Identifying CD36 as a Receptor for Albumin Transcytosis by Dermal Microvascular Endothelial Cells

By

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ABSTRACT

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Albumin is the most abundant serum protein. Under basal physiological conditions the movement of albumin in and out of the vasculature occurs by transcytosis. Little is known regarding the mechanism of albumin transcytosis and its variability across tissue beds. Using a novel TIRF microscopy based assay, in this present study I have shown that albumin transcytosis is saturable in dermal (HDMECs) but not in lung microvascular endothelial cells (HPMECs). I have identified the scavenger receptor CD36 as necessary for albumin transcytosis across HDMECs, in contrast to HPMECs where macropinocytosis dominates. Mutations in the apical bundle of CD36 prevented albumin internalization. A modified Miles assay in mice with an endothelial CD36 deficiency exhibited lower basal permeability to albumin. These mice also developed a smaller subcutaneous fat layer despite comparable body weights and circulating FA levels as wild-type animals. These findings shed light on the mechanism and functional importance of albumin transcytosis.

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I have always been a firm a believer in the famous proverb "*Do what you love, and you'll never work another day in your life"*, so I have always pursued endeavors which genuinely interest me. Thus my decision to enter the world of research and obtain a Master's degree was both a rational and emotional decision. I began this journey with passion and love for scientific discovery, and that passion still remains, the love has matured more into a great respect for the complex world of research. The challenges I have faced over the two and half years have helped me grow both as a scientist and a human being. There are many people who have helped me overcome the challenges I faced and have been integral in my academic and personal development. Some of these individuals were with me before I began my Master's, most I have met as I progressed through this journey, and hope to carry these relationships into the future.

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both were a natural fit for the lab. I know you will continue the collegial and enthusiastic motto of the Lee Lab.

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LIST OF ABBREVIATIONS

AF	Alexa Fluor
AFM	Afamin
AFP	α-Fetoprotein
AJ	Adherens Junction
Alb-Au	Gold-Tagged Albumin
ALK1	Activin receptor-Like Kinase 1
AOPP	Advanced Oxidation Protein Products
AP2	Adaptor Protein 2
APC	Antigen Presenting Cell
ARDS	Acute Respiratory Distress Syndrome
Aβ	β-amyloid
BBB	Blood Brain Barrier
BSA	Bovine Serum Albumin
Cav-1	Caveolin-1
CD36	Cluster of Differentiation 36
CHO Cells	Chinese Hamster Ovary Cells
COP	Colloid Osmotic or Oncotic Pressure
CSF	Cerebrospinal Fluid
Cx	Connexions
DBP	Vitamin D Binding Protein
EC-CD36KO	Endothelial Specific CD36 Knockout
ECs	Endothelial Cells

ELISA	Enzyme-Linked Immunosorbent Assay
FA	Fatty Acid
FABP	Fatty Acid Binding Protein
FAT	Fatty Acid Translocase
FcRn	Neonatal Fc Receptor
FITC	Fluorescein Isothiocyanate
GAG	Glycosaminoglycans
GFP	Green Fluorescent Protein
GI Tract	Gastrointestinal Tract
GJ	Gap Junction
Gp18	Glycoprotein 18
Gp30	Glycoprotein 30
Gp60	Glycoprotein 60
GTP	Guanosine Triphosphate
HDL	High Density Lipoprotein
HDMEC	Human Dermal Microvascular Endothelial Cells
HPMEC	Human Pulmonary Microvascular Endothelial Cells
HSA	Human Serum Albumin
IEJ	Inter-Endothelial Junction
Ig	Immunoglobulin
IgA	Immunoglobulin A
IGF	Insulin-like Growth Factor
IGF-R	Insulin-like Growth Factor Receptor
IR	Insulin Receptor

JAM	Junctional Adhesion Moleculues
JNK	c-Jun N-terminal Kinase
K_{fc}	Coefficient of Vascular Permeability
LCFA	Long Chain Fatty Acid
LDL	Low-Density Lipoprotein
LDLR	Low-Density Lipoprotein Receptor
LIMPII	Lysosomal Integral Membrane Protein II
LRP2	Low-Density Lipoprotein-Related Protein 2
MAPK	Mitogen Activated Protein Kinase
MTX	Methotrexate
NEM	N-ethylmaleimide
NHS	N-Hydroxysuccinimdyl
NSAID	Non-Steroidal Anti-Inflammatory Drugs
NSF	NEM-Sensitive Factor
ox-LDL	Oxidized Low-Density Lipoprotein
PAF	Platelet Activating Factor
P_c	Capillary Hydrostatic Pressure
P*f*EMP-1	*P. Falciparum* Erythrocyte Membrane Protein 1
pI	Isoelectric Points
P_{IF}	Interstitial-Fluid Hydrostatic Pressure
PM	Plasma Membrane
PPAR	Peroxisome Proliferator Activated Receptor
S1P	Sphingosine-1-Phosphate
SA	Serum Albumin

SPARC	Secreted Protein, Acidic and Rich in Cysteine
SR-B1	Scavenger Receptor class B type 1
SSO	Sulfo-N-Succinimidyl-Oleate
TEER	Trans-Endothelial Electrical Resistance
TGFβ-type 1	Transforming Growth Factor Beta 1
TIRF	Total Internal Reflection Fluorescence
TJ	Tight Junctions
TNF-α	Tumor Necrosis Factor Alpha
TSP-1	Thrombospondin 1
VE-Cadherin	Vascular Endothelial Cadherin
VEGF	Vascular Endothelial Growth Factor
ZO-1	Zona Occulden 1
π_{if}	Interstitial-Fluid Oncotic Pressure
π_c	Capillary Oncotic Pressure

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CHAPTER 1: LITERATURE REVIEW

1.1 ENDOTHELIAL CELLS

The circulatory system, comprised of the cardiovascular and lymphatic system, serves as the primary transportation route for molecules in nearly all animals (K. L. Moore et al., 2014). The cardiovascular system is organized into a network of hundreds of millions of blood vessels, which supply organs with fresh oxygen and nutrients, and remove metabolites and waste products, using the blood as the vehicle (K. L. Moore et al., 2014). The lymphatic system, a network of filtration vessels, on the other hand is responsible for collecting excess fluids from the interstitial space, the space located between organ tissues and blood vessels (K. L. Moore et al., 2014). The fundamental component common to both blood and lymphatic vessels is the presence of an endothelium, a type of cell that lines the lumen or interior surface of every vessel. The endothelium is comprised of a monolayer of squamous cells, known as endothelial cells (ECs) that create a barrier, and play a key role in regulating exchange between vessels and the surrounding compartments (K. L. Moore et al., 2014).

1.1.1 THE VASCULATURE

The cardiovascular system can be generally divided into macrocirculation and microcirculation, dictated by the size and structure of blood vessels. Blood vessels can be categorized into three main types, arteries, veins, and capillaries. Arteries and veins form the macrovasculature, whereas arterioles, venules, and capillaries are a part of the microvasculature (K. L. Moore et al., 2014). These vessels essentially form a circuit responsible for conducting blood, the heart being the pump which propels the blood throughout the body. Arteries are responsible for carrying blood away from the heart, they become gradually smaller in diameter with increasing distance from the heart, eventually diverging into vast networks of capillaries. The high pressure of blood faced by a few large arteries close to the heart, is quickly distributed through the divergence into hundreds of small capillaries, which supply organs with nutrients through circulating blood (K. L. Moore et al., 2014). After supplying organs, blood exits

capillary beds, collecting in the post-capillary venules, which subsequently converge into veins which return deoxygenated blood back to the heart. The structure of these vessels varies greatly, dictated by the physiological function they serve (K. L. Moore et al., 2014).

Arteries and veins share a similar wall structure, which can be divided into three layers: the tunica externa, also known as the tunica adventitia, tunica media and tunica intima **(see Figure 1)**. The tunica externa forms the outer most layer of these vessels, it is composed of collagen and connective tissue that functions to stabilize the vessel (K. L. Moore et al., 2014). Blood flows from the left-ventricle of the heart with immense force directly into arteries, thus these vessels are thick-walled allowing them to withstand the immense pressure of the blood. Since arteries face higher blood pressure than veins, they contain an additional layer of support between the adventitia and tunica media known as the external elastic lamina, giving these vessels extra elasticity. The middle layer, the tunica media, is composed of elastic tissue and smooth muscles cells which are responsible for the control of the vascular tone of blood vessels. Once again because of the high blood pressure experienced by arteries, they contain a much thicker layer of smooth muscle cells, when compared to veins. The tunica intima is the innermost layer of blood vessels, and is simply composed of a single layer of endothelial cells, that are directly in contact with flowing blood. The endothelial cell lining of the tunica intima serves several key functions, such as regulating vascular smooth muscle tone, host-defence reactions, development of new blood vessels (angiogenesis), and tissue fluid hemostasis (Mehta & Malik, 2006).

Veins differ from arteries in that they must move blood against gravity back to the heart, thus they contain special valves attached to the intima the prevent the reverse flow of blood. As you move further from the heart, both arteries and veins become smaller in diameter, with the tunica externa and media being significantly thinner, and the elastic tissue being absent, these vessels are referred to as arterioles and venules, respectively. Eventually, at the site of organs, arterioles diverge into numerous capillaries, forming a bed or network that supplies the organ tissues with nutrients and oxygen, while also collecting the wastes produced. Capillaries, lack the tunica externa and media completely, and are composed only of a single layer of endothelial cells, supported by an extracellular matrix referred to as the basal lamina. The simple structure of these vessels is what makes them primary site of metabolic exchange between all organs and our

blood. Without the exchange capacity and the unique structure of capillaries, organs would quickly fail.

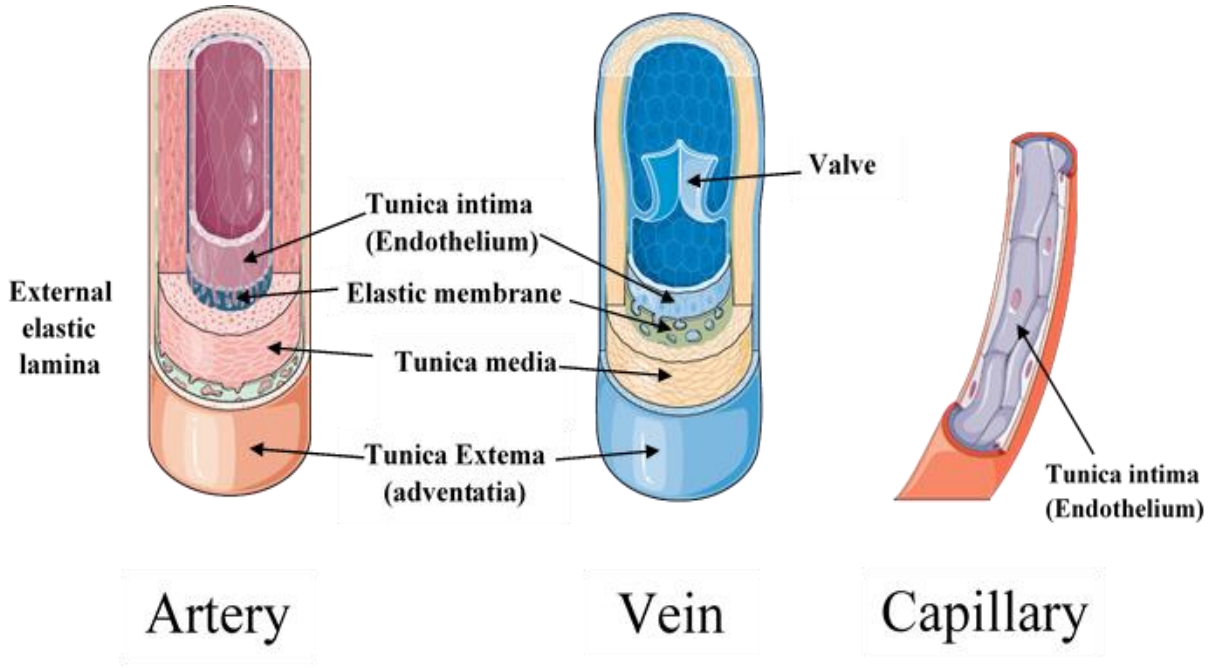


Figure 1. Structure of Arteries, Veins and Capillaries

Arteries, veins, and capillaries greatly vary in structure. Arteries and veins are large vessels containing many different layers of cells. A major component of arteries are the smooth muscle cells found in the tunica media that contribute to the elasticity and vasomotor tone of the vessel. Veins are thinner structures, containing fewer smooth muscle cells. Capillaries are the smallest component of the vasculature and contain only a layer of endothelial cells and their basement membrane. Note: Vessels are not drawn to scale. Figure was created using templates from Servier Medical Art.

1.1.2 MICROVASCULAR CIRCULATION

The capillaries of the microcirculation can be further categorized based on structure into three types: continuous, fenestrated, and discontinuous. Continuous capillaries can be found in most organs, including lungs, skin, heart, muscle and adipose tissue. As the name suggests continuous capillaries are composed of a continuous layer of endothelial cells that are perfectly aligned, forming a tight barrier. The tight monolayer of continuous capillaries allows only the passage of very small molecules such as water, hexose sugars, amino acids and ions, between the intercellular junctions. Fenestrated capillaries are composed of endothelial cells that have numerous fenestrations or pores within their membranes, creating channels that allow small molecules and some proteins to diffuse through. These capillaries are typically found in the intestines, endocrine glands, kidney and pancreas. Discontinuous or sinusoidal capillaries are composed of an endothelium in which the endothelial cells do not form a tight seal, instead have large gaps between cells that allow the free diffusion of blood cells and proteins. These capillaries are typically found in the organs where constant exchange of materials takes place, such as the liver, endocrine organs, bone marrow, spleen and lymphoid tissues (Aird, 2007b). Of the three types of capillaries, the continuous form is most abundant. The capillary density present in tissues varies according to metabolic activity, such that tissues with high metabolic activity like the skeletal muscles have a high capillary density, while those with low energy needs, such as cartilage, have fewer capillaries. The tight endothelial layer of these vessels form a thin, but tight barrier. In this thesis we focus on the role of the endothelium in regulating permeability, since it is the only structure segregating the circulating blood from the underlying organs.

1.1.3 THE STARLING FORCES

Despite the tight barrier, continuous capillaries constantly facilitate life sustaining nutrient and waste exchange. Due to the thin structure of capillaries, the exchange of water, oxygen, ions and other small molecules, between the circulation and tissues occurs primarily by diffusion (Sawdon & Kirkman, 2017). The interstitial fluid that bathes organs is generally poor in oxygen, ions and proteins, whereas the arterial blood supply in capillaries is rich these molecules. Thus, as per the law of diffusion, molecules move from an area of high concentration

to low concentration, pushing these small molecules from out of the circulation and into interstitial space. Since molecules in the vasculature are dissolved in the aqueous environment of the blood, majority of the molecular transport occurs through the movement of fluid, known as bulk flow. The regulation of bulk flow between the vasculature and interstitium is essential for organ health. The movement of fluid across capillary walls is dynamically controlled by four pressure based forces, collectively known as the Starling forces **(see Figure 1.2)**. The Starling forces can be grouped into two categories, hydraulic or hydrostatic pressures, and colloid osmotic or oncotic pressures (COP). The relationship of these forces has been summarized by the Starling equation: $J_v = (L_pS)[(P_c - P_{IF}) - \sigma(\pi_c \ \pi_{IF})]$. J_v represents the volume flux of fluid (ml/min), L_p is hydraulic conductivity ($cm \cdot min^{-1} \cdot mmHg^{-1}$), S is capillary surface area (cm^2), and σ symbolizes the osmotic reflection coefficient of a vessel wall (Mehta & Malik, 2006). Hydraulic pressure refers to the force of fluid, and in the case of the Starling forces can be divided into two opposing forces: capillary hydrostatic pressure (P_c) and interstitial-fluid hydrostatic pressure (P_{IF}). Capillary hydrostatic pressure forces fluid out of capillary blood, and into the interstitial space, conversely P_{IF} forces interstitial fluid into capillaries. Osmotic pressure on the other hand is the force caused by dissolved solutes. Unlike water and other small molecules, large molecules, such as proteins, are more greatly restricted in their movements across capillaries. Due to their large size and vast abundance, proteins dissolved in blood plasma and interstitial fluid generate their own opposing forces, designated as capillary oncotic pressure (π_c), and interstitial-fluid oncotic pressure (π_{if}), respectively. Proteins more abundant in the plasma, such as albumin (**see section 3**), exert π_c which draws water from the interstitial space into capillaries, opposing this force are proteins present in the interstitium, which pull blood fluid from capillaries into interstitial space. The protein that largely dictates osmotic pressure across capillaries is albumin, since it is the most abundant protein in the blood.

