Staining of retinal tissue for albumin was performed using a peroxidase-conjugated goat anti-albumin antibody, which yields a brown reaction product. Compared with control (non-induced single transgenic is shown) age-matched mice (A), endothelial specific- Tbdn-knockdown eyes (B) showed

(B) showed significant leakage or extravasation of albumin (brown staining) from retinal blood vessels. Brown albumin staining is confined mainly to blood vessel lumens in control retinas (A), whereas brown albumin staining is observed in extravascular locations both in and around blood vessels and in neural retinal tissues in Tbdn knockdown eyes (B). (C,D) Control (C) and Tbdn-knockdown (D) sections stained with negative-control horse-radish-peroxidase-conjugated goat anti rabbit IgG at the same concentration as the anti-albumin reagent showed no staining. All images show the inner and some of the outer layers of the neural retina (most are visible in E) and are oriented with the vitreous cavity (v) of the eye at the bottom of the panel. The ganglion cell layer and inner limiting membrane, which are immediately adjacent to the vitreous (v), are arrowed near the bottom of panels A-D. (E,F) Hematoxylin and Eosin staining of adjacent sections, revealing thickening of the retina and abundant abnormal blood vessels in Tbdn-knockdown retina (F) compared with control retinal tissues (E). Arrowheads in A,B,E and F point to blood vessels; brackets indicate the inner retinal layers (inner limiting membrane and ganglion cell layer). Representative images are shown. Magnification is 400X. A-D are not counterstained in order to emphasize brown albumin staining in A and B and lack of staining in C and D.

