12-9-2020 12:00 PM

The Role of TIMP3 in Microvascular Endothelial Cell-Extracellular Matrix Interaction and Regulation of Microvascular Barrier Function

Nidhi Kulkarni, *The University of Western Ontario*

Supervisor: Gill, Sean E., *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Physiology and Pharmacology

Abstract

Pulmonary microvascular endothelial cell (PMVEC) interactions with the extracellular matrix (ECM) mediates PMVEC barrier function. Further, data suggests an association between decreased PMVEC-ECM interactions under proinflammatory conditions and PMVEC barrier dysfunction. Tissue inhibitor of metalloproteinases 3 (TIMP3) may also regulate barrier function, as PMVEC from *Timp3*$^{-/-}$ mice show increased leak. Studies in the developing lung also showed TIMP3 regulating PMVEC-ECM interactions. Based on this, I hypothesized that TIMP3 maintains PMVEC barrier function by promoting PMVEC-ECM interactions.

Using the XperT-permeability assay, *Timp3*$^{-/-}$ PMVEC demonstrated enhanced leak vs. WT PMVEC, particularly under proinflammatory conditions, and this was associated with decreased phosphorylated focal adhesion kinase (pFAK). Furthermore, FAK inhibition increased leak in WT PMVEC, and enhanced leak in *Timp3*$^{-/-}$ PMVEC under proinflammatory conditions. Finally, MMP inhibition attenuated leak in *Timp3*$^{-/-}$ PMVEC under both basal and proinflammatory conditions.

These results suggest a role for TIMP3 in mediating PMVEC-ECM interactions and promoting PMVEC barrier function.

Keywords

Microvascular endothelial cells, extracellular matrix, tissue inhibitor of metalloproteinase, metalloproteinases, intercellular adhesion molecules, focal adhesion kinases, pulmonary microvascular barrier function, leak, cell-matrix interactions.

Summary for Lay Audience

Endothelial cells (EC) line the blood vessels and are important in maintaining their function. EC normally form a barrier by interacting with each other and with the supporting scaffold found beneath the EC, known as extracellular matrix (ECM). A specific protein called tissue inhibitors of metalloproteinases 3 (TIMP3) may help support EC in their role of maintaining a barrier within the lungs as an absence of TIMP3 is shown to increase fluid buildup in the lungs due to EC losing their ability to form a barrier. While exactly how TIMP3 regulates EC in the lung is still unclear, it is possible that TIMP3 regulates the interaction of EC with their environment. As such, I hypothesized that TIMP3 regulates lung function by supporting the interaction of EC with the ECM.

By comparing normal mouse lung cells with cells that contained no TIMP3 (*Timp3*$^{-/-}$ cells), I found that the *Timp3*$^{-/-}$ cells had fewer focal adhesion proteins in them, as compared to the normal cells. Focal adhesions are important proteins found inside cells that help maintain the interaction of cells with their environment. Additionally, the *Timp3*$^{-/-}$ cells seemed to be leakier than the normal cells, as shown by a fluorescent protein that marked where gaps would form between the cells and their environment. Addition of inhibitors that targeted metalloproteinases, proteins that TIMP3 is shown to inhibit within the tissue, rescued the increased leak in normal cells as well as in the *Timp3*$^{-/-}$ cells. Furthermore, after adding inhibitors to block focal adhesion formation, both normal cells and *Timp3*$^{-/-}$ cells showed an increase in leak. These results were especially prominent in groups where an inflammatory environment was induced.

These results show a promising start to understanding the role of TIMP3 in mediating cellular interaction with each other and with the surrounding environment in the lungs. Importantly, they suggest that TIMP3 and EC-ECM interactions may be a possible therapeutic target for supporting the EC barrier function under inflammatory conditions in the lungs.

Co-Authorship Statement

All studies and data included in this thesis report were generated by Nidhi P. Kulkarni. In addition, Chapter 1 contains content from: Jayawardena DP, Kulkarni NP, and Gill SE. (2019) The role of tissue inhibitors of metalloproteinases in microvascular endothelial cell barrier dysfunction during sepsis. *Metalloproteinases in Medicine.* 6: 1-12. Nidhi P. Kulkarni was the second author of the article. Figures are reproduced with permission from Dove Press (Appendix A).

Acknowledgments

First and foremost, I want to thank Dr. Sean E. Gill, my supervisor and mentor, for taking a leap of faith when accepting me as a graduate student. His continuous support over the past two years has molded me into the capable research student that I am today, and I am forever grateful. I would also like to thank my lab members, Cynthia Pape and Dr. Lefeng Wang, for providing me with the hands-on expertise and sound advice that has allowed me to pursue my research goals and taught me lifelong skills along the way. Thank you to my fellow graduate students as well, for being the supporting friends that we all hope for during the ups and downs of graduate life. Also, a huge thank you to Dr. Sanjay Mehta and to my advisory committee, Dr. Ruud Veldhuizen, Dr. John Di Guglielmo, and Dr. Geoffrey Pickering, for guiding me through my project and challenging me to think outside of the box. I also would not have been able to acquire some my results if not for the help and training from the Robarts staff- I will always appreciate the time and help provided. Last but certainly not least, I would like to thank all my loved ones who have stood behind me since day one- I would not be here without your constant encouragement (and the occasional tough love). You guys are my light and my motivation to keep growing into the best version of me.

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List of Abbreviations

ADAM	A Disintegrin And Metalloproteinase
ADAMTS	A Disintegrin And Metalloproteinase with Thrombospondin Motifs
AT2	Angiotensin 2
ALI	Acute Lung Injury
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium ion
CO_2	Carbon Dioxide
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
EB	Evans Blue
EC	Endothelial Cells
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
FAC	Focal Adhesion Complexes
FAK	Focal Adhesion Kinase

GAG	Glycosaminoglycan
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
ICAM	Intercellular Adhesion Molecule
IFN	Interferon
IL	Interleukin
LDL	Low Density Lipoprotein
LPS	Lipopolysaccharide
MAPK	Mitogen Activated Protein Kinase
MMP	Matrix Metalloproteinase
MOD	Multi-Organ Dysfunction
mRNA	Messenger Ribonucleic Acid
MT-MMP	Membrane Type-Matrix Metalloproteinase
MVEC	Microvascular Endothelial Cells
NOS	Nitric Oxide Synthase
PBS	Phosphate Buffered Saline
PECAM	Platelet Endothelial Cell Adhesion Molecule
pFAK	Phosphorylated Focal Adhesion Kinase

PI3K	Phosphoinositide 3-Kinase
PKB	Protein Kinase B
PKC	Protein Kinase C
PMVEC	Pulmonary Microvascular Endothelial Cells
RGD	Arginine Glycine Aspartic Acid
TACE	TNFα Converting Enzyme
TEER	Trans-endothelial Electrical Resistance
TGF	Transforming Growth Factor
TIMP	Tissue Inhibitor of Metalloproteinase
TNF	Tumor Necrosis Factor
VCAM	Vascular Cell Adhesion Molecule
VE	Vascular Endothelial
VEGF	Vascular Endothelial Growth Factor
WT	Wild Type
Zn^{+}	Zinc ion

Chapter 1

1 Introduction

1.1 The vascular endothelium

The vascular system plays an important role in nutrient-provision and waste removal from tissues and organ systems of the body [1]. Additionally, it regulates important homeostatic processes such as inflammation, barrier maintenance, and wound repair [2]. The vascular wall is made up of three distinct layers: the tunica intima which directly interacts with blood, the tunica media which alters vascular tone, and the tunica externa which provides structural and other (i.e. nutritional, neurogenic) support to the vessels [1,3].

The tunica intima is comprised of the endothelium, a single-cell layer composed of a heterogeneous population of endothelial cells (EC). While the endothelium is present in blood and lymphatic vessels throughout the body, the structure, function, and response of these cells differ depending on the type of vessel, location and the stimulation they are exposed to [3]. The endothelium can be further segregated into macrovascular endothelial cells, which line large blood vessels like the umbilical cord, and the microvascular endothelial cells (MVEC), which line the capillaries and are regulators of tissue perfusion and microcirculation [3–5]. These vessels not only vary in size but also express

heterogeneity in gene expression and functional processes like angiogenesis and protein synthesis [3–5].

The endothelium is highly dynamic in its function, and its roles extend beyond maintaining vessel structure and adequate circulation [6]. For instance, the EC form a tightly regulated, semi-permeable barrier, which controls the movement of molecules and cells, allowing for compartmentalization within the body [6]. Furthermore, the EC secrete a variety of autocrine, paracrine, and endocrine factors and also regulates vascular function through their interaction with circulating compounds (i.e. hormones, neurotransmitters, and vasoactive factors) [3,6]. Through these interactions, EC are critical mediators of platelet and leukocyte interactions, thrombosis and coagulation, regulating vascular tone, and angiogenesis [7]. Due to the extensive role of the endothelium in maintaining key homeostatic processes, disruption of this layer is associated with numerous pathologies, like atherosclerosis, chronic kidney disease, and sepsis [6].

1.2 Pulmonary microvasculature

The pulmonary endothelium differs widely in structure and function, depending upon the location within the lungs. For instance, the pulmonary arterial EC are situated on a thick membrane, whereas the capillary EC are separated from sub-endothelial tissues only by a thin basement membrane [8]. Additionally, capillary EC express different lectin-binding proteins and cell adhesion molecules compared to EC from larger pulmonary vessels [5]. Not to mention, various stimuli (i.e. bacterial insult, proinflammatory cytokines, etc.) result in varied responses

amongst the different types of pulmonary endothelium, with diverse underlying mechanisms mediating their response [5,8].

These site-specific differences are important for functional compartmentalization within the lungs- for instance, the capillary EC form tight barriers that mediate gas exchange and greatly limit the passage of fluid and solutes between the blood and interstitial compartments of the lungs [5,8]. Due to these intrinsic differences in the endothelium throughout the lungs, capillary EC and those of small arterioles and venules are often collectively referred to as pulmonary microvascular EC (PMVEC).

PMVEC act as an interface between the circulating blood and the alveoli of the lungs, making them critical in processes such as gas exchange, maintaining barrier integrity, and regulating vascular tone through secretion of vasoactive factors (i.e. nitric oxide, prostacyclin, endothelin) [9]. These cells are highly dynamic and depend on interactions with adjacent PMVEC, circulating factors and cells, as well as signals from the surrounding extracellular matrix to maintain a healthy, functional endothelium and blood vessel [9].

1.2.1 Endothelial barrier function

The ability of PMVEC to maintain a structurally functional barrier is key for lung function. PMVEC form a semi-permeable monolayer that regulates the efflux of fluid and molecules from the lumen into the pulmonary interstitial compartment, allowing for adequate gas exchange [10].

The EC barrier is regulated through cell-cell and cell-matrix interactions [10]. These interactions are modulated by numerous factors- for instance, the Rho GTPases are shown to regulate EC barrier permeability by altering the formation of actin stress fibers, interaction of intercellular junctional proteins, and focal adhesion complexes (FAC).

There are multiple complexes that support inter-PMVEC adhesion, specifically adherens junctions, tight junctions, and gap junctions [11,12]. These complexes are crucial in forming interactions between adjacent PMVEC to maintain the intact endothelial monolayer [5]. Tight junctions, formed through the association of claudins with occludins, regulate the exchange of ions and solutes between microvascular EC [11,12]. Additionally, junctional adhesion molecules, a family of transmembrane proteins, also contribute to the formation and maintenance of tight junctions [12]. However, the main regulators of inter-PMVEC adhesion are the adherens junctions [11,13]. These complexes are composed of a specialized protein called vascular endothelial cadherin (VE-cadherin) that is involved in cell stability, monolayer integrity, and cell signaling [11,13].

While PMVEC interact with adjacent PMVEC via junctional complexes, they also interact with the underlying extracellular matrix (ECM) via integrins [11,14]. Integrin activation and binding to the ECM leads to the recruitment of signaling proteins such as focal adhesion kinase (FAK) and the tyrosine kinase Fyn, leading to the formation FAC [11,14]. The establishment of FAC and the interaction of PMVEC with the ECM further promotes endothelial barrier function and regulation of microvascular permeability [11,15].

The surrounding ECM, comprised largely of collagen, fibronectin, and glycosaminoglycans (GAGs), adds to the restrictive property of the endothelium required for normal tissue functioning [11,16]. Collagen is the main structural component of the ECM that helps regulate cell adhesion, cell migration, and elasticity of the tissue by providing tensile strength [11,17,18]. Heparan sulfate, one of the most abundant GAGs, has numerous biological functions, including cell–matrix interactions, chemokine activation, and regulation of degradative enzymes [11,16,19]. Fibronectin is important not only for the organization of ECM components but also plays a role in cell adhesion and migration, and as a mechano-regulator [11,20,21]. These ECM components are intertwined in a complex network that maintains the structural integrity of the matrix while allowing it to resist tensile stresses and regulate movement of molecules under healthy conditions [11,16].

An intact endothelium, regulated through inter-PMVEC and cell-matrix interactions, is crucial for maintaining functional blood-lung homeostasis. Healthy EC interactions promote anti-thrombotic processes by limiting platelet and leukocyte adhesion to the endothelial wall, and by deterring platelet activation and aggregation [5]. Additionally, resting PMVEC promote an anti-inflammatory environment by limiting interaction with proinflammatory cytokines, mitigating leukocyte extravasation into pulmonary tissue, and suppressing transcription of adhesion and proinflammatory biomarkers [5]. As such, any compromise to the pulmonary endothelium results in EC dysfunction and impaired barrier regulation.

1.2.2 Endothelial barrier dysfunction

Disruption of the pulmonary endothelium impairs the homeostatic balance within the lungs, leading to a shift from an anti-inflammatory phenotype to an activated proinflammatory phenotype [22]. This causes acute inflammation within the lung parenchyma through an accumulation of proinflammatory leukocytes and platelets, activation of procoagulant pathways, and disruption of the PMVEC barrier, ultimately resulting in pulmonary oedema and hypoxemia [22]. This can often result in acute lung injury (ALI) and if more severe, acute respiratory distress syndrome (ARDS), which can be associated with multi-organ dysfunction (MOD), and has a high risk of death [9].

PMVEC dysfunction is largely associated with proinflammatory pathologies- the lung's innate immune response recruits leukocytes to the pulmonary microvasculature and promotes the extravasation of immune cells into the alveolar space [9]. This is mediated through the release of proinflammatory markers (i.e. cytokines), which are initially secreted by activated leukocytes in the circulation and within the pulmonary tissue [11]. These cytokines direct leukocyte-PMVEC interaction, and stimulation of PMVEC with bacterial products (i.e. lipopolysaccharides (LPS)) results in PMVEC activation and subsequent dysfunction [11].

PMVEC activation leads to further downstream expression of PMVEC-dependent cytokines, cell surface leukocyte receptors (i.e. ICAM1 and VCAM1), and of extracellular enzymes known to regulate inflammatory signaling pathways,

such as the matrix metalloproteinases (MMPs) and closely related a disintegrin and metalloproteinases (ADAMs) [11,23].

There are multiple mechanisms that can mediate the loss of PMVEC barrier function under proinflammatory conditions. For instance, PMVEC retraction due to actin cytoskeleton rearrangement and microtubule-dependent PMVEC contraction is thought to promote gap formation and subsequent barrier dysfunction [11,24]. Additional mechanisms, such as enhanced transcytosis and PMVEC apoptosis, may also play a role; however, the direct impact of these mechanisms on the loss of PMVEC barrier function is unclear [11].

Loss of PMVEC intercellular junctions, including adherens and tight junctions, has also been found to occur under proinflammatory conditions and is thought to be a critical driver of PMVEC barrier dysfunction [11]. The disruption of PMVEC intercellular junctions is initiated by multiple mechanisms and is characterized by the functional loss of key junctional proteins, such as VE-cadherin [11,25]. Loss of functional membrane VE-cadherin can be due to VE-cadherin phosphorylation, targeted cleavage by extracellular proteases, including MMPs and ADAMs, and loss of attachment to submembrane cytoplasmic adaptor proteins (i.e. β-catenin), which link VE-cadherin to the actin cytoskeleton [13,24,25].

Moreover, it has been suggested that alterations to the basement membrane leading to altered PMVEC–ECM interactions may also lead to disruption of intercellular junctions and PMVEC barrier dysfunction [11]. This may be associated with focal adhesion kinase (FAK) activation, which promotes the

assembly of FAC [22]. FAC are shown to interact with integrin receptors on the cell surface to promote PMVEC-ECM adhesion, and to help stabilize adherens junctions to promote inter-PMVEC interactions [22]. Meanwhile, activated FAK is also a Rho-inhibitor, and its action may act to inhibit Rho-regulated barrier dysfunction [22]. The net effect of FAK activation could help regulate PMVEC barrier stability and, subsequently, a lack thereof may be a critical mediator in promoting barrier dysfunction.

1.3 Cellular interactions with the extracellular matrix (ECM)

The ECM is a meshwork of proteins secreted by cells that establishes a micro-environment for the cells [26]. It has numerous functions, from providing structural support to mediating mechanical and biochemical signaling pathways between cells [26]. For instance, vascular development depends on the correct interaction of the vascular progenitor cells with the surrounding basement membrane and the underlying matrix proteins [27]. While these interactions may differ depending on the vessels and the microenvironment, in quiescent states, the EC are non-proliferative and stably attached to the basement membrane [27].

Additionally, studies have shown that maintaining functional differentiation of EC is dependent on providing them with an adequate environment, part of which is the provision of a physiologically relevant basement membrane, including specific components of the ECM [28].

Another role of an adequate environment is providing extracellular signals that maintain the proper orientation of EC [29]. In order to form and maintain a functioning endothelium, EC have shown to secrete their own extracellular matrix, which is deposited at the basal surface of these cells, allowing for polarized adhesion [29]. As such, cell-ECM interactions are critical in developing and maintaining EC polarity and function [26,28,29].

1.3.1 Composition of the ECM

The vascular ECM is composed of a variety of proteins, including laminins, collagens, fibronectin, and proteoglycans [30,31]. During angiogenesis, the secretion of specific biochemical molecules by the endothelium, including transforming growth factor-β (TGFβ), stimulates ECM deposition, while environmental cues (i.e. blood flow, hypoxia, circulating factors) determine the dynamic interaction between established EC and the surrounding membrane [18]. However, the precise mechanisms regulating ECM assembly by EC and its supporting roles are still unknown [18,32].

Each of the ECM proteins plays a different role in the endothelium. For instance, collagens are a class of matrix protein, with at least 27 subtypes [33]. Collagen IV is the predominant subtype associated with the basement membrane layer beneath EC [33]. Collagens mediate stabilization of an established basement membrane, particularly under mechanical stress, by forming fibrillar structures that connect elastin and other matrix proteins to the basement membrane [18,33,34]. In fact, the deletion of collagen IV proves to be embryonically lethal due to defects

in cell-matrix contacts in regions of high mechanical stress (i.e. in the heart and blood vessels). As such, collagen IV seems to be a crucial player in basal lamina assembly and vessel development [34]. On the flip side, excess collagen is the vasculature can lead to vessel fibrosis and increased stiffness [34]. Gelatin, a product of degraded insoluble collagen, is a commonly used biomaterial in the research field, with uses ranging from tissue engineering to producing a cell-interactive coating for culturing cells [35]. This is possible due to the similarity of gelatin to collagen, in terms of adhesion molecules and receptors, while reducing the cost of production and immunogenic effects, as compared to pure collagen [35].

An important proteoglycan found throughout the ECM is fibronectin. This 440kDa protein is often implicated in regulating cell-matrix interactions and cell function, due to its interaction with vascular cells, including EC, and regulation of cellular adhesion, growth, and differentiation [33,34]. Additionally, fibronectin deposition promotes synthesis, organization, and stability of other matrix proteins (i.e. collagen I and III, and thrombospondin), and vascular remodeling through leukocyte infiltration and expression of adhesion molecules [34].

Laminin, a class of glycoproteins, is thought to be the main biologically active component of the vascular ECM and plays a critical role in maintaining the integrity of the endothelium [18]. The two main isoforms of laminin in the endothelium are laminin 8 and laminin 10, each expressed in varying degrees depending on the state of the vessels (new vs. established) and the environmental cues [36]. Laminins are crucial for dictating cell migration and vessel stability during angiogenesis and neovascularization [36]. In fact, during development,

laminins are the primary determinants of EC plasma membrane assembly and critical for vessel maturation and branching [32,36]. Additionally, laminin can regulate transmigration of circulating leukocytes and biomolecules across the endothelium, alluding to a critical role in barrier stability [36]

Vitronectin is another glycoprotein found in both the ECM and circulation [34]. While there is limited data on the role of vitronectin in cell-matrix interactions, it has been shown to regulate vessel remodeling by regulating vascular cell migration and may also contribute to thrombosis [34]. Interestingly, activation of vitronectin has been shown to decrease EC adhesion and results in poor EC spreading on vitronectin-coated matrices [37]. Furthermore, activated vitronectin may increase vascular permeability by promoting internalization of inter-cellular adhesion proteins (i.e. VE-cadherin) [38]. These studies imply a significant role of vitronectin in regulating barrier stability and repair under pathological conditions.