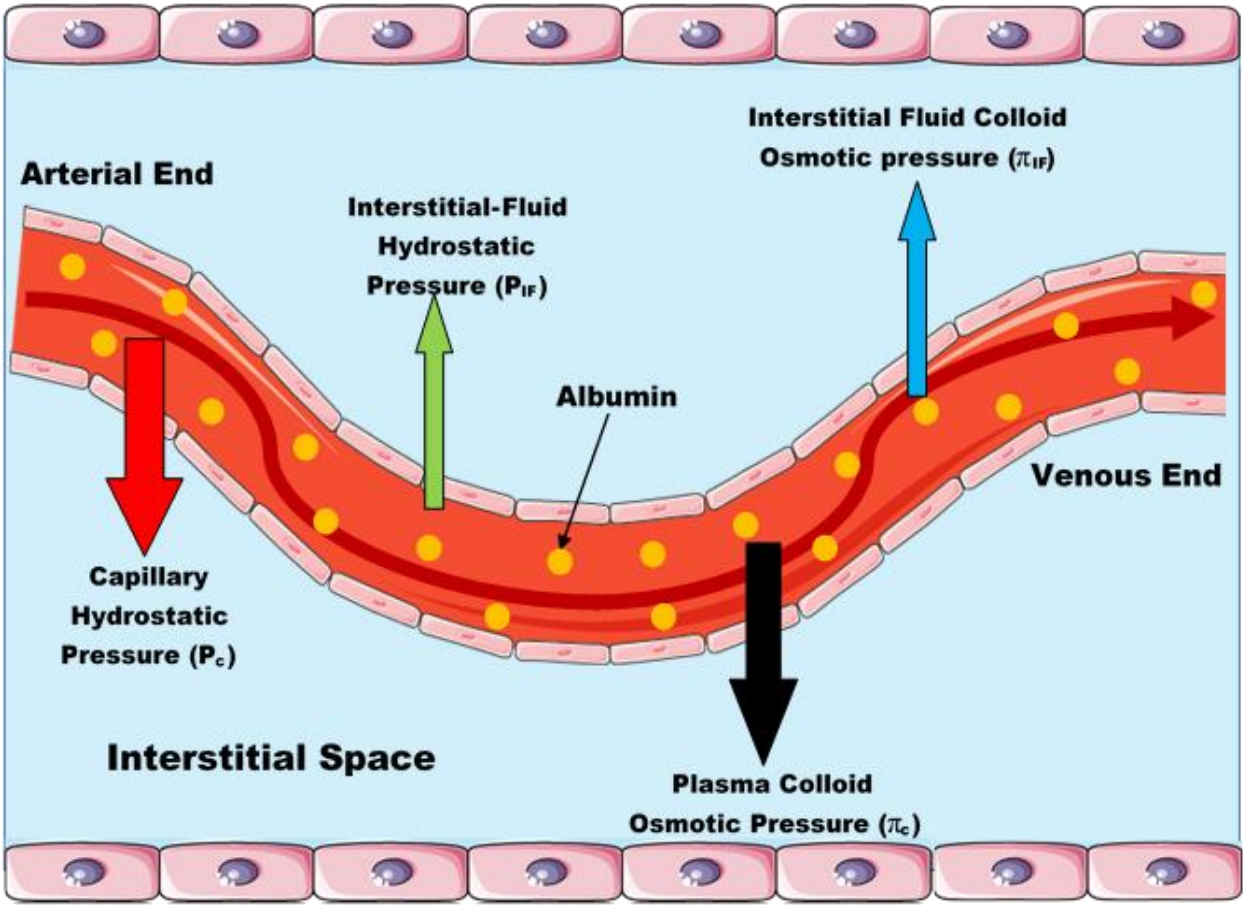


Figure 1.2 The Starling Forces

Blood enters through the arterial end of capillaries, where, P_c is greater than π_c, forcing net fluid filtration out of the vessel and into the interstitial space. As blood reaches the venous end however, P_c decreases, while π_c remains fairly constant, such that there is a net fluid flirtation into of the vessel and out of the interstitial space. Both P_{IF} and π_{IF} remain generally stable under basal physiological states, thus do not impact fluid filtration rates greatly at either ends of the vessels. Figure was created using templates from Servier Medical Art

1.1.4 ENDOTHELIAL HETEROGENEITY ACROSS TISSUE BEDS

The endothelium is essential in regulation of the Starling forces. Without this barrier the concentration gradient responsible for maintaining the four pressure based forces would quickly dissipate. It serves a selective barrier between the inner content of blood vessels and tissues, regulating the transport of molecules. Endothelial cells, ranging in length from 70 μm to 200 μm, depending on their location in the vascular branches (Adamson, 1993), are selective for both the size and charge of molecules that pass through them. In a healthy monolayer, endothelial cells are linked together by multiple cell surface proteins that create inter-endothelial junctions (Yuan & Rigor, 2011). These junctions essentially create a semi-permeable barrier, which is permeable to small solutes, including ions, and impermeable to large macromolecules, greater than 3 nm in molecular radius (Michel & Curry, 1999). The charge selectivity of the endothelial barrier is determined by the negative electric charge on the luminal surface of vessels. The negative charge across the endothelium is maintained by the presence of a glycocalyx on the luminal surface of microvascular endothelial cells. The glycocalyx is composed of a network of glycoproteins and polysaccharides that help generate the negative charge. The negative charge on endothelial cells functions to repel blood cells, and attracts or repels plasma components based on charge (Yuan & Rigor, 2011).

The selectivity of endothelial cells for molecules varies from organ to organ, depending on the physiological needs of the tissue. In fact, the overall phenotype of the endothelium exhibits significant heterogeneity across tissue beds. Endothelial cells vary in both structure and function in order to adapt to the differing needs of organs and the location in the vasculature (Aird, 2007b, 2012). The structure of endothelial varies depending on the type of blood vessels. Microvascular endothelial cells generally are very flat, with a thickness of less than 0.3 μm, whereas those from the macrovasculature can be 1 μm thick, and cuboidal in shape (Aird, 2007b; Florey, 1966; Wallez & Huber, 2008). Furthermore, studies on endothelial cells from different tissue sources have reported tissue specific differences. In a study on rat blood vessels, aortic endothelial cells were reported to be elongated and narrow, while pulmonary artery endothelial cells were wider and shorter, and rectangular in shape. The same study reported pulmonary vein endothelial cells were large and round, whereas those from the inferior vena cava were long, narrow, forming a rectangular shape (Kibria et al., 1980). There is increasing evidence that site-

specific difference in endothelial cell structure is not fixed. Instead it is dynamically regulated throughout life, due to forces such as blood flow, shear stress, and hormones, such as vascular endothelial growth factor (VEGF) (Aird, 2007a).

Since structure and function are interrelated, the function of endothelial cells is also very heterogeneous. As the primary function of microvascular endothelial cells is to act as a barrier and to regulate molecular exchange between blood and tissue, endothelial cells vary greatly to the extent to which they internalize (endocytose) large molecules. Endothelial cells possess clathrin-coated pits, clathrin-coated vesicles, caveolae, multivesicular bodies, and lysosomes, which are structural components of the endocytic pathway. Endothelial cells from certain tissue beds have a high propensity for endocytosis; for example liver sinusoidal endothelial cells have high rates of clathrin-mediated endocytosis (Aird, 2007a). On the other hand endothelial cells in microvessels supplying tissues with high metabolic needs, such as the heart, lung, and skeletal muscle have a higher density of specialized endocytic vesicles known as caveolae, rather than clathrin-coated pits (Bendayan, 2002). Blood vessels supplying the brain however have been reported to contain very few caveolae (M. Simionescu, Gafencu, & Antohe, 2002). The endothelium also aids in maintaining blood in a fluid state, and preventing clot formation when damage to blood vessel walls occur. Endothelial cells produce and release anti and pro-coagulant molecules into the blood, however it is well established that endothelial cells from different vascular beds express distinct repertoires of coagulation factors. Due to this differential expression of hemostatic factors, certain organs are more susceptible to blood clot formation in states of vascular dysregulation (Aird, 2007a).

In addition to shape and size, endothelial cells also vary in the number of inter-endothelial junctions (IEJs) they possess, effecting the permeability of the endothelium. The relative presence of different types (**see section 1.1.4**) of junctions in inter-endothelial clefts varies along the vascular tree. The endothelium of large arteries displays a well-developed system of tight junctions, as would be expected since these vessels experience significantly higher rates of blood flow (Aird, 2007a). In the microvasculature however, tight junctions are relatively lower in numbers and/or strength, when compared to large arteries and veins. A study that measured the coefficient of vascular permeability (K_{fc}) across the endothelium under basal conditions in isolated perfused rat lungs, showed a gradient in permeability across the

microvascular bed. The gradient was distributed such that the capillary region contributed to 42% of the permeability, whereas the arterial site added only 19% of the total permeability, and the venous area constituting 37% (Parker et al., 2006). The findings of this study, along with many others, support the notion that the arterial system is more restrictive than both the venous and capillary vessel linings (Sukriti et al., 2014). The microvasculature of the brain is an exception to the weak tight junctions' phenotype. In fact, the cerebral microvasculature is particular rich in inter-cellular junctions, forming a very tight barrier, known as the blood brain barrier, which functions to protect the brain from fluctuations in the blood composition (Aird, 2007a).

1.1.5 ENDOTHELIAL PERMEABILITY

As stated above, the microvascular endothelium serves as a semi-permeable barrier between the blood stream and tissues. The integrity of this barrier is chiefly regulated by various cell-surface proteins, referred to as junctional proteins, which serve as connections between adjacent endothelial cells. Endothelial cells can possess three types of intercellular junctions: Tight Junctions (TJ), Adherens Junctions (AJ), and gap junctions (GJs), also known as connexions (Cx) (Sukriti et al., 2014). The first two types of junctions, TJ and AJ, facilitate cell-to-cell adhesion through the linking of transmembrane proteins in adjacent cells, thereby regulating permeability of the endothelial barrier. A greater number of junctions results in a less permeable endothelial barriers, and less junctions generally means more permeable and leaky blood vessels (Aird, 2007a). Gap junctions on the other hand, facilitate intercellular communication, thus are less important for endothelial permeability (Bazzoni, 2004).

Tight junctions, also termed as zona occludens, constitute about 20% of the total number of junctional proteins (Dejana, 2004; Giannotta, Trani, & Dejana, 2013; Vogel & Malik, 2012). They create a barrier that prevents the transport of large solutes, proteins and cells between adjacent endothelial cells, a process known as paracellular transport (Vogel & Malik, 2012). This type of junction also helps to maintain endothelial cell polarity, such that the surface of the cell facing the lumen of the blood vessels is distinct from the end facing the interstitial space (Aird, 2007a). Tight junctions are primarily formed through the interaction of claudins, occludin, and

junctional adhesion molecules (JAMs). These membrane-spanning junctional proteins are anchored to the actin cytoskeleton of cells through cytoplasmic interactions with zona-occludens (ZO-1), a peripheral membrane protein (Vandenbroucke et al., 2008; Vogel & Malik, 2012). Each junctional protein links to its equivalent in adjacent cells to create extra-cellular interactions that form the TJs, with claudins linking to a claudins, occludin to occludin, and JAMs to JAMs. To date, several isoforms of each TJ proteins have been identified, with claudin-5 being the predominant endothelial claudin isoform, and JAM-B the endothelial-specific JAM (Aurrand-Lions et al., 2001; Morita, Sasaki, Furuse, & Tsukita, 1999). The presence of tight junctions was shown to be essential for survival in a study on mice deficient in claudin-5, which found that these null mice died 10 hours after birth due to increased permeability to small molecules in the blood brain barrier (Nitta et al., 2003). However, not all claudins prevent vascular leakage, some have been shown to augment paracellular permeability. Of the 27 different members of the claudin family, claudins 2, 10, 15, and 17, have been shown to form channels or pores, that allow for the paracellular diffusion of small charged molecules across cellular barriers (Gunzel & Yu, 2013). Claudin-1, the most ubiquitously expressed of all claudins is known to allow for paracellular water transport (Furuse et al., 2002). Mice with a claudin-1 knockout were shown to die due to dehydration during the neonatal stage, and a downregulation of claudin-1 is associated with dry skin in patients with skin diseases such as dermatitis and psoriasis (De Benedetto et al., 2011; Watson et al., 2007). Moreover, recent studies on Caludin-3 function in alveolar epithelial cells have demonstrated that its expression is greatly upregulated under conditions of a weakened cell barrier (Chen et al., 2005; Mitchell, Overgaard, Ward, Margulies, & Koval, 2011). The overexpression of Claudin-3 was found to decrease trans epithelial resistance, and increase the flux of paracellular markers, calcine and dextran (Mitchell et al., 2011). Adherens Junctions (AJ), are formed through the calcium-dependent, interaction of vascular endothelial (VE) cadherin proteins in adjoining endothelial cells (Komarova & Malik, 2010; Mehta & Malik, 2006). The cytoplasmic domains of VE-cadherin interact with a group of catenin proteins, β, γ, and p-120, which in turn interact with α-catenin, which links the entire VE-cadherin complex to the actin-cytoskeleton of the endothelial cell (Dejana, Tournier-Lasserve, & Weinstein, 2009; Giannotta et al., 2013). The presence of VE-cadherin is crucial for the normal development of the vasculature, as a loss of VE-cadherin is known to be embryonically lethal (Carmeliet et al., 1999; D. Mehta & Malik, 2006). Moreover, the depletion of extracellular

calcium leads to endothelial barrier leak, and endothelial cell detachment, by breaking the attachment between VE-cadherin molecules from contiguous endothelial cells (Leckband & Sivasankar, 2000; Sukriti et al., 2014). Just as the extra-cellular interaction of VE-cadherin domains is important, various studies have shown that cytoplasmic anchoring of junctional proteins is essential for maintaining endothelial barrier integrity. Studies in which β and p-120-catenin have been inactivated, mutated, and/or depleted, show endothelial barrier disruption, with increased permeability (Broman et al., 2006; Tian et al., 2013). AJs are the most ubiquitous type of endothelial cell junction, and are significantly more abundant then TJs in the microvasculature. Due to their relative abundance, AJs contribute most greatly to the size exclusion of the endothelial barrier to macromolecules, restricting their access to organs and tissues.

Cellular junctions are dynamic structures, with distinct steps of maturation during the formation of an endothelial monolayer. Research in epithelial cells indicates that membrane proteins of AJs and TJs initially form adhesion complexes at sites of cell-to-cell contact on the plasma membrane (PM). Following the intitial step, they organize into zipper-like structures through adhesion along the lateral cell border (Chitaev & Troyanovsky, 1998; Yap et al., 1997). A process similar to epithelial cell junction formation is likely to be the case for endothelial junction development. Even after reaching a stable formation, junctions continuously undergo remodeling, with adhesion proteins being constantly recycled between the PM and intercellular compartments. Under basal physiological states, TJs and AJs are intact, with strong extra-cellular and cytoplasmic interactions of junctional proteins. They act as a barrier to macromolecules with a radii greater than 3 nm, while allowing small hydrophilic molecules through paracellular transport. However, many macromolecules essential to cell survival, such as hormones, fatty acids (FA), cholesterols and most proteins are too large (greater than 3 nm in size), and/or too hydrophobic, to leak through IEJs. Relatively recent studies suggest that under steady-state physiological conditions, endothelial vesicles mediate the transport of larger proteins and small hydrophobic solutes, through a process known as transcellular transport or transcytosis (M. Simionescu, Popov, & Sima, 2009; Tuma & Hubbard, 2003). Counter to paracellular transport, transcellular transport involves molecules being actively taken-up by endothelial cells at the luminal membrane by cellular vesicles. The molecules are trafficked through the cytoplasm, and released into the interstitial space by the fusion of the transport vesicle to the basal membrane.

In addition to IEJs, the limited permeability of the endothelium to macromolecules is also contributed by the glycocalyx. As stated earlier the glycocalyx is a negatively charged network of proteoglycans, glycosaminoglycan (GAG), and absorbed plasma proteins, which lines the luminal surface of endothelial cells. This surface matrix physically repels both red blood cells, and leukocytes, as well as negatively charged proteins. It has been reported that albumin, with a molecular mass of 67 kDa, and fibrinogen, with a molecular mass of 340 kDa, permeate the glycocalyx at the same rate, despite fibrinogen being significantly greater in size (Vink & Duling, 2000). This difference is attributed to the isoelectric points (pI) of the proteins, with albumin having a pI value of 4.9, making it more acidic and negatively charged under physiological conditions, and fibrinogen having a pI of 6.1, making it more basic and positively charged.

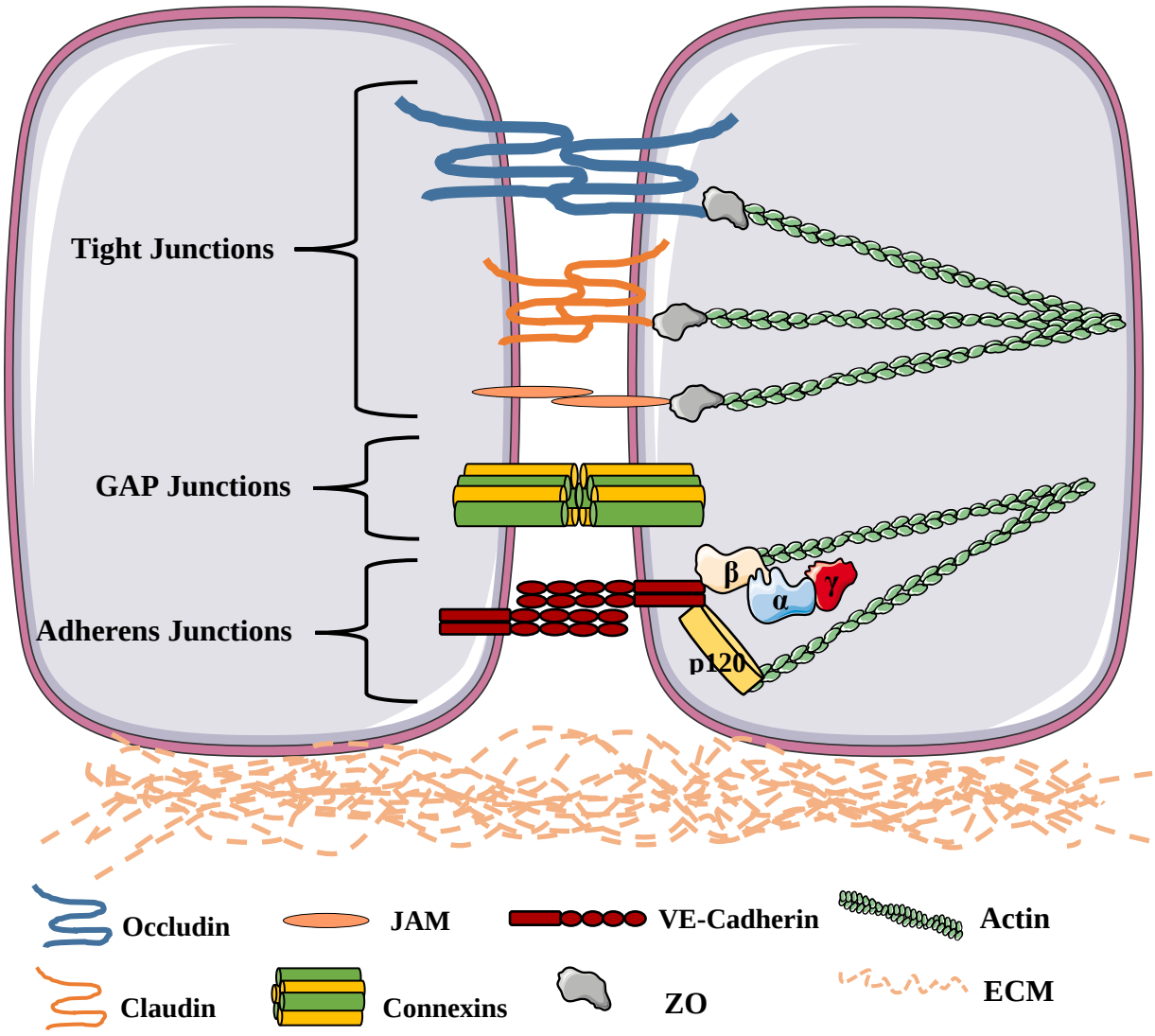


Figure 1.3 Molecular Composition and Organization of Endothelial Cell Junctions. *Interendothelial junctions are comprised of tight junctions, gap junctions, and adherenes junctions. The molecular components of these junctions interact with complimentary proteins in adjacent cells and the actin cytoskeleton in order to regulate endothelial barrier permeability.* ***See section 1.1.5*** *for a detailed description of the structure and function of each of the three forms of junctions. Figure was created using templates from Servier Medical Art.*