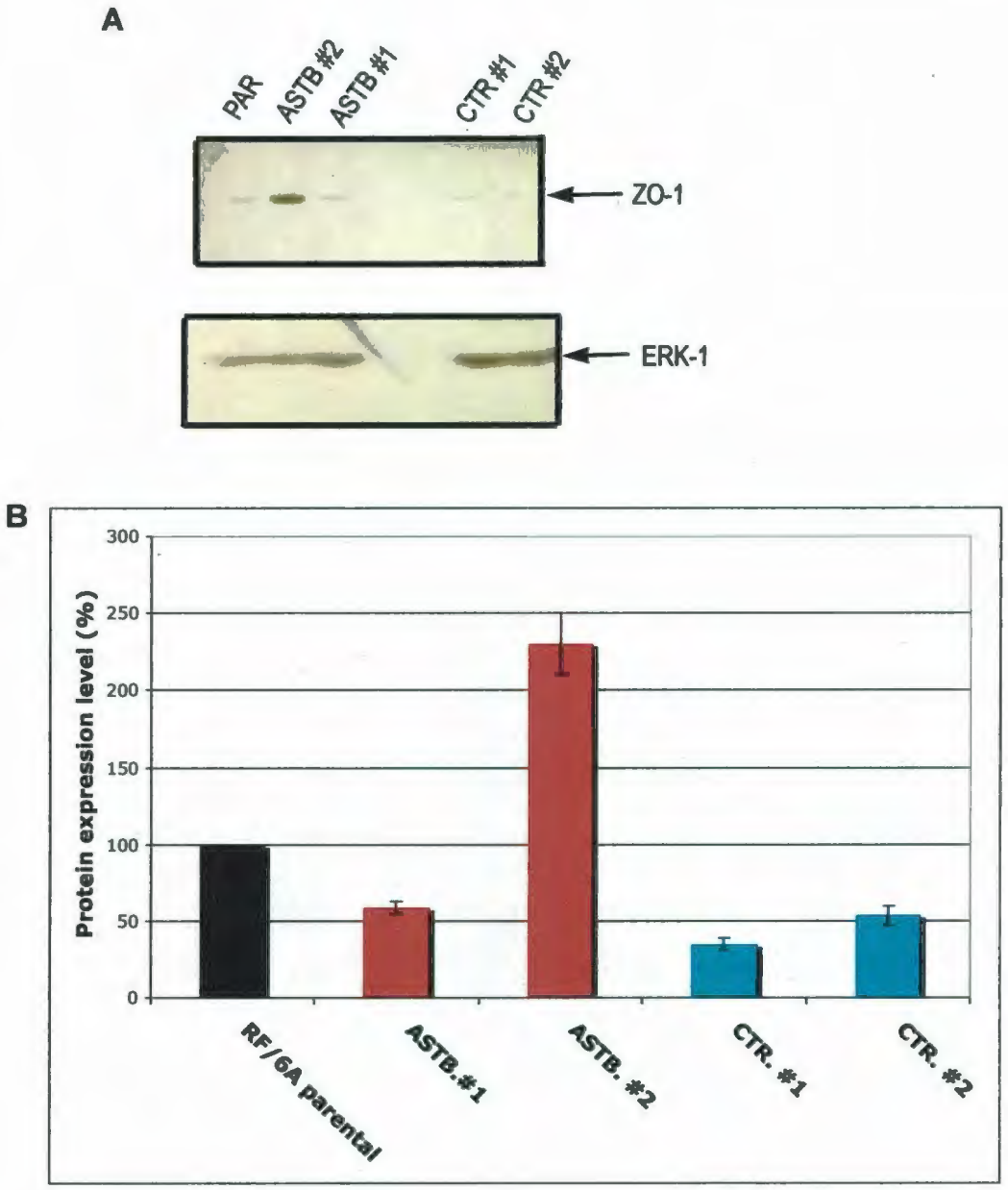

Figure 3-8. Protein expression level of ZO-1 in retinal endothelial cells. A, RF/6A parental endothelial cells (Par), Tbdn knockdown clones AS-*TBDN* (ASTB#1 and #2) and control clones (CTR#1 and #2) analyzed by Western blot for ZO-1 expression (Top panel). Blots were reprobed and analyzed for ERK-1 as loading control (Bottom panel). A representative experiment is shown in **A**, while the average of ZO-1 levels +/- s.e.m. are shown in **B**, n=3 separate experiments.

CHAPTER 4

Discussion

The Tbdn protein sequence contains several tetratricopeptide (TPR) repeats which are known to mediate protein-protein interactions (Gendron et al., 2000; Willis et al., 2002; Main et al., 2005). This information suggests that Tbdn may associate specifically with other proteins. Tbdn associates with the acetyltransferase ARD1 (Sugiura et al., 2003; Arnesen et al., 2005) to form the NATA complex and with the putative acetyltransferase NAT5 (Arnesen et al., 2006c; Hou et al., 2007) forming a complex of still unknown function. Paradis et al. (2008) reported that Tbdn binds to the actin binding protein cortactin. Cortactin has been implicated in regulating the dynamics of the actin cytoskeleton such as cell migration, endocytosis and intracellular movement of vesicles and plays a role in the regulation of the endothelial barrier and endothelial cell permeability (Weed and Parsons, 2001; Daly, 2004; Mehta and Malik, 2006).

Studies describing the role of cortactin and the actin cytoskeleton in post-Golgi mediated vesicle traffic and processing have emerged (Cao et al., 2005; Kessels and Qualmann, 2005; Egea et al., 2006). However, only a single study linking vesicle transport with Tbdn protein function has been reported by Asaumi and co-workers (2005) who presented data that the endocytosis of β–amyloid precursor transmembrane protein from cell surface was inhibited by transient overexpression of the active NATA complex

consisting of Tbdn and ARD1 in transformed embryo kidney cells (HEK293). In addition, secretion of amyloid-β 1-40 which likely depends on the endocytosis of the β-amyloid precursor protein was also suppressed by overexpression of NATA and this suppression required the overexpression of both ARD1 and Tbdn (Asaumi et al., 2005). These results are consistent with our findings that Tbdn suppression increased the transit of FITC-labeled albumin through a cellular monolayer. Our present results show that the transit of FITC-albumin across confluent monolayers of clones of Tbdn knockdown RF/6A endothelial cells was higher than that of control cells. Interestingly, knockdown of Tbdn in diverse cellular systems including the RF/6A endothelial cells (Fig. 3-1) has been associated with a reduction in ARD1 levels (Hou et al, 2007; Polevoda et al, 2007). This observation suggests that Tbdn and ARD1 together regulate FITC-albumin transit across endothelial cell monolayers.

To further understand the mechanism of FITC-albumin transit the intracellular localization of FITC-albumin taken up by RF/6A cells was examined by fluorescence microscopy. Using phalloidin, an F-actin marker, it was observed that the FITC-albumin formed punctate like structures which were partly co-localized with the F-actin (Fig. 3-4). This observation was consistent with previously reported transcytotic mechanism for the permeability of FITC-albumin in retinal endothelial cells (Vinores et al., 1993a; Vinores et al., 1998). We did not see any co-localization between Tbdn and FITC-albumin (Fig. 3-5) in the RF/6A cells. This finding indicates that Tbdn most probably is not involved in the regulation of the cargo of molecule that is found inside the cells but may interact with the vesicle on the outside (Fig.4-1).

The fluorescence microscopy data were further supported by the measured uptake of FITC-albumin in the RF/6A cells (Fig. 3-6). The uptake of FITC-albumin increased gradually up to 120 min, but no significant difference was observed between the intracellular uptake amounts of FITC-albumin in Tbdn knockdown RF/6A cells and the control cells. This result may be explained by either a differential rate of uptake at the entry (endocytosis) or the exit (exocytosis) of the cargo or may be caused by receptor saturation in the vesicle, which limits the uptake of material after a certain time point. The specificity of the assay was confirmed by competing FITC-labeled albumin with excess unlabeled albumin (data not shown).