Components of the ECM are highly dynamic and often work in conjunction with one another. Previous studies analyzing the structure and function of ECM proteins under basal and pathological conditions suggest collagen type IV provides structural support to the basal membrane, laminins convey biological signals to surrounding cells, and proteoglycans act principally to cross-link the collagen type IV and laminin networks, bind soluble factors such as growth factors, and are important for the filtration properties of the ECM [36]. Furthermore, the interaction of vascular cells with the basement membrane maintains endothelial homeostasis by reducing inflammatory gene expression, inhibiting matrix calcification, and enhancing contractile genes [34]. Additionally,

basement membrane stiffness seems to play a crucial role in determining the endothelial phenotype- studies have revealed that the differential EC phenotypes in arterial vs. venous capillaries may be due to the differential stiffness of the underlying ECM, regardless of differences in flow [39].

Environmental factors, in turn, influence the expression of matrix proteins and how they interact with cells. Previous studies have shown an upregulation of laminin under proinflammatory conditions, as well as alterations in other matrix proteins, alluding to the dynamic nature of the ECM in relation to environmental conditions [36].

1.3.2 Integrins

Cell-matrix interactions are mediated by the binding of matrix proteins to cell surface receptors- the most common endothelial receptors that interact with the matrix are integrins [40]. Integrins are a class of heterodimeric proteins comprised of an α subunit and a β subunit [40]. These proteins recognize a sequence of amino acids (arginine, glycine, aspartic acid (RGD)) found on multiple matrix proteins, including fibronectin, collagen, laminin, and vitronectin, as well as certain plasma proteins [40].

Integrins mediate differential functions depending on receptor subtype and cell type [40]. For instance, the α2β1 integrin interacts with laminin in EC but binds to collagen in most other cell types [40]. There are seven integrin subclasses found in human EC that can bind with varying affinity to ECM components, with α2β1, α3β1, and αvβ3 having the broadest affinity [40].

Integrins play an important role in vasculogenesis, angiogenesis, and maintaining vascular integrity [41]. They mediate EC-ECM adhesion and mediate cell migration through the basement membrane while providing intracellular signaling cues via tyrosine kinases [41]. Modulation of integrin synthesis is highly dependent on environmental factors- for instance, a paper reported that combination of tumor necrosis factor α (TNFα) and interferon γ (IFNγ) induced a 70% decrease in the number of αvβ3 integrin molecules [40]. In EC, integrins are typically activated by growth factors (i.e. vascular endothelial growth factor (VEGF)) and mechanical stress, resulting in the intracellular activation of focal adhesion kinase (FAK)- this downstream signaling is crucial for dictating cytoskeletal organization and signal transduction [41].

There is still a limited understanding of the regulation of integrins in EC and the impact of biochemical signaling induced by integrin-ECM contacts on EC barrier function. However, clustering of certain integrins (α2β1 and αvβ1 specifically) has been observed around confluent EC borders, with αvβ1 being associated with focal contacts [40]. Furthermore, antibodies targeted to integrin receptors have been shown to increase EC permeability, leading to barrier dysfunction [42,43]. These results suggest a crucial role of integrins in regulating EC-ECM interactions and endothelial barrier function, though further work is needed to elucidate the underlying mechanisms.

1.3.3 Focal adhesions

Interactions of certain integrins with specific basement proteins promote the formation of specialized structures called focal adhesions [44]. Focal adhesions are important in regulating the intracellular cytoskeletal structure via stress fiber formation, which in turn mediates important cell processes, such as mitosis, ECM deposition, and interactions with cell-binding molecules (i.e. growth factors) [44].

Focal adhesions typically form at regions with specialized interactions between matrix components, integrins, and the cytoskeleton- typically, these regions are characterized by strong binding interactions between the cell and the basement membrane, leading to the belief that focal adhesion may act as the primary structure for stable cell-ECM attachment, particularly in quiescent cells [44].

Focal adhesion assembly requires the interaction of multiple binding proteins, which are all allosterically linked to form large multiprotein complexes [45]. There are more than sixty focal adhesion proteins discovered in vertebrates so far, and due to the complexity of players involved, the exact mechanism of focal adhesion regulation is still unclear [14].

Studies have elucidated that the formation of focal adhesions is initiated through integrin-matrix interactions [14]. These initial interactions lead to the establishment of early cell-ECM adhesion structures called focal complexes (transient), which can later mature into the more stable FAC [14]. Integrin-ECM interactions lead to the recruitment and activation of numerous downstream focal

adhesion proteins (i.e. vinculin, talin), which in turn stabilize the newly formed focal complexes [14,45]. Cell-ECM adhesion structures are regulated through Rac and other members of the Rho GTPase family, possibly through increased actin polymerization and stabilization of nascent focal complexes [14]. Another important regulatory process in focal adhesion formation is protein phosphorylation of focal adhesion proteins, namely paxillin and focal adhesion kinases (FAK) [14].

Integrin-matrix interactions regulate FAK recruitment to focal adhesion sites, where it undergoes autophosphorylation into the active form (i.e. phosphorylated FAK (pFAK)). pFAK exhibits regulatory functions via interactions with the plasma membrane and is a crucial linker to the F-actin scaffold, suggesting an important role in intracellular signaling [45]. The involvement of these multi-domain proteins present in FAC allows for the formation of an extensive "web" within the cell, that has broad impacts on cellular processes, including cell-cell interactions [14].

Focal adhesion disassembly can be mediated through intracellular signaling and degradation of focal adhesion components (i.e. talin degradation), and proteolytic degradation of the ECM [45]. The loss of matrix proteins affects mechanical tension, while the soluble matrix fragments can signal the activation of different cellular processes (i.e. angiogenesis) [45]. Interestingly, proteases are often present in proximity to focal adhesion regions, indicating that the levels of proteases, and the balance between protease activators and inhibitors, may be important in modulating EC-ECM adhesion by regulating the formation and stability of FAC [44].

While the exact function of FAC remains unclear, one potential role of these complexes may be to mediate stable adhesion of cells to the basement membrane *in vivo* (or adhesion to a planar substrate, collagen, or collagen-derivatives *in vitro*) [44]. This is suggested by the finding that cells that depend on cell-matrix adherence to promote function (i.e. fibroblasts, EC) form the more stable and prominent FAC, whereas migratory cells typically contain fewer and more transient focal complexes [44]. FAC may also play a role in stress fiber formation and wound repair- during the proliferative stage of wound repair, the force exerted by the cytoskeleton via focal adhesions can aid in wound contraction and remodeling of the basement membrane [44].

1.3.4 Function of cell-matrix interactions in endothelial barrier homeostasis

Under homeostatic conditions and during tissue regeneration, remodeling of the ECM enables EC to interact with each other, circulating leukocytes, and the surrounding tissue- these interactions have important implications for cellular processes, such as development, tumorigenesis, and tissue repair [30]. Adhesion of the EC to the underlying matrix progresses in stages: attachment, spreading, and finally the organization of the cytoskeleton [44]. While growing cells on physiologically-relevant matrix components (i.e. fibronectin-coated wells) has been shown to aid initial adhesion, EC can also synthesize and secrete their own endogenous matrix proteins, which is eventually followed by physiologically comparable cell spreading and cytoskeletal organization [44]. Furthermore, the

presence of large FAC, primarily situated at cell borders, in quiescent cells (i.e. EC) compared to smaller, less distinguished, transient focal adhesions in motile cells suggest an important role of focal adhesions as a mechanism to confer stability and strength in cellular adhesion [44].

Multiple mechanisms have been implicated in the contribution of cell-ECM interactions in conferring cell barrier stability. Changes in matrix composition have been shown to alter the adhesive capacity of cells under different physiological states (i.e. age, wound healing) [44]. For instance, differential synthesis of certain matrix proteins (i.e. chondroitin) has been observed in highly migratory cells, which may interfere with binding of cells to adhesion-promoting matrix proteins (i.e. fibronectin). Changes in membrane-associated components, specifically integrins, may also affect the capacity of cells to form focal adhesions [44]. Studies have shown that these membrane components are primary targets for regulating changes associated with cellular transformation decreased adhesion, resulting in reduced capacity for cells to form FAC.

In other cases, while ECM proteins stayed constant, changes in the cells and their receptor affinity to matrix proteins or cytoskeleton have been implicated in altered cellular adhesion [44]. Activation of proteases at focal adhesion sites may also be one potential mechanism by which cells disrupt adhesion, leading to detachment from the basement membrane [44]. Specifically, extracellular proteases, produced during cellular transformation or released during tissue damage or inflammatory response, may cleave matrix proteins, interfering with cell-ECM interactions. Furthermore, proteases may also act on FAC proteins directly,

interfering with the ability to cross-link subunits within the FAC [44]. For instance, talin can be targeted by calcium-dependent proteases, and cleavage of this protein interferes with downstream interactions with other focal adhesion proteins (i.e. vinculin) [44].

Extracellular signals, such as proinflammatory cytokines, may also affect cell-ECM interactions to promote the infiltration of leukocytes into the underlying tissue [46]. Previous studies have shown that under basal conditions, T cell extravasation is inhibited by endothelial focal adhesions by acting as a physical barrier for trans-endothelial migration of leukocytes. However, treatment with TNFα, a proinflammatory cytokine, revealed larger subendothelial spaces and fewer focal adhesions near EC junctions [46]. This indicates an important role of cell-ECM and cell-cell interactions, mediated through focal adhesions, in remodeling the basal layer to promote leukocyte migration [46].

The process through which cell-matrix interactions can mediate PMVEC barrier function is summarized in Figure 1-1.

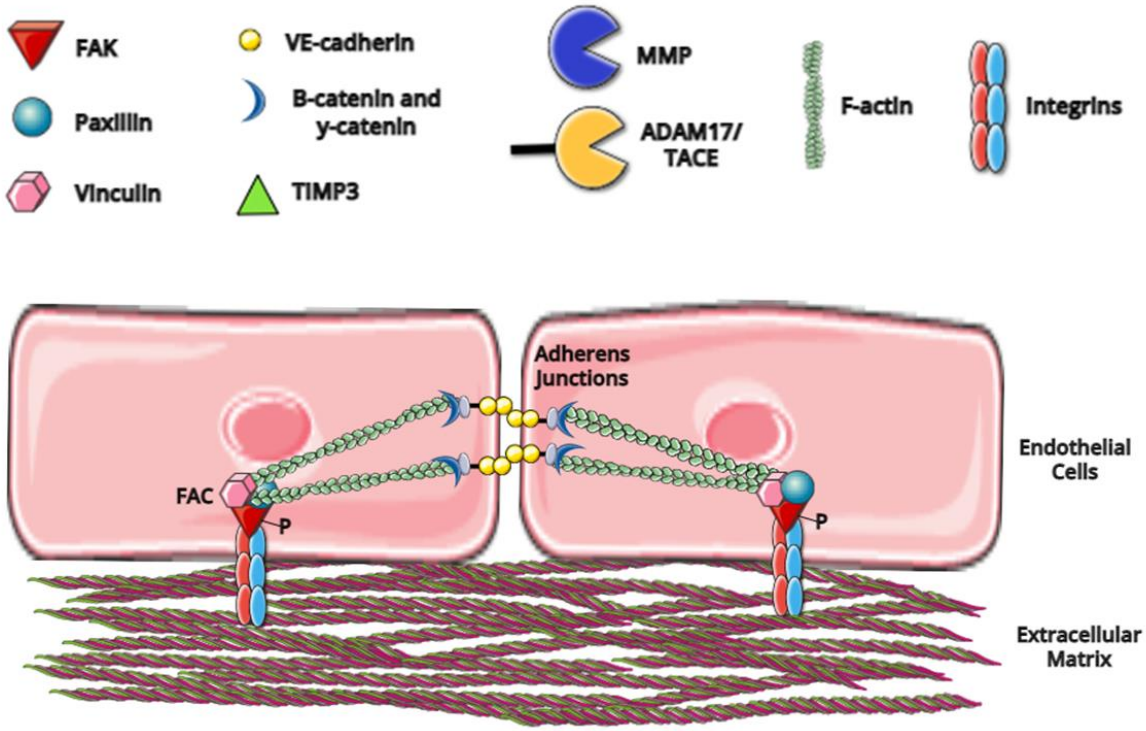


Figure 1-1. Regulation of PMVEC barrier function through PMVEC-ECM and inter-PMVEC interactions. Under basal conditions in the endothelial monolayer, integrin receptors interact with ECM proteins to form cell-matrix adhesions. This interaction promotes the assembly of focal adhesion complexes (FAC) through phosphorylation of focal adhesion kinase (pFAK) which recruits further downstream proteins. FAC strengthens integrin-ECM interactions, and interacts with intracellular components, like F-actin filaments, to stabilize adherens junctions. The cumulative effect of these pathways mediates PMVEC-ECM and inter-PMVEC interactions, supporting PMVEC barrier function.

1.4 Metalloproteinases

Metalloproteinases are a family of Ca^{2+}- and Zn^{+}-dependent endopeptidases found in the tissue microenvironment [47]. These include matrix metalloproteinases (MMPs), the membrane-bound a disintegrin and metalloproteinases (ADAMs), and their close derivatives, ADAM with thrombospondin motifs (ADAMTSs) [47]. While first discovered through their function in remodeling the extracellular matrix, later studies have suggested immunomodulatory roles of metalloproteinases in the body, including those related to EC barrier function (i.e leukocyte extravasation) [47].

1.4.1 Metalloproteinase structure and activity

Metalloproteinases are highly preserved in animals, with studies identifying at least 23 unique MMPs and 22 ADAMs in humans- of these, at least 12 demonstrate proteolytic activity [47]. Most metalloproteinases, whether soluble or membrane-bound, share a common structural phenotype, including a signaling peptide, a pro-peptide domain, and a catalytic domain [48]. The pro-peptide is responsible for maintaining the enzymes in an inactive conformation by binding to the Zn^{+} or Ca^{2+} present in the active site until an external stimulus disrupts the interaction [48].

Different subtypes of the metalloproteinase family may have a slight variation in structure, including MMPs and ADAMs/ ADAMTSs. For instance, membrane-type MMPs (MTMMPs), in addition to the base structures mentioned

above, contain a transmembrane domain to anchor the protein to the cell surface membrane [47]. ADAMs have a similar organization as MTMMPs due to being primarily membrane-bound, while also containing a disintegrin domain and some unique regions to allow for substrate interaction with adjacent cells [47].

Both soluble and membrane-anchored metalloproteinases process their substrates by the clipping or shedding of specific surface proteins, and by regulating intramembrane proteolysis [47]. Metalloproteinases are capable of interacting with circulating cytokines, chemokines, and growth factors, and can alter their bioactive state and local delivery [47]. Additionally, metalloproteinases can mediate shedding of cell surface receptors, which in turn alters the surface composition of cells and their ability to interact with extracellular signals [47]. Furthermore, receptor cleavage results in intra-membrane proteolysis, causing downstream intracellular signaling and post-translational modifications [47].

Due to the ubiquitous role of metalloproteinases in cells and tissue, their expression is highly regulated. Metalloproteinases are often secreted as inactive zymogens and are catalytically activated only after the cleavage of the inhibitory pro-peptide domain [47]. Additionally, compartmentalization of certain MMPs in specific intracellular and extracellular regions may be crucial for controlling MMP specificity and activity through substrate-specific degradation of the basement membrane [47,48]. Multiple factors, including cytokines, growth factors, and hormones, can also interact with metalloproteinases and mediate their expression, as can cell-ECM interactions via integrins [48]. Furthermore, activated

metalloproteinases can be inhibited by a family of unique inhibitory proteins termed the tissue inhibitors of metalloproteinases (TIMPs) [47].

1.4.2 Function of metalloproteinases in endothelial barrier homeostasis

Metalloproteinases are involved in numerous biological processes, including matrix remodeling, cell proliferation, migration, and apoptosis [49]. For instance, MMP13 plays an important role in bone development and repair, as demonstrated by elevated levels of MMP13 in chondrocytes and osteoblasts, which promotes normal bone remodeling via ECM degradation [50]. MMPs have also been shown to play an important role in angiogenesis (both physiological and pathological) through their contribution to EC tube formation [51]. Additionally, MMPs can regulate immune functions- previous studies have shown that these proteases facilitate leukocyte transmigration through the vascular wall and may also be involved in negative feedback mechanisms to regulate the inflammatory response [52]. As such, metalloproteinases demonstrate functions that extend beyond ECM degradation- through their ability to cleave cytokines, chemokines, and cell surface proteins, these enzymes are critical mediators in regulating immune function, tissue repair, cell differentiation, and transformation [53].

In mature, healthy adult tissue, metalloproteinase levels are generally low- however, when subjected to pathologic stimuli (i.e. injury, disease), increased expression of certain metalloproteinases has been observed [49]. For instance, patients with ALI demonstrate elevated MMP9 expression, which may be a

contributor to oedema via degradation of tight junction proteins between adjacent brain EC (i.e. occludins and claudin-5) [54]. Other studies have implicated MMPs and ADAMS (i.e. MMP7, ADAM10, ADAM12) in cleaving VE-cadherin, an important component of adherens junctions [11,25]. These findings suggest that metalloproteinases can regulate MVEC interactions and affect vascular permeability [11,23,55].

While some metalloproteinases target intercellular junctions, others act notably on the ECM, playing a role in its maturation, turnover, and assembly [53]. In fact, the first metalloproteinase, MMP1 (initially termed collagenase for its action on collagen) was discovered through its degradative properties of the ECM during tadpole tail metamorphosis [56]. ADAMTSs, similar to ADAMs except for the fact that they are secreted, have also recently been shown to be key participants in ECM turnover and matrix assembly during morphogenesis, via activation of matrix precursor proteins [53]. However, while metalloproteinases have important roles in physiologic ECM remodeling, excessive protease expression can be detrimental to tissue functioning.

Metalloproteinase expression can become aberrant under certain disease conditions, particularly in response to an inflammatory stimulus, leading to excessive proteolysis and disease progression if not controlled [47]. MMPs and ADAMs are involved in vascular remodeling under pathological conditions and can affect endothelial barrier function through a disruption of cell-matrix interactions [11,23,25]. For instance, both MMP2 and -9 have been associated with increased PMVEC permeability via degradation of matrix proteins (i.e.

fibronectin, laminin, and collagen IV) [11]. Metalloproteinase overexpression can be mediated through multiple factors, including proinflammatory cytokines (i.e. TNFα), certain pathogens (i.e. *Streptococcus pneumoniae*), or a dysregulation of the metalloproteinase and TIMP balance [11,57]. As such, metalloproteinases can influence endothelial function through multiple mechanisms, and aberrations in their homeostatic activity may significantly alter barrier integrity, particularly under proinflammatory conditions.

1.5 Tissue inhibitors of metalloproteinases (TIMPs)

Excessive ECM degradation and shedding of surface molecules are associated with numerous pathologies, including inflammation, degenerative diseases, and cancer [58]. As such, the balance between metalloproteinases and their regulators is crucial for maintaining the equilibrium between matrix deposition and remodeling [58]. Among the most prominent endogenous metalloproteinase inhibitors are the TIMPs [59].

1.5.1 TIMP structure, localization, and activity

TIMPs are a family of proteins, with four mammalian subtypes- TIMP1 to -4 [58]. Each of the TIMPs varies in its binding affinity to the various metalloproteinases, with TIMP3 having the broadest inhibitory profile by efficiently targeting most MMPs, and several ADAMs and ADAMTSs [59]. Human TIMPs are comprised of 2 domains, an N-terminal domain and a C-terminal domain [60]. Generally, the N-domain interacts with metalloproteinases at their

active site and causes inhibition, while the C-domain is responsible for protein-protein interaction [11]. However, some studies have revealed that the C-terminus may be involved in activating certain proMMPs (i.e. proMMP2 and -9) and may regulate cellular activities independent of metalloproteinase action [11,61].

Much like metalloproteinases, TIMPs demonstrate tissue-specific activity, and can be constitutively active or expressed following transcriptional signaling under different stimuli (i.e. cytokines, growth factors) [59]. TIMP1 and -2 are expressed in most mammalian tissue. However, TIMP1 expression may be restricted to certain regions within the organ- for instance, in the central nervous system, TIMP1 activity is restricted to regions of high neuronal plasticity, such as the cerebellum and the hippocampus [59]. TIMP3 is also expressed in many tissues and is particularly important as a matrix protein in the basement membrane of certain organs (i.e. the eyes and the kidneys) [59]. Unlike the other TIMPs, TIMP4 has a very limited and specific distribution (the heart, kidneys, pancreas, colon, testes, brain, and adipose tissue) and its functionality remains to be fully elucidated [59].