1.1.6 ENDOTHELIAL DYSFUNCTION AND ALBUMIN LEAKAGE

The stability of the IEJs can be regulated by external stimuli acting on endothelial cells, in response to the needs of underlying tissue and overall physiological state of the body (Komarova, Mehta, & Malik, 2007; Mehta & Malik, 2006). Inflammatory mediators such as thrombin, bradykinin, histamine, VEGF, platelet activating factor (PAF), and tumor necrosis factor alpha (TNF-α), can bind to their respective receptors on endothelial cells and trigger the disruption of endothelial junctions (Mehta & Malik, 2006). The gap formation in the endothelium allows for the unrestricted passage of cells, plasma proteins, including albumin, and large amounts of fluid. As previously stated AJs are primarily responsible for regulating endothelial permeability, as such, their disruption is the likely mechanism of enhanced endothelial permeability. The mechanism is believed to occur through the phosphorylation of AJ complex proteins, which trigger the internalization of VE-cadherin, thereby reducing the extent of cell-to-cell contact in the endothelium (Komarova & Malik, 2010). The weakened cell-to-cell contact, is accompanied by the activation of the contractile actin-myosin machinery in endothelial cells. This leads to the reorganization of the actin cytoskeleton; the mechanical forces of the cytoskeleton retract adjacent cells to widen inter-endothelial gaps (Mehta & Malik, 2006). The above changes are a hallmark of endothelial dysfunction and are identified by a shift in the endothelium towards vasodilation, pro-inflammatory and prothrombic states. Endothelial dysfunction is associated with nearly all inflammation associated disease states, including most forms of cardiovascular diseases, such as hypertension, coronary artery disease (atherosclerosis), chronic heart failure, peripheral vascular disease, diabetes, chronic kidney failure, and severe bacterial and viral infections (Davignon & Ganz, 2004; Deanfield et al., 2007; Rajendran et al., 2013). During these states of inflammation the blood vessels becomes inevitably leaky, primarily to allow the transmigration of immune cells from the blood into the affected tissue, allowing the initiation of an immune response (Dejana, Orsenigo, & Lampugnani, 2008; Vestweber, 2008; Vestweber, Winderlich, Cagna, & Nottebaum, 2009). In addition to allowing for cellular migration, these inflamed and leaky vessels become permeable to plasma proteins and liquids, resulting in a condition known as protein-rich edema. Albumin being the most abundant protein in the blood, is the primary indicator of a disruption in paracellular endothelial permeability, with its increased interstitial presence during states of inflammation.

1.2 TRANSCYTOSIS

1.2.1 PARACELLULAR VS. TRANSCELLULAR TRANSPORT

The relative importance of paracellular and transcellular transport in regulating endothelial permeability has long been debated and actively studied amongst vascular biologists. Paracellular transport has been the more widely studied process, however this mode of transfer occurs primarily under states of increased inflammation, leading to the disruption of IEJs. Under basal physiological conditions endothelial transport of most macromolecules is reported to occur through the cytoplasm of endothelial cells by transcytosis (Predescu, Predescu, & Malik, 2007; Tuma & Hubbard, 2003). It has been reported that a significant amount of water, up to 40% of total hydraulic content, crosses the endothelial barrier through transcellular means, specifically through the formation of water-transporting membrane channels called aquaporins (Michel & Curry, 1999). Liquids, water, and small molecules, like glucose, fructose, urea, amino acids, and ions, cross the endothelial layer primarily through paracellular routes, but can also be transcytosed (Mehta & Malik, 2006). Under steady-state physiological conditions many essential metabolic micronutrients, macromolecules, immune components, hormones, and proteins regulating oncotic pressure, chiefly albumin, traverse the endothelial barrier by transcytosis (Tuma & Hubbard, 2003). Transcytosis, in the simplest definition is the transport of molecules within membrane-bound vesicles from one end of the cell to the opposing end. The term transcytosis was first coined by N. Simionescu, in 1979; he used it to describe the vectoral transfer of macromolecular cargo across capillary endothelial cells to the interstitium of tissues (Simonescu, 1979). During this period, another form of transcytosis was also being discovered by immunologists comparing the different types of immunoglobulins (Ig) found in various bodily secretions, such as serum, milk, saliva, and in the intestinal lumen (Tomasi et al., 1965). They speculated that the externally secreted form immunoglobulin A (IgA), was transported across the epithelial cell barrier through transcellular transport (Tomasi & Zigelbaum, 1963). The transcytotic systems of both endothelial and epithelial cells were the first to be identified and studied, and are still actively investigated today. It is now known that transcytosis occurs much more ubiquitously, it is a transport process used by a variety of cell types, including osteoclasts (Nesbitt, 1997; Salo, 1997) and neurons (Hémar et al., 1997).

The existence of a transcytosis like process however, was first postulated a decade prior to the Ig studies, by Nobel Prize winning cell biologist George Palade, in the 1950s, during his studies on capillary permeability (Palade, 1953). In a short abstract Palade described the presence of many small vesicles, many of which were continuous with the plasma membrane (Palade, 1953). Shortly after, in one of his early electron microscopy studies Palade coined the term plasmalemmal when referring to the vesicles (Palade, 1953). Two years prior to the findings of Palade, physiologists Pappenheimer and his colleagues, during a study on the permeability of micro-vessels, hypothesized the existence of large pores within cells. They used the existence of these large pores to explain the high permeability of the microvessels towards macromolecules typically too large to traverse the inter-endothelial junctions (Pappenheimer, Renkin, & Borrero, 1951). Palade hypothesized that his plasmalemmal vesicles were morphologically equivalent to the large pores described by Pappenheimer (Tuma & Hubbard, 2003). These plasmalemmal vesicles are now more commonly known as caveolae, a term coined by Yammada in 1955 (Yamada, 1955a, 1955b). There is growing evidence suggesting that paracellular and transcellular pathways of transport may in fact have in interdependent function in regulating tissue fluid homeostasis (Komarova & Malik, 2010). Studies have shown that the absence of caveolin-1, the primary protein constituting caveolae, greatly reduces transcytosis, but is also associated with increased vascular permeability (Miyawaki-Shimizu et al., 2006; Schubert et al., 2002), due to either increased paracellular leak or to changes in capillary pressure of the glycocalyx (Rosengren et al., 2006). A more recent study has shown that the link between increased paracellular leak when transcytosis is inhibited maybe due to a loss of cortical actin from the PM, and the displacement of active Rac from the plasmalemma (Armstrong et al., 2012). They also demonstrated the reverse relation, where inhibition of paracellular leakage by sphingosine-1-phosphate (S1P), an activator of Rac and inducer of cortical actin, caused a significant increase in the transcytosis of albumin (Armstrong et al., 2012). Though the fundamentals of the transcytosis process have been identified, much is yet to be elucidated regarding the mechanisms of molecule-specific transcytosis.

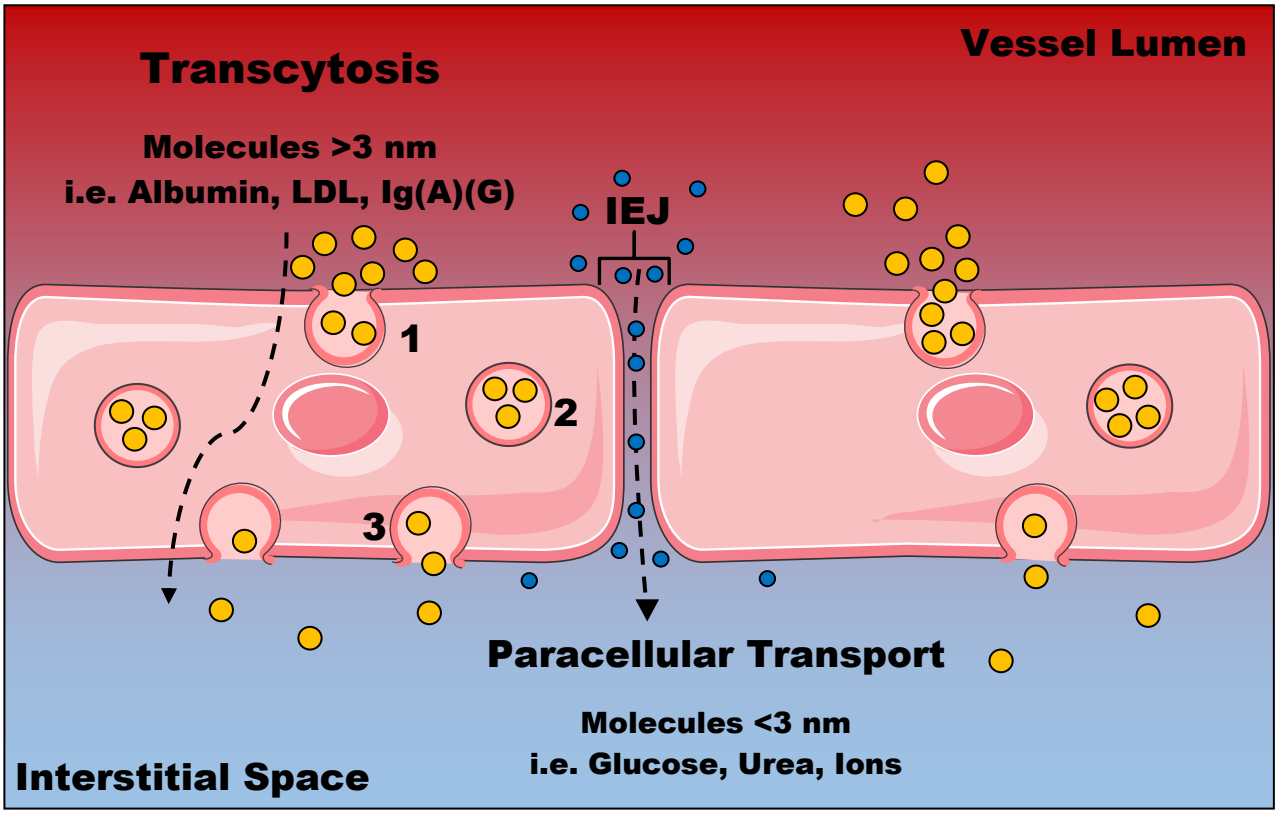


Figure 1.4 Paracellular and Transcellular Transport

Molecules cross the endothelial barrier through two main routes: ***paracellular transport:*** *passive diffusion of small molecules through the inter-endothelial junction (IEJ), or by* ***transcytosis:*** *active transport of large molecules through individual cells. Transcytosis is a three step process:* ***1) Internalization*** *into vesicles,* ***2) Vesicle Trafficking*** *through the cell,* ***3) Exocytosis*** *of vesicle cargo extracellularly. Figure was created using templates from Servier Medical Art*

1.2.2 TRANSCYTOSIS BY ENDOTHELIAL CELLS

Transcytosis is a transport process known to occur across many cell types, including osteoclasts and neurons, as well as epithelial and endothelial cells, the latter two being the most studied cell types for transcytosis. Prior to the discovery of caveolae mediated endocytosis, clathrin-coated vesicles were known as the main intracellular transport vesicles (Schmid, 1997). As the name suggestes, these vesicles are characterized by the presence of cytosolic, lattice-like, coat of proteins, the primary proteins being clathrin and AP2 (Adaptor Protein) which interact in a way to create hexagonal and pentagonal lattices around the vesicle. Receptor-mediated transcytosis was believed to be solely facilitated by clathrin-coated vesicles, allowing for selective transcellular transport of a receptors specific ligand (Frank, Woodman, Park, & Lisanti, 2003; Minshall, Tiruppathi, Vogel, & Malik, 2002). However, research starting in the 1990s revealed several clathrin-independent mechanisms of endocytosis, including caveolae based endocytosis. The receptors which initiate the latter mode of transcytosis are often localized within caveolar regions within the PM, located at the luminal surface of endothelial cells (Komarova & Malik, 2010). Caveolae comprise approximately 15% of the total endothelial cell volume, with about 10,000 to 30,000 caveolae/cell, ranging in diameter between 50 to 100 nm (70 nm on average) (Bruns & Palade, 1968; Johansson, 1979; Palade, 1960; Predescu, 2004; M. Simionescu, Simionescu, & Palade, 1974). The caveolae are now the most well characterized vehicles of transcytosis in endothelial cells, with clathrin-mediated transcytosis being more predominant in epithelial cells. Comprised of caveolin-1 (cav-1, a 22-kDa protein), caveolae are cholesterol-rich, flask-shaped pits, present on and continuous with the apical and basal PM of all endothelial cells (Tuma & Hubbard, 2003). Cav-1 assists in the assembly of caveolae into lipid microdomains rich in sphingolipids and cholesterol. During the initiating stage of transcytosis, cav-1 assembles into oligomers to mediate the characteristic flask-shaped invaginations of the membrane (Sargiacomo et al., 1995; Schlegel & Lisanti, 2000). The scission of the caveolar vesicle is then triggered by dynamin, a GTPase that oligomerizes at the neck of the invagination (Minshall et al., 2002; S. A. Predescu et al., 2001; Tuma & Hubbard, 2003). This step frees the caveolae to fuse with other vesicles within the cell, or to traffic to the basolateral membrane where it can deliver its contents to the interstitial space via exocytosis. Endothelial cells comparatively possess more caveolae than clathrin-coated pits. Thus the transport of albumin, the most abundant protein in blood plasma, through endothelial cells occurs exclusively in

caveolae (Ghitescu & Bendayan, 1992; John et al., 2003). Studies of caveolin-1 knockout mice (*Cav-1* $^{-/-}$), causing total loss of caveolae formation, showed inhibition of vesicular albumin transport. Nonetheless, albumin transcytosis has been shown to occur through both receptor mediated and fluid phase mechanisms, depending on the nature of the tissue endothelium (Frank et al., 2003; Tuma & Hubbard, 2003)

1.2.3 CHALLENGES IN STUDYING TRANSCYTOSIS

The lack of mechanistic knowledge regarding transcytosis is evident by the limited literature available on the topic. The scarcity in research can be attributed to both long-standing technical difficulties in studying transcytosis and persistent scrutiny regarding the physiological importance of this process (Rippe et al., 2002). The primary technical limitation of studying transcytosis *in vitro* arises from the phenotypic variability that occurs in primary cells in culture, and a gradual reduction in caveolae with each passage of the cell culture (Aird, 2007a; Lacorre et al., 2004). Since caveolae are required for the transcytosis of many molecules, this variability makes studying transcytosis difficult in cultured cells (Mehta & Malik, 2006). A second challenge in studying this process is the difficulty in distinguishing transcellular transport from paracellular leakage between cells in monolayers, which are susceptible to gap formation. Historically transwells (Boyden chambers) have been used to study endothelial permeability (Maruo et al., 1992), however in this technique the extent of apical-to-basal transcytosis can easily be overpowered by unintended intercellular gaps and paracellular diffusion from the top chamber to the bottom of the transwell. These paracellular gaps may result from improper seeding or unequal growth of cells, or from an experimental treatments such as transfections, gene silencing, or molecular inhibition (Armstrong et al., 2012). This leads to the next complication in the mechanistic studies using transwells, which require high transfection rates of the cellular monolayer in order to obtain reliable data. Obtaining a high transfection efficiency, whether of plasmids or siRNA, in most primary cell cultures, especially endothelial cells, is known to be technically challenging. Prior to the use of transwell systems, much of the early research on transcytosis as stated above was electron microscopy based, and provided the fundamental knowledge available today on this process. Despite the immense relevance and importance of electron microscopy in early transcytosis studies, such as those by Palade and

Simionescu, the data obtained from this method is primarily qualitative, making its application limited for elucidating precise regulatory mechanisms.

Recent advances in microscopy have allowed scientists to overcome the challenges in studying transcytosis. Through the use of total internal reflection fluorescence microscopy (TIRF), real-time transcytosis events can be imaged live in individualized cells (Armstrong et al., 2015). This method requires the use of fluorophore-tagged ligands, such as, DiI-LDL or Alexa-Fluor-conjugated albumin, which are internalized at the apical surface of a confluent monolayers of cells and their exocytosis at the basolateral membrane is visualized by a TIRF microscope. Significant work has been done to validate this technique for studying transcytosis accurately. The guiding principles for identifying and measuring exocytosis of vesicles using microscopy were established in chromaffin cells in a study published in a letter to Nature from 1997 (Steyer et al., 1997), this paper provided insight into vesicle size and the timing of exocytosis events. In order to distinguish actual exocytosis events distinctly from photobleaching and/or endocytic traffic in cells, the rate of loss of fluorescence over time of particles that have become stationary was measured, an event which indicates that the vesicle has docked at the terminal membrane. Using the values published in the 1997 study along with some modification there is now a MATLAB script developed that can allow for the analysis of TIRF microscopy data, including tracking and quantification of transcytosing particles, in an automated and a semi-blinded fashion. The TIRF-microscopy based assay circumvents the issues associated with both transwell and electron microscopy based studies. It minimizes concerns regarding poor transfection rates, as it allows for the imaging of individual cells in the monolayer and provides quantitative data. In addition to *in vitro* research techniques, *ex vivo* assays such as aortic perfusion and *in vivo* intravital microscopy can be used as complementary methods to study transcytosis, and for the validation of molecular insights obtained from cell culture studies. A combination of these approaches have been used to study the transcytosis of many molecules, including albumin, insulin, and low-density lipoprotein (LDL), these three being the ones for which there is currently the most knowledge regarding the endothelial transcytosis.