The regulation of permeability of endothelial cells is known to be functionally important for maintaining proper blood-retina barrier and retinal function (Mehta and Malik, 2006). This property of retinal endothelial cells limits serum proteins such as albumin to intravascular spaces (blood vessel lumens) (Pino and Thouron, 1983; Liao and Gonzalez-Fernandez, 2004). Conversely, extravasation or leakage of serum albumin and other materials out of retinal blood vessels has been described as a marker of retinal blood vessel damage in humans with retinopathy (Vinores et al., 1990; Vinores, et al., 1993b) and has been reported in several rodent models of retina-blood barrier breakdown (Vinores et al., 1999; Liu et al., 2004; Tomasek et al., 2006). The breakdown of retinal-blood barrier function has been shown to involve changes in permeability of retinal endothelial cells (Tomasek et al., 2006) and is associated with edema and thickening of retinal tissues (Gardner et al., 2002; Sander et al., 2007). Our previous studies have shown that the loss of retinal endothelial Tbdn expression is associated with retinal

fibrovascular growth and thickening and occurs in a range of retinopathies including diabetic retinopathy in humans, retinopathy of prematurity in infants, oxygen-induced retinopathy in mice and in proliferative vitreoretinopathy in TGFβ-null mice (Gendron et al., 2001; Paradis et al., 2002; Wall et al., 2004; Gendron et al., 2006). These specific types of retinopathies are known, in some cases, to involve not only retinal angiogenesis but also breakdown of the blood-retinal barrier and changes in retinal endothelial cell permeability and integrity which are associated with extravasation of albumin (Vinores et al., 1990; Vinores, et al., 1993b). In the present study examination of endothelial specific Tbdn-knockdown mice revealed significant leakage of albumin from retinal blood vessels compared to control age matched mice (Fig. 3-7). These results are consistent with our in vitro data showing that the knockdown of Tbdn in retinal endothelial cells leads to increased permeability to FITC-albumin. Moreover, we have previously demonstrated that the same Tbdn knockdown RF/6A retinal endothelial cell clones showing increased permeability here also display significant increases in capillary formation in vitro (Paradis et al., 2002). Overall, our results raise the possibility that increased transfer of serum proteins such as albumin across endothelial cells in Tbdn suppressed blood vessels is a contributing factor to the retinal pathology present in the Tbdn knockdown mouse model of neovascular retinopathy.

In light of cortactin's known role in regulation of the dynamics of the actin cytoskeleton, it was speculated that Tbdn might participate in an acetyltransferase complex with cortactin in a range of processes involving the regulation of the F-actin cytoskeleton in endothelial cells (Paradis et al., 2008). Our present results on the effects

of Tbdn on the transit of FITC-albumin across cellular monolayers support a model in which Tbdn is involved in at least one of these cytoskeletal-mediated structural processes in endothelial cells (Mehta and Malik, 2006). The transport of FITC-labeled albumin has been used to assess endothelial permeability in vitro (Vogel at al., 2001; Tuma and Hubbard, 2003; Irwin et al., 2005). Endothelial cell permeability and the transit of molecules across the endothelial cell barrier are dependent upon both paracellular pathways and receptor mediated transcellular processes. The paracellular pathway involves intercellular junctions while the transcellular pathway utilizes a vesicular system (Mehta and Malik, 2006). Previous studies have shown that albumin utilizes the transcellular pathway in retinal endothelial cells in vivo (Vinores et al, 1993a; Vinores et al., 1998). Our results suggest that FITC-labeled albumin transit across RF/6A endothelial cell monolayers occurs at least via intracellular vesicular transport (Fig. 3-4) as previously reported for albumin in retinal endothelial cells (Vinores et al., 1993a; Vinores et al., 1998). Our results together with previous studies (Asaumi et al., 2005) implicating Tbdn in the regulation of endocytosis which is necessary for transcellular transport of albumin, support the notion that Tbdn is involved in the regulation of transcellular permeability. Both the para- and transcellular pathways are dependent upon the actin cytoskeleton and cortactin likely plays a role in both processes (Tuma and Hubbard, 2003; Mehta and Malik, 2006). Cortactin has been demonstrated to interact with proteins which regulate intercellular junction dynamics while also interacting with proteins participating in the vesicular flux of molecules across endothelial layers (Weed and Parsons, 2001; Daly, 2004; Mehta and Malik, 2006; Huang et al., 2006). Moreover,

both pathways are important for vascular homeostasis (Tuma and Hubbard; Mehta and Malik, 2006). The effects of Tbdn on endothelial cell permeability are consistent with the role of Tbdn in the regulation of retinal vascular homeostasis (Wall et al., 2004). Nevertheless, the possibility of a role for Tbdn in paracellular permeability of endothelial cells will require further studies. We have looked to see if Tbdn regulates the level of ZO-1, a protein involved in the regulation of paracellular permeability, by measuring the expression level of ZO-1 in vitro using RF/6A cell clones with suppressed levels of Tbdn. There does not appear to have been any correlation between the expression level of ZO-1 and Tbdn levels although some clonal variability was observed (Fig. 3-8). This finding does not support a role for Tbdn in regulating the paracellular permeability. However, Tbdn knockdown clones could still influence the sub-cellular localization of ZO-1, which is essential for the functional assembly of tight junctions (Stevenson et al. 1986) and therefore, indirectly affect the paracellular permeability. Based on our findings, we propose a model to explain the mechanism by which FITC-albumin permeability occur in RF/6A retinal endothelial cells and the possible role of Tbdn in mediating this permeability is shown in Fig.4-1.