TIMPs are highly multifunctional proteins with various biological functions, such as modulation of cell proliferation, cell migration and invasion, anti-angiogenesis, anti- and pro-apoptosis, and neuronal plasticity [60]. Due to their influence on metalloproteinase activity, TIMPs can mediate cellular function through signaling pathways related to ECM catabolism, cell adhesion molecules, cytokines, and growth factors [59]. However, the exact role of TIMPs in regulating ECM remodeling is still debated, largely due to contradictory evidence from

studies showing opposing effects of respective TIMPs [11]. For instance, a previous study revealed that knocking out the *Timp3* gene increased collagen accumulation and fibrosis post-bleomycin-induced lung injury [11]. While it was suggested to be due to persistent inflammation, these findings contradict the initially accepted belief that decreased TIMP expression leads to ECM degradation, while excess ECM deposition would be due to increased TIMP levels [11].

Interestingly, recent studies have indicated some metalloproteinase-independent functions of TIMPs through their interaction with specific cell surface receptors [59]. While the exact mechanisms are still unknown, these findings highlight the complex mechanisms involved in the TIMP-metalloproteinase balance and the need for further studies to elucidate the role of this balance depending on the specific underlying process.

1.5.2 MMP-dependent function of TIMPs

The function of TIMPs is highly varied, depending on the specific TIMP, the microenvironment, and the metalloproteinase in question [11,58,62]. For instance, TIMP1 is more restricted in its inhibitory range on metalloproteinases than the other TIMPs, with activity limited to membrane-type MMPs, MMP6, -14, and -24 [60]. However, its affinity for specific MMPs (i.e. MMP-3, MMP-7) is superior to TIMP3 and TIMP4 [60].

Most of the previous work investigating TIMP function is a product of gene knockout studies in mice [60]. While ablating *Timp1* or *-2* respectively is not associated with any severe physiological aberrations, *Timp1* and *-2*-deficient mice

do manifest abnormalities related to matrix remodeling (i.e. impaired neurological and motor-function) following a pathological challenge [59]. Interestingly, *Timp3*-null mice have significantly higher abnormalities, even under basal conditions, than other *Timp* knockout mice, with the ablated mice showing emphysema-like alveolar damage and apoptosis of mammary epithelial cells [59]. These results have been associated with increased metalloproteinase activity (i.e. MMP9), strongly suggesting that TIMP3 may be a critical regulator of MMP activity [59].

All TIMPs have been shown to inhibit MMPs to some degree [48]. TIMP1 may regulate cell signaling by targeting specific MMPs (i.e. MMP7, MMP9) [11]. A gene knockout study revealed that *Timp1*-null mice had increased MM7 expression in the airway epithelium upon naphthalene exposure, suggesting a strong relationship between TIMP1 and MMP7 [48]. Furthermore, TIMP1 can inhibit MMP7-dependent syndecan-1 shedding which may restrict epithelial cell migration [11]. TIMP1 may also inhibit MVEC migration by limiting the action of MMP9 on cell surface proteins, reducing VE-cadherin and platelet endothelial cell adhesion molecule 1 (PECAM1) shedding [11].

Some TIMPs may have contradictory actions on metalloproteinases, including activation of proMMPs [11,63,64]. For instance, latent MMP2 can bind to TIMP2 for complete activation of the protease [11,63]. The N-domain of TIMP2 binds to MMP14, while its C-domain binds to the inactive MMP2, which mediates the interaction between MMP14 and MMP2, leading to the activation of MMP2 [11].

Certain TIMPs can also interact with the ADAM and ADAMTS subtypes of the metalloproteinase family, particularly TIMP3 [11,65]. An important substrate of TIMP3 is ADAM17, also known as the TNFα converting enzyme (TACE) [11]. TACE is an important enzyme in mediating inflammation through its actions on membrane proteins [11]. For instance, ADAM17 catalyzes the shedding of membrane-bound TNFα and other pro-inflammatory cytokines, and transforming growth factor α (TGFα) [11,66,67]. TGFα is a ligand of the epidermal growth factor receptor (EGF), which, when activated, leads to cell proliferation, expression of IL8, and leukocyte migration [11]. *Timp3* knockout studies have shown that a lack of TIMP3 is associated with increased inflammation following an insult (i.e. bleomycin-induced lung injury)- this may be due to higher ADAM17 expression, resulting in increased TNFα shedding [48]. As such, TIMP3 may play an important role in mediating the immune response by regulating the processing of TNFα via TACE [59]. These studies have also revealed an association between lack of TIMP3 and increased substrate degradation (i.e. aggrecan cleavage), alluding to the role of TIMP3 in inhibiting ADAMTS-dependent degradation of matrix components and ECM turnover [11,48].

1.5.3 MMP-independent function of TIMPs

While most studies focus on the role of TIMPs in relation to metalloproteinase inhibition, recent data support multifunctional properties of TIMPs in regulating processes unrelated to or only partially related to metalloproteinase activity [68]. These studies indicate that TIMP-receptor binding is

involved in numerous important cellular processes, such as regulating cell proliferation, apoptosis, and angiogenesis [68].

TIMP1 and -2 have been implicated in regulating mitogenic activities in various cells, including mesenchymal and epithelial cells, through independent receptor signaling [11,69,70]. It has been thought that TIMP1 promotes cell growth by targeting the tyrosine kinase/ mitogen activated protein kinase (MAPK) pathway, while TIMP2 targets protein kinase A to activate the Ras/ phosphoinositide 3-kinase (PI3K) complex [68]. Conversely, TIMP1 and -2 can suppress cell proliferation and apoptotic signaling in certain cells by targeting specific antibodies (i.e. CD63) and integrin complexes (i.e. $\alpha_3\beta_1$) [68]. In fact, overexpression of TIMP1 and -2 has been linked to various cancers, including ductal breast cancer development, apoptotic-resistant Burkitt's lymphoma, and murine melanoma [68]. Interestingly, TIMP2 has shown some pro-apoptotic activity in specific cells (i.e. apoptosis of activated T cells), indicating the need for further research on establishing the exact role of TIMP2 in apoptosis [68].

TIMP3 has also shown some apoptotic function in specific cell types- for instance, overexpression of TIMP3 in pulmonary artery EC is associated with increased apoptosis, likely be due to inhibition of a FAK-dependent survival pathway [7111]. Additionally, recent studies have demonstrated TIMP3's function as a potent inhibitor of angiogenesis [68]. TIMP3 can bind to the VEGF receptor on EC, subsequently blocking the interaction of its endogenous ligand, VEGF, and inhibiting the downstream pathway [68]. Meanwhile, TIMP3 can also target the angiotensin II type 2 receptor, and the combined effect of TIMP3 on inhibiting the

VEGF pathway and upregulating angiotensin II type 2 receptor activity results in cumulative inhibition of angiogenesis [68].

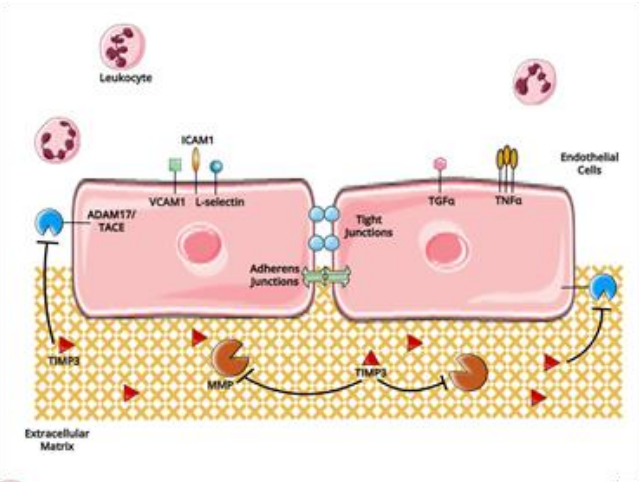
TIMP3 may also regulate cell proliferation, though whether it promotes or inhibits the process depends on the cell type [72]. Some studies have demonstrated an association between TIMP3 and enhanced proliferation in chicken fibroblasts and bronchiole epithelial cells during embryonic development, while others have indicated that TIMP3 inhibits vascular smooth muscle cell and EC proliferation [72]. Interestingly, the inhibition of cellular proliferation may be mediated through metalloproteinase-independent processes, as studies have demonstrated that overexpression of TIMP3 inhibited cell proliferation without significantly altering inhibition of protease activity- this may be due to the role of TIMP3 on p21 and p27, both of which inhibit cyclin-dependent kinases, at least as demonstrated in cardiomyocyte proliferation [72].

The metalloproteinase-dependent and metalloproteinase-independent roles of TIMP3 are further summarized in Figure 1-2.

Metalloproteinase-Dependent

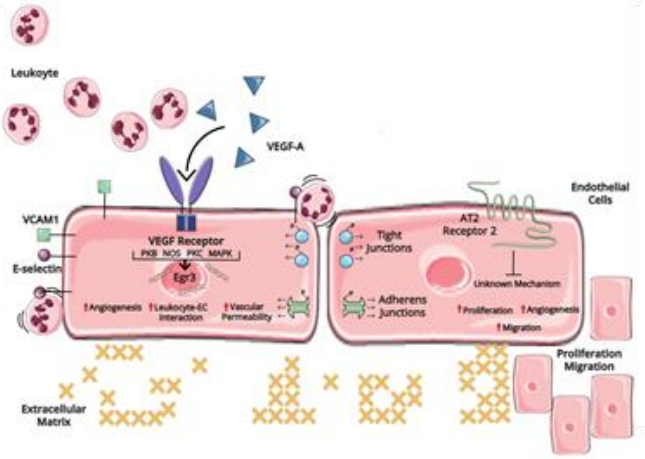
A)

TIMP3 Present



B)

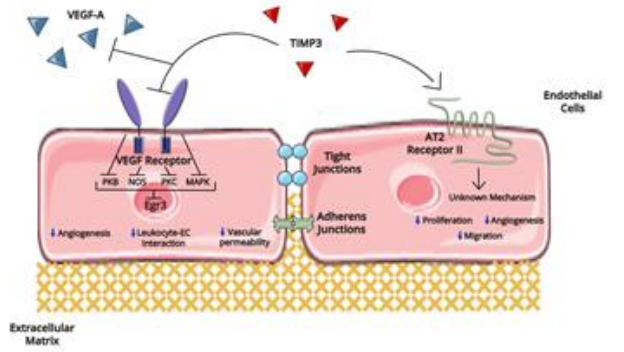
TIMP3 Absent



Metalloproteinase-Independent

C)

TIMP3 Present



D)

TIMP3 Absent

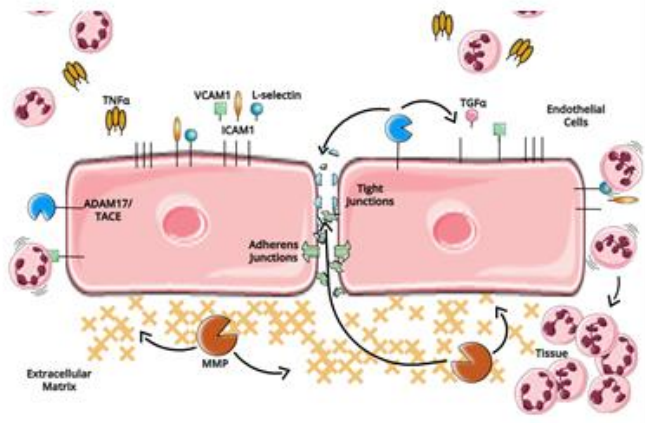


Figure 1-2. Proposed metalloproteinase-dependent and metalloproteinase-independent functions of tissue inhibitor of metalloproteinases 3 (TIMP3) in microvascular endothelial cells [11]**.** A) In the presence of TIMP3, MMPs and ADAM 17/ TACE are inhibited. The structure and composition of the ECM and MVEC intercellular junctions are maintained, while leukocyte adhesion and extravasation are limited. B) In the absence of TIMP3, increased MMP activity leads to degradation of the ECM and proteins associated with intercellular junctions. Increased ADAM17 activity promotes the release of TNFα, enhancing the proinflammatory response. Expression of leukocyte receptors, such as ICAM1 and VCAM1, is increased on the surface of MVEC. Additionally, the shedding of these receptors by metalloproteinases may promote leukocyte extravasation into the underlying tissue. Membrane-bound TGFα is also shed from the cell surface by ADAM17, resulting in binding to and activation of epidermal growth factor (EGF) receptors to promote cell proliferation, cell migration, and angiogenesis. C) TIMP3 binds the VEGF receptor, inhibiting its downstream signaling cascade by preventing interaction with and binding of its ligand, VEGF-A. TIMP3 also binds to and activates the Angiotensin 2 (AT2) receptor II. The combined effects of VEGF receptor inhibition and AT2 receptor II activation decreases cell proliferation and migration, inhibits angiogenesis, reduces vascular permeability, and inhibits leukocyte-EC interaction. D) The absence of TIMP3 allows VEGF to bind to its receptor, activating the MAPK, PKB, PKC, and NOS pathways and leading to increased expression of the Egr3, and phosphorylation of proteins associated with MVEC intercellular junctions. Moreover, the AT2 receptor II remains inactivated due to the lack of TIMP3 binding. Overall, the combined effects of VEGF activation and AT2 receptor II inactivation result

in increased cell proliferation and migration, increased angiogenesis, increased expression of cell surface adhesion molecules, leading to enhanced leukocyte-EC interaction, and increased vascular permeability.

1.5.4 The role of TIMPs in EC barrier function

Due to the impact of TIMPs in various cellular functions, these inhibitors may be crucial regulators of MVEC interaction and barrier function [11,73]. There is some evidence suggesting that the metalloproteinase-dependent role of TIMPs is responsible, as increased metalloproteinase activity has been associated with enhanced cerebral microvascular permeability, and the inhibition of metalloproteinase activity partially rescues the leak [74]. Other studies have shown that the addition of synthetic metalloproteinase inhibitors or recombinant TIMP1 appears to reduce VE-cadherin cleavage [74].

Inflammation could be a driving force of TIMP dysfunction and the subsequent increase in metalloproteinase activity. While TIMPs contribute to cytokine and cytokine receptor turnover, proinflammatory cytokines themselves (TNFα, interleukin 1β (IL1β), and IFNγ) can differentially regulate MMP and TIMP expression [55]. For instance, previous studies have shown that proinflammatory cytokines up-regulate TIMP1 expression in both rat brain microvascular EC and astrocytes, while TIMP2 remained unchanged [55].

TIMP3, in particular, is of great research interest due to its significant presence within vascular tissue- emerging data suggests that TIMP3 can regulate microvascular barrier function, as mice lacking TIMP3 have increased leak of Evans blue (EB) dye-labeled albumin within the snout and kidneys, due to the absence of TIMP3 in pericytes [74]. Other studies have shown that mesenchymal

stem cell-derived TIMP3 decreases cerebral microvascular leak in a model of traumatic brain injury [74].

Additionally, TIMP3 is shown to be crucial in maintaining lung homeostasis. *Timp3*$^{-/-}$ lungs in murine embryos demonstrated reduced matrix protein abundance due to increased activity of TIMP3-inhibited metalloproteinases and decreased FAK signaling [75]. Furthermore, treatment with a broad-spectrum metalloproteinase inhibitor increased FAK signaling and matrix protein abundance, restoring appropriate bronchiole branching in the developing lungs [75]. Under inflammatory conditions, *Timp3*$^{-/-}$ mice demonstrated enhanced neutrophil influx in the lungs and a longer duration of inflammation, as compared to WT mice [76]. These findings indicate an important role of TIMP3 for maintaining normal cell-ECM interactions in the lungs- understanding the contributing mechanisms under basal and proinflammatory conditions may be crucial for targeting disruption of the EC barrier function under pulmonary dysfunction.

1.6 Rationale

While numerous studies have focused on specific aspects of the endothelium and its function in the body, the precise role of cell-matrix interactions in regulating pulmonary microvascular endothelial barrier function remains a mystery. Some studies have suggested that decreased EC-ECM interactions, as indicated by altered pFAK abundance and distribution, is associated with an impaired endothelial function, resulting in oedema and lung

dysfunction [15,77,78]. However, many studies have shown conflicting and often opposing results in pFAK change, with the results being highly dependent on the stimulus, cell-type, and duration of study [15,78–82]. There is a persistent need to study the role of cell-ECM interactions, via pFAK analysis, in PMVEC barrier function, particularly under cytokine treatment, as proinflammatory conditions like sepsis are closely associated with accumulation of pulmonary oedematous fluid and lung dysfunction.

Interestingly, very few, if any, studies, have looked at the role of TIMPs in relation to cell-matrix interactions and EC barrier stability. Previous work in our lab has shown that TIMP3 plays an important regulatory role in PMVEC barrier function, as lack of TIMP3 via murine knockout studies revealed a compromised PMVEC barrier, causing increased protein leak in the pulmonary vasculature- this was particularly prominent under proinflammatory conditions [74]. The metalloproteinase-dependent action of TIMP3 is thought to be the main contributor to PMVEC barrier stability, as studies using a broad-spectrum metalloproteinase inhibitor (GM6001) in PMVEC derived from *Timp3*$^{-/-}$ mice rescued the augmented leak seen in the knockout mice [11].

Many studies trying to elucidate the mechanism by which TIMP3 and metalloproteinase activity affect PMVEC interactions explored the disruption of intercellular junctions. Prior studies have demonstrated that multiple metalloproteinases (i.e. MMP7, ADAM10, and ADAM12) cleave VE-cadherin, one of the primary junctional proteins responsible for PMVEC barrier stability [11]. However, few studies have focused on the impact of TIMP3 in relation to cell-

ECM interactions, including via metalloproteinase activity. There is some research suggesting that degradation of the matrix proteins (i.e. fibronectin, laminin, type IV collagen) by MMP2 and -9 is associated with increased PMVEC permeability both *in vivo* and *in vitro* [11,83]. Additionally, increased FAK phosphorylation has been associated with MMP expression in some cancer cell lines, particularly under cytokine treatment [84]. Furthermore, some studies indicated that while certain metalloproteinases (i.e. MMP2, -9) decreased cell survival post-injury, and that inhibition of these metalloproteinases enhanced cell survival, ECM-integrin-dependent FAK activation rescued cell survival, even when MMPs remained activated [85]. While these studies suggest an important association between metalloproteinase activity and integrin-mediated pFAK activation, there is a limited understanding of the overall role of TIMP3 in regulating cell-ECM interactions in PMVEC barrier function, and how this may be altered under proinflammatory conditions.

1.7 Objectives

Considering the lack of data on the role of TIMPs in metalloproteinase-mediated EC-ECM interactions in the pulmonary vasculature, I proposed to:

1. Examine the role of TIMP3 in regulating PMVEC permeability.

2. Examine the role of TIMP3 in regulating PMVEC-ECM interaction.

To achieve my objectives, I employed the novel XperT-permeability assay to visualize individual PMVEC-specific localized protein (i.e. avidin) leak in

relation to VE-cadherin dissociation, and compare the specific regions and extent of PMVEC permeability in WT vs. *Timp3*$^{-/-}$ PMVEC under basal and proinflammatory conditions.

To better understand potential differences in PMVEC-ECM interactions and permeability between WT and *Timp3*$^{-/-}$ PMVEC, I also examined basal adhesion of WT and *Timp3*$^{-/-}$ PMVEC to commonly used cell-culture adhesion coatings (i.e. gelatin) and matrix proteins (collagen, fibronectin, vitronectin, laminin). Additionally, I employed the use of confocal microscopy to assess potential differences in pFAK aggregations between WT and *Timp3*$^{-/-}$ PMVEC under basal vs. proinflammatory stimuli.

Lastly, I repeated the individual PMVEC leak studies in WT and *Timp3*$^{-/-}$ PMVEC using broad-spectrum metalloproteinase and FAK inhibitors respectively, to better assess the role of metalloproteinases and pFAK in PMVEC barrier permeability, and the impact of TIMP3 in mediating metalloproteinase-dependent PMVEC-ECM adhesion.

1.8 Hypothesis

Based on the prior data regarding TIMP3 and metalloproteinase function, and the importance of PMVEC-ECM interactions in normal cell function, I hypothesized that TIMP3 maintains PMVEC barrier function by promoting PMVEC-ECM interactions.

Chapter 2

2 Methodology

2.1 Isolation of murine pulmonary microvascular endothelial cells (PMVEC) for *in vitro* analysis

In order to study the role of TIMP3 in regulating the pulmonary microvascular barrier function, under both basal conditions and a proinflammatory environment, PMVEC were isolated from healthy male WT and *Timp3*$^{-/-}$ mice and cultured as performed routinely within out lab [86,87].