1.2.4 PHYSIOLOGICAL ROLE OF TRANSCYTOSIS

As stated earlier, under normal physiological conditions the endothelial monolayer is impermeable to molecules greater in radius then 3 nm, however many of these large molecules are essential for regulating important physiological functions and maintaining homeostasis. Thus in order to travel from the vascular lumen to the tissue interstitial space, and vice versa, many molecules have now been shown to rely on transcytosis to varying degrees. Cells are able to distinguish between the various cargos that are internalized by transcytotic vesicles, and decide the fate of the cargo, whether it is to be degraded or transcytosed (Tuma & Hubbard, 2003). They do so through the use of different receptors localized to different entry regions in the PM. Below, is a brief review of three of the more well-characterized molecules in endothelial transcytosis research.

1.2.4.1 *Low-Density Lipoprotein (LDL)*

LDL, low-density-lipoprotein is the major carrier of cholesterol in the bloodstream, and is made up of a single apolipoprotein B-100 molecule, with a hydrophobic core of fatty acids, cholesterol and triglycerides, as well as additional supporting proteins (Feingold & Grunfeld, 2015). It is primarily synthesized in the liver, then transported to tissues throughout the body, supplying cells with the cholesterol necessary for PM synthesis, modifications in PM fluidity, regulations in cell signaling, and cholesterol based hormone synthesis (Goldstein & Brown, 1977). As a supplier of cholesterol, LDL, must cross the endothelial cell layer, in order for it to be available to tissues which require it. The process by which circulating LDL traverses the endothelial barrier has not been well understood. The diameter of LDL is 22 nm (radius of approximately 11 nm), making it much too large to be traverse the paracellular junctions between endothelial cells. Early electron microscopy studies of LDL transport, using tagged-LDL injected into the circulation of rats, revealed LDL accumulation in the vascular intima and in intracellular vesicles, targeting it to either lysosomes or the basolateral membrane of the cell, but it was never observed at the intercellular junctions (Snelting-Havinga et al., 1989; Vasile et al., 1983). Taken together, these two facts support the notion that LDL crosses an intact endothelial monolayer through transcytosis, however, the mechanism behind LDL transcytosis is poorly understood.

Despite the limited knowledge on LDL transcytosis, the receptor-mediated endocytosis of LDL, via the high-affinity receptor LDLR, is a fairly well characterized process (Goldstein & Brown, 2009). Accumulation of LDL in the sub-endothelial intima of large blood-vessels is an early step in atherosclerotic plaque formation, thus the knowledge of this transcytosis process holds large implications for cardiovascular health. In the blood brain barrier (BBB), LDLR has previously been reported to mediate LDL transcytosis, however it is not believed to be ubiquitously involved in LDL internalization in the systemic circulation, such as in the coronary artery, where atherosclerotic lesions typically form (Dehouck et al., 1997). Studies in patients with certain forms of familial hypercholesterolemia, found a mutation in the LDLR which impairs cholesterol internalization (Karayan et al., 1994), and associate it with very high circulating cholesterol levels, and significantly greater risk of heart disease. This finding proposes that LDLR is in fact not required for LDL transcytosis. Supporting this notion further, it was recently reported that total degradation of LDLR did not inhibit LDL transcytosis by primary human coronary endothelial cells (Armstrong et al., 2015). The same study went on to show that LDL transcytosis is actually mediated by the scavenger receptor SR-B1, and is dynamin-dependent, through the use of TIRF-microscopy and an *ex-vivo* perfusion assay (Armstrong et al., 2015). However, the involvement of SR-B1 in LDL transcytosis is also called into question; SR-B1 deficient mice have accelerated atherosclerosis, indicating that other receptors must also be capable of performing transcytosis, and may actually perform compensatory upregulation in the absence of SR-B1. A group from Yale University recently conducted a genome-wide siRNA-based screen in order to identify the endothelial-specific genes which regulate LDL internalization (Kraehling et al., 2016). Through the screening, they found Activin receptor-Like Kinase 1 (ALK1), a receptor for TGFβ-type1, as also being able to mediate LDL transcytosis. They showed high levels of ALK1 expression in endothelial cells and when it was knocked-down there was a significant decrease in Di-LDL (but not oxidized-LDL) internalization and transcytosis. LDL, like albumin, has been shown to require caveolae for endothelial transcytosis. This was shown through the disruption of caveolae formation in the brain endothelial cells, which were treated with filipin, a cholesterol-binding agent (Dehouck et al., 1997). Moreover, the aorta from caveolin-1-deficient mice was found to have significantly lower LDL internalization compared to the aortas from wild-type animals (Frank, Pavlides, Cheung, Daumer, & Lisanti, 2008). As stated previously (sub-section 1.2.1) loss of caveolin-1,

specifically in mice, leads to increased endothelial paracellular leakage (Schubert et al., 2002), suggesting that this leak does not attribute to the accumulation of sub-endothelial LDL. Predictably, increased expression of caveolin-1 in endothelial cells results in enhanced rates of atherosclerosis (Fernández-Hernando et al., 2009, 2010) although these studies do not directly monitor the transcellular transport of LDL. Beyond the receptors identified, very little is known about the downstream signaling cascade required for LDL transcytosis.

1.2.4.2 *Insulin*

Another essential molecule long debated to require transcytosis for transport from the vasculature into metabolic tissue sites is insulin. After being produced in the pancreas, insulin relies on the systemic circulation for delivery to its target tissues. The process of insulin delivery is a two-step process. First, the blood carrying the hormone must perfuse the vessels supplying the tissue, after which insulin must exit the vasculature to complete its delivery to tissues such as the skeletal muscles and adipose tissues. At these tissues, insulin is then able to elicit its effect on glucose metabolism through binding to cell surface receptors and triggering a signal cascade within the cells. The delivery of insulin is jointly regulated by hemodynamic factors and by the permeability of the endothelium to insulin. The endothelium severs as the final interface which insulin must traverse to reach its target tissues.

Whether insulin relies on paracellular transport or transcytosis is a more complicated matter then that of LDL. The molecular radius of monomeric insulin is 1.34 nm (Shorten et al., 2007), making it small enough to pass through paracellular junctions in the capillary endothelium. On the contrary, an electron microscopy study in retinal vascular endothelial cells showed that insulin was present in clathrin-coated pits, but never at intercellular junctions (Stitt et al., 1994). However, it must be remembered that size is not the only factor that regulates the transport of molecules across the endothelium; characteristics like charge and pH also play a large part in regulating its transport. For example, the net negative charge on insulin may result in repulsion by the negatively charged glycocalyx present on the endothelium (Lee & Klip, 2016). The structure of the endothelial barrier in blood vessels also determines the mode and rate of insulin transcytosis. For example, the fenestrated endothelium of the liver is highly permeable to insulin and allows passage through the fenestrations. Whereas the continuous endothelium of

vessels supplying skeletal muscles and adipose tissue is much less permeable, thereby constituting a hindrance to insulin delivery and action.

Further support for the transcytosis route of insulin egress from blood vessels has been demonstrated by the receptor dependent nature of insulin transport through endothelial monolayers. The binding and transport of radioactive insulin across a retinal endothelial monolayer in vitro was inhibited by an antibody to the insulin receptor (IR) and was significantly attenuated by the addition of an excess of unlabeled insulin (King & Johnson, 1985). In another in vitro study, this time in bovine aortic endothelial cells, fluorescent insulin was shown to partially co-localize with both IR and the receptor for insulin-like growth factor (IGF) grown in culture (Wang, Liu, Li, & Barrett, 2006). The flux of fluorescent insulin across the endothelial cell monolayer was attenuated by both unlabeled insulin and the addition of IGF-I. Together, these data suggest that insulin transit across endothelial monolayers in vitro is likely receptor-mediated rather than occurring by simple diffusion, and that both the IR and receptor for IGF (IGF-1R) may participate.

The IR, like LDL receptors SR-B1 and ALK1, are also localized to caveolae regions of the plasma membrane, thus making insulin transcytosis also a caveolae mediated process (Kima, Dawesb, & Jessup, 1994; Vasile et al., 1983; H. Wang, Wang, & Barrett, 2011). Caveolae-mediated transcytosis of insulin has been observed in macrovascular endothelial cells derived from the aorta of both bovine and humans (Strålfors, 2012; H. Wang et al., 2011). A study showed that with the knockdown of cav-1, or the disruption of caveolae through filipin treatment, the uptake of FITC labelled insulin was reduced, and that overexpression of cav-1 increased uptake (Schnitzer, Oh, Pinney, & Allard, 1994). Another group has shown that endothelial cells derived from cav-1 knockout mice have a reduced capacity for insulin uptake (Schubert et al., 2001). In human microvascular endothelial cells derived from adipose tissue however, insulin transcytosis has recently been found to be mediated by clathrin-coated pits, rather than caveolae (Azizi et al., 2015). This study showed that the knockdown of clathrin, but not, caveolin-1, partly attenuated insulin transcytosis. The variability in the findings of these studies can largely be attributed to the endothelial heterogeneity between tissues and structural differences between macrovascular and microvascular vessels (Aird, 2007a, 2007b). Nearly all *in*

vitro data on insulin transcytosis to date, with some exceptions (Samuel & Shulman, 2012) suggests that insulin crosses endothelial cells by transcytosis.

1.2.4.3 *Albumin*

Albumin, was once one of the most actively studied molecule in transcellular transport, often considered the model molecule for understanding the mechanism of transcytosis. There exists a large collection of studies from the 1990's and early 2000's investigating albumin transcytosis, however these studies have still left large gaps in our understanding of the mechanism behind albumin transcytosis. Albumin exits the circulation through several mechanisms, which vary depending on the tissue involved. Organs with discontinuous capillaries, such as the liver and bone marrow, can transport plasma, and albumin through the large gaps in the endothelium. Other organs such as the pancreas, small intestine, and adrenal glands, have fenestrated endotheliums, thereby allowing unrestricted passage of plasma proteins, including albumin. Together, transport through sinusoidal and fenestrated capillaries accounts for about 50% of albumin transport from the bloodstream. The remaining 50% of albumin transported out of the capillary lumen of vessels with a continuous endothelium occurs through active transcytosis. The molecular size of serum albumin has been reported to be around 3.8 nm in diameter and 15 nm in length, just slightly too large to pass through the 1 to 3 nm gap between adjacent endothelial cells (Tojo & Kinugasa, 2012). Transcytosis of albumin occurs primarily through caveolae vesicles, a fact proven by the presence of gold-tagged albumin at the plasma membrane of caveolae and inside the vesicles (Millici et al., 1987; S. A. Predescu et al., 2007). The formation of caveolae is essential for albumin transcytosis. Mice with an inability to form caveolae, due to a knockout of Cav-1, were unable to transcytosce albumin, and demonstrated an increased paracellular permeability to albumin across the endothelium of small veins and capillaries (Miyawaki-Shimizu et al., 2006; Razani et al., 2001). The transcytosis of albumin has been reported to occur through fluid phase and receptor mediated processes (Frank et al., 2003; Tuma & Hubbard, 2003). The receptor-mediated process has been shown to require the binding of albumin to a saturable 60 kDa cell surface protein, called Gp60 or albondin, which triggers clustering of the receptor and interaction with caveolin-1. These events trigger a signal transduction, leading to the fission of caveolae vesicles, and intiating albumin transport

(Minshall et al., 2000; Shasby & Peterson, 1987; Tiruppathi, Finnegan, & Malik, 1996; Tiruppathi, Song, Bergenfeldt, Sass, & Malik, 1997). Cav-1 in turn interacts with multiple partners through its scaffold domain, it recruits members of Src-kinase family of proteins, c-Src and Fyn, and heterotrimeric G proteins Gαi and Gαq, into the caveolae region (Li, Couet, & Lisanti, 1996). Therefore, Cav-1 serves not only as a structural, but also a scaffold protein, that allows for the dynamic interaction of signaling molecules into multi-protein complexes at the sites of caveolae formation. The prevalence of albumin, due to its vast abundance in the circulation, makes it one of the most actively transcytosed molecules, thus a great candidate molecule to develop a better understanding of the transcytosis mechanism. Though there have been countless studies on albumin to date, there is much we still lack in our understanding of its functional importance.

1.3 ALBUMIN – STRUCTURE AND FUNCTION

1.3.1 SYNTHESIS AND STRUCTURE

1.3.1.1 *History*

Human serum albumin (HSA) is the most abundant protein in the blood plasma of all vertebrates, with the concentration in human serum being 35–50 g/L (Peters, 1995). It is a monomeric multi-domain macromolecule, and due to its abundance HSA is the main determinant of plasma oncotic pressure and the key modulator of fluid distribution between body compartments. The word albumin was derived from the old German word albumen, a term generally referring to proteins. This German term albumen can be then traced back to the Latin word albus (white) indicating the white part of the cooked egg surrounding the yolk (Peters, 1995). The word albumen is still used to refer to the white of an egg, which is made up of a heterogeneous mixture of albumin proteins. Albumin on the other hand, with the "in" ending refers to the specific protein from the blood plasma, or to proteins with similar properties (Fanali et al., 2012). Albumin, along with hemoglobin and fibrin, were likely some of the first proteins in the body to be studied (Peters, 1995). The presence of proteins, or albumen, in the blood

serum was recognized in the seventeenth century (Harvey & Sigerist, 1628). The physiological importance and presence of albumen in other tissues and bodily fluids, including the lymph, chyle, in the aqueous and vitreous humors of the eye was determined in early nineteenth century (Ancell, 1840). Shortly after the protein component was formally named serum albumin (Fanali et al., 2012). Chemists in the late nineteenth, and early twentieth century also began to crystalize serum albumin from animals, including horses and bovine (Peters, 1995). In 1932, HSA was finally isolated from plasma proteins (Race, 1932), and was crystalized shortly after (Hewitt, 1937). It was not until 1979, that the HSA gene was isolated (Sargent et al., 1979), and in 1981, the nucleotide sequence of HSA cDNA was reported (Lawn et al., 1981). By 1986, the complete gene sequence of HSA had been determined (Minghetti et al., 1986). Over the following decade, the expression, cleavage, and secretion of HSA was identified and many HSA mutations were localized (Fanali et al., 2012; Minchiotti et al., 2008; Otagiri & Chuang, 2009; Peters, 1995). Finally in 1992, the "heart-shaped" three-dimensional structure of HSA was elucidated (He & Carter, 1992). Serum albumin is now broadly categorized into a family of homologous proteins grouped by their unique structural features and distinctive ligand-binding properties. The other members of this family including α-fetoprotein (AFP), afamin (AFM; also named α-albumin), and vitamin D binding protein (DBP) (Fasano et al., 2007; Peters, 1995).

1.3.1.2 *Synthesis*

HSA is coded by a single copy gene, located on the long arm of chromosome four, at position q13.3, along with the genes of other members of the SA family (AFP, AFM, and DBP), in a region proximate to the centromere, at positions q11-22 (Harper & Dugaiczyk, 1983; Mikkelsen et al., 1977; Minghetti et al., 1986). The gene is expressed in a co-dominant manner, with both alleles being transcribed and translated (Hawkins & Dugaiczyk, 1982; Peters, 1995) The genes for the SA family of proteins also occur in a group in other organisms, however on different chromosomes across species, in chromosome 14 in rats (Cooke et al., 1987), chromosome 5 in mice (Yang et al., 1990), on chromosome 8 in pigs (Johansson et al., 1992), and chromosome 6 in chickens (Palmer & Jones, 1986). The *ALB* gene contains 22,303 nucleotides, starting from the first poly(A)-cap site, with 15 exons, separated by 14 introns. The *ALB* gene shows several DNA mutations that lead to the synthesis of genetic variations of HSA

(Peters, 1995). Following the transcription of the gene, the mRNA transcript of HSA contains about 2250 nucleotides. The final structure of HSA, after translation and the associated post-translational modification contains 585 amino acids, consisting of three repeating domains (labelled I-III), which of the three domains is further divided into two sub-domains (A and B), possessing common structural motifs (**see Figure 1.4**) (Curry, 2004). The secondary protein structure of the domains is primarily comprised of α-helices, 4 in the A domains, and 6 in the B domains, along with a large number of disulphide bonds (17 in total) (He & Carter, 1992; Quinlan et al., 2005), which provides albumin with its durable and heart-shaped three-dimensional structure. HSA structure shows extensive structural variations due to pH, environmental Ca^{2+} concentration, and FA binding (Curry, 2004) Several mutant allele forms of HSA have been identified to date (more than 77). The majority of mutant alleles arise due to a point mutation at charged amino acid residues, resulting in the protein having a net charge which is different from that of normal HSA, at a physiological pH (Minchiotti et al., 2008). Despite the variety of mutations, most of them are benign, and only detected upon clinical electrophoretic studies (Minchiotti et al., 2008). The structure of albumin makes it an acidic, very soluble and robust protein, it is stable at a pH range of 4-9, soluble in 40% ethanol, and can be heated at 60°C for up to 10 hours without disrupting the structure.