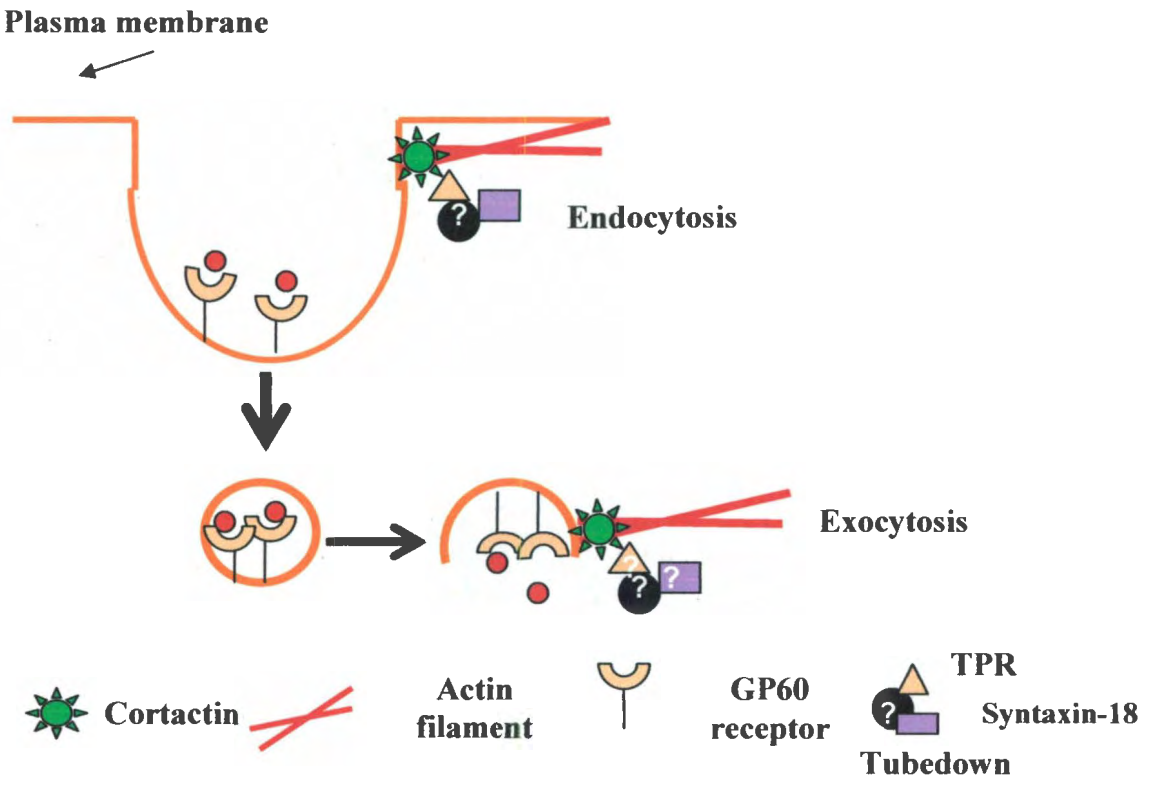

Figure 4-1 Proposed mechanism for transcytosis of FITC-albumin in the retinal endothelial cells and the role of Tbdn. Transcytosis pathway consists of both endocytosis and exocytosis. While it is not clear if Tbdn is involved in the endocytosis or the exocytosis pathway, the interaction of Tbdn with cortactin could mediate albumin transcytosis via either the TPR or syntaxin conserved binding domains found in the Tbdn protein sequence.

Conclusions

Neovascular retinopathies including PDR, ROP and AMD are leading cause of blindness in the general population affecting the quality of patient's life significantly. It is therefore essential to get a thorough understanding of the molecular mechanisms underlying the cause of neovascular pathologies to prevent and treat these diseases. Previous investigations indicate that Tubedown is an important homeostatic factor for maintaining a healthy retinal vascular network (Gendron et al., 2000; Gendron et al., 2001; Paradis et al., 2002; Wall et al., 2005). Ocular diseases with neovascularization are often accompanied by a change in permeability (Erickson, 2007). Therefore, to provide a mechanistic view of the involvement of Tbdn in neovascular retinal diseases, we investigated here the role of Tbdn on the permeability of retinal endothelial cells.

We have gathered evidence indicating that the level of Tubedown affects the permeability of RF/6A retinal endothelial cells in vitro and of retinal blood vessels in vivo to albumin. Immunofluorescence data indicate a transcellular pathway for shuttling FITC-albumin across the cell monolayer. Based on our results we have proposed a mechanism where Tbdn may play an important in regulating the permeability of retinal endothelial cells to albumin by interacting with the actin binding protein, cortactin via its conserved domains. This work implicates a functional role for Tbdn in blood-vessel permeability dynamics that are crucial for vascular homeostasis. Loss of Tbdn could have clinically relevant significance and Tbdn may be used as a future drug target.

References

Andreoli C.M. and Miller J.W. Anti-vascular endothelial growth factor therapy for ocular neovascular disease. *Current Opinion in Ophthalmology*. 2007; **18**:502–508.

Arnesen T., Anderson D., Baldersheim C., Lanotte M., Varhaug J.E., and Lillehaug J.R. Identification and characterization of the human ARD1-NATH protein acetyltransferase complex. *Biochem J.* 2005; **386**, 433-443.