Briefly, isolated lung tissue was minced and digested using 0.3% collagenase in Hank's Balanced Salt Solution (HBSS, #14170-112, Invitrogen). The cells were filtered through a 100μm pore mesh sieve, followed by a period of incubation with magnetic microbeads (Dynabeads M 450 sheep anti-rat IgG, #11035, Dynal Biotech Inc., Lake Success, NY) that were coupled to anti-platelet endothelial cell adhesion molecule (PECAM) antibodies (Rat anti-mouse CD31 monoclonal antibody, #557355, BD Pharmingen, Franklin Lakes, NJ). Following the magnetic capturing of PMVEC (MPC magnet, Dynal Biotech Inc., Lake Success, NY), cells were washed, suspended in a growth medium (Dulbecco's modified Eagle's medium [DMEM] supplemented with 20% heat-inactivated fetal bovine serum [FBS], #11885-092, Invitrogen, Carlsbad, CA; 1% Penicillin/Streptomycin [10 000 U/mL], #15140122, Invitrogen; 2% [4-2[2-

hydroxyetyl]-1-piperazineethanesulfonic acid] [HEPES] buffer [1M], #15630-080, Invitrogen) and seeded into a 1% gelatin-coated cell culture flask. The cells were incubated at 37°C with 5% carbon dioxide (CO2), with weekly assessments to ensure appropriate morphology. Once achieving approximately 90% confluency, the cultured PMVEC were stained with fluorescent acetylated-low density lipoprotein (LDL) (Biomedical Technologies, # BT902, Stoughton, MA) and assessed by immunofluorescence, as well as stained with fluorescently-labeled antibodies against endothelial cell markers (i.e. CD31, CD34, CD146, and CD202) and assessed by flow cytometry to ensure the purity of cell type. Collectively, these processes resulted in a PMVEC culture that was 99% homogeneous. PMVEC were then transferred to cell culture plates and grown in supplemented DMEM growth medium and incubated at 37°C with 5% CO2 until forming a monolayer of 90% to 95% confluency (approximated). All experiments were conducted on PMVEC between passages 5 to 10.

2.2 Assessment of PMVEC barrier permeability

2.2.1 Assessing PMVEC localized permeability using the XperT-permeability assay

Localized barrier permeability around murine PMVEC under basal and proinflammatory conditions was studied by implementing a derivation of the XperT-permeability assay as previously used in our lab [88,89]. In brief, WT and *Timp3*$^{-/-}$ PMVEC were grown on a layer of biotinylated gelatin in 48-well tissue culture plates. The cells were maintained in supplemented DMEM growth

medium until a 90% to 95% confluent monolayer was formed. PMVEC were then stimulated with phosphate-buffered saline (PBS) (basal conditions) or cytomix (an equimolar mixture of TNFα, IL1β, and IFNγ to mimic proinflammatory conditions, 30 ng/mL) for 5hr at 37°C with 5% CO_2. Following stimulation, fluorescently-labeled avidin (Invitrogen, Avidin, green 488, lot 1888323, 2.5mg/ml) was added into all wells (control and treatment) (Figure 2-1). After 3 min, the avidin was removed from each well and PMVEC were washed 3 times with PBS.

PMVEC were fixed in ice-cold methanol and stained following an established immunostaining protocol used in our lab [74]. In brief, 0.1% Triton-X was added to each well to permeabilize cells. After 30min incubation at room temperature, PMVEC were washed with PBS 3 times and 3% bovine serum albumin (BSA) in PBS was then added to each well to block off-target sites. Following blocking, PMVEC were stained with rabbit anti-VE-cadherin antibody suspended in 1% BSA/PBS (1:100; Invitrogen, ab33168, 0.4ug/mL). After 2hr, PMVEC were washed with PBS 3 times and then stained with goat anti-rabbit antibody conjugated to Alexa Fluor 594 in 1% BSA/PBS (1:500; Invitrogen, cat # A-11012, 2mg/mL). After 1hr, PMVEC were again washed with PBS 3 times and then stained with Hoechst counterstain (Hoechst 33342, 10mg/mL) for 10min. PMVEC were then washed with PBS 5 times and placed at 4°C in PBS. Cells were imaged within two days of fixing using fluorescent microscopy (Zeiss Axiovert 200M Inverted Microscope; Carl Zeiss Canada, Toronto, Canada) at 20X magnification.

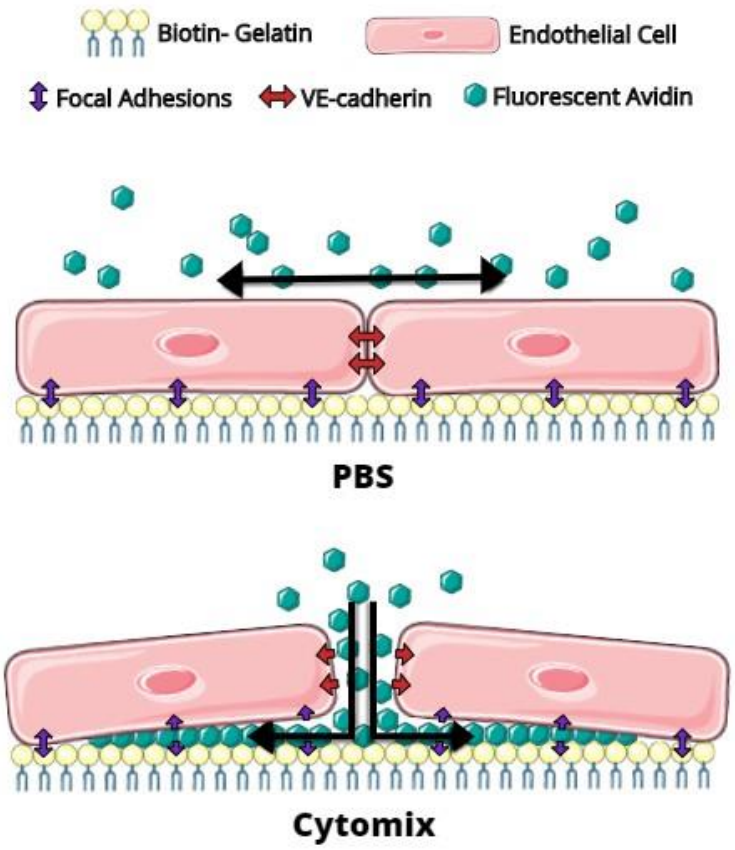


Figure 2-1. Graphic representation of the XperT-permeability assay.

2.2.2 Quantification of total avidin leak and localized avidin diffusion

All raw images were analyzed using ImageJ software. A central section of the image was selected within ImageJ (parameters preset by software) to analyze the avidin signal in the given region- this was applied to all 5 images acquired per well (Figure 2-2). To quantify total avidin using ImageJ, the regions of avidin signal were selected and all background fluorescence was subtracted (Figure 2-2). The highlighted avidin regions were converted to binary; using an ImageJ plugin (Morpholib, set at Quasi-Euclidean), the regions were converted to grayscale and the small avidin leak spots selected individually. (Figure 2-2). This selection criteria allowed ImageJ to differentiate between avidin regions that were in proximity and count them as separate signals (Figure 2-2). The selected region was then highlighted and all 'particles' were analyzed (threshold was set at a pixel range of 100-infinity, and any cut-off regions at the edge of the selected image were excluded) (Figure 2-2). The regions separated by the MorphoLib plugin had to be individually selected and added to the total count (Figure 2-2). The total number of spots was measured, as was the total avidin area.

To assess localized avidin diffusion with respect to the VE-cadherin-stained cell border, the final image showing the outline of the total avidin regions was merged with the image from the VE-cadherin channel (Figure 2-2). This provided an outline of all avidin spots superimposed over the VE-cadherin image (Figure 2-2). To quantify avidin diffusion, the distance from the VE-cadherin border to the edge of the avidin outline was measured perpendicular to the VE-

cadherin border (Figure 2-3). This ensured representative measurement of the extent to which the PMVEC-ECM interaction was disrupted to allow for avidin to leak beneath the cells (Figure 2-3). Additionally, only one measurement was made per avidin region (Figure 2-3). This protocol was repeated sequentially for all avidin regions in the selected region of the VE-cadherin image, and the length of each measurement calculated (Figure 2-3).

The above protocols were followed for all raw images with results per image being averaged within the treatment groups. Per treatment group, 5 images were acquired, and the experiments repeated 3 times.

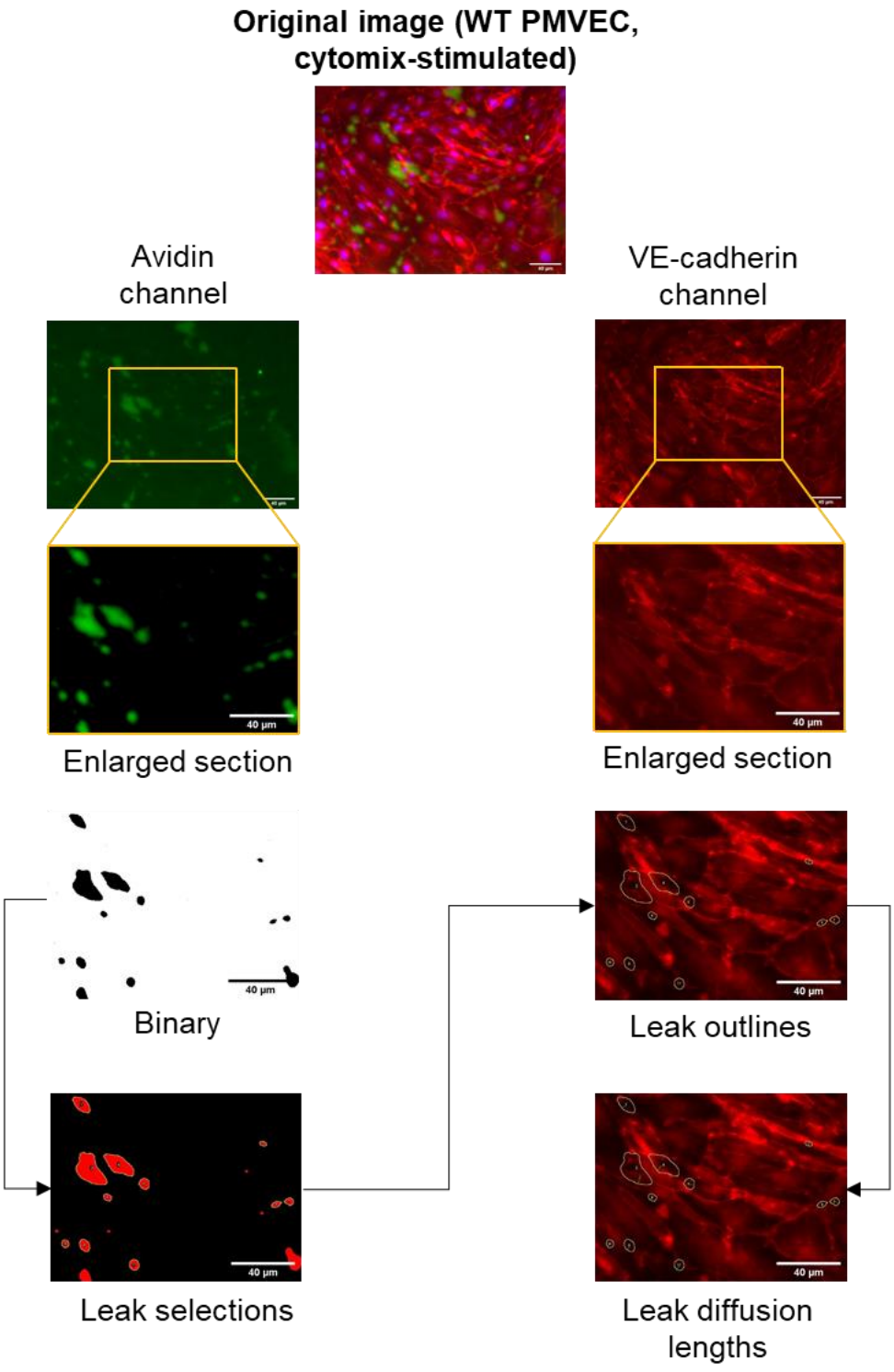


Figure 2-2. An example of the quantification of total avidin fluorescence and avidin diffusion length.

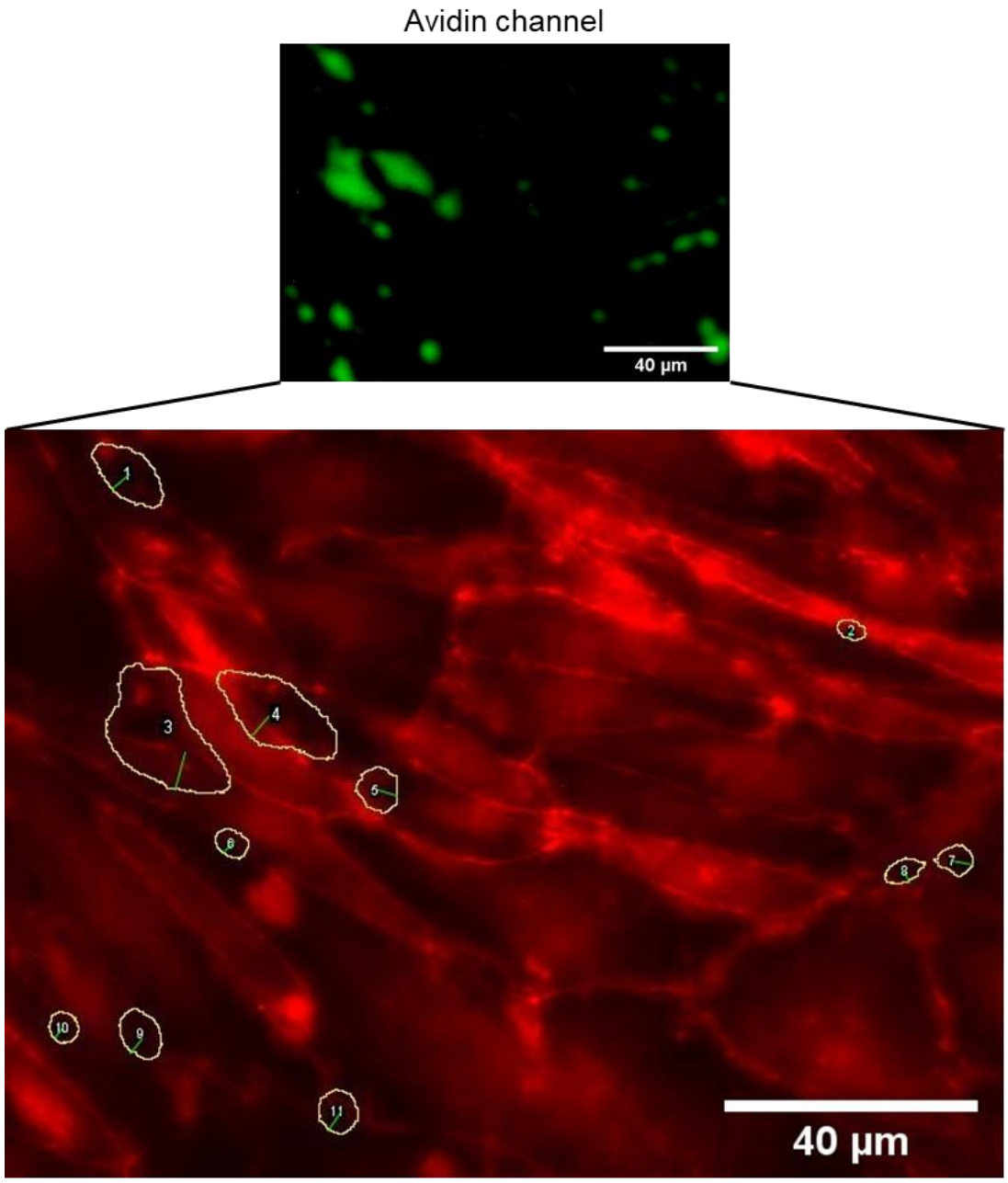


Figure 2-3. An enlarged example of measuring avidin diffusion length.

2.3 Assessment of PMVEC-ECM adhesion

EC adhesion to matrix proteins was assessed in male murine WT and *Timp3*$^{-/-}$ PMVEC under basal conditions, using previously established adhesion protocols. The cell-matrix interactions were assessed under static conditions, using two types of assays : a) gelatin-coated adhesion assays, where the PMVEC were seeded onto a gelatin-coated 96-well plate, and b) ECM cell culture optimization arrays, where the PMVEC were seeded into a commercially acquired 96-well plate containing four common matrix proteins at varying concentrations.

2.3.1 Assessing PMVEC adhesion to gelatin

Assays testing adhesion of different cell types under various stimuli have been previously established [90–92]. Thus, a generally accepted adhesion assay format was adopted and optimized to test PMVEC adhesion to gelatin (Molecular Probes, Vybrant Cell Adhesion Assay Protocol (V-13181).

Under sterile conditions, 0.1% gelatin was added to each well of a black-walled 96-well tissue culture-treated plate with four wells per plate left empty to provide gelatin-free control wells. The plate was incubated for 60min at room temperature, after which time, the wells were washed 3 times with sterile PBS. The final wash was aspirated before use.

Before seeding, PMVEC were suspended in phenol red-free DMEM containing 0.1µM calcein-AM and placed on tube rotator for 20min at 37°C and 5% CO_2. Following calcein AM incubation, PMVEC were seeded in quadruplets

into the 96-well plate at 30,000 and 60,000 cells per well. An initial measurement of the calcein fluorescence was recorded (excitation: 485nm; emission: 520nm) using a Victor 3 multilabel fluorescence microplate reader. The plate was then incubated at 37ºC and 5% CO_2 for 6hr and 16hr, in addition to pilot experiments at 1hr, 2hr, 4hr, and 24hr, to provide a comprehensive overview of cell-matrix adhesion.

Following the respective incubation period, the cells were washed 3 times with sterile PBS. The initial adhesion experiments altered the force of the wash (gentle vs. strong), the number of washes (1, 2, or 4 washes), and wash media (PBS vs. phenol red-free media). Following aspiration of the final wash, 200uL of phenol red-free DMEM was added to all wells and the final calcein fluorescence was measured, as per the above parameters.

2.3.2 Assessing PMVEC adhesion to ECM proteins

Commercially available ECM optimization kits (Millipore, Cat. No. ECM541) were used to assess PMVEC adhesion to collagen I, fibronectin, laminin, and vitronectin. ECM proteins were plated within the 96-well plates in triplicate at concentrations ranging from 20 μg/mL to 0.125 μg/mL. The plate also contained BSA coated wells (negative controls) and non-coated wells (blank control). Cell adhesion was assessed according to the manufacturers recommended protocol.

Briefly, all 96 wells of the ECM plate were blocked with 30% BSA in PBS for 1hr at room temperature. The BSA was aspirated before adding cells. WT

and *Timp3*$^{-/-}$ PMVEC in serum-free DMEM were then added to each well at a final concentration of 50,000 cells per well. The plates were incubated for 2hr at 37°C and 5% CO_2. Following incubation, all wells were washed gently 3 times with serum-free DMEM. The provided cell stain was then added to each well to stain and fix the PMVEC. After 10min, the cell-stain solution was aspirated, and the wells were washed 5 times with PBS. Extraction buffer (provided by the manufacturer) was then added to all wells and the plate was placed on a gentle rotator for 10min at room temperature. Finally, the absorbance measured using a Thermo Ascent plate reader at 570nm.

2.4 Assessment of phosphorylated focal adhesion kinase (pFAK) content in PMVEC

2.4.1 Assessing the impact of gelatin on pFAK abundance in PMVEC

To study pFAK protein in murine PMVEC under basal vs. proinflammatory stimuli, WT and *Timp3*$^{-/-}$ PMVEC were seeded on glass coverslips that were coated with gelatin. These coverslips were placed in 24-well plates, and the PMVEC were seeded at 50k/ well. The cells were grown in supplemented DMEM growth medium until reaching 90%- 95% confluency, followed by treatment with either 4uL of PBS (basal conditions) or 4uL of cytomix (proinflammatory conditions) and left to incubate in a cell incubator for 5hr (37°C, 5% CO_2). Following incubation, the solution was siphoned, and all cells were fixed in 250uL of ice-cold 4% paraformaldehyde.

The fixed cells were stained following a similar immunostaining protocol as outlined in the XperT-permeability section, with minor changes in the volume of solutions with respect to the larger wells. The primary antibody used was pFAK 397 rabbit, SAB4504181, following the same ratios as outlined previously. Following the last PBS wash after immunostaining, the coverslips containing the fixed and stained cells were transferred onto glass slides containing antifade mounting medium and sealed. The slides were stored covered at -20°C until imaged. The samples were imaged using confocal microscopy (Leica TCS SP8) as Z-stacks.