Like most plasma proteins, HSA is synthesized in the liver, and secreted almost immediately following synthesis as a non-glycosylated protein, with a molecular weight of 66.5 kDa. In healthy, young adults, it is produced at a rate of approximately 0.4 mg/h for every gram of liver, or 194 mg/kg/day, for an average of 12-25 g daily, and has an average half-life of 19 days (Peters, 1995). In the hepatocytes, the biosynthesis of albumin begins with the production of preproalbumin, which is then converted into proalbumin in the lumen of the endoplasmic reticulum. Successively, proalbumin is converted to albumin in the trans-Golgi network by the cleavage of a six amino acid peptide at the N-terminal of the protein (Peters, 1995). After being synthesized in the liver, albumin is released into the circulation. Due to the liver having discontinuous (sinusoid) capillaries, albumin along with the plasma, can pass through the large gaps in the endothelium. After being released into circulation, albumin travels to and accumulates in various tissue beds. Surprisingly, albumin is more abundantly present in extravascular locations such as the skin, muscles, and gut, as well as fluids, such as cerebrospinal

fluid (CSF), pleural, peritoneal, and pericardial fluids, as well as sweat, for a total of approximately 242 g. In the intravascular space there is about 118 g of albumin (Evans, 2002; Peters, 1995). Within cells however there is a very low concentration of albumin, if any at all. The cytosol of hepatocytes contains no albumin, the secretory channels of hepatocytes on the other hand store about 0.5 g of albumin, and another 0.2 g is stored within lysosomes as a part of the degradation process. In an average sized adult of about 70 kg, approximately 14 g of albumin is degraded daily, comprising 5% of the daily protein turnover. The breakdown of albumin occurs in almost all organs, with the skin and muscle degrading 40 to 60%, the liver breaking down 15% or less, the kidney 10%, and the remaining 10% of albumin leaks through the stomach and into the GI tract (Peters, 1995; Yedgar et al., 1983). In order to enter back into the circulation from its extravascular deposits albumin relies on the filtration capacity of the lymphatics system, reportedly making about 28 trips in and out of the lymphatics during its lifetime (Evans, 2002; Peters, 1995).

1.3.1.3 *Function*

With its unique structure and vast abundance, albumin serves essential roles in the circulatory system including maintaining the COP of the blood plasma (**see Figure 1.2**), transportation of metabolites, and now recently being harnessed by pharmaceutical companies to carry exogenous therapeutics. While comprising 60% of the total mass of plasma proteins present within our blood, albumin is responsible for 80% of the COP in blood plasma (Peters, 1995). Even though albumin has a significantly smaller molecular mass as compared to the average plasma globulin protein, 67 kDa versus 170 kDa, its net negative charge and low isoelectric point, provide albumin with the capacity to contribute to the COP in the plasma to a much greater extent (Figge et al., 1991; Lundsgaard-Hansen, 1986). The structure of albumin lends itself as a carrier protein, with the ability to bind many endogenous metabolites, primarily long-chain fatty acids, but also to lesser degrees steroid hormones, and L-tryptophan as well (Evans, 2002; U Kragh-Hansen, 1981; Peters, 1995). HSA is also an important physiological transporter of the essential metal ions, copper (Cu^{2+}) and zinc (Zn^{2+}), throughout the circulation (Bal et al., 2013). Furthermore, albumin has the ability to bind exogenous molecules and drugs, such as warfarin, ibuprofen, chlorpromazine, naproxen, among many other compounds (Evans,

2002; Fasano et al., 2005; Kragh-Hansen, 1981; Peters, 1995). The affinity of these drugs to albumin significantly affects their activity and half-life. In addition to transportation, albumin helps in detoxification by binding to bilirubin, the product of heme breakdown, and carrying it to the liver for hepatic excretion (Peters, 1995; Weisiger et al., 2001). Albumin is also reported to act as an anti-oxidant, by protecting the compounds bound to it, whether FA and lipoprotein, from peroxidative damage (Evans, 2002; Taverna et al., 2013).

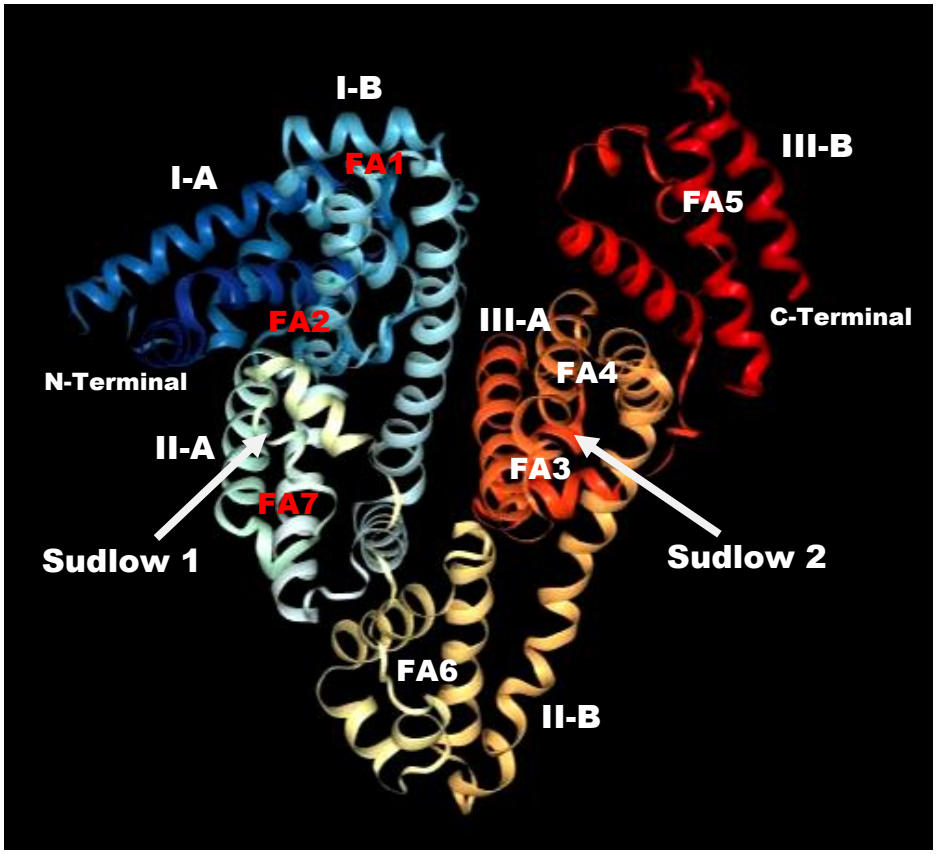


Figure 1.5. 3D Structure of Human Serum Albumin – A Potent Fatty Acid and Drug Binding Protein. *The structure of human serum albumin is comprised of primarily α-helices in three connected domains. Each domain is grouped into subdomains A and B (Subdomain I-A, Dark Blue; I-B Light Blue; II-A, Pale Blue; II-B, Yellow; III-A, Orange; III-A, Red). The primary drug binding sites: Sudlow 1, subdomain II-A; Sudlow 2, subdomain III-A (**see subsection 1.3.3**). Albumin contains seven fatty acid (FA) binding sites (**see subsection 1.3.2**). Structure downloaded from RCSB Protein Data Bank using NGL 3D viewer*(Rose et al., 2016)*; PDB ID: 1AO6; Structure published by Sugio et al. 1999* (Sugio et al., 1999).

1.3.2 FATTY ACID TRANSPORTER

From the above listed functionalities of HSA, its role as a FA carrier is believed to be the most important and best studied. Fatty acids are required mainly for the chemical storage of energy, some are used as the substrates for the synthesis of membrane phospholipids, and some for synthesizing immune signaling molecules such as eicosanoids and leukotrienes (Ger J. Van Der Vusse, n.d.). The primary source of FAs are dietary lipids available through digestion in the GI tract. Some of the FAs are also produced using carbohydrate substrates in the liver. Most FAs are stored in adipose cells, in adipose tissue deposits throughout the body. Under standard physiological conditions, each day about 0.3 mM of FA are transported from adipose tissue to FA consuming organs, such as skeletal and cardiac muscles, through the blood plasma (Vorum et al., 1992). Due to their hydrophobic structure however, FAs have poor solubility in aqueous solutions, such as the blood plasma, interstitial fluid, and lymph. Thus there arises a need for a carrier molecule to ensure its sufficient transport to tissue sites in metabolic need for FAs. This is where HSA's impressive structure is of great use, it has a total of 7 natural binding sites for fatty acids with moderate to high affinity. Albumin increases the circulating concentration of FAs several folds. In fact, it is reported that HSA binds FAs with such high affinity that it results in physiological plasma concentration of FAs in the range of 5 to 10 nM (Kragh-Hansen, 1981). Initial reports on the FA-binding properties of albumin arose in the 1940's (Forrest E. Kendall, 1941). It was the pioneering work of D.S. Goodman in 1958 however, which demonstrated the presence of 2 high affinity ($1.1 \times 10^8\ M^{-1}$), 5 moderate affinity ($4 \times 10^6\ M^{-1}$), and more than 20 low-affinity ($1 \times 10^3\ M^{-1}$), binding sites on the structure of HSA for FAs (Goodman, 1958). More recent studies, using newer crystallographic techniques reported seven common, high affinity FA-binding sites on HSA, distributed asymmetrically throughout the protein structure (Curry, 2004) The first binding site is located in subdomain IB, the second exists at the interface between subdomains IA and IIA, sites 3 and 4 are both located within subdomain IIIA, site 5 is located in subdomain IIIB, site 6 is at the interface between IIA and IIB, and site 7 is associated with subdomain IIA (Curry, Brick, & Franks, 1999). Despite a number of studies in this area, there has been great difficulty in identifying the precise number of high affinity FA binding sites, primarily due to methodological challenges. There is however general agreement that there are at least two to three high affinity binding sites, and 4 to 5 sites with intermediate affinity (Curry et al., 1999). The affinity is not only affected by the structure of the albumin binding sites, but is

also dependent on the length of the fatty acid chain. The affinity of HSA for FA increases as the length of the FA carbon chain increases, from laurate acid (12 carbon atoms) to oleate acid (18 carbon atoms). For oleate acid the association constant have been reported in the order of 10^8 M^{-1}, at the two high affinity binding sites on HSA.

Since albumin has been shown to bind FAs with a high affinity, it raises that question as to how tissues such as the heart, skeletal muscle, and the liver, are able to efficiently extract the FA from albumin in the plasma. In the case of the liver due to the fenestrated structure of the endothelium there is no real barrier for hepatocytes in accessing the plasma FA bound to HSA. The endothelium in cardiac and skeletal muscle however, is continuous therefore acts as a barrier for these tissues in obtaining plasma FA. There are four predicted methods by which FA can exit the vascular lumen and enter the interstitial space where they are readily available to the muscle cells. The first route predicts that FA-HSA complex diffuses through the inter-endothelial clefts into the interstitium. Mechanism number two presents the possibility that the FA bound HSA is endocytosed and transcytosed through endothelial cells in vesicles. The third route involves the dissociation of FA from HSA in the capillary lumen followed by diffusion of free FA through the inter-endothelial cell gaps. The last mechanism predicts the transcytosis of free FA through endothelial cells and their subsequent exocytosis into the interstitial compartment. The scientific evidence for route two, the transcytosis of the HSA-FA complex through the cardiac endothelium has been suggested by Simionescu and colleagues (Simionescu et al., 2002). In this route of FA transport, it is believed that specific HSA binding proteins (receptors) at the luminal surface of endothelial cells facilitate endocytosis of the FA-HSA complex (Schnitzer & Oh, 1994) Both routes 1 and 3, are deemed not likely to occur, because as stated previously the space between IEJs is too narrow to allow for little, if any, diffusion of FA, whether on detached or bound to HSA (Bassingthwaighte et al., 1989). There is also high likelihood of the fourth route as the mechanism of FA transport into tissue sites. A study from 1993 investigating the transfer of palmitate across the endothelium of intact, isolated rat hearts, found that the endothelium did in fact act as a barrier for plasma FA (Tschubar et al., 1993). They suggested that a reason for the barrier is that the disassociation of FA from HSA in the vascular lumen serves as a rate limiting step in transcytosis through the cardiac endothelium (Tschubar et al., 1993). Recent studies, using more sophisticated techniques revealed that the release rate of FA from HSA is significantly faster (3 to 8/second) (Demant et al., 2002; Massey et al., 1997) than previously

reported (0.003 to 0.14/second) (Scheider, 1978, 1979; Weisinger & Ma, 1987). Therefore it is highly unlikely that the inadequate release of FA from HSA is the bottle-neck in the overall access of cardiac and skeletal muscle for FA. Some studies also suggest the existence of a fatty acid binding protein (plasmalemmal FABP) on the luminal membrane of endothelial cells which facilitates FA uptake and transcytosis in cardiac tissues (Cechetto et al., 2002; Goresky et al., 1994; Pohl et al., 2004). There is a need for further experimentation to better understand the mechanism of FA transport, release and uptake by cardiac and skeletal muscle, and the role played by HSA in this process.

1.3.3 ALBUMIN AS A DRUG DELIVERY TOOL

The binding properties of HSA is not limited to FAs or only endogenous ligands. Due to its structural properties and vast bioavailability, within the last two decades HSA has been manipulated by pharmaceutical companies as a drug delivery tool. A unique property of HSA is that it preferentially accumulates in tumors and inflamed tissues, this is due in part to the development of paracellular leak in the capillary endothelium and defective or absent lymphatic drainage, under inflammatory states. Proof of this concept was shown in a study where animals with tumors were injected with Evans blue dye, which rapidly and tightly binds to circulating albumin, and thereby turns the tumor blue within a few hours post-injection (Kratz & Beyer, 1998). The leaky blood vessels of tumor tissues make its vasculature permeable to large macromolecules such as HSA. Whereas blood vessels in healthy tissue beds has a healthy, tight endothelium, therefore only permeable to small molecules. This property increases the specificity of using albumin as a transporter for anti-cancer therapeutics, by allowing accumulation into tumor sites but not healthy tissues. The leaky vasculature of tumors is insufficient on its own to explain the enhanced uptake of large molecules such as HSA, since this would also affect the uptake of smaller molecules to a similar or greater degree. The enhanced accumulation of large molecules in tumors is also partly attributed to the dysfunction in the lymphatic drainage, which reduces the clearance of molecules greater in size then 40 kDa (Noguchi et al., 1998). A study on macromolecule accumulation in tissues, it was shown that smaller molecules were rapidly cleared from the tumor interstitial space, while large molecules

were retained this showing higher concentrations in the tumor even 100 hours post administration (Noguchi et al., 1998). In tumors the excess albumin is believed to be catabolized and serve as a major energy source for tumor growth (Stehle, Sinn, Wunder, Schrenk, Stewart, et al., 1997). The consumption of albumin by tumors often leads to abnormally low plasma albumin levels in cancer patients, a condition known as hypoalbuminemia (Nazha, 2015; Stehle, Sinn, Wunder, Schrenk, Stewart, et al., 1997). Analogous to patients with cancer, patients with active rheumatoid arthritis have been shown to frequently develop hypoalbuminemia (Ballantyne et al., 1971; Niwa et al., 1990; Wilkinson et al., 1965). This is caused by high levels of albumin consumption at sites of inflammation by synovial cells, which face high metabolic and nitrogen demands during bouts of inflammation. The permeability of blood vessels is markedly increased in the inflamed joints of rheumatoid arthritis patients, thereby giving access to albumin accumulation (Levick, 1981; Wunder et al., 2003). One of the first albumin conjugated drugs to go to clinical trials was methotrexate (MTX-HSA), the most common drug used for arthritis treatment, it was synthesized by directly coupling the drug to lysine residues of HSA (Stehle, Sinn, Wunder, Schrenk, Schutt, et al., 1997). Therefore albumin serves as an attractive drug carrier to target both cancer and a variety of other immune conditions.

Albumin helps overcome several challenges associated with the successful application of therapeutic, bioactive compounds, such as insufficient stability and shelf-life, immunogenic and allergic potential, as well as bioavailability, non-specific accumulation, susceptibility to degradation and renal clearance. The two main drug binding sites on HSA are named Sudlow site I, located in subdomain IIA, and Sudlow site II positioned at subdomain IIIA (Sudlow et al., 1975). Sudlow site I was originally identified by its ability to reversibly bind the anticoagulant drug warfarin, site II on the other hand is known for its high-affinity for binding benzodiazepines, which are used to treat anxiety (Kragh-Hansen et al., 2002; Petitpas, Bhattacharya, Twine, East, & Curry, 2001). Though these two sites were the originally identified locations for drug binding, it has since been found that some drugs can bind in other sites on HSA (Bhattacharya et al., 2000; Sjöholm et al., 1979; Sudlow et al., 1975). Drugs can also be covalently linked to albumin through acyl-glucuronidation reactions to an NH_2, OH, or SH moiety in the protein, or through the reaction of an aldehyde in the open tautomer structure reacting with a lysine group in HAS (Benet et al., 1993; Ulrich Kragh-Hansen et al., 2002; Williams & Dickinson, 1994). Drug metabolites such as furosemide, salicylic acid, Non-

Steroidal Anti-Inflammatory Drugs (NSAIDs) such as ibuprofen, covalently react with HSA, which naturally affects their clearance and metabolic fate (Kragh-Hansen et al., 2002). One of the strategies pharmaceutical companies use to develop HSA-associated drugs is to incorporate components which mimic endogenous HSA ligands such as FAs, in their drug structure in order to potentiate the association with HSA. Some drugs currently on the market using this method include two diabetes treatments, Levemir®, which is an insulin analog modified with myristic acid (14 carbon FA) and Victoza®, a glucagon-like-peptide-1 agonist attached to palmitic acid (16 carbon FA) (Dornhorst et al., 2008; Marre et al., 2009; Nauck et al., 2009; Zinman et al., 2009). The benefits of these HSA-conjugated diabetes treatments, over standard treatments is enhanced bioavailability and distribution, specifically slower release, therefore prolonging the need for subsequent doses.

Another category of drugs utilizing HSA are nanoparticles, which are simply comprised of an outer shell of albumin molecules, with an inner core that contains a water insoluble drug (Elzoghby et al., 2012). HSA based nanoparticles are being studied extensively by pharmaceutical companies as cancer therapeutics, which are often highly toxic in their free forms. One HSA-based nanoparticle drug currently available in the market is Abraxine, which is a nanoparticle about 130 nm in size, housing the chemotherapeutic agent paclitaxel in its core. Abraxine is shown to be not only less toxic then paclitaxel alone, but to also exhibit higher anti-tumor activity (Desai et al., 2006). There are many other HSA based nanoparticles currently in the final stages of clinical trials involving chemotherapeutic drugs, including docetaxel and rapamycin. In addition to therapeutic uses, HSA-based nanoparticles are also used for diagnostic applications (Larsen et al., 2016). Instead of pharmaceutical compounds, these HSA aggregates contain the metastable nuclear isotope technetium-99, which can be used for the diagnosis of cancer, infectious diseases, and rheumatoid arthritis (Liberatore et al., 1992; Rink et al., 2001; Y.-F. Wang et al., 2007). Though there are many albumin associated pharmaceuticals already available on the market, and many under clinical trials, the mechanism by which they enter the affected tissue sites is poorly understood and not heavily investigated. An understanding of the mechanism by which albumin is transported out of the circulation and into specific tissue beds, may help in improving the specificity and delivery of albumin-bound drugs.