Arnesen T., Betts M.J., Pendino F., Liberles D.A., Anderson D., Caro J., Kong X., Varhaug J.E., and Lillehaug J.R. Characterization of hARD2, a processed hARD1 gene duplicate, encoding a human protein N-alpha-acetyltransferase. *BMC Biochem.* 2006; **7**, 13.

Arnesen T., Gromyko D., Pendino F., Ryningen A., Varhaug J.E., and Lillehaug J.R. Induction of apoptosis in human cells by RNAi-mediated knockdown of hARD1 and NATH, components of the protein N-alpha-acetyltransferase complex. *Oncogene*. 2006b; **25**, 4350-4360.

Arnesen T., Anderson D., Torsvik J., Halseth H.B., Varhaug J.E., and Lillehaug J.R. Cloning and characterization of hNAT5/hSAN: an evolutionarily conserved component of the NatA protein N-alpha-acetyltransferase complex. *Gene*. 2006c; **371**, 291-295.

Asaumi M., Iijima K., Sumioka A., Iijima-Ando K., Kirino Y., Nakaya T., and Suzuki T. Interaction of N-terminal acetyltransferase with the cytoplasmic domain of beta-amyloid precursor protein and its effect on A beta secretion. *J. Biochem* (Tokyo). 2005; **137**, 147-155.

Bazzoni G. Endothelial tight junctions: permeable barriers of the vessel wall. *Thromb Haemost.* 2006; 95: 36–42.

Bhisitkul RB. Vascular endothelial growth factor biology: clinical implications for ocular treatments. *Br J Ophthalmol.* 2006 Dec ; **90**(12):1542-7.

Bradley J., Ju M. and Robinson G.S. Combination therapy for the treatment of ocular neovascularization. *Angiogenesis.* 2007; **10**:141-148.

Cao, H., Weller, S., Orth, J.D., Chen, J., Huang, B., Chen, J.L., Stamnes, M., and McNiven, M.A.Actin and Arf1-dependent recruitment of a cortactin-dynamin complex to the Golgi regulates post-Golgi transport. *Nat. Cell Biol.*2005; 7:483-492.

Chawla D., Agarwal R., Deorari A.K. and Paul V.K. Retinopathy of prematurity. *Indian J Pediatr.* 2008 Jan; **75**(1):73-6.

Chen J. and Smith L.E. Retinopathy of prematurity. *Angiogenesis.* 2007; **10** (2):133-40.

Cines D.B., Pollak E.S., Buck C.A.; Loscalzo J., Zimmerman G.A., McEver R.P., Pober J.S.,Wick T.M., Konkle B.A., Schwartz B.S., Barnathan E.S., McCrae K.R., Hug B.A., Schmidt AM and Stern DM. Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood.*1998. **91** (10): 3527–3561.

Clark P.R., Manes T.D., Pober J.S., Kluger M.S. Increased ICAM-1 expression causes endothelial cell leakiness, cytoskeletal reorganization and junctional alterations. *J Invest Dermatol.* 2007 Apr; 127(4):762-74.

Clements R.T., Minnear F.L., Singer H.A., Keller R.S. and Vincent P.A. RhoA and Rho-

kinase dependent and independent signals mediate TGF-β induced pulmonary endothelial cytoskeletal reorganization and permeability. *Am J Physiol Lung Cell Mol Physiol.* 2005. **288**: L294-L306.

Daly, R.J. Cortactin signaling and dynamic actin networks. *Biochem. J.* 2004 ; **382**:13-25.

Dorrell M., Jarvinen-Uusitalo H., Aguilar E. and Friedlander M. Ocular neovascularization: basic mechanisms and therapeutic advances. *Survey of Opthalmology*. 2007; **52** (1):S3-S19.

Egea, G., Lazaro-Dieguez, F., and Vilella, M. Actin dynamics at the Golgi complex in mammalian cells. *Curr. Opin. Cell Biol.* 2006; **18**:168-178.

Erickson K.K., Sundstrom J.M. and Antonetti D.A. Vascular permeability in ocular disease and the role of tight junctions. *Angiogenesis.* 2007; **10**:103–117.

Esper R.J., Nordaby R.A., Vilariño J.O., Paragano A., Cacharrón J.L., Machado R.A. Endothelial dysfunction: a comprehensive appraisal. *Cardiovasc Diabetol.* 2006 Feb 23;**5**:4.

Fluge, O., Bruland, O., Akslen, L.A., Varhaug, J.E., and Lillehaug, J.R. NATH, a novel gene overexpressed in papillary thyroid carcinomas. *Oncogene.* 2002. **21**; 5056-5068.

Gardner T.W., Antonetti D.A., Barber A.J., LaNoue K.F. and Levison S.W. Diabetic Retinopathy: More Than Meets the Eye. *Survey of Ophthalmology*. December, 2002; **47**(Supplement 2): S253-S262.

Gautschi M., Just S., Mun A., Ross S., Rucknagel P., Dubaquie Y., Ehrenhofer-Murray A., and Rospert S. The yeast N(alpha)-acetyltransferase NatA is quantitatively

anchored to the ribosome and interacts with nascent polypeptides. *Mol. Cell. Biol.* 2003; **23**: 7403-7414.