2.4.2 Quantification of total pFAK abundance and differential pFAK particulate sizes

Initial analysis of the raw images acquired from confocal microscopy was performed using the LAS-X proprietary software associated with the Leica TCS SP8 microscope. The optimal plane was selected when scrolling through the Z-stacks per image, with the selection criteria focusing on the pFAK signal at the basal layer of the cell. Once selected, the snapshot images at the basal plane were exported to the ImageJ software for pFAK particle analysis.

In ImageJ, exported images were split to extract the red channel, indicative of the pFAK signal. The red channel image was converted to binary to reduce the background signal and the remaining particles analyzed according to size (Figure 2-4). The size selection criteria were as follows: pixels from 0-1 (background or nonselective antibody staining), 2-10 (small), 11-50 (moderate),

51-100 (large), 101-infinity (background or nonselective antibody staining; NOTE: 1 pixel= 0.566μm; Figure 2-4). These subsequent results for the small, moderate, and large pixel categories per image were averaged within the specific treatment groups and graphed to allow comparison between groups (Figure 2-4).

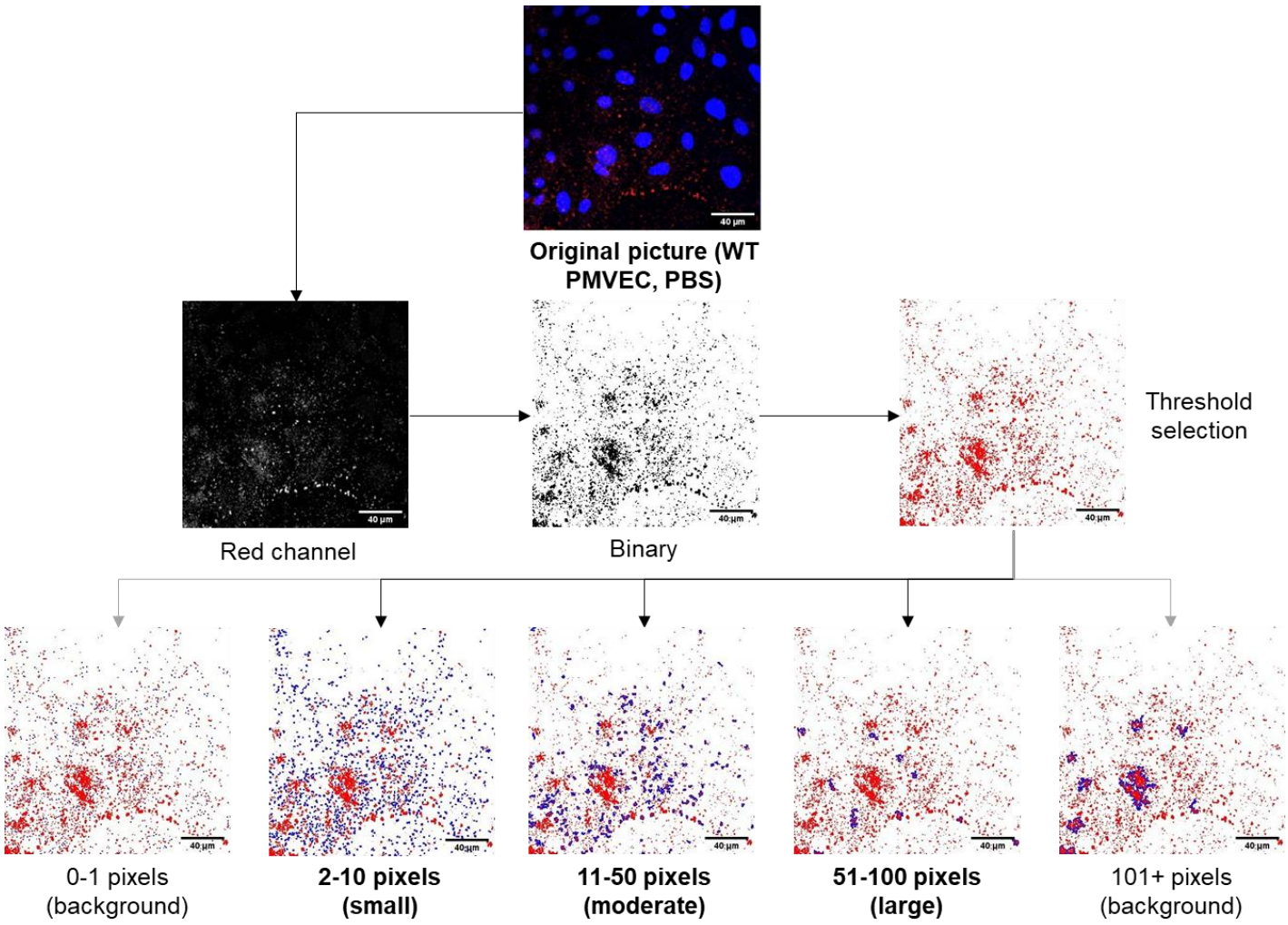


Figure 2-4. An example of the pFAK quantification methodology (NOTE: 1 pixel= 0.566µm).

2.5 Assessment of PMVEC barrier function post-inhibitors

2.5.1 Assessing the impact of PF-573228 (PF-228) on barrier permeability

To assess the role of FAK activity in local leak under basal and proinflammatory conditions, WT and *Timp3*$^{-/-}$ PMVEC were treated with 5, 10, and 25μM PF-228 (Sigma, cat#324878), a synthetic FAK inhibitor that inhibits FAK phosphorylation at Tyr397 (concentrations were based on established literature; Cabrita et al. 2011; Lederer et al. 2018). For these studies, PF-228 or an equal volume of DMSO (vehicle control for PF-228) was added to PMVEC for 16hr. After 16hr, WT and *Timp3*$^{-/-}$ PMVEC were stimulated with PBS or cytomix (30ng/mL) for 5hr and local leak was assessed with using the protocol described above for the XPerT-permeability assay.

2.5.2 Assessing the impact of BB94 on barrier permeability

To examine the role of metalloproteinases in local leak under basal and proinflammatory conditions, WT and *Timp3*$^{-/-}$ PMVEC were treated with 10 and 25μM BB94 (Batimastat, Calbiochem, cat#196440), a synthetic global metalloproteinase inhibitor, and local leak was examined as above. For these studies, BB94 or an equal volume of DMSO (vehicle control for BB94) was added to PMVEC for 16hr. After 16hr, WT and *Timp3*$^{-/-}$ PMVEC were stimulated with PBS or cytomix (30ng/mL) for 5hr and local leak was assessed with using the protocol described above for the XPerT-permeability assay.

2.6 Statistical Analysis

Depending on the number of variables, differences between groups were assessed using a paired t-test (one-tailed or two-tailed), a one-way ANOVA with a Dunnett post hoc test (one independent variable), or a two-way ANOVA with a Bonferroni post hoc test (two independent variables) using GraphPad Prism 5. The significance threshold was set at an α of 0.05.

Chapter 3

3 Results

3.1 Localized paracellular avidin leak in PMVEC

To visualize and assess the localized disruption of inter-PMVEC junctions and PMVEC-ECM interactions, the use of the novel XperT-permeability assay and immunofluorescent microscopy was implemented. The XperT-permeability assay was previously used in our lab to assess VE-cadherin disruption in human PMVEC under basal vs. proinflammatory conditions. To translate this method into the mouse model, the assay was optimized for murine WT and *Timp3*$^{-/-}$ PMVEC, under basal vs. cytomix conditions. The cells were cultured on biotinylated gelatin containing biotin, and the resultant interaction of the fluorescent avidin which leaked between adjacent PMVEC and bound to sub-cellular biotin was visualized. The number of total avidin-leak regions per field was quantified, and we measured the perpendicular distance of avidin diffusion from the PMVEC borders (as identified by VE-cadherin-staining) beneath the cells. The studies revealed that trans-PMVEC avidin leak was paracellular, rather than transcellular, and associated with sites of VE-cadherin disruption (Figure 3-1A). Additionally, the results indicated a significant increase in the number of avidin regions in *Timp3*$^{-/-}$ PMVEC vs. WT PMVEC, under proinflammatory conditions (Figure 3-1B). Furthermore, there were significant differences in avidin diffusion distance in WT PMVEC vs. *Timp3*$^{-/-}$ PMVEC, under basal

conditions- this trend, while not statistically significant, was reflected in the cytomix cohorts. (Figure 3-1D).

3.1.1 *Timp3*$^{-/-}$ PMVEC exhibited enhanced number of avidin regions under proinflammatory conditions vs. *Timp3*$^{-/-}$ PMVEC under basal conditions

When analyzing the visual data as presented by immunofluorescent imaging, *Timp3*$^{-/-}$ PMVEC appeared to exhibit increased avidin leak under basal conditions vs. WT PMVEC under basal conditions and appeared enhanced in the PMVEC cytomix cohorts (Figure 3-1A). While quantification of the total number of leak regions revealed no significant differences in total number of leak regions under PBS-treatment between PMVEC genotypes, the results revealed a trend towards a greater number of leak regions in *Timp3*$^{-/-}$ PMVEC vs. WT PMVEC (Figure 3-1B). Furthermore, *Timp3*$^{-/-}$ PMVEC demonstrated significant enhancement in leak regions under cytomix-stimulation vs. PBS-treated *Timp3*$^{-/-}$ PMVEC (Figure 3-1B). However, there was no significant difference in total number of leak regions between PBS-treated WT PMVEC vs. WT PMVEC under cytomix-stimulation, though there was a general trend towards a great number of leak regions in the cytomix-stimulated WT PMVEC vs. PBS-treated WT PMVEC (Figure 3-1B).

These trends were reflected following quantification of total leak area, though not statistically significant (Figure 3-1C).

3.1.2 *Timp3*$^{-/-}$ PMVEC exhibited longer avidin diffusion lengths vs. WTPMVEC, under basal conditions

Timp3$^{-/-}$ PMVEC appeared to exhibit greater total avidin fluorescence at basal conditions vs. WT PMVEC under basal conditions (Figure 3-1A). While quantification of the avidin diffusion distance beneath the PMVEC from points of VE-cadherin disruption at PMVEC margins did not reveal a significant difference between cytomix-stimulated WT PMVEC vs. PBS-treated WT PMVEC, the results indicated significantly greater diffusion in *Timp3*$^{-/-}$ PMVEC vs. WT PMVEC, under PBS-treatment (Figure 3-1D). While this difference in diffusion lengths was present between PMVEC genotypes in cytomix-stimulated cohorts, the results were not statistically significant (Figure 3-1D).

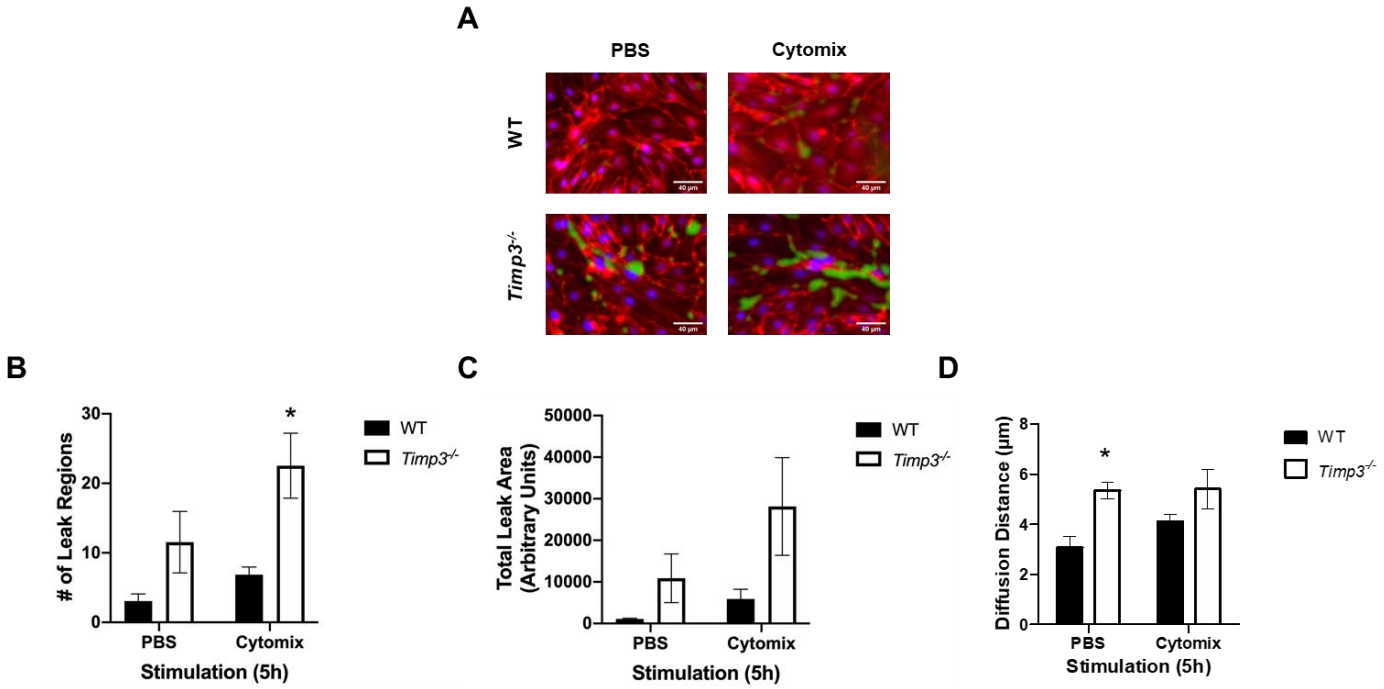


Figure 3-1. Localized protein leak in WT vs. *Timp3*$^{-/-}$ murine PMVEC. (A) XperT immunofluorescent staining of WT and *Timp3*$^{-/-}$ PMVEC treated with PBS (basal) or stimulated with cytomix (proinflammatory) for 5hr indicated greater avidin leak (green) in WT PMVEC following cytomix stimulation vs. PBS. Further, *Timp3*$^{-/-}$ PMVEC appeared to have greater overall avidin leak vs. WT PMVEC under basal and septic conditions. Scale bar= 40µm. (B) The total number of avidin leak regions was significantly greater in cytomix-stimulated *Timp3*$^{-/-}$ PMVEC vs. WT PMVEC. *$p<0.05$ vs. cytomix-treated WT PMVEC, two-way ANOVA with Bonferroni post hoc test, n=3. (C) Quantification of total leak area revealed no significant differences between PMVEC genotypes or between treatment groups. n=3. (D) The diffusion distance of avidin below PBS-treated PMVEC was significantly greater in *Timp3*$^{-/-}$ PMVEC vs. WT PMVEC. *$p<0.05$ vs. PBS-treated WT PMVEC, two-way ANOVA with Bonferroni post hoc test, n=3.

3.2 Basal adhesion of WT vs. *Timp3*$^{-/-}$ PMVEC to gelatin

Previous studies have indicated that mice lacking TIMP3 demonstrated an increased basal permeability compared to WT mice, which was also reflected at the cellular level [74]. As such, WT and *Timp3*$^{-/-}$ PMVEC were seeded on a gelatin-coated membrane to assess potential differences in cell-matrix adhesion between PMVEC genotypes as a contributing factor for the enhanced basal permeability observed. Gelatin was used based on previous studies having established the adequacy of a gelatin matrix coating for PMVEC adhesion and culture [35].

3.2.1 WT PMVEC and *Timp3*$^{-/-}$ PMVEC demonstrated no significant differences in adhesion to gelatin

Analysis of PMVEC adhesion on gelatin revealed no significant differences between WT vs. *Timp3*$^{-/-}$ PMVEC. Multiple incubation timepoints were tested (6hr and 16hr), and both cell types were seeded at 30k and 60k cells/ well (Figure 3-2A, Figure 3-2B). While there seemed to be a possibility of lower cellular adhesion in *Timp3*$^{-/-}$ PMVEC vs. WT PMVEC when incubated for 6hr and 16hr, these results were not statistically significant.

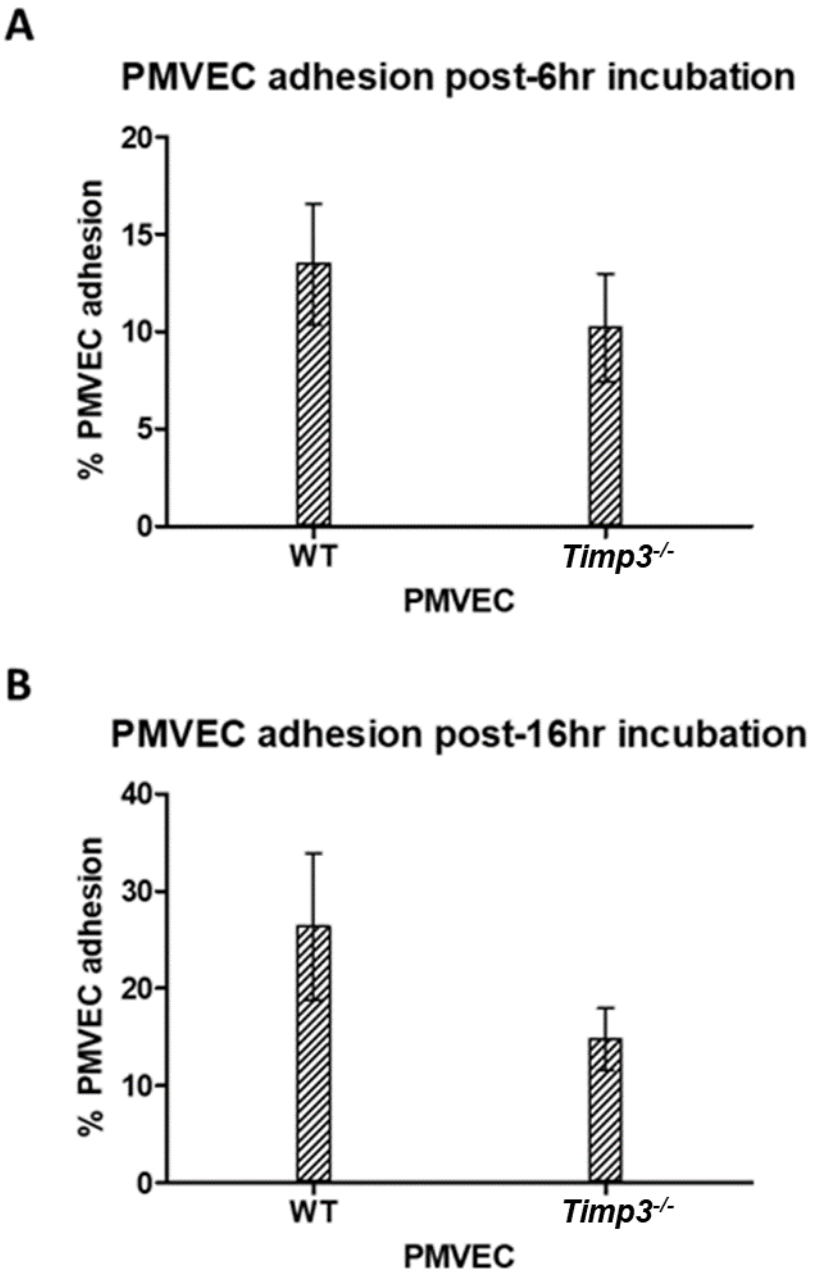


Figure 3-2. Percentage cellular adhesion of PMVEC to gelatin under basal conditions. (A) WT and *Timp3*$^{-/-}$ murine PMVEC were seeded at 60k cells/ well and incubated for (A) 6hr or (B) 16hr before washing and acquiring total cellular fluorescence, revealing no significant differences in cellular adhesion between PMVEC cell types. n=3.

3.3 Basal adhesion of WT vs. *Timp3*$^{-/-}$ PMVEC to ECM proteins

While the adhesion assays revealed no significant basal differences in WT and *Timp3*$^{-/-}$ PMVEC adhesion to both gelatin-coated and bare tissue-culture treated wells, this begged the questions of 1) whether the differences were maintained when the cells were grown on physiologically-relevant components of the ECM, 2) whether there were differential interactions of PMVEC depending on the underlying ECM protein, and 3) whether there was a dose-dependent interaction of PMVEC to different concentrations of ECM proteins.

As such, a commercial ECM adhesion assay was used to assess the average percent adhesion of WT and *Timp3*$^{-/-}$ PMVEC to four common ECM proteins (collagen I, fibronectin, laminin, vitronectin) coated at varying doses. The results revealed that PMVEC adhesion between PMVEC genotypes was comparable per ECM protein (Figure 3-3) However, both PMVEC types demonstrated differential adhesion to the ECM proteins depending on the underlying substrate and the concentration of the substrate (Figure 3-3).