1.3.4 ALBUMIN ASSOCIATED ILLNESSES

Just as albumin can serve a therapeutic role, changes in serum albumin levels are also associated with many pathophysiological states. As mentioned above low serum albumin levels, or hypoalbuminemia, is a common occurrence in cancer patients. In fact it is associated with so many disease states, that many survival studies over the years have found that more than a quarter of all hospitalized patients suffer from hypoalbuminemia (Akirov et al., 2017; Hannan et al., 2012; Nazha, 2015; Reinhardt et al., 1980). The counter-condition, high serum albumin levels, or hyperalbuminemia, on the other hand is fairly uncommon, typically on seen in patients with acute dehydration (Mutlu et al., 2006; Throop et al., 2004). The changes in HSA levels can be due to protein catabolism (hypoalbuminemia), anabolism (hyperalbuminemia), excretion, or redistribution and accumulation of albumin in interstitial spaces or tissue beds. Overall, alterations in HSA levels in the blood serves as an indicator of physiological stress going on in the body. These stresses can include malnutrition, injury, infection, and inflammation. Due to the wide range of disease states associated with changes in HSA levels, measurements of serum albumin levels in blood, urine and other bodily fluids are heavily relied upon in clinical settings for both diagnostic and prognostic purposes (Anderson & Wochos, 1982; Bistrian, Blackburn, Sherman, & Scrimshaw, 1975; Buzby, Mullen, Matthews, Hobbs, & Rosato, 1980; Metgud & Patel, 2014; C. Yang et al., 2012). Serum albumin levels below 22 g/L are indicative of hypoalbuminemia, and are indicative of anergy, and are strongly correlated with higher incidences of infection mortality (Akirov et al., 2017; Goldwasser & Feldman, 1997; Reinhardt et al., 1980). It is well established that there is a linear correlation between the level of albumin and survival of hospitalized patients, with also a 12-fold increase in the mortality of patients with hypoalbuminemia, relative to those with normal levels (30-50 g/L) (Akirov et al., 2017; C. F. Anderson & Wochos, 1982; Goldwasser & Feldman, 1997; Hannan et al., 2012; K. B. Harvey et al., 1981). The highest rates of mortality occurs when serum albumin falls below 20 g/L, and an inability to raise albumin levels bodes a poor patient prognosis (Ching et al., 1980; McCauley & Brennan, 1983; Reinhardt et al., 1980). Thus, hypoalbuminemia is characterized as a hallmark of critical illnesses.

Specific medical conditions which are deemed to be in the critical stages due to the dangerously low HSA levels include, malnutrition, diabetes, renal disorders, liver disease,

cardiovascular disease, cancer, traumatic injury, sepsis, most infectious diseases, and autoimmune conditions, such as arthritis and HIV/AIDS (Dominguez de Villota et al., 1980; Don & Kaysen, 2004; Doweiko & Nompleggi, 1991; Mehta, Astemborski, Sterling, Thomas, & Vlahov, 2006). A general commonality in all of these diseases is that they are associated with an inflammatory response, which is believed to be the cause of changes in serum albumin levels (Doweiko & Nompleggi, 1991; Nicholson et al., 2000). The cytokines released during inflammatory states have been shown through numerous research studies to affect the synthesis, degradation, and distribution of albumin in the body. The release of cytokines interleukin-6 (IL-6), and tumor necrosis factor α (TNF-α), during acute-phase immune reactions, have been shown to decrease albumin gene transcription, indicated by a reduction in mRNA levels (Brenner et al., 1990; Castell et al., 1990; Perlmutter et al., 1986). Hormones, both steroidal and peptides, have also been demonstrated to affect albumin mRNA levels (Nicholson et al., 2000). Insulin is required for adequate albumin synthesis, as patients with diabetes have decreased rates of synthesis, which improves after insulin administration (P. D. Feo et al., 1991). During states of nutritional depravation certain amino acids can signal degradation of albumin in tissues, such as muscles, to serve as an energy source, and provide amino acids for synthesis of essential proteins (P. De Feo et al., 1992; Nicholson et al., 2000; Oratz et al., 1976).

1.3.4.1 *Role in Tissue Edema*

Altering the synthesis and degradation of albumin represents a less efficient and more chronic method of changing serum albumin levels. A more dramatic and immediate way by which cytokines and hormones disrupt circulating HSA levels is by affecting its distribution between intravascular and extravascular compartments. Proinflammatory molecules, cytokines, chemokines, leukotrienes, histamine, histamine, platelet activating factor, elicit their effect by weakening endothelial barrier integrity, through modifications of IEJs and endothelial cell transport, allowing HSA to pass the endothelium with ease (Nicholson et al., 2000). The increase in capillary leakage leads to a loss of HSA and fluids from the circulation, and subsequently accumulate into the interstitial space and organ beds, a condition known as edema. Edema has long served as a marker of an ongoing inflammatory response, especially in patients suffering from sepsis and traumatic injury (Scallan et al., 2010).

Due to its high concentration in the plasma, in healthy individuals HSA contributes up to 80% of the normal COP, which is about 25 mmHg. As per the Starling forces (**see subsection 1.1.3**), an increase in the permeability of capillary vessels reveres the COP gradient, by decreasing the plasma COP (π_c), while increasing the interstitial COP (π_{IF}), therefore forcing fluid out of vasculature and into the interstitial space. Under normal conditions the lymphatic system would serve to drain the excess fluid and protein out of the interstitial space of the organ, however in many severe inflammatory responses edema formation occurs so rapidly that it overwhelms the lymphatic clearance (Scallan et al., 2010). Critically ill patients typically have decreased serum COP, a study in 200 critically ill patients reported a mean serum COP of 19 mmHg (Weil et al., 1979). Moreover, during inflammatory states vasoactive molecules lead to further capillary recruitment and vasodilation which further increases the net filtration pressure, leading to even greater fluid and albumin flux out of the vessels and into tissues.

The accumulation of excessive fluid into interstitial spaces, or edema, is more than just an indicator of hypoalbuminemia and critical illness, it how these physiological states can become detrimental. Excessive interstitial fluid is harmful to tissue function primarily because it increase the distance oxygen and other nutrients much diffuse across in order to access tissues, which in turn compromises the cellular metabolism of edematous organ. Conversely, edema formation also restricts the diffusional removal of potential toxic metabolites from organs and into the capillaries. Regardless of the organ site where edema occurs it renders the tissue hypoxic, susceptible to oxidative stress and damage from reactive oxygen species (Nicholson et al., 2000). Edema is especially problematic when it develops in the lungs, where fluid accumulations significantly impairs gas exchange, and if the condition exacerbates can lead to respiratory failure. Pulmonary edema is a hallmark of acute respiratory distress syndrome (ARDS), which can occur due to either a lung specific disease such pneumonia, or systemic illness such as severe sepsis.

Edema formation is equally detrimental in the brain and kidneys, where anatomical structures limit their swelling in response to fluid buildup. The inability of these organs to readily expand their interstitial volume, results in large increases in interstitial fluid pressure, even with small changes in capillary filtration. Abnormally high interstitial fluid pressure reduces the vascular transmural pressure gradient, and physically compresses capillaries, thereby lowering

tissue perfusion of nutritive blood flow (Jerome et al., 1994). Cerebral edema can occur as a result of traumatic brain injury or ischemic stroke, cancer, meningitis and encephalitis. In all conditions, damage to the cerebral vasculature and blood brain barrier leads to fluid accumulation in the cranial vault. Another form of edema is ascites, which is the accumulation of protein rich fluid in the peritoneal cavity, and occurs in patients with cirrhosis (Doweiko & Nompleggi, 1991). In this form of edema, albumin accumulation in the peritoneum of patients with liver disease is due to leakage from the hepatic lymphatics and non-hepatic splanchnic organs, caused by insufficient and overwhelmed lymphatic drainage (Henriksen et al., 1983; C. M. Moore & Van Thiel, 2013). Edema formation is also common in severely diabetic patients, where fluid buildup typically occurs in the skin or eyes, conditions known as peripheral and macular edema, respectively. Macular edema is the leading cause of blindness in individuals with diabetes, and is caused by damage to the retinal microvasculature which in turn leads to the fluid collection in the macula of the retina. The occurrence of peripheral edema can easily be identified by swelling of the area, which is typically in the lower limbs, legs and feet of diabetic patients (Scallan et al., 2010).

Certain research studies suggest that albumin itself may have a role in limiting the stress-induced increases in capillary permeability and the associated fluid leakeage (International Albumin Workshop. Grindelwald, 1986). The negative charge on albumin may function in protecting the glycocalyx distribution across the endothelium, which is essential in maintaining endothelial permeability (**see sections 1.1.4 and 1.1.5**). A direct protective effect of albumin was suggested by a study that demonstrated that albumin inhibits apoptosis of cultured endothelial cells (Zoellner et al., 1996). Undoubtedly albumin is essential to maintain fluid balance in all compartments in the body and a disruption in its steady-state concentration can lead to edema formation in the afflicted organ beds. As stated above, low serum albumin levels have been associated with poor clinical outcomes, therefore it would seem a good rationale to infuse patients with more albumin in their circulation in attempts to reestablish normal COP. In order to treat edema and associated hypoalbuminemia, critically ill patients have long been treated with infusions of solutions containing HSA as early as the 1940s, however the efficacy of this treatment has become heavily debated in the last two decades. A heavily publicized meta-analysis from 1998 reported increased mortality rates in critically ill patients who received albumin solutions (Cochrane Injuries Group Albumin Reviewers, 1998). More recent clinical

studies conducted in much large and diverse patient populations however, have shown that albumin solutions do not result in more mortality then simple saline solutions (Finfer et al., 2004, 2011; Myburgh et al., 2007). But such clinical trials also show that albumin supplementation does not confer any improvement in patient conditions. The benefit of albumin based solutions is likely to vary based on the specific patient condition, and the underlying causes of hypoalbuminemia (Vincent et al., 2014). The therapeutic benefit of the solution is also likely to depend on the concentration of albumin in the solution, relative to albumin levels in the patients, as well as what other components are present in the albumin infusion (Vincent et al., 2014). Further clinical evaluations are necessary to determine which type of critical illnesses are more likely to benefit from albumin administration, and what composition of such a solution would be most therapeutic (Vincent et al., 2014).

1.3.5 EVIDENCE OF ALBUMIN TRANSCYTOSIS

Albumin is one of the first and the most studied macromolecule which undergoes transcytosis. The earliest evidence that albumin is transported out of blood vessels through transcellular routes rather than paracellularly was in the 1951 study by Pappenheimer and colleagues (Pappenheimer et al., 1951). In this seminal research the scientists put forth the “Pore Theory” to explain the permeability of capillary endothelial cells towards water and dissolved molecules. The theory suggests the presence of ultramicroscopic openings in the capillary walls of different sizes to allow for the passage of different sized molecules (Pappenheimer et al., 1951). Capillaries with a greater numbers of small pores restrict the passage of plasma proteins while allowing the movement of water and small solutes, whereas vessels with larger pores are more permeable to large proteins such as serum albumin (Pappenheimer et al., 1951). It was not until the 1980s when researchers had definitive proof of the trans-endothelial transport of albumin through capillaries and into interstitial spaces. These early studies were primarily morphological in nature, relying on electron microscopy to visualize the localization of gold-tagged albumin (Alb-Au) in perfused into mice capillary beds (Ghitescu et al., 1986; Millici et al., 1987). The two studies were published a year apart, in 1986 and 1987, by two of the leading groups in transcytosis research, one headed by Nicolae Simionescu, and the latter by George

Palade. Both showed that albumin was transported out of the vessel lumen through endothelial cells in intracellular vesicles or pits, through transcytosis, and never at intercellular junctions. At this time the term transcytosis was newly coined by Simionescu himself (Simonescu, 1979), and the transcytotic pits were referred to as plasmalemmal vesicles, which are now known as caveolae. Nonetheless these studies independently proved that under basal physiological states, with the endothelium intact, albumin is transported exclusively through transcytosis. Despite reporting the same principle finding, the exact results of the two papers were different in terms of the kinetics of albumin transport. The 1986 study by Simionescu reported the existence of a slow, high-affinity, receptor-mediated process of albumin uptake and transcytosis. Whereas the Palade group in 1987 suggested albumin transcytosis is much faster than previously suggested, and its binding to the capillary endothelium occurs with low-affinity (Ghitescu et al., 1986; Millici et al., 1987). Following these groundbreaking papers, numerous functional studies, both *in vitro* and *in vivo*, have supported the transcellular route of albumin transport (Mehta & Malik, 2006). However, the debate and controversy regarding the exact mechanism of albumin transcytosis continued for more than a decade, reaching a peak in the late 1990's, after which there has been a gradual decline in transcytosis research. Very few research groups now study albumin transcytosis. This decline is likely attributable to limitations in the experimental techniques for studying transcytosis (**see subsection 1.2.3**).

1.3.6 MECHANISM OF ALBUMIN TRANSCYTOSIS

Over the three decades since it was confirmed that albumin is transported transcellularly through the microvascular endothelium, not much detail has been elucidated with regards to the downstream signaling mechanism necessary for its transcytosis. The transcytosis of albumin through endothelial cells is widely believed to occur through caveolae vesicles, rather than clathrin-coated pits (Mehta & Malik, 2006; Rosengren et al., 2006; Schubert et al., 2001; Tuma & Hubbard, 2003) (**see subsection 1.2.2**). There is some literature however, which suggests some albumin trafficking through epithelial cells may occur via clathrin-coated vesicles (Lambot et al., 2006; Li et al., 2013; Monks & Neville, 2004), while majority of the research supports the caveolae route. Thus the focus of this section will be to describe what is currently known with

regards to the mechanism of caveolae based transcytosis of albumin in ECs. To initiate transcytosis, albumin must first be internalized into the cell, how exactly this occurs has long been debated, but is believed to involve either or both fluid-phase uptake or receptor-mediated internalization. In fact, studies have shown that both modes of internalization may occur in conjunction, with receptor-mediated internalization triggering fluid-phase uptake (John et al., 2003; Tiruppathi et al., 1997) (**see subsection 1.3.7.1**). Two independent studies in rat pulmonary ECs have shown that fluid-phase internalization, or pinocytosis, dominates as the internalization method, with almost 98% of albumin being internalized in this manner (John et al., 2003; Li et al., 2013). Several potential receptors involved in albumin internalization have been identified over three decades of research, and the most well characterized ones are discussed in detail in the following section. Though a significant amount of literature has focused on how albumin is internalized during the initiation of transcytosis, there still remains a lack of consensus and clarity on the mechanism and receptor involved.

Regardless of how albumin is endocytosed, the subsequent events of transcytosis are fairly consistent, involving the budding or fission of caveolae from the apical plasma membrane, followed by the trafficking of the albumin containing vesicle through the cell, and terminating by the fusion of the caveolae to the basal plasma membrane, and ending with exocytosis of albumin into the interstitial space. A lot research has been conducted on determining the protein machinery involved in facilitating each step of transcytosis, and this process has been well characterized in ECs (Mehta & Malik, 2006). Some of the key proteins include Src family kinases, dynamin, the SNARE complex, Rab proteins, actin and microtubules. The Src family of tyrosine kinases, is believed to regulate the first step in caveolae mediated endocytosis. Inhibitors of Src kinase, herbimycin and PP2 have been shown to prevent albumin internalization and transcytosis and reduced the total number of transport vesicles in ECs (Shajahan, Timblin, et al., 2004; Tiruppathi et al., 1997). Src phosphorylates both dynamin and caveolin-1 within minutes of adding albumin, this is believed to trigger the complexing of the two proteins, which is necessary for the fission of the caveolae (Minshall et al., 2000; Shajahan, Tiruppathi, et al., 2004). Another important function of Src phosphorylation activity may be to regulate the GTPase activity of dynamin. The GTPase function of dynamin is crucial to the fission or pinching of the caveolae from the plasma membrane. A 2004 study by Shajahan *et al*., showed

that the expression of mutants forms of dynamin-2, at specific tyrosine residues, blocked albumin endocytosis and transcytosis (Shajahan, Timblin, et al., 2004).

Dynamin is also known to interact with actin-polymerizing proteins, cortactin and profilin, and adaptor protein Grb2, thus it is likely that protein interactions with dynamin have roles in modifying the actin dynamics necessary for the trafficking of released caveolae (Krueger, 2003; Orth & McNiven, 2003; Schafer, 2002). Actin filaments are the building blocks of the cellular cytoskeleton, and are necessary for maintaining the shape of ECs. The proteins involved in regulating actin polymerization also interact with endocytic proteins, including caveolin-1 and dynamin (Krueger, 2003). To date pharmacological methods to studying the role of actin in endocytic vesicle trafficking have not been clear, however it is possible that localized, rather than global, actin polymerization at plasma membrane regions concentrated with caveolae may play part in vesicle trafficking (Mehta & Malik, 2006). Microtubules are another filamentous component of the cytoskeleton that assist in the spatial organization of cellular organelles, as well as, vesicle trafficking. A study on the role of the cytoskeleton in caveolae trafficking, used Chinese hamster ovary (CHO) cells expressing fluorescent-tagged caveolin-1 and showed the movement of caveolae along microtubules. They also demonstrated that disruption of microtubules with the drug nocadozole led to the disappearance of cell surface caveolae in COS cells expressing GFP-caveolin-1 (Mundy, 2002). Furthermore, knockdown of microtubule motor proteins kinesin or dynein in ECs was reported to decrease albumin transcytosis (Mehta & Malik, 2006).