Geissenhoner A., Weise C., and Ehrenhofer-Murray A.E. Dependence of ORC silencing function on NatA-mediated N-alpha acetylation in Saccharomyces cerevisiae. *Mol. Cell. Biol.* 2004; **24**: 10300-10312.

Gendron R.L., Adams L.C., and Paradis H. Tubedown-1, a novel acetyltransferase associated with blood vessel development. *Dev. Dyn.* 2000; **218**: 300-315.

Gendron R.L., Good W.V., Adams L.C., and Paradis H. Expression of tubedown-1 is suppressed in retinal neovascularization of proliferative diabetic retinopathy. *Invest. Opthalmol. Vis. Sci.* 2001; **42**: 3000-3007.

Gendron R.L., Good W.V., Miskiewicz E., Tucker S., Phelps D.L., and Paradis H. Tubedown-1 (Tbdn-1) suppression in oxygen-induced retinopathy and in retinopathy of prematurity. *Mol. Vis.* 2006; **12**: 108-116.

Good W.V. and Gendron R.L. Genomics and proteomics of retinopathy of prematurity: DNA-based prevention and treatment. *British Journal of Opthalmology.* 2007; **91**(12):1577.

Gregory P.D., Wagner K., and Horz W. Histone Acetylation and Chromatin Remodeling. *Experimental Cell Research.* 2001; **265:** 195–202.

Harhaj N.S. and Antonetti D.A. Regulation of tight junctions and loss of barrier function in pathophysiology. *The International Journal of Biochemistry & Cell Biology.* 2004; **36**(7): 1206-1237.

Hou F., Chu, C.W., Kong X., Yokomori K., and Zou H. The acetyltransferase activity of San stabilizes the mitotic cohesin at the centromeres in a shugoshin-independent manner. *J. Cell. Biol.* 2007; **21**, 587-597.

Huang, R., Cao, G.J., Guo, H., Kordowska, J., and Albert Wang, C.L. Direct interaction between caldesmon and cortactin. *Arch. Biochem. Biophys.* 2006; **456**:175-182.

Hosoya K. and Tomi M. Advances in the cell biology of transport via the inner blood-retinal barrier: establishment of cell lines and transport functions. *Biol.Pharm.Bull.* 2005; **28**(1):1-8.

Irwin D.C., Tissot van Patot M.C., Tucker A., Bowen R. Direct ANP inhibition of hypoxia-induced inflammatory pathways in pulmonary microvascular and macrovascular endothelial monolayers. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 2005; **288**: 849-859.

Ishibashi T., Tanaka K. and Taniguchi Y. Disruption of the blood-retinal barrier in experimental diabetic rats: An electron microscopic study. *Experimental Eye Research.*1980; **30**: 401–410.

Kessels, M.M., and Qualmann, B. Extending the court for cortactin: from the cortex to the Golgi. 2005. *Nat. Cell. Biol.* 7: 448-449.

Kimura Y., Saeki Y., Yokosawa H., Polevoda B., Sherman F., and Hirano H. N-Terminal modifications of the 19S regulatory particle subunits of the yeast proteasome. *Arch. Biochem. Biophys.* 2003; **409**: 341-348.

Kowalski J.R., Egile C., Gil S., Snapper S.B., Li R., and Thomas S.M. Cortactin regulates cell migration through activation of N-WASP. *J. Cell Sci.* 2005; **118**: 79-87.

Kvanta A. Ocular Angiogenesis: the role of growth factors. *Acta Opthalmologica Scandinavica*. 2006; **84** (3):282-288.

Lamalice L., Le Boeuf F., and Huot J. Endothelial cell migration during angiogenesis. *Circ Res*. 2007 Mar 30; **100** (6):782-94.

Liao, R., and Gonzalez-Fernandez, F. Albumin is not present in the murine interphotoreceptor matrix, or in that of transgenic mice lacking IRBP. *Mol Vis*. 2004; **10**: 1038-1046.

Lim J.H., Park W., and Chun Y.S. Human arrest defective 1 acetylates and activates beta-catenin, promoting lung cancer cell proliferation. *Cancer Res*. 2006; **66**: 10677-10682.

Liu H., Demetriades A.M., Xiao W.H., Campochiaro P.A., and Vinores S.A. Mouse model of post-surgical breakdown of the blood-retinal barrier. *Curr Eye Res*. 2004; **28**:42142-42146.

Madara J.L., Barenberg D. and Carlson S. Effects of cytochalasin D on occluding junctions of intestinal absorptive cells:further evidence that the cytoskeleton may influence paracellular permeability and junctional charge selectivity. *J.Cell Biol*. 1986; **102**: 2125-2136.

Martin D.T., Gendron R.L., Jarzembowski J.A., Perry A., Collins M.H., Pushpanathan C., Miskiewicz E., Castle V.P. and Paradis H. Tubedown expression correlates with the differentiation status and aggressiveness of neuroblastic tumors. *Clin Cancer Res*. 2007 Mar 1;**13**(5):1480-7.

Main E.R., Stott K., Jackson S.E., and Regan L. Local and long-range stability in

tandemly arrayed tetratricopeptide repeats. *Proc Natl Acad Sci U S A.* 2005. **102**; 5721-5726.