3.3.1 Both PMVEC demonstrated comparable adhesion to ECM proteins

When comparing percent adhesion of PMVEC genotypes to ECM proteins, the data revealed that WT PMVEC had similar levels of overall adhesion

vs. *Timp3*$^{-/-}$ PMVEC for each of the respective ECM proteins (Figure 3-3). This trend was maintained at all concentrations of the ECM proteins (Figure 3-3).

3.3.2 Both PMVEC demonstrated preferential adhesion to fibronectin, and the lowest adhesion preference for collagen I

When comparing percent adhesion of PMVEC to different ECM proteins, both cell types demonstrated greater adhesion to fibronectin vs. other ECM substrates (Figure 3-3A, 3C). Furthermore, WT PMVEC presented with a concentration-dependent increase in percent adhesion to fibronectin- however, this trend plateaued at 10ug/mL, the second highest substrate concentrations (Figure 3-3A). Additionally, *Timp3*$^{-/-}$ PMVEC paralleled the WT PMVEC trend by demonstrating highest adhesion with fibronectin vs. other ECM proteins (Figure 3-3B, 3C, 3D, 3E).

Both PMVEC demonstrated the lowest adhesion to collagen I substrate (Figure 3-3B). Interestingly, collagen I seemed to have an inhibitory role on PMVEC adhesion, as indicated by a dose-dependent decrease in PMVEC adhesion of both cell types as the concentration of collagen I increased (Figure 3-3B). This decrease seemed to plateau at a concentration of 2.5ug/mL of collagen I (Figure 3-3B).

Both cell types demonstrated moderate adhesion to laminin and vitronectin (Figure 3-3D, 3E). The adhesion of both PMVEC was higher for laminin and vitronectin than collagen I, but lower than fibronectin, particularly

when comparing PMVEC adhesion to higher concentration of fibronectin vs. PMVEC adhesion to the respective laminin and vitronectin concentrations (Figure 3-3A). Neither cell types indicated a concentration-dependent relationship to laminin or vitronectin (Figure 3-3D, 3E).

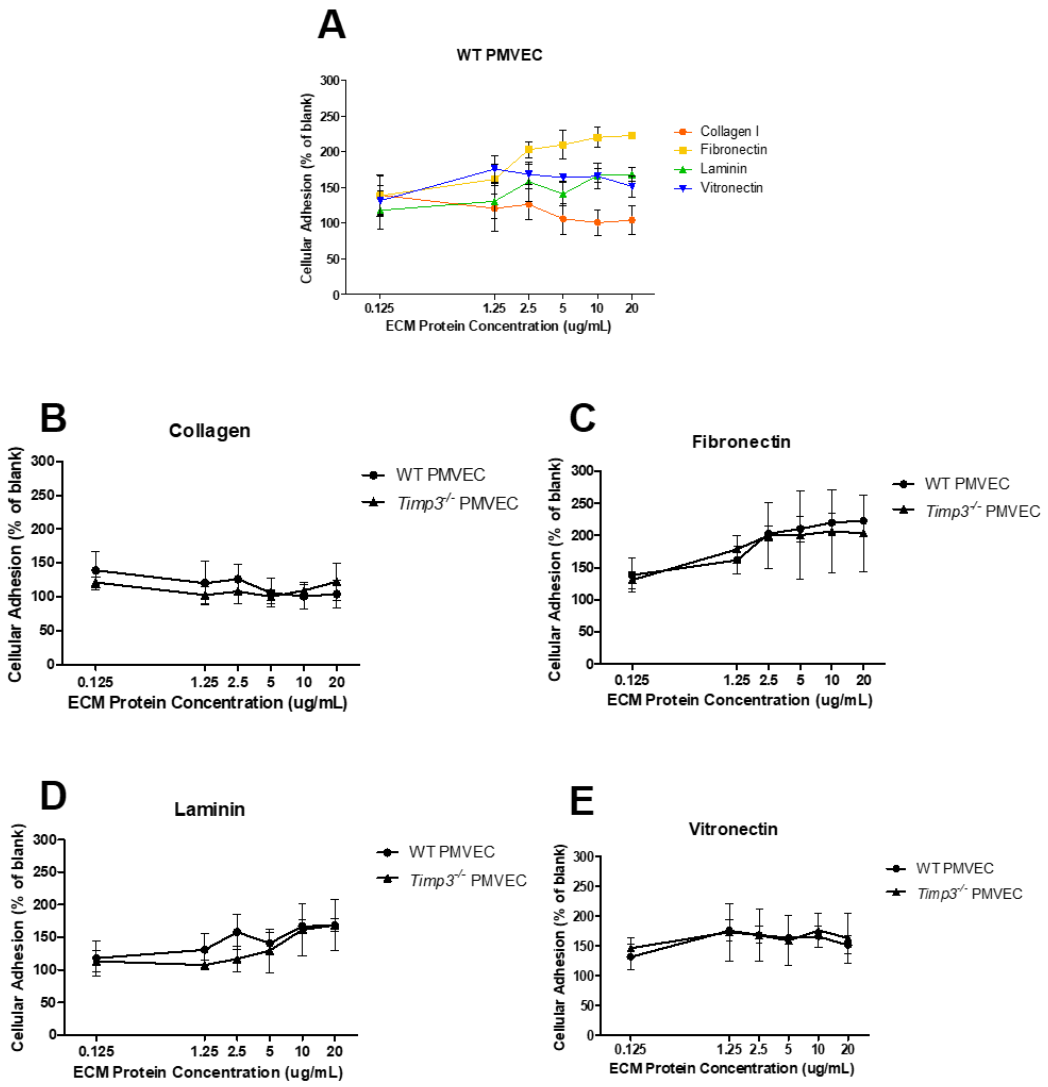


Figure 3-3. Cellular adhesion of WT vs. *Timp3*$^{-/-}$ murine PMVEC to differing concentrations of ECM proteins (collagen I, fibronectin, laminin, vitronectin). (A) WT PMVEC seeded on varying concentrations of four common ECM proteins and incubated for 2hr before washing, staining, and measuring absorbance revealed a dose-dependent, highest affinity for fibronectin, and the lowest affinity towards collagen I. n=3. (B, C, D, E) Cellular adhesion of WT and *Timp3*$^{-/-}$ PMVEC to individual ECM proteins revealed similar adhesive affinity to each ECM protein at different concentrations. n=3.

3.4 pFAK abundance in PMVEC

pFAK is commonly used to assess the stability of cell-matrix interactions [15,77,78,80,82,93]. However, many of these studies often show contradicting results in the association between pFAK abundance and cell-matrix interactions, largely due to variable factors amongst the experiments (i.e. type of stimuli and time points, cell type, cellular process being assessed). Interestingly, some studies have also alluded to the regulatory role of the ECM on barrier stability under inflammatory conditions [83,94,95].

In order to assess PMVEC-matrix interactions under basal and inflammatory conditions, pFAK content of WT and *Timp3*$^{-/-}$ PMVEC seeded on gelatin was assessed, in response to PBS vs. cytomix treatment. Cell culture monolayers were stained for pFAK via immunocytochemistry and imaged using confocal microscopy.

3.4.1 WT PMVEC exhibited a lower number of pFAK particulates under proinflammatory conditions vs. WT PMVEC under basal conditions

When assessing the impact of a proinflammatory environment on pFAK abundance in PMVEC, WT PMVEC demonstrated an enhanced pFAK signal under basal conditions vs. cytomix treatment. This was reflected when comparing all pFAK particulate sizes, with WT PMVEC under basal conditions having a

significantly greater number of pFAK particulates per size category (small, moderate, large), vs. WT PMVEC under cytomix treatment (Figure 3-4A).

3.4.2 *Timp3*$^{-/-}$ PMVEC exhibited a lower number of pFAK particulates vs. WT PMVEC, under basal conditions

Timp3$^{-/-}$ PMVEC exhibited a significantly lower number of small and moderate particulates vs. WT PMVEC, in the PBS-treated groups (Figure 3-4B). However, there were no significant differences in abundance of respective pFAK particulates between the PMVEC genotypes following cytomix-stimulation (Figure 3-4C).

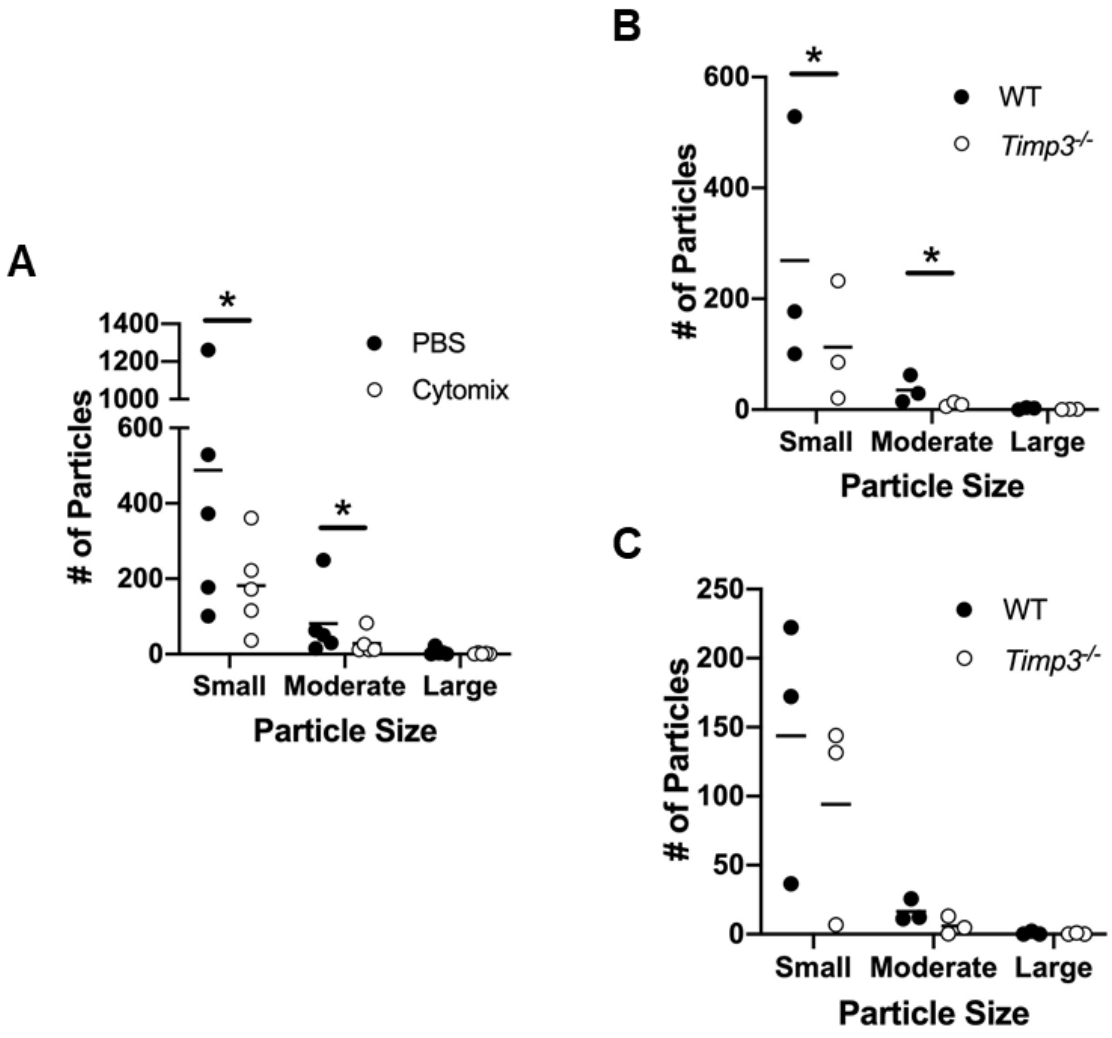


Figure 3-4. Quantification of pFAK in PMVEC under basal vs. proinflammatory conditions. (A) Quantification of pFAK particulates categorized by size (small= 2-10 pixels, moderate= 11-50 pixels, large= 51-100 pixels; NOTE: 1 pixel= 0.566μm) in WT PMVEC indicated significantly higher levels of small and moderate pFAK particulates in the PBS-treated vs. cytomix-treated cohort. *$p<0.05$ vs. PBS-treated WT PMVEC respective to particulate cohort, two-tailed paired t-test, n=3. (B) Quantification of pFAK particulates categorized by size indicated higher levels of small and moderate pFAK particulates in PBS-treated WT PMVEC vs. PBS-treated *Timp3*$^{-/-}$ PMVEC. *$p<0.05$ vs.

PBS-treated WT PMVEC respective to particulate cohort, one-tailed paired t-test, n=3. (C) Quantification of pFAK particulates categorized by size revealed no significant difference in pFAK particulates between WT PMVEC vs. *Timp3*$^{-/-}$ PMVEC, under cytomix stimulation

3.5 Effect of FAK inhibitor, PF228, on localized avidin leak

To elucidate the role of FAK in cellular processes, FAK inhibitors have been tested in numerous cell adhesion, migration, and transformation studies. Multiple cell types (i.e. platelets, mesenchymal stem cells) and various treatment protocols (i.e. proinflammatory conditions, VEGF stimulation) have been assessed [96–98]. In recent years, there has been an emphasis on the anti-tumorigenic potential of FAK inhibitors to treat various cancers [99–101]. However, few studies have looked at the relationship of FAK and PMVEC-ECM adhesion in maintenance of barrier stability, particularly under proinflammatory conditions. As such, PF228, a commonly used FAK inhibitor, was used to assess the impact of inhibiting phosphorylation of FAK on WT vs. *Timp3*$^{-/-}$ PMVEC barrier integrity, under basal vs. proinflammatory conditions.

3.5.1 PF228 enhanced localized avidin leak in WT PMVEC under basal and proinflammatory conditions

WT PMVEC were incubated with various concentrations (5µM, 10µM, and 25µM respectively) of the PF228 inhibitor for 16hr prior to PBS vs. cytomix-treatment. This was followed by the previously outlined XperT-permeability assay and immunofluorescent protocol. The results indicated that inhibition of FAK phosphorylation was associated with an increased level of localized avidin leak in WT PMVEC under cytomix conditions vs. WT PMVEC control (Figure 3-5A). Quantifications of the number of avidin leak regions supported this observed trend under proinflammatory conditions, with cytomix-treated WT PMVEC

demonstrating a significantly increased number of avidin regions at 25μM of PF228 vs. WT PMVEC control (Figure 3-6B). Furthermore, quantification of total avidin leak area revealed a significant increase in total avidin leak in WT PMVEC when exposed to 25μM of PF228 vs. WT PMVEC control, in both PBS-treated and cytomix-stimulated cohorts.

3.5.2 PF228 enhanced total avidin leak area in *Timp3*$^{-/-}$ PMVEC under proinflammatory conditions

Immunofluorescent imaging revealed enhanced avidin leak in *Timp3*$^{-/-}$ PMVEC in response to PF228 exposure, particularly under proinflammatory conditions (Figure 3-5B). While there were no significant differences in number of avidin leak regions in *Timp3*$^{-/-}$ PMVEC under basal or proinflammatory conditions (Figure 3-6C, 6D), quantifications of total avidin area revealed that cytomix-stimulated *Timp3*$^{-/-}$ PMVEC exposed to 25μM of PF228 demonstrated enhanced total avidin area vs. cytomix-stimulated *Timp3*$^{-/-}$ PMVEC vehicle control (Figure 3-7D). However, there were no significant differences in total avidin area in the PBS-treated *Timp3*$^{-/-}$ PMVEC cohort (Figure 3-7C).

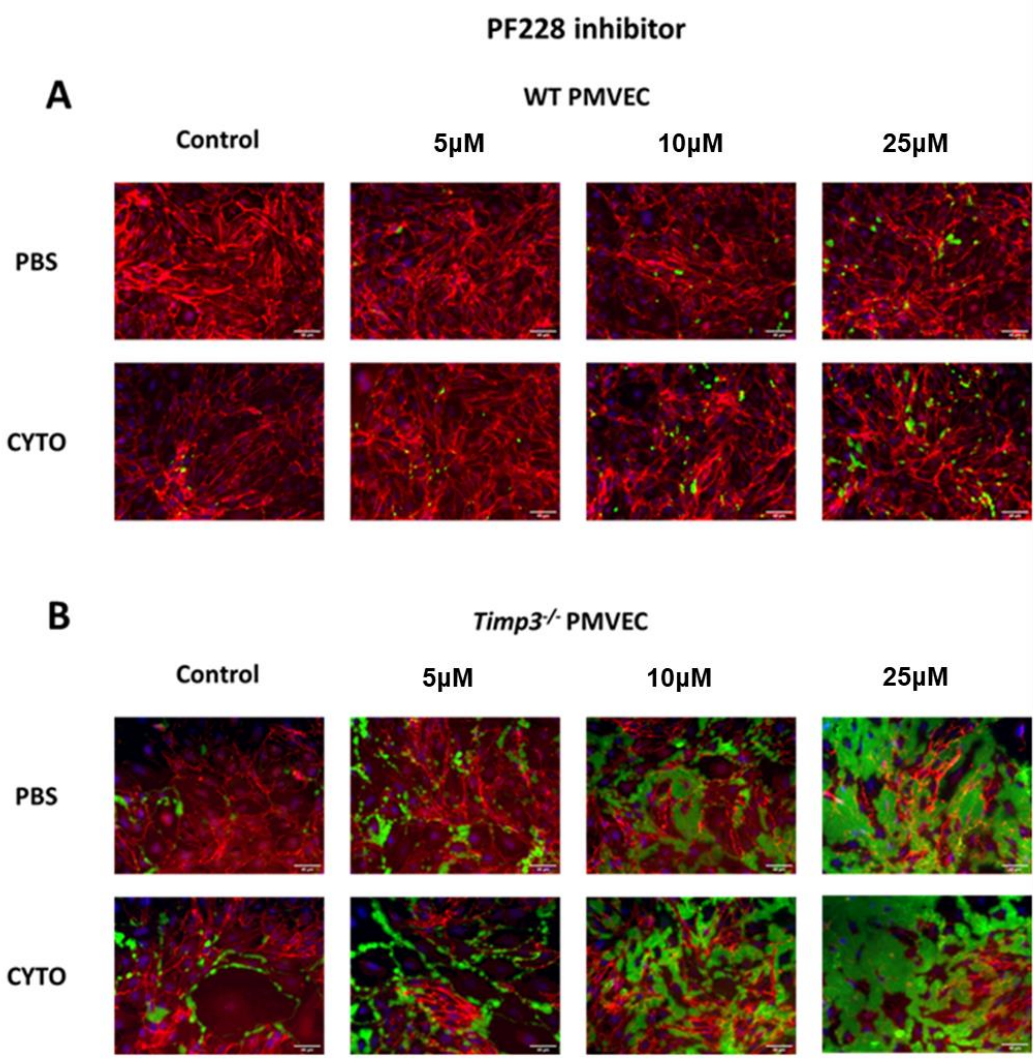


Figure 3-5. Localized avidin leak (green) in WT and *Timp3*$^{-/-}$ murine PMVEC, stained for VE-cadherin (red), following incubation with PF-573228 (PF228), a FAK inhibitor. (A) WT PMVEC demonstrated a dose-dependent increase in avidin leak to PF228 in the PBS cohort vs. vehicle control. This was further enhanced in the cytomix cohort vs. PBS n=3. (B) *Timp3*$^{-/-}$ PMVEC demonstrated increased avidin leak in the PBS cohort vs. vehicle control and WT PMVEC at respective PF228 concentrations. This was further enhanced in the cytomix cohort vs. vehicle control and WT PMVEC at respective PF228 concentrations. n=3.