An additional set of proteins essential for the trafficking of membrane bound vesicles in almost all cell types, including ECs, are the Rab family of GTPases. With more than 60 GTPases identified in the human genome as a part of this family, they are involved in nearly all signaling vesicle formation, motility, docking and fusion, which are all events critical to transcytosis in ECs. At least 15 different Rabs, Rab 1–9, 11, 13, 14, 15, 18, 22, and 30, have been proven to be expressed by ECs (Mehta & Malik, 2006), thus it possible these ubiquitous proteins play some part in albumin transcytosis. Out of these Rabs, Rab5 has specifically been shown to localize in caveolae in ECs, while its role in transcytosis remains to elucidated (S. A. Predescu et al., 2001).

Lastly, the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor, or SNARE proteins for short, are the main proteins facilitating the final step of transcytosis, the exocytosis of the vesicle cargo. SNAREs are responsible for the fusion of caveolae with the target membrane, usually the basolateral cell membrane, thereby allowing for the release of albumin and other macromolecules into the interstitial space (Gerst, 2003). Within the SNARE family of proteins there are two distinct groups, one is of proteins which are attached to the target plasma membrane and the other is proteins which localize on the vesicle membrane. The former group is referred to as t-SNAREs, and includes the proteins syntaxin and SNAP-25, and the latter referred to as v-SNAREs include VAMP (Mehta & Malik, 2006). The mechanism by which SNAREs facilitate exocytosis is fairly well characterized in neurons, and there is evidence supporting that a similar process occurs in ECs. When a caveolae approaches the membrane where it wishes to fuse, t-SNAREs and v-SNAREs interact to form a SNARE complex, which anchors the vesicle to the membrane and allows for fusion of the vesicle and cell membrane. This complex must then be recycled in order to facilitate future exocytosis events, this task is carried out by NEM-sensitive factor (NSF), and α-SNAP. N-ethylmaleimide a chemical inhibitor of NSF, has been shown to inhibit albumin transport in endothelial cells both *in situ* and *in vitro*, confirming the role of both NSF and SNAREs in albumin transcytosis (D. Predescu et al., 1994; Schnitzer et al., 1995). Evidently, the mechanism of the later stages of transcytosis are understood with much greater clarity then the earlier stages, with the process of ligand internalization being the most poorly understood.

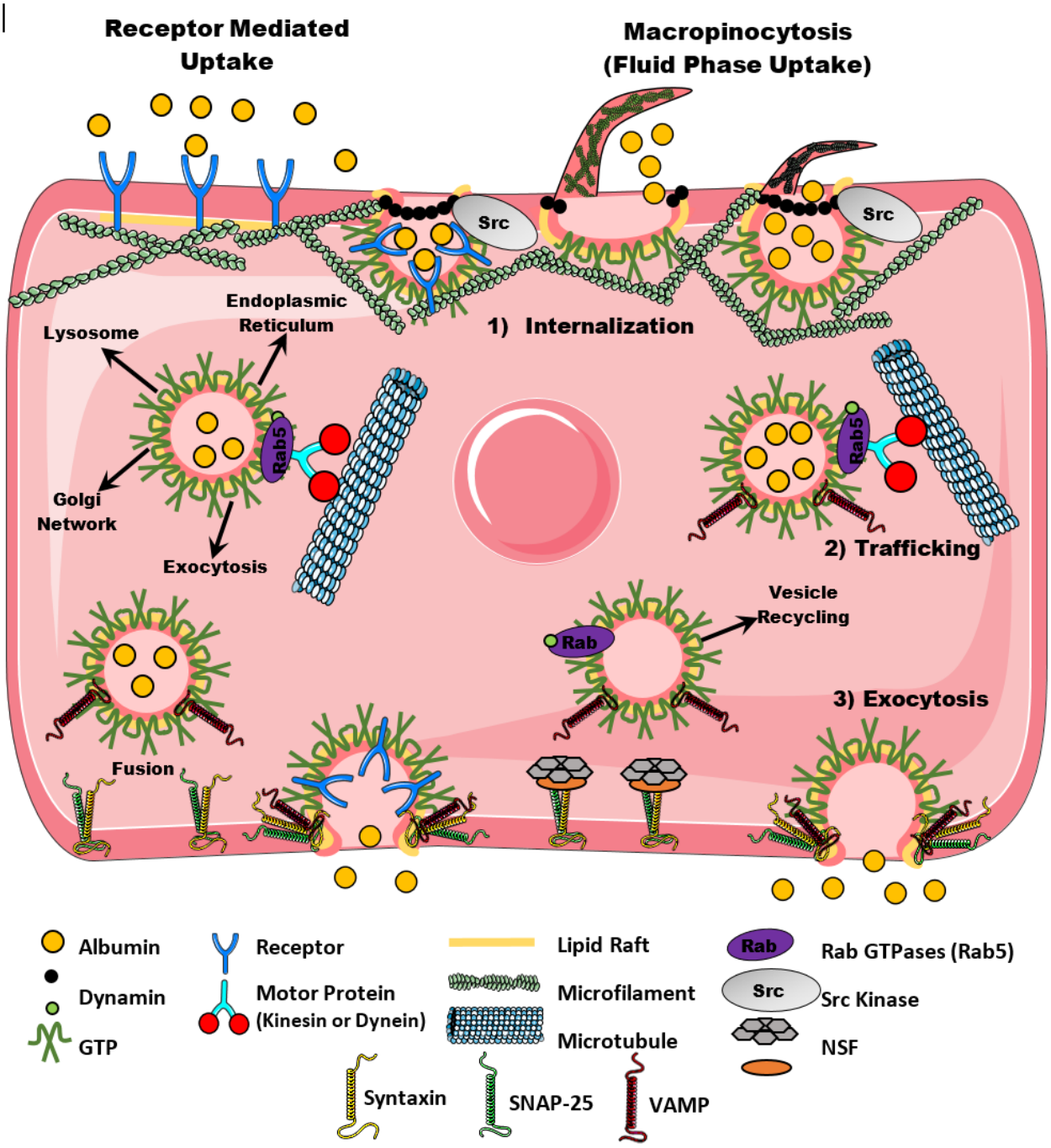


Figure 1.6 Signaling Pathway for Caveolae Mediated Albumin Transcytosis in Endothelial Cells. Transcytosis of albumin occurs in three steps: 1) Ligand internalization into caveolae; 2) Caveolae Trafficking; 3) Exocytosis. **See section 1.3.6** for a detailed description of the protein mediators involved. Figure was created using templates from Servier Medical Art.

1.3.7 RECEPTORS FOR ALBUMIN INTERNALIZATION

Considering the physiological importance of albumin, numerous putative receptors and albumin-binding proteins have been identified in various tissues and cell lines. Albumin receptors have been detected in the kidney and intestinal epithelial cells, endothelial cells, fibroblasts, smooth muscle cells and cancer-cell surfaces (Merlot et al., 2014). Due to the vast abundance of albumin it is possible that multiple receptors may be involved in its internalization in any given cell type, including ECs. The key albumin receptors discovered to date are glycoprotein 60 (gp60), glycoprotein 18 (gp18), glycoprotein 30 (gp30), the neonatal Fc receptor (FcRn), secreted protein, acidic and rich in cysteine (SPARC), cubilin, megalin, and most recently, scavenger receptor CD36. Regardless of the numerous candidate receptors, none can be conclusively said to internalize albumin in ECs. Below is a detailed description of some the best studied albumin receptors (**see Table 1.1 for summary**).

1.3.7.1 *Glycoprotein 60 (Gp60)*

The first receptor identified for albumin internalization and transport is gp60, a glycoprotein (Antohe, Dobrila, Heltianu, N. Simionescu, & Simionescu, 1993; Ghinea et al., 1988; Ghinea, Eskenasy, M. Simionescu, & Simionescu, 1989; Schnitzer, 1992), also known as albondin. The search for a receptor for albumin began following the initial observations of Ghitescu *et al* in 1986, reporting the saturability, and high affinity binding of albumin to the surface of the microvascular endothelium of mice lung, heart and diaphragm. Shortly after, in 1988 Palade and colleagues identified this 60 kDa glycoprotein as being bound to albumin in ECs. Since then gp60 has become the most studied albumin receptor, with dozens of studies published during the 1990's and early 2000's evaluating its albumin binding properties (Schnitzer, Carley, & Palade, 1988). Gp60 is named as such because of its molecular weight, is believed to be selectively expressed on the membrane of continuous endothelium, with the exception of the brain (Ghinea et al., 1988, 1989; Schnitzer & Oh, 1994; Schnitzer, 1992; Schnitzer et al., 1988; Tiruppathi et al., 1996). It was detect by immunoblotting endothelial cell lysates rat epididymal fat pad microvasculature, and observing the binding of labelled rat serum albumin at molecular weight of 60 kDa on the blot (Schnitzer et al., 1988). Later studies further

characterized the role of gp60 in albumin binding, internalization and its role in transendothelial transport. The work of Schnitzer and Oh, showed that approximately 50% of albumin transport was facilitated by binding to Gp60, while fluid-phase internalization in vesicles or paracellular transport accounted for the remaining transport (Merlot et al., 2014; Schnitzer & Oh, 1994; Schnitzer, 1993). The role of gp60 in albumin transport was demonstrated *in situ* and *in vitro* studies in bovine and rat adipose and lung microvascular endothelium. Many studies generated antibodies against rabbit gp60 in-house, to block gp60 function, and observed that albumin binding to the EC membrane was almost completely inhibited by the anti-Gp60 antibodies (Schnitzer, 1993; Schnitzer & Oh, 1992, 1994; Tiruppathi et al., 1997). Studies have also showed that the initiation of gp60-mediated and fluid-phase internalization of albumin are linked processes (John et al., 2003; Tiruppathi et al., 1997). In 2003 work from John *et al* on rat lung microvascular endothelial cells showed that the binding of albumin to gp60 triggered clustering of the receptor, which in turn initiates fission of caveolae for the cell membrane (John et al., 2003). During the budding of caveolae from the membrane, albumin localized freely in the extracellular fluid of that region is trapped within the vesicles and internalized. Based on the measurements of this study approximately 98% of albumin was reported to be internalized in fluid-phase (John et al., 2003). Gp60 is believed to not only specifically bind and uptake albumin, but also subsequently mediate caveolae-dependent transcytosis (Schnitzer, 1992; Schnitzer et al., 1994; Tiruppathi et al., 1996).

Despite gp60 being the first endothelial specific receptor for albumin identified, as of yet, very little is known about this protein. The gene encoding this protein has not been mapped in the animal or human genome, nor has its structure been determined (Bern, Sand, Nilsen, Sandlie, & Andersen, 2015; Mehta & Malik, 2006). There are no commercially available pharmacological reagents or genetic tools (siRNA or DNA plasmids) which can be used to modify gp60 activity and expression, therefore making the direct study on its role in albumin transport nearly impossible. The use of in-house generated antibodies against gp60, as done in earlier studies, (John et al., 2003; Schnitzer & Oh, 1994; Tiruppathi et al., 1997), to block its function, is not only technically challenging, but may also lead to confounding results due to antibody cross-reactivity.. The same issue arises in studying the role of gp18 and gp30, the other glycoproteins shown to bind albumin (Ghinea et al., 1988, 1989). Papers published in 1990s described this smaller molecular weight glycoproteins as binding modified, ligand carrying

albumin, rather than the native form, on a variety of cells, such as macrophages, fibroblasts and different EC types (Schnitzer et al., 1992). However, just like gp60, no gene or protein sequence and structure have been identified for gp18 and gp30, thereby making their existence a mystery and role in albumin transport speculative. Due to the methods by which these glycoproteins were first implicated in binding albumin, it is possible their existence is artefactual. For this reason, until the precise identity of gp60 is known, and better tools for its study become available, its role and mode of action as a receptor for albumin transcytosis cannot be irrefutably be accepted, leaving the quest for a true albumin receptor in endothelial cells open.

1.3.7.2 *Megalin*

Megalin or low density lipoprotein-related protein 2 (LRP2), is a very large, 600 kDa, trans-membrane glycoprotein that is known to facilitate albumin uptake in the kidney proximal tubule cells, and absorptive intestinal cells, and a variety of other absorptive epithelial cell types (Merlot et al., 2014). A study by Cui *et* al., in 1996 suggested that megalin may be directly involved in albumin binding and reabsorption by the kidney proximal tubule epithelial cells (Cui et al., 1996). Research since then however has demonstrated megalin must form a complex with the protein cubilin in order to mediate albumin transport (Birn et al., 2000). Cubilin is also a large glycoprotein of 460 kDa, that has been shown to bind an array of ligands including, the intrinsic factor-vitamin B_{12} complex, HDL, transferrin, hemoglobin, albumin and megalin (Bern et al., 2015). Like megalin, cubilin is also expressed in the kidney proximal tubule cells, and absorptive intestinal cells, and a few other cell types, though not as widely as its counterpart (Christensen & Birn, 2002). The complexing between the two proteins is necessary because cubilin lacks a transmembrane and cytoplasmic region, therefore is unable to transmit signals to initiate endocytosis. It relies on its interaction with megalin, which has extracellular, transmembrane, and cytoplasmic domains, to facilitate ligand internalization (Christensen & Birn, 2002; Moestrup et al., 1998).

The role of the megalin-cubilin complex in the reabsorption of albumin in the kidneys has been extensively studied. A knockout of megalin in mice or humans with a mutation in their megalin gene results in decreased tubular reabsorption of albumin, leading to albuminuria

(Moestrup et al., 1998; Storm et al., 2013). Similarly, humans with Imerslund-Gräsbeck syndrome, a rare condition caused by a mutation in the cubilin gene, generally suffer with proteinuria, and decreased renal albumin uptake (Gräsbeck, 2006). Interestingly, mice with a knockout of both megalin and cubilin did not show greater albuminuria as compare to mice lacking only cubilin (Amsellem et al., 2010). this finding supports the notion that cubilin is the main albumin binding protein, with megalin driving the internalization of albumin bound cubilin (Amsellem et al., 2010). In a 2011 study by Weyer et al, the group generated a mouse model of endocytic dysfunction in the proximal tubule by knocking out both megalin and cubilin in the kidney (Weyer et al., 2011). They looked at albumin uptake, degradation and its urinary excretion, and found that both uptake and degradation was decreased, while urinary excretion of whole albumin was increased, in the mice lacking megalin and cubilin relative to control mice. Undoubtedly there is significant functional data supporting the role of megalin in conjunction with cubilin, as being receptors for albumin in the kidney and intestinal epithelium. As of yet however, the full-length crystal structure of cubilin has yet to be resolved, and nor has an albumin binding site been identified on the structure of megalin. Whether these receptors, either individually or in complex, are expressed on ECs is not known. If in fact they are found to be expressed on the endothelium, megalin and/or cubilin may possibly serve as mediators of albumin transcytosis in ECs.

1.3.7.3 *FcRn*

The primary role of the FcRn is the transport of IgG across the placenta, from mother the fetus, and in the proximal small intestine (Roopenian & Akilesh, 2007). It not only serves as a carrier of IgG, it also protects it from lysosomal degradation by binding to it with high affinity only at a pH less than 6.5, in acid endosomes, and facilitates its transport and exocytosis (Chaudhury et al., 2003; Ober, Martinez, Lai, et al., 2004). FcRn is also a glycoprotein, but structurally unique due its extracellular domain being comprised of an MHC-class-1 heavy chain, with three distinct ligand binding domains (α1, α2, α3). Also a part of its extracellular region is a β2-microglobulin light chain, which non-covalently links to the heavy chain and is required for the function of FcRn (Zhu et al., 2002). The receptor also contains a transmembrane

region along with a cytoplasmic tail at the C-terminal end of the protein. Research in both humans and rodents suggest FcRn is very broadly expressed in various cell types and tissues, including antigen presenting cells (APCs), such as monocytes, macrophages, dendritic cells and lymphocytes (Bern et al., 2015). It is also reportedly found in hepatocytes, the microvascular endothelium of the BBB, skin (Ober, Martinez, Vaccaro, et al., 2004), and retina, in choroid plexus cells, and the gut, lung and kidney epithelium (Bern et al., 2015). The initial indication that FcRn may also bind albumin came through research in 2004 which reported the co-elution of BSA with soluble human FcRn, during a column chromatography experiment with IgG (Chaudhury et al., 2003). The same publication also stated that the concentration of serum albumin was 3-fold reduced and its half-life was shortened in mice with an FcRn knockout, as compared to the wild-type strain. The co-elution results of this seminal study alluded to the possibility that FcRn simultaneously bound IgG and albumin. Subsequent studies conducted *in vitro*, further analyzed the FcRn-albumin interaction. These studies confirmed the concurrent binding of albumin and IgG by FcRn, and that a conserved histidine residue on the α2 extracellular domain of FcRn (H166), was required for albumin binding (Andersen et al., 2006; Chaudhury et al., 2006). A mutation in the β2-microglobulin subunit of FcRn has been implicated in causing a rare familial disorder, hypercatabolic hypoproteinemia, which is characterized by extremely low serum levels of albumin and IgG (Waldmann & Terru, 1990; Wani et al., 2006). The reason for the low serum albumin and IgG levels in the absence of FcRn, in both mice and humans, is due to increased susceptibility to degradation. Just as FcRn binds to IgG with high affinity under acidic cellular conditions and protects it from lysosomal degradation, it has been proven to serve the same protective function for albumin, thereby extending its half-life in the circulation (Anderson et al., 2006; Chaudhury et al., 2003).

The mechanism proposed in the binding and transcytosis of albumin by FcRn, does not involve cell surface interactions (Bern et al., 2015). Rather, extracellular albumin in combination with IgG is believed to be internalized through fluid-phase uptake. Once inside the cell the early endosomes containing the proteins fuse with acidic endosomes, it is in the membrane of this endosome where FcRn primarily localizes (Knudsen Sand et al., 2015). Once in the acidic endosome the receptor can bind albumin and IgG. Following binding, the FcRn-albumin-IgG complex has three potential fates (Knudsen Sand et al., 2015). One outcome is that the complex can be degraded if the endosome further acidifies and becomes a lysosome. The second possible

result is that the endosome can be recycled back to the apical surface of the cell and FcRn can release the albumin and IgG back into the circulation (Knudsen Sand et al., 2015). Alternatively the vesicle can be transcytosed to the basolateral membrane and exocytose the protein cargo into the interstitial space (Knudsen Sand et al., 2015). The mechanism of albumin binding and trafficking by FcRn in intracellular endosomes is clear, however it is not known whether this receptor interacts with albumin at the cell surface, and if it is involved in the caveolae mediated transcytosis of albumin.