Mehta D. and Malik A.B. Signaling mechanisms regulating endothelial permeability. *Physiol Rev.* 2006; **86**: 279-367.

Montezuma S.R., Sobrin L. and Seddon J.M. Review of Genetics in Age Related Macular Degeneration. *Seminars in Ophthalmology.* 2007; **22**:229–240.

Mullen J.R., Kayne P.S., Moerschell R.P., Tsunasawa S., Gribskov M., Colavito-Shepanski M., Grunstein M., Sherrnan F. and Sternglanz R. Identification and characterization of genes and mutants for an N-terminal acetyltransferase from yeast. *EMBO J.* 1989 Jul;**8** (7): 2067-75.

Nagpal M., Nagpal K. and Nagpal P.N. A comparative debate on the various anti-vascular endothelial growth factor drugs: pegaptanib sodium (Macugen), ranibizumab (Lucentis) and bevacizumab (Avastin). *Indian J Ophthalmol.* 2007 Nov-Dec; **55**(6):437-9.

Neely K.A. and Gardner T.W. Ocular neovascularization: Clarifying complex interactions. *The American Journal of Pathology.* 1998; **153**:665-670.

Packer S., Jampol L.M. An ethical view of the ranibizumab and bevacizumab controversy. *Arch Ophthalmol.* 2008 Feb;**126**(2):286.

Paradis H., and Gendron R.L. LIF tranduces contradictory signals on capillary outgrowth through induction of Stat3 and $^{P41/43}$MAP kinase. *J. Cell Sci.* 2000; **113**: 4331-4339.

Paradis H., **Islam T.**, Tucker S., Tao L., Koubi S., Gendron R.L. Tubedown associates with cortactin and controls permeability of retinal endothelial cells to albumin. *J Cell Sci.* 2008 Jun 15; **121**(Pt 12):1965-72

Paradis H., Liu C.Y., Saika S., Azhar M., Doetschman T., Good W.V., Nayak R., Laver N., Kao C.W., Kao W.W., and Gendron R.L. Tubedown-1 in TGF-b2 mediated remodeling of the developing vitreal vasculature in vivo and regulation of capillary outgrowth in vitro. *Dev. Biol.* 2002; **249**: 140-155.

Park E.C., and Szostak J.W. ARD1 and NAT1 proteins form a complex that has N terminal acetyltransferase activity. *EMBO J.* 1992; **11**: 2087-2093.

Pino, R.M., and Thouron, C.L. Vascular permeability in the rat eye to endogenous albumin and immunoglobulin G (IgG) examined by immunohistochemical methods. *J Histochem Cytochem.* 1983; **31**:411-416.

Polevoda B., and Sherman F. N-terminal acetyltransferases and sequence requirements for N-terminal acetylation of eukaryotic proteins. *J. Mol. Biol.* 2003; **325**: 595-622.

Polverini P.J. The pathophysiology of angiogenesis. *Crit Rev Oral Biol Med.* 1995; **6**(3): 230-47.

Predescu S.A., Predescu D.N. and Malik A.B. Molecular determinants of endothelial transcytosis and their role in endothelial permeability. *Am J Physiol Lung Cell Mol Physiol.* 2007 Oct; **293**(4):L8.

Polevoda B., Brown S., Cardillo T.S., Rigby S., and Sherman F. Yeast N(alpha)-terminal acetyltransferases are associated with ribosomes. *J. Cell. Biochem.* 2007; May 31, Epub ahead of print.

Sander, B., Thornit, D.N., Colmorn, L., Strøm, C., Girach, A., Hubbard, L.D., Lund-Andersen, H., and Larsen, M. Progression of diabetic macular edema: correlation with blood retinal barrier permeability, retinal thickness, and retinal vessel diameter. *Invest Ophthalmol Vis Sci.* 2007; **48**:3983-3987.

Schneeberger E.E., Lynch R.D. The tight junction: a multifunctional complex. *Am J Physiol Cell Physiol.* 2004; **286**: C1213-C1228.

Shukla D., Namperumalsamy P., Goldbaurn M. and Cunningham ET Jr. Pegaptanib sodium for ocular vascular disease. *Indian J Ophthalmol.* 2007 Nov-Dec; **55**(6):427-30.

Simionescu M. and Antohe F. Functional ultrastructure of the vascular endothelium: changes in various pathologies. *Handb Exp Pharmacol.* 2006;(176 Pt 1):41-69.

Simionescu N. The Microvascular Endothelium: Segmental Differentiations, Transcytosis, Selective Distribution of Anionic Sites. New York: Raven, 1979.

Stevenson, B.R., Siliciano, J.D., Mooseker, m.S., Goodenough, D.A. Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J Cell Biol.* 1986; **103**(3):755-766.

Sugiura, N., Adams, S.M., and Corriveau, R.A. An evolutionarily conserved N-terminal acetyltransferase complex associated with neuronal development. *J. Biol.*

*Chem.*2003; **278**:40113-40120.

Swaroop A., Branham K., Chen W. and Abecasis G. Genetic susceptability to age-related macular degenration : a paradigm for dissecting complex disease traits. *Human Molecular Genetics.* 2007; **16** (R2) : R174-182.