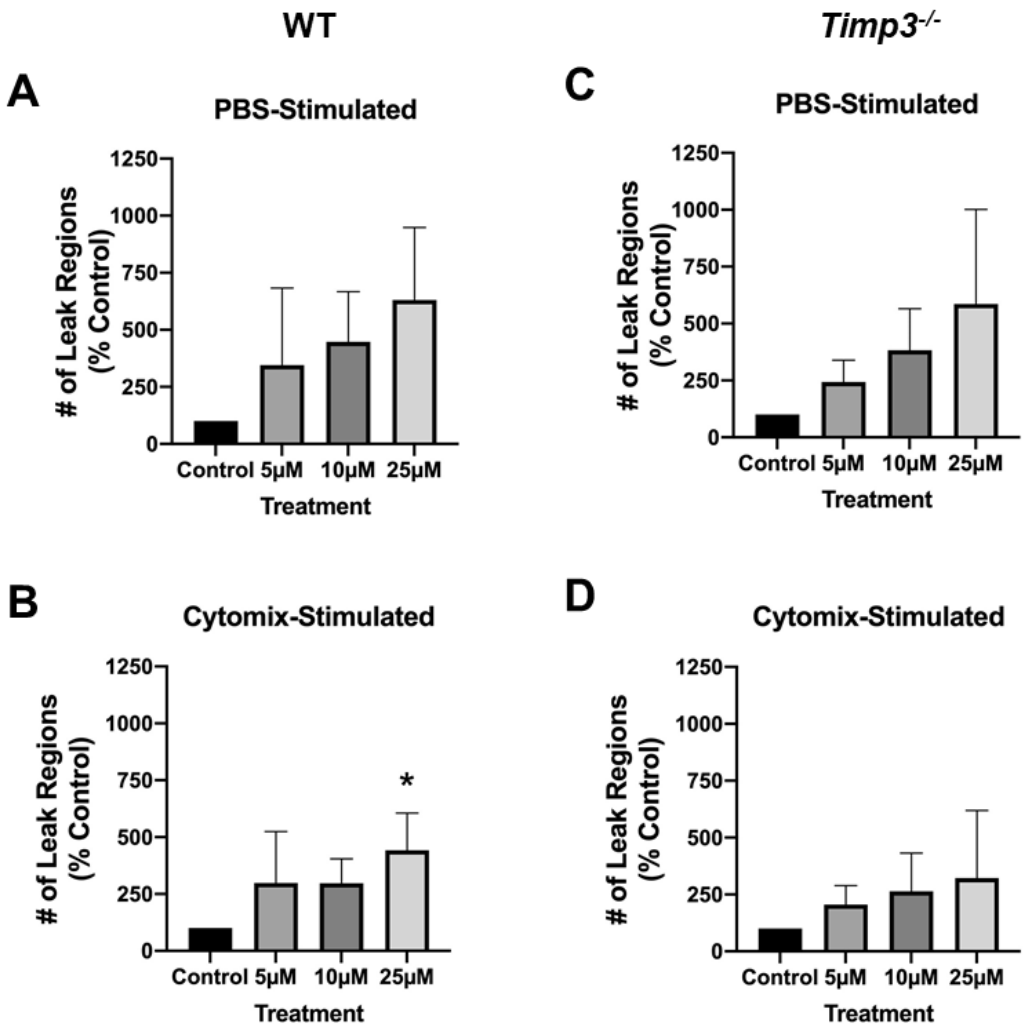


Figure 3-6. Quantification of the number of localized avidin leak regions in WT and *Timp3*$^{-/-}$ murine PMVEC, following incubation with PF-573228 (PF228), a FAK inhibitor. (A) FAK inhibition with PF228 had no significant effect on the number of avidin leak regions in PBS-treated WT PMVEC. n=3. (B) FAK inhibition (PF228 25μM) enhanced the number of avidin leak regions in cytomix-stimulated WT PMVEC. *$p<0.05$ vs. other cytomix-stimulated WT PMVEC groups, one-way ANOVA with Dunnett's post hoc test, n=3. (C) PF228 had no significant effect on the number of avidin leak regions in *Timp3*$^{-/-}$ PMVEC in the PBS-treated cohorts or (D) in the cytomix-stimulated cohorts.

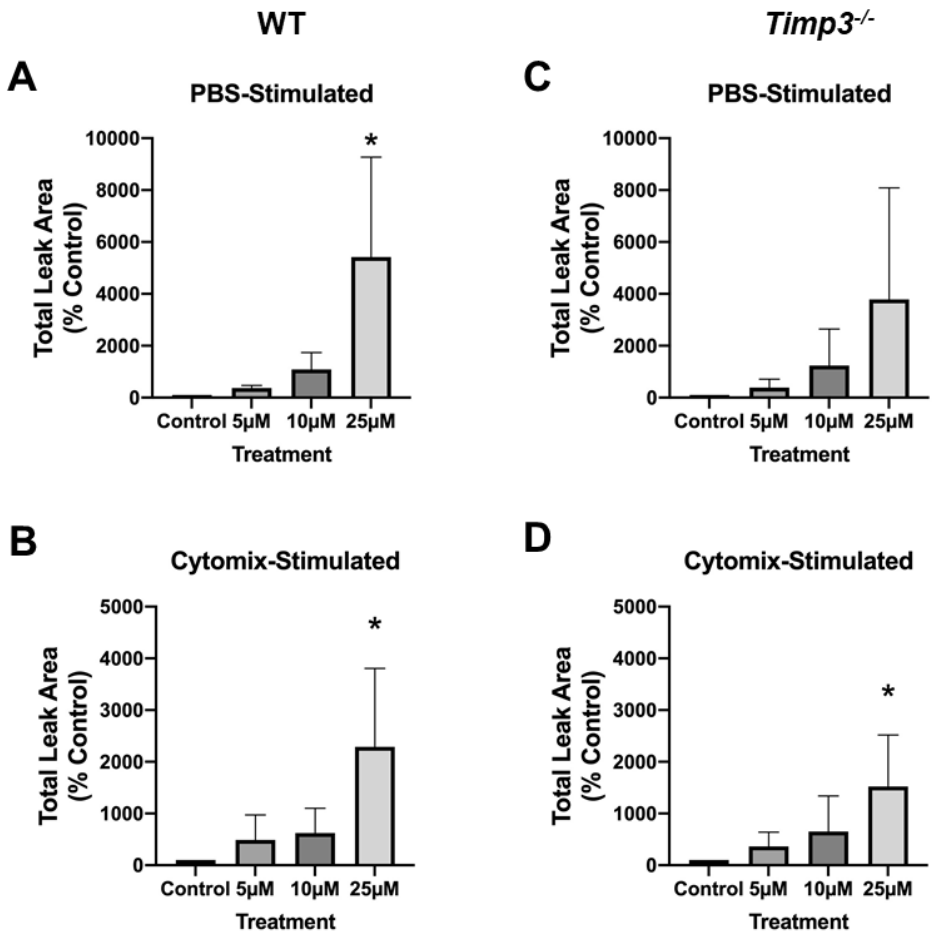


Figure 3-7. Quantification of localized avidin leak area in WT and *Timp3*$^{-/-}$ murine PMVEC, following incubation with PF-573228 (PF228), a FAK inhibitor. (A) WT PMVEC demonstrated significant enhancement in total avidin leak area when treated with 25μM of PF228 vs. WT PMVEC control, in the PBS-treated cohorts and (B) in the cytomix-stimulated cohorts. *p<0.05 vs. WT PMVEC control, one-way ANOVA with Dunnett's post hoc test, n=3. (C) *Timp3*$^{-/-}$ PMVEC demonstrated no significant enhancement in the number of avidin leak regions in the PBS-treated cohorts. (D) In the cytomix-stimulated cohorts, *Timp3*$^{-/-}$ PMVEC demonstrated significant enhancement in total avidin leak area when treated with 25μM of PF228 vs. *Timp3*$^{-/-}$ PMVEC control. *p<0.05 vs. WT PMVEC control, one-way ANOVA with Dunnett's post hoc test, n=3.

3.6 Effect of metalloproteinase inhibitor, BB94, on localized avidin leak

Previous studies from our lab have shown an attenuation of endothelial leak following broad-spectrum metalloproteinase inhibition, with higher TEER (lower permeability) in cells treated with the inhibitor vs. control, particularly under proinflammatory conditions [74]. While certain factors, such as VE-cadherin disruption, have been implicated in metalloproteinase-dependent endothelial leak, the underlying mechanism, including the effect of metalloproteinases on cell-ECM interactions and subsequent impact on PMVEC barrier stability is yet to be elucidated. As such, the use of a broad-spectrum metalloproteinase inhibitor, BB94, was implemented to assess localized avidin leak in the murine PMVEC model, under basal vs. proinflammatory conditions.

3.6.1 BB94 attenuated leak in *Timp3*$^{-/-}$ PMVEC under basal and proinflammatory conditions

PMVEC were incubated with various concentrations (10μM and 25μM respectively) of the BB94 inhibitor for 16hr prior to PBS vs. cytomix-treatment. This was followed by the previously outlined XperT-permeability assay and immunofluorescent protocol. The results revealed a limited response of WT PMVEC to the BB94 inhibitor vs. WT PMVEC control, under both basal and proinflammatory conditions (Figure 3-8A, Figure 3-9). Furthermore, WT PMVEC demonstrated no significant attenuation in total avidin leak in response to BB94,

in either the PBS-treated or cytomix-stimulated cohorts (Figure 3-9C, 9D). Additionally, quantification of the number of localized leak regions revealed no significant differences between WT and *Timp3*$^{-/-}$ PMVEC, nor any significant differences between PBS vs. cytomix-stimulated cohorts (Figure 3-9A, 9B).

However, *Timp3*$^{-/-}$ PMVEC treated with BB94 appeared to show a decrease in localized avidin leak in response to BB94 concentrations vs. control PMVEC, under both basal and proinflammatory conditions (Figure 3-8B). Quantification of total avidin leak area supported this observation, with *Timp3*$^{-/-}$ PMVEC treated with 25μM of BB94 demonstrating an attenuation in total avidin area vs. control, in both PBS-treated and cytomix-stimulated *Timp3*$^{-/-}$ PMVEC cohorts (Figure 3-9C, 9D).

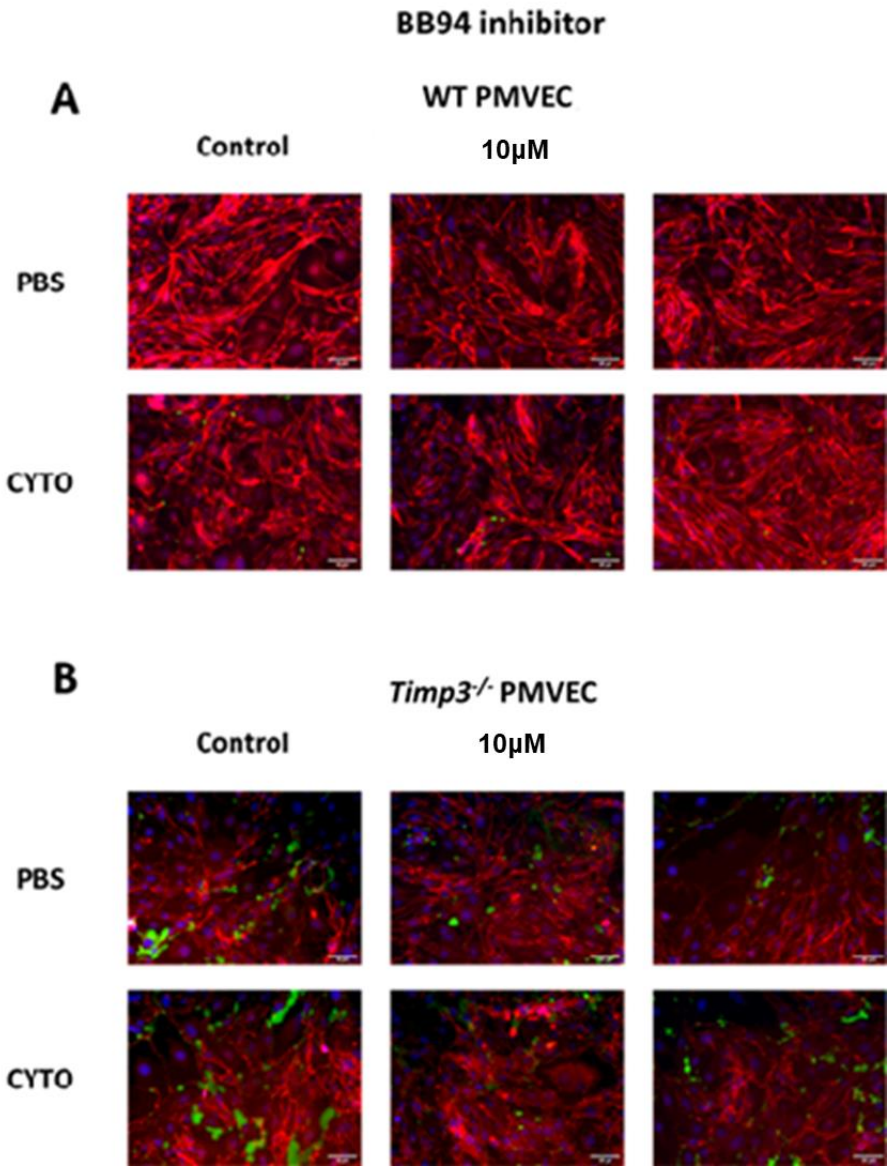


Figure 3-8. Localized avidin leak (green) in WT and *Timp3*$^{-/-}$ murine PMVEC, stained for VE-cadherin (red), following incubation with BB94, a broad spectrum MMP inhibitor. (A) WT PMVEC demonstrated limited attenuation in localized avidin leak in response to BB94 concentrations of 10μM and 25μM, in the PBS vs. cytomix cohort. n=3. (B) BB94-treated *Timp3*$^{-/-}$ PMVEC demonstrated attenuation in localized avidin leak in the PBS cohort vs. vehicle control. This attenuation was especially enhanced in the cytomix cohort vs. vehicle control. n=3.

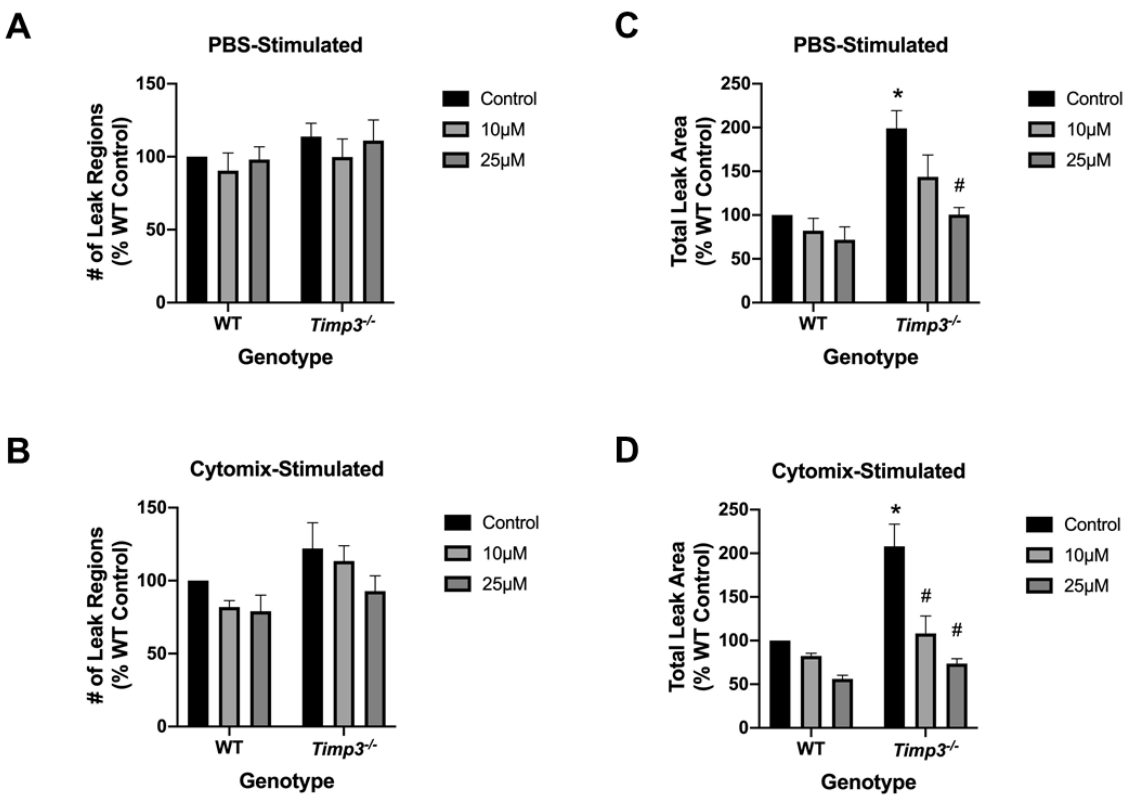


Figure 3-9. Quantification of localized avidin leak area in WT and *Timp3*$^{-/-}$ murine PMVEC, following incubation with BB94, a broad spectrum MMP inhibitor. (A) (B) Quantification of the number of total avidin leak regions revealed no significant differences between PMVEC genotypes, or between treatment groups. n=3. (C) In the PBS cohorts, *Timp3*$^{-/-}$ PMVEC demonstrated significantly enhanced total avidin leak under control conditions vs. WT PMVEC control. At a 25μM concentration of BB94, the total leak area was significantly attenuated in *Timp3*$^{-/-}$ PMVEC vs. the *Timp3*$^{-/-}$ PMVEC control group. *$p<0.05$ vs. WT PMVEC control, #$p<0.05$ vs. *Timp3*$^{-/-}$ PMVEC control, two-way ANOVA with Bonferroni post hoc test, n=3. (D) In the cytomix-treated cohorts, *Timp3*$^{-/-}$ PMVEC demonstrated significantly enhanced total avidin leak under control conditions vs. WT PMVEC control. At both 10μM and 25μM concentrations of BB94, the total leak area was significantly attenuated in *Timp3*$^{-/-}$ PMVEC vs. the *Timp3*$^{-/-}$

PMVEC control group. *p<0.05 vs. WT PMVEC control, #p<0.05 vs. *Timp3*$^{-/-}$ PMVEC control, two-way ANOVA with Bonferroni post hoc test, n=3.

Chapter 4

4 Discussion

4.1 Summary of Findings

The pulmonary endothelium is vital in maintaining the homeostatic function of the lungs by providing a highly regulated barrier for the controlled movement of macromolecules [102]. Dysfunction of the PMVEC barrier, which is associated with inflammatory pathologies like sepsis, results in an uncontrolled accumulation of protein-rich edema fluid within the distal airspaces of the lungs, impeding gas exchange [103]. The loss of pulmonary function has a broad effect on the body and is ultimately associated with multi-organ failure and subsequent death [9]. Thus, understanding the mechanisms that regulate PMVEC barrier function are critical for developing adequate treatments to target endothelial dysfunction under proinflammatory conditions in the lungs.

PMVEC barrier function is dependent on interaction between adjacent PMVEC, as well as interaction between PMVEC and the underlying ECM leading to the formation of FAC [15,77,78]. Further, it has been suggested that loss of cell-ECM interactions can promote endothelial cell barrier dysfunction [15,77,78]. Previous studies have demonstrated that TIMP3 appears to have the ability to regulate inter-PMVEC interactions and subsequently, PMVEC barrier function [11,74]. Further, in the developing lung, TIMP3 has been shown to regulate cell-ECM interactions [75]. The underlying mechanism through which TIMP3 regulates

PMVEC barrier function and whether this mechanism is dependent on PMVEC-ECM interactions is unknown.

In this study, I was able to identify a potential role for TIMP3 in regulating PMVEC-ECM interactions leading to formation of focal adhesions and PMVEC barrier stability (Figure 4-1). Specifically, *Timp3*$^{-/-}$ PMVEC had significantly decreased pFAK vs. WT PMVEC under basal conditions and this was associated with increased local leak and leak diffusion beneath *Timp3*$^{-/-}$ PMVEC. Moreover, the increased leak around *Timp3*$^{-/-}$ PMVEC was rescued by treatment with BB94 suggesting that TIMP3 regulation of PMVEC-ECM interaction appeared to be metalloproteinase-dependent. I was also able to demonstrate that formation of FAC and subsequent FAK activation was required for PMVEC barrier function. Specifically, inhibition of FAK activity resulted in local leak around WT PMVEC under basal conditions and significantly exacerbated cytomix-induced leak. Inhibition of FAK also exacerbated the basal and septic leak around *Timp3*$^{-/-}$ PMVEC. Collectively, these findings suggest that PMVEC dysfunction under proinflammatory conditions may occur via disruptions in the TIMP3-mediated PMVEC-ECM interactions, and may offer novel therapeutic approaches to treat endothelial barrier dysfunction in the lungs, as associated with proinflammatory pathologies (Figure 4-1).