1.3.7.4 *CD36*

In a study from 2012 on proteinuric nephropathies, the scavenger receptor, CD36 was shown to be involved with albumin endocytosis, specifically in proximal tubule epithelial cells of the kidneys (Baines et al., 2012). This study, along with a few others which suggest CD36 as a receptor for albumin, will be presented in detail in the following section (**see section 1.4.4**). The role of CD36 to bind and facilitate the uptake of fatty acids, serves a commonality between it and albumin, which well-characterized as the main carrier of FAs in the circulation. Furthermore, expression of CD36 in endothelial cells is well established, as is its role in mediating FA transport through the endothelium (Mehrotra et al., 2014). There is a gross lack of research into whether CD36 directly binds albumin and facilitates its transcytosis. In the subsequent section I will describe this intriguing receptor in much greater detail, and present the current knowledge of its general function, and its potential as a receptor and mediator of albumin transcytosis.

Table 1.1 Summary of key albumin binding proteins

Candidates	Expressed In	Additional Function
Gp60	Continuous endothelium	Suggested primary receptor for native albumin; caveolae mediated transcytosis
Gp18/Gp30	ECs, macrophages, fibroblasts	Bind modified albumin, target for lysosomal depredation
Megalin/LRP2	Absorptive epithelial tissues (kidney, intestine, lungs)	Endocytosis for degradation or transcytosis; LDL receptor
FcRn	ECs, BBB, Epithelial cells, Podocytes, APCs	Transcytosis of IgG in epithelial cells
CD36	Platelets, monocytes, ECs, Epithelial cells	Scavenger receptor; binds ox-LDL, FA transport

1.4 CLUSTER OF DIFFERENTIATION 36 (CD36)

1.4.1 STRUCTURE AND PHYSIOLOGICAL SIGNIFICANCE

CD36, also known as fatty acid translocase (FAT), is a heavily glycosylated 80 kDa integral membrane protein, that is the founding member of the scavenger receptor family of proteins. Other members of the scavenger receptor family include the HDL receptor, SR-B1, and lysosomal integral membrane protein LIMPII. It is a fairly ubiquitously expressed receptor, having been identified on the surface of megakaryocytes, precursor erythroid cells, platelets, monocytes, dendritic cells, adipocytes, myocytes, epithelial cells, adipocytes, and even on endothelial cells of the microvasculature and small intestine. Contingent on the cell type, the function of CD36 may vary, it can act as an adhesion molecule, or a class B scavenger receptor, however its primary role is for FA internalization. The primary ligand for CD36 are long-chain FA, to which it binds with very high affinity, thereby acts as primary facilitator of FA uptake in

muscle and adipose tissues. It can also function as an adhesion molecule through its binding capacity for the extracellular matrix proteins thrombospondin 1 (TSP-1) and collagens type I and IV. The binding of TSP-1 to CD36 on ECs induces apoptosis, thereby inhibiting angiogenesis (Dawson et al., 1997). As an adhesion molecule CD36 can modulate platelet aggregation and the cell to cell interactions necessary for the recruitment and trafficking of monocytes to damaged tissues. In a disease context, CD36 is the microvascular endothelial cell receptor for the *Plasmodium falciparum* protein, which is expressed on the surface of malaria-infected erythrocytes and thus increases the virulence of this form of malaria. In macrophages and dendritic cells, CD36 is a scavenger receptor required for the recognition and phagocytosis of apoptotic cells (Coburn et al., 2000). CD36 also has a secondary function in macrophages, especially those cells located at sites of atherosclerotic lesions, where it becomes a key receptor for binding and internalization of oxidized low-density lipoproteins (oxLDL). This role links back to its ability to bind anionic phospholipids, such as FAs, as well as lipids or proteins modified by lipid peroxidation. Due to the variety of functions served by CD36, there has been an extensive amount of studies which have contributed to detailed model for its primary protein structure.

The human CD36 gene is located on the q11.2 band of chromosome 7, consisting of 15 exons, and spanning approximately 46 kilobases (Yang et al., 2017). The transcription factors, peroxisome proliferator activated receptor (PPARα, PPARγ, and PPARδ) have been shown to ubiquitously regulate CD36 gene transcription. Metabolites of lipids, including fatty acids (Hua et al., 2015) and ox-LDL (Han et al., 1997), have also been shown to upregulate CD36 gene expression. Human cDNA of CD36 estimates a 472 amino acid sequence of the full-length CD36 protein, with a 30 amino acid long N-terminal signal peptide directing transcription to the endoplasmic reticulum (ER), which remains uncleaved throughout the life of the protein. Like other members of the scavenger receptor family of proteins, CD36 is believed to share a similar "hairpin" structure, defined by two transmembrane domains, with both terminal ends in the cytoplasm. Because of this unusually rare configuration, the intervening amino acids direct the proteins either to the cell surface in the structure of CD36, or to the lumen of lysosome vesicles, in the structure of LIMPII. Through the crystal structure of CD36 the existence of a large cavity spanning the entire length of the molecule was identified (Pepino et al., 2014). This cavity has been proposed to serve as a tunnel for lipid transfer through the cell membrane (Pepino et al.,

2014). The cytoplasmic domain of CD36 consists of only 19 amino acids, 7 at the N-terminus, and 12 at the C-terminus (C. Martin et al., 2011). In these cytoplasmic domains are cysteine residues which allow for palmitoylation of CD36, an increase in this modification is believed to signal insulin or energy depletion in adipocytes (Coburn & Abumrad, 2003). The remaining amino acid residues comprise the extracellular domain, where the binding sites of each of the many ligands of CD36 are located on distinct residues. For example, the TSP-1 binding site resides in amino acids 93 to 155, whereas oxLDL binds at residues 157 to 171 (Puente Navazo et al., 1996). On the extracellular domains of CD36 there are ten possible sites for glycosylation, which can increase the apparent protein mass from 53 kDa in a non-glycosylated form, to between 78 to 88 kDa, varying for cell type to cell type (Coburn & Abumrad, 2003; C. A. Martin et al., 2007). The extensive glycosylation of CD36 and its family of proteins likely serves as a protection technique from degradation in protease-rich environments such as lysosomes, or sites of injury and inflammation (Coburn & Abumrad, 2003; Pepino et al., 2014). In many cell types CD36 is found in caveolae or lipid-raft regions of cell membrane, however it lacks the caveolin scaffold recognition sequences present in many caveolae-localized proteins(Coburn & Abumrad, 2003; Pohl et al., 2005). It is believed however, that the palmitoylation of the cytoplasmic domains of CD36 may serve as a transient signal to regulate its association with rafts or caveolae. Signal transduction associated with CD36 is believed to involve the Src family kinases, Fyn, Lyn and Yes (Febbraio et al., 2001; Huang et al., 1991; Silverstein et al., 2010). When CD36 is activated, it is believed to undergo oligomerization, and subsequent transphosphorylation, at which point these kinases disassociate from the cytoplasmic domain and begin phosphorylating downstream proteins (Febbraio et al., 2001; Silverstein et al., 2010). CD36 is involved in a variety of physiological functions from lipid metabolism, atherogenesis, thrombosis, angiogenesis, inflammation, platelet function, and even the pathogenesis of malaria (Coburn & Abumrad, 2003; Febbraio et al., 2001).

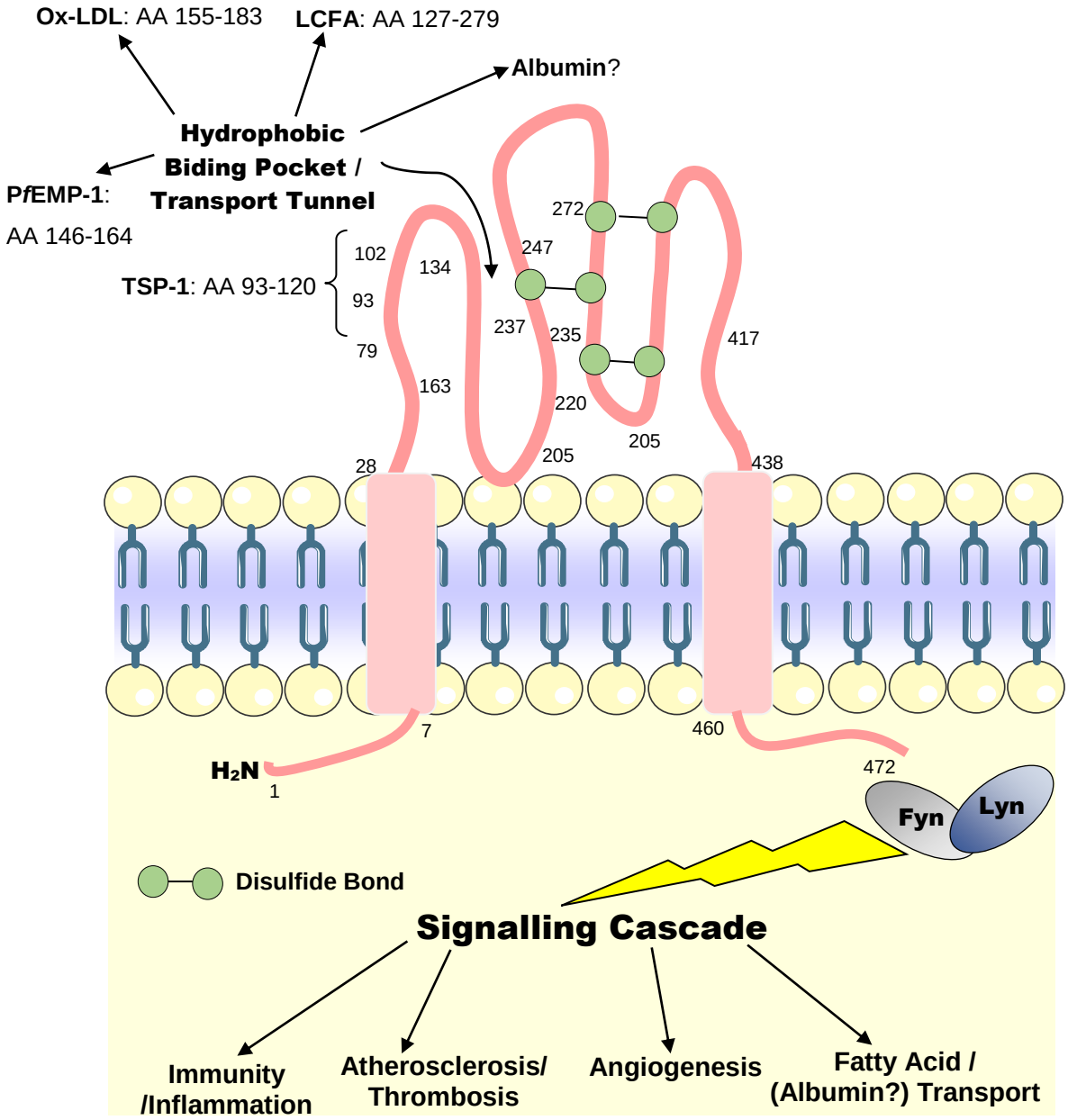


Figure 1.7 Structure and Function of CD36 – A Versatile and Ubiquitous Receptor

CD36 has 2 transmembrane domains, two small cytoplasmic tails, and contains a looped extracellular domain.. It typically localizes in lipid raft on the plasma membrane. Many ligands bind to CD36 in the hydrophobic binding pocket. The binding of these ligands initiates a signaling cascade through Src kinases, Fyn and Lyn, and leads to a variety of physiological consequences. AA: Amino Acid Residue, LCFA: Long Chain Fatty Acid,. Figure was created using templates from Servier Medical Art. Reference for binding site location Martin et al. (C. Martin et al., 2011).

1.4.2 CD36 – A FATTY ACID TRANSLOCASE

As its alternate name suggests CD36/FAT, a primary biological role of this receptor is to facilitate the transport of long-chain FAs into cells. is The discovery of CD36 as a fatty acid transporter were first made in 1993, during a kinetic study on FA transport in isolated rat adipocytes, which reported the process to be saturable and specific for long-chain FAs, suggesting the existence of a high-affinity receptor (Abumrad et al., 1993). The membrane protein was isolated by labelling with reactive FA analogs and sequenced, the cDNA showed 85% homology to human CD36 (Abumrad et al., 1993). Conversely, CD36 isolated from adipose tissue had long-chain FAs bound to it with high affinity and specificity (Baillie et al., 1996). In a line of fibroblasts, transfection of CD36 was associated with increased uptake of long-chain FAs and increased incorporation of FAs into cellular phospholipids (Ibrahimi et al., 1996). Further proof of the role of CD36 in FA uptake is that its expression levels are highest in tissue with high metabolic needs, such as adipose tissue, heart and skeletal muscle (Abumrad et al., 1993). Expression of CD36 has also been demonstrated to be high in tissues which experience large fluxes of FA such as the microvascular endothelium, mammary secretory epithelia, enterocytes of the small intestine (Bull et al., 1994; Clezardin et al., 1993; Poirier et al., 1996). CD36 distribution is highest in the jejunum of the small intestine as it is the site of greatest dietary FA absorption, and the expression is upregulated by high-fat diet (Poirier et al., 1996). In the muscle CD36 expression in red oxidative fibers is increased during continuous muscle stimulation to allow for an increase in the maximal rate of FA transport, its Vmax (Van Nieuwenhoven et al., 1995). A study by Bonen *et al.*, in 2000 illustrated that during muscle contraction, the translocation of CD36 from an intracellular pool, to the sarcolemma was necessary to mediate increase FA uptake (Bonen et al., 2000). Moreover, overexpression of CD36 in muscle, results in an increased rate of FA uptake and metabolism(Azeddine Ibrahimi et al., 1999), and mice with a whole-body CD36 knockout show reduced FA uptake (Febbraio et al., 1999). Interestingly, CD36 has also been shown to be involved in FA accumulation in the skin. It was shown to be weakly expressed in the lower epidermis, hair follicles and subcutaneous fat of mice, however its expression was strongly upregulated in the dermis and epidermis following skin permeability barrier disruption (Schmuth et al., 2005). Under basal physiological conditions CD36 is not detected in human skin, though it is shown to be induced in the skin lesions of patients with

dermatological diseases such as psoriasis, dermatitis, and skin wounds (Harris et al., 1998; Lin & Khnykin, 2014).

The generation of well-defined mice models of CD36 deficiency and tissue specific overexpression has greatly enhanced the understanding of the physiological role of CD36 as a mediator of FA uptake. For example a line of transgenic mice with a muscle-specific overexpression of CD36 were shown to have less total body fat, lower serum FA, triglycerides and cholesterol, consistent with an increased FA utilization in metabolism (Azeddine Ibrahimi et al., 1999). Conversely, mice lacking CD36 exhibited an increase in fasting serum FA, ketone bodies, triglycerides and cholesterol, surprisingly these mice also have lower body fat, likely due to the absence of high-affinity FA transport by adipocytes (Febbraio et al., 1999). Taken together these studies provided strong functional evidence that CD36 is intimately involved in the facilitation of FA internalization across the plasma membrane and the subsequent metabolism of this essential energy substrate.

The ability of CD36 to directly bind FAs was confirmed by the identification of specific binding sites on its extracellular domain. In order to identify the exact binding sites researchers used the same inhibitors that were initially used to determine that CD36 binds FAs and regulates there transport in adipocytes (Harmon & Abumrad, 1993). The existence of the FA binding sites on CD36 was determined by locating the target residues for the specific inhibitors of FA uptake. The inhibitors in question were the N-hydroxysuccinimdyl (NHS) esters of long-chain FAs, palmitate, myristate, and oleate, which were shown to effectively inhibit FA uptake by adipocytes (Harmon & Abumrad, 1993). These esterified FAs irreversibly preclude the CD36 mediated internalization and signaling of unmodified FAs, by forming stable bonds with primarily the amino groups in the lysine side chains (Harmon & Abumrad, 1993). A further modified form of long-chain FAs are sulfo-NHS ester of FAs, which contain a negatively charged sulfonate group that limits membrane permeability (Harmon & Abumrad, 1993). The sulfo-NHS ester of the 18-carbon FA oleate, also known as sulfo-N-succinimidyl-oleate (SSO), is the most widely used inhibitor of CD36 (Harmon & Abumrad, 1993). SSO has been shown to inhibit both CD36 mediated uptake of FAs (Coort et al., 2002; Su & Abumrad, 2009) and signaling cascades initiated by FAs (Dramane et al., 2012; Kuda et al., 2011), in a variety of cell types. The exact binding site at which SSO inhibits CD36 function was determined in a 2013

study which conducted proteomics analysis on CHO cells expressing human CD36, treated with SSO (Kuda et al., 2013). They found the location of SSO attachment in human CD36 to be at the lysine 164 (Lys-164) residue, a site situated within a pocket on the extracellular loop. This studied used site specific mutations and expression in CHO cells to show Lys-164 as a FA binding site. CHO cells transfected with CD36 mutated at Lys-164, showed reduced FA uptake and a decrease in FA-induced calcium release from the endoplasmic reticulum (Kuda et al., 2013). The binding of FA to this residue is suggested to induce a conformational change in CD36, that would allow the FAs to slide into the CD36 channel that leads the molecules to the plasma membrane (Pepino et al., 2014). A surprising coincidence of Lys-164 is that it is also the proposed binding site of oxLDL and oxidized phosphatidylcholine on CD36 (Kar et al., 2008).

1.4.3 **CD36 AND DISEASES**

Due to its essential metabolic role, along with other widespread physiological functions, CD36 is responsible for mediating many disease states. The illnesses which CD36 is involved in can broadly be categorized into metabolic and/or inflammatory conditions. The most common disease state which CD36 is known to exacerbate is atherosclerosis, which is one of the leading causes of death in the world. CD36 is attributed in the development and early progression of atherosclerotic plaque formation, owing to its ability to bind and