Szaflik J.P., Wysocki T., Kowalski M., Majsterek I., Borucka A.I., Blasaiak J. and Szaflik J. An association between vascular endothelial growth factor gene promoter polymorphisms and diabetic retinopathy. *Graefes Arch Clin Exp Ophthalmol.* Epub 2007. **246** (1): 39-43.

Takano M., Nakanishi N., Kitahara Y., Sasaki Y., Murakami T. and Nagai J. Cisplatin-induced inhibition of receptor-mediated endocytosis of protein in the kidney. *Kidney International.* 2002; **62**:1707–1717.

Tomasek, J.J., Haaksma, C.J., Schwartz, R.J., Vuong, D.T., Zhang, S.X., Ash, J.D., Ma, J.X., and Al-Ubaidi, M.R. Deletion of smooth muscle alpha-actin alters blood-retina barrier permeability and retinal function. *Invest Ophthalmol Vis Sci.* 2006; **47**:2693-2700.

Trepels T., Zeiher A.M. and Fichtlscherer S. The endothelium and inflammation. *Endothelium.* 2006 Nov-Dec; **13**(6): 423-9.

Tuma, P. L. and Hubbard, A. L. Transcytosis: crossing cellular barriers. *Physiol. Rev.* 2003; **83**:871-932.

Tyagi N., Roberts A.M., Dean W.L., Tyagi S.C., Lominadze D. Fibrinogen induces endothelial cell permeability. *Mol Cell Biochem.* 2008 Jan; **307**(1-2):13-22.

Vinores, S.A., Campochiaro, P.A., Lee, A., McGehee, R., Gadegbeku, C., and Green, W.R. Localization of blood-retinal barrier breakdown in human pathologic specimens by immunohistochemical staining for albumin. *Lab Invest.*1990; **62**:742-750.

Vinores, S.A., Van Niel, E., Swerdloff, J.L., Campochiaro, P.A. Electron microscopic immunocytochemical evidence for the mechanism of blood-retinal barrier breakdown in galactosemic rats and its association with aldose reductase expression and inhibition. *Exp Eye Res.* 1993a; **57**:723-735.

Vinores, S.A., Van Niel, E., Swerdloff, J.L., and Campochiaro, P.A. Electron microscopic immunocytochemical demonstration of blood-retinal barrier breakdown in human diabetics and its association with aldose reductase in retinal vascular endothelium and retinal pigment epithelium. *Histochem J.* 1993b; **25**:648-663.

Vinores, S.A., Derevjanik, N.L., Mahlow, J., Berkowitz, B.A., and Wilson, C.A. (1998) Electron microscopic evidence for the mechanism of blood-retinal barrier breakdown in diabetic rabbits: comparison with magnetic resonance imaging. *Pathol Res Pract.* **194**, 497-505.

Vinores, S.A., Derevjanik, N.L., Ozaki, H., Okamoto, N., and Campochiaro, PA. Cellular mechanisms of blood-retinal barrier dysfunction in macular edema. *Doc Ophthalmol.*1999; **97**:217-228.

Vogel, S.M., Easington, C.R., Minshall, R.D., Niles, W.D., Tiruppathi, C., Hollenberg, S.M., Parrillo, J.E., and Malik, A.B. (2001) Evidence of transcellular permeability pathway in microvessels. *Microvasc. Res.* 2001; **61**:87-101.

Wall D.S., Gendron R.L., Good W.V., Miskiewicz E., Woodland M., Leblanc K., Paradis H. Conditional knockdown of tubedown-1 in endothelial cells leads to neovascular retinopathy. *Invest Ophthalmol Vis Sci.* 2004 Oct; **45**(10):3704-12.

Wang, X., Connelly, J.J., Wang, C.L., and Sternglanz, R. (2004) Importance of the Sir3 N-terminus and its acetylation for yeast transcriptional silencing. *Genetics* **168**, 547-551.

Weed, S.A., and Parsons, J.T. (2001) Cortactin: coupling membrane dynamics to cortical actin assembly. *Oncogene* **20**, 6418-6434.

"What is macular degeneration?". American Macular Degeneration Foundation (AMDF). [Online]. Accessed on May 7th.2008 < http://www.macular.org>.

Whiteway M., Freedman R., Van Arsdell S., Szostak J.W., Thorner J.The yeast ARD1 gene product is required for repression of cryptic mating-type information at the HML locus. *Mol Cell Biol.* 1987 Oct; 7(10):3713-22.

Whiteway M., Szostak J.W. The ARD1 gene of yeast functions in the switch between the mitotic cell cycle and alternative developmental pathways. *Cell.* 1985 Dec; **43**(2 Pt 1):483-92.

Willis, D.M., Loewy, A.P., Charlton-Kachigian, N., Shao, J.S., Ornitz, D.M., and Towler, D.A. (2002) Regulation of osteocalcin gene expression by a novel Ku antigen transcription factor complex. *J. Biol. Chem.* **277**, 37280-37291.

Yu P.K., Yu D.Y., Cringle S.J. and Su E.N. Endothelial F-actin cytoskeleton in the retinal vasculature of normal and diabetic rats. *Current Eye Research*.2005; **30**(4): 279-290.