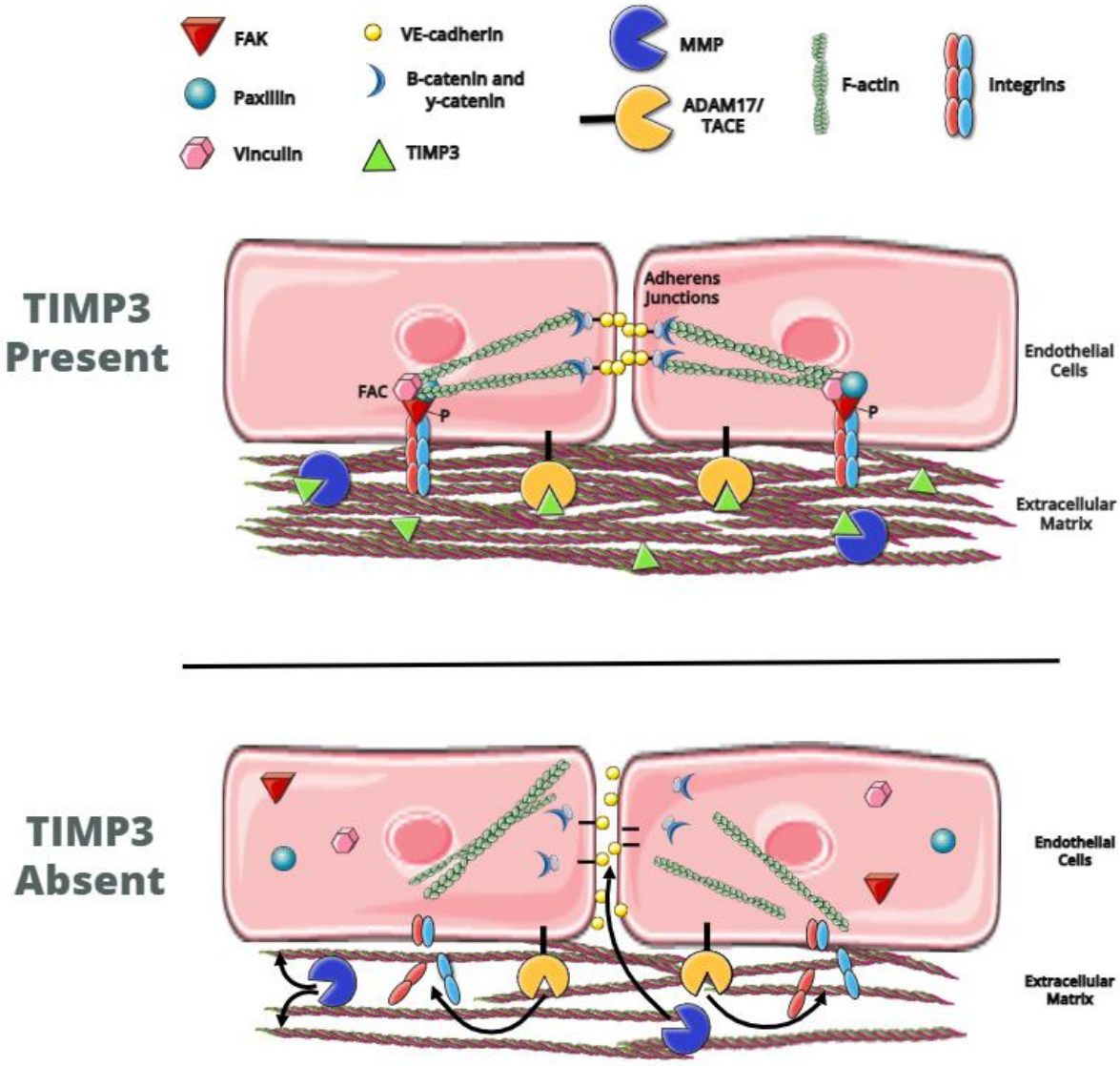


Figure 4-1. TIMP3 promotes PMVEC barrier function through stabilizing focal adhesion formation and FAK activation. UPPER PANEL: Under basal conditions, TIMP3 appears to inhibit metalloproteinases, allowing for adequate integrin-ECM interactions. This promotes phosphorylation of focal adhesion kinase (FAK) and allows for assembly of focal adhesion complexes (FAC), which stabilize integrin-ECM interactions. Adherens junctions are maintained between adjacent PMVEC, and further stabilized by FAC signaling via F-actin filaments. LOWER PANEL: In the absence of

TIMP3, metalloproteinase activity is enhanced, allowing for the degradation of the ECM, integrins, and VE-cadherin. The loss of ECM and integrins leads to disassembly of FAC. VE-cadherin cleavage leads to loss of adherens junctions. Together, these events lead to a loss of PMVE-ECM and inter-PMVEC interactions.

4.2 Contributions of Research to Current Knowledge

4.2.1 TIMP3 regulates local leak around PMVEC

Previous studies demonstrated reduced TEER and enhanced EB albumin leak across *Timp3*$^{-/-}$ PMVEC demonstrating greater overall leak [74]. To examine the local leak around individual cells, I utilized the XPerT-permeability assay in the murine WT and *Timp3*$^{-/-}$ PMVEC, which also enabled me to assess leak in relation to VE-cadherin disruption, and assess the potential impact of interaction of PMVEC with the underlying matrix.

Under basal conditions, the avidin leak diffusion was greater under the *Timp3*$^{-/-}$ PMVEC vs. WT PMVEC, which also appeared to have increased VE-cadherin disruption. Furthermore, the number of avidin leak regions was greatly enhanced in *Timp3*$^{-/-}$ PMVEC exposed to proinflammatory conditions vs. *Timp3*$^{-/-}$ PMVEC under basal conditions.

These results support previous studies that have associated VE-cadherin disruption with increased levels of leak, due to loss of inter-PMVEC interactions [88]. They also confirm previous finding that have demonstrated enhanced albumin leak and reduced TEER across *Timp3*$^{-/-}$ PMVEC vs. WT PMVEC, supporting the idea that TIMP3 is a crucial component for EC barrier regulation. Furthermore, the enhanced diffusion distance under basal *Timp3*$^{-/-}$ PMVEC vs. WT PMVEC, combined with previous reports of the importance of cell-ECM interactions in maintaining EC function, alludes to a potential disruption in PMVEC-ECM

interactions in the absence of TIMP3 [26,28,29]. Thus, these results suggest that there were an altered number of localized avidin leak and altered avidin leak diffusion beneath the cells that had reduced cell-matrix interactions further highlighting the importance of PMVEC-ECM interactions in supporting PMVEC barrier function.

Overall, these results provide a novel approach for studying barrier dysfunction through direct visualization and subsequent quantification of localized avidin leak in the murine PMVEC model, in addition to the previously established methods of measuring TEER and EB albumin leak. This is especially useful for visually assessing leak in relation to VE-cadherin disruption or for future studies looking at other proteins-of-interest in relation to murine PMVEC barrier integrity.

4.2.2 TIMP3 does not appear to regulate initial PMVEC-ECM adhesion

TIMP3 has the ability to mediate cell-ECM interaction and there is evidence that PMVEC-ECM interactions promote PMVEC barrier function [44,104]. Based on this, I began to investigate whether alterations in initial adhesion of WT vs. *Timp3*$^{-/-}$ PMVEC were present that may explain the enhanced leak and barrier dysfunction associated with loss of TIMP3.

Previous studies have shown that TIMP3 can regulate cell-matrix adhesion through metalloproteinase-dependent processes [11,48,75]. For example, suppression of TIMP3 in cancer cells has been associated with increased metalloproteinase

expression (i.e. MMP9, ADAM17) reduced cell adhesion and increased cell migration [105,106]. My data, however, found no significant differences in initial cellular adhesion between WT and *Timp3*$^{-/-}$ PMVEC to their underlying substrate. Furthermore, both WT and *Timp3*$^{-/-}$ PMVEC demonstrated a similar adhesion response to each of the commonly used ECM proteins. Collectively, these findings suggest a limited role for TIMP3 in initial PMVEC cell adhesion and appears to indicate that TIMP3 does not confer an adhesion preference to specific matrices.

One potential reason for these observed differences may be the nature of the cell types being examined. Most studies assessing the role of TIMP3 on cellular adhesion were looking at highly-proliferative, migratory cells, as opposed to a quiescent PMVEC monolayer under basal conditions [107–109]. Furthermore, many of these studies specifically implemented cancerous cell lines, which demonstrate a differential genotype and phenotype as compared healthy PMVEC [110,111]. These variations in study design may explain the differential results of my cell adhesion studies using a PMVEC monolayer as compared to other studies using migratory, tumorigenic models.

It is also important to note that my cell adhesion data only suggests that TIMP3 is not required for the initial adhesion of PMVEC to the underlying ECM. However, the observed increase in leak diffusion beneath *Timp3*$^{-/-}$ PMVEC vs. WT PMVEC, as well as the significant reduction in pFAK within *Timp3*$^{-/-}$ PMVEC vs. WT PMVEC both suggest that TIMP3 has the ability to regulate PMVEC interaction with the ECM. This is supported by previous studies in

Timp3$^{-/-}$ mice. Specifically, lungs from mice lacking TIMP3 had increased metalloproteinase activity leading to enhanced fibronectin degradation [75]. This degradation of fibronectin resulted in decreased epithelial cell-ECM interaction and a significant reduction in FAK phosphorylation [75].

Initial cell attachment to the ECM is driven by specific integrin-mediated interactions with the matrix- these start off as single receptor-ligand pairs and over time, as the monolayer forms, more bonds are initiated to confer greater adhesive strength. However, interaction of an established monolayer with the underlying ECM is mediated largely by the presence of metalloproteinases, which can interfere with the established cell-ECM interactions- by regulating metalloproteinase activity, TIMPs are particularly important to maintain basal conditions in an established endothelial monolayer [53]. Thus, while not examined within the context of my study, this may be one explanation for why TIMP3 does not appear to have a role in regulating the initial PMVEC-ECM adhesion but does appear to be a critical mediator of PMVEC-ECM interaction in the PMVEC barrier.

4.2.3 FAK activation promotes PMVEC barrier function and is regulated by TIMP3

FAK has been studied extensively in proliferating and migrating cells, with substantial implications for cancer research [112]. However, there is limited research on the role of focal adhesions in relation to endothelial barrier function. Furthermore, the data currently present regarding focal adhesions and EC is often

opposing [15,77,78,81,82,84]. For instance, one study showed that thrombin-stimulation enhanced FAK phosphorylation in HUVEC, while another study revealed that thrombin exposure reduced FAK expression in human pulmonary arterial EC [15,81].

Interestingly, WT PMVEC exposed to proinflammatory stimuli were found to have reduced levels of pFAK vs. WT PMVEC under basal conditions. Additionally, inhibition of FAK activity in WT PMVEC resulted in a significant increase in localized avidin leak under both basal and proinflammatory conditions. Collectively, these two lines of evidence suggest that FAK activation is crucial in maintaining adequate barrier function in PMVEC and support previous work in human pulmonary arterial EC.

My data in combination with other studies also suggests that the role of FAK in barrier function may be dependent on the specific EC being studied, the stimulation used, and the timespan examined. For example, FAK may promote barrier function in microvascular EC, such as PMVEC, but have a different role in macrovascular EC, such as HUVEC. In fact, previous work has demonstrated differential responses to albumin leak between macrovessels (i.e. HUVECs) and microvessels (i.e. human pulmonary MVEC), which is not surprising given the heterogeneity between the vessels of vasculature [3–5,113].

Timp3$^{-/-}$ PMVEC were also found to have significantly reduced levels of pFAK vs. WT PMVEC under basal conditions, and were found to have enhanced avidin leak in response to FAK inhibition when exposed to proinflammatory conditions. This supports previous work demonstrating that a loss of TIMP3 leads

to reduced pFAK and highlights the potential role for TIMP3 in regulating PMVEC-ECM interaction [75]. It also emphasizes the importance of both TIMP3 and FAK in maintaining a functioning PMVEC barrier, and suggests that reduction of both TIMP3 and pFAK, as associated with inflammation, is highly detrimental to PMVEC barrier function (Figure 4-1).

In face of opposing studies showing differential pFAK response to a proinflammatory stimuli, my results provide a more concrete understanding of the effect of inflammation on pFAK abundance in PMVEC, the subsequent importance of pFAK in maintaining the PMVEC barrier, and the potential role of TIMP3 regulating pFAK. Furthermore, based on our previous understanding of the importance of pFAK in maintaining functional cell-matrix interactions and consequently a functional barrier, this response to an inflammatory environment suggests a potential link between reduced focal adhesion signaling and enhanced barrier dysfunction.

4.2.4 Metalloproteinase-dependent role of TIMP3 on PMVEC barrier stability

Metalloproteinases have previously been found to mediate EC barrier function. For example, both MMP2 and -9 have been associated with increased PMVEC permeability via degradation of matrix proteins, while ADAM15 has been implicated in altering EC barrier permeability by disrupting adherens junctions and promoting monocyte transmigration [11,25]. Moreover, the increased leak observed in the absence of TIMP3 is thought to be due, at least in part, to

increased metalloproteinase activity as treatment of *Timp3*$^{-/-}$ PMVEC with a synthetic metalloproteinase inhibitor (GM6001) while in culture rescued the observed decrease in TEER [74].

Previous studies have shown that loss of TIMP3 is associated with enhanced metalloproteinase activity [11,59]. Furthermore, degradation of the ECM due to metalloproteinase activity is associated with impaired focal adhesion signaling, which is problematic on multiple fronts. Firstly, loss of focal adhesions impairs the ability of cells to form adequate integrin-ECM interactions, compromising cell-matrix interactions [45,114]. Furthermore, focal adhesions have been shown to interact with and potentially stabilize adherens junctions via intracellular F-actin signaling [14,115]. As such, impaired focal adhesions prevents stabilization of adherens junctions between adjacent PMVEC, which, with the addition of enhanced metalloproteinase activity, leads to cleavage of VE-cadherin and loss of inter-PMVEC junctions [11,14]. Additionally, pFAK signaling is further altered under proinflammatory stimuli [15,46,77].

My findings, in combination with these studies, allude to the potential link between metalloproteinases, TIMP3, and FAK in regulating PMVEC barrier function, as *Timp3*$^{-/-}$ PMVEC had significantly increased leak associated with significantly reduced pFAK vs. WT PMVEC under both basal and proinflammatory conditions. Moreover, inhibition of metalloproteinases rescued the observed leak in *Timp3*$^{-/-}$ PMVEC under both basal and proinflammatory conditions. This is potentially due to inhibition of metalloproteinase-dependent degradation of the matrix and adherens junctions, which stabilizes integrin-ECM

interactions, allowing for formation of focal adhesions. This in turn supports intracellular signals that maintain inter-PMVEC junctions and PMVEC-ECM interactions (Figure 4-1).

These results indicate a potential link between the action of TIMP3 on PMVEC barrier function via metalloproteinase-dependent processes, possibly through downstream actions on FAK. Some studies support the idea that metalloproteinase inhibition is associated with FAK activation, though no present data exists for the murine PMVEC model under proinflammatory conditions [85]. As such, these results provide a promising avenue for future studies to assess the role of metalloproteinase-dependent TIMP3 action on PMVEC barrier function through FAK regulation.

4.3 Limitations and Future Directions

All experiments were conducted under *in vitro* conditions, with most experiments utilizing murine PMVEC grown on gelatin-coated, cell culture-treated plates. While cells were grown under physiologically-relevant conditions (37°C, 5% CO_2) with supplemented DMEM growth medium, the conditions were not completely representative of an *in vivo* vascular environment. Under physiological conditions, the endothelium is exposed to various haemodynamic stimuli (i.e. shear stress, vasoactive compounds), which stimulates the EC and surrounding cytoskeleton to constantly reshape the EC monolayer in response to *in vivo* signals [40]. Future studies should incorporate the use of a model with shear

stress to better assess the response of the PMVEC monolayer under physiological conditions.

In addition, most of my studies, with the exception of the cell adhesion assay, were conducted using gelatin as the underlying matrix. While our lab has historically utilized gelatin as a substrate for human and mouse PMVEC, gelatin may not be considered physiologically relevant and it may be beneficial to use matrices that are more representative of *in vivo* conditions [74,88,116]. Specifically, repeating the studies utilizing fibronectin could be beneficial as that is the matrix substrate that PMVEC appeared to have the highest affinity in my cell adhesion assays. It is important to note, however, that my cell adhesion studies with gelatin showed similar results to those obtained with other ECM proteins. Moreover, I also performed pilot studies examining pFAK in PMVEC grown on fibronectin with very similar results to those obtained from PMVEC grown on gelatin.

When studying the role of FA, a potential pitfall of my experiments is that only FAK, specifically pFAK, was used to assess focal adhesion signaling in the PMVEC. While pFAK has been shown to be a major component of focal adhesions through recruitment of various proteins to form FAC and promote further downstream effects, future studies should assess other upstream and downstream proteins that may be involved in the focal adhesion pathway (i.e. integrins, paxillin, and vinculin) [14,45,117]. This may help better understand the effect of differential pFAK expression in PMVEC on downstream signaling pathways and help elucidate a potential mechanism for the link between cell-ECM interactions, FA, and PMVEC barrier function. Furthermore, future studies

should use western blot analysis to look at protein levels of total FAK in relation to pFAK in PMVEC to better understand the mechanism that leads to altered pFAK abundance in PMVEC, in relation to loss of TIMP3, and under cytomix stimulation. Further, while my studies using a FAK inhibitor strongly suggest that FAK is critical to PMVEC barrier function, future studies could examine the effects of overexpressing pFAK in PMVEC, via FAK gene amplification, to assess a potential means of rescuing enhanced PMVEC permeability. This could be applied to other barrier studies using TEER or EB albumin leak to assess the replicability of my results in other methods of measuring PMVEC barrier integrity.

While my experiments have revealed a link between TIMP3, FA, and PMVEC barrier function, the underlying mechanism remains unclear. The metalloproteinase inhibitor data suggests that the role of TIMP3 in regulating FA stability and subsequently, PMVEC monolayer integrity, is dependent on metalloproteinase inhibition. However, future studies need to measure pFAK content in PMVEC treated with BB94 to assess whether there is a change in pFAK abundance following metalloproteinase inhibition. Furthermore, it will also be important to determine how TIMP3 is linked to FAK activation and focal adhesion formation, which could be through metalloproteinase-dependent processes that enhance degradation of the underlying ECM or through cleavage of cell surface receptors, such as integrins [11,53]. To address this, future studies could utilize a technique such as terminal amine isotopic labelling of substrates (TAILS)

to examine protein degradation in WT and *Timp3*$^{-/-}$ PMVEC under basal and proinflammatory conditions.

Finally, while some of my experiments revealed striking trends, they did not reach the level of significance. For example, while quantification of total leak area showed that *Timp3*$^{-/-}$ PMVEC had greater under cytomix stimulation vs. under PBS treatment, this trend was not statistically significant (Figure 3-1). Similarly, quantification of leak regions following FAK inhibition (i.e. addition of PF228 at 25uM) showed increased leak in both WT and *Timp3*$^{-/-}$ PMVEC cohorts- however, these results were not statistically significant. This was likely due to a low n-value (n=3) combined with high variability due to the use of two biologically different murine PMVEC isolations. The use of different PMVEC isolations is absolutely critical as it demonstrates that the observed differences between WT and *Timp3*$^{-/-}$ PMVEC are not due to isolation effects. To address this, these studies should be repeated to increase the n-value. Furthermore, my studies were performed in mouse PMVEC. It would be beneficial to repeat all conducted and proposed experiments with human PMVEC to assess the clinical relevance of my findings.

4.4 Summary and Conclusions

Collectively, my data indicates that TIMP3 may regulate PMVEC barrier function through metalloproteinase-dependent regulation of PMVEC-ECM interaction and focal adhesion formation. Moreover, PMVEC barrier dysfunction under inflammatory conditions is associated with disrupted cell-matrix

interactions and the loss of TIMP3 may be a contributing factor in this process. Overall, this study enhances our understanding of PMVEC barrier stability by providing an insight of the physiological processes that regulate endothelial barrier function, and how these may be affected under pathological conditions.

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Curriculum Vitae

Name:	Nidhi Kulkarni
Post-secondary Education and Degrees:	University of Guelph Guelph, Ontario, Canada Bachelor of Science Honors Major in Biomedical Sciences, Minor in English 09/2014-04/2018
	University of Western Ontario London, Ontario, Canada Master of Science Physiology and Pharmacology 08/2018-Current
Honors and Awards:	Canadian Institutes of Health Research (CIHR), Travel Award 01/2020-01/2021
	Lawson's Internal Research Fund (IRF), Studentship Award 07/2019-06/2020

Related Work Experience

Graduate Teaching Assistant- Pharm 4320
University of Western Ontario
09/2020-Current

Graduate Teaching Assistant- Pharm 3620
University of Western Ontario
09/2019-04/2019

Graduate Teaching Assistant- Pharm 3620
University of Western Ontario
09/2018-04/2020

Publications:

Kulkarni N, Mehta S, Wang L, Pape C, Gill S. (2020). The Role of TIMP3 in Microvascular Endothelial Cell-Extracellular Matrix Interaction and Regulation of Microvascular Barrier Function. Federation of American Societies for Experimental Biology. 34: 1-1.

Jayawardena D, Kulkarni N, Gill S. (2018). The role of tissue inhibitors of metalloproteinases in microvascular endothelial cell barrier dysfunction during sepsis. Metalloproteinases in Medicine. 6: 1-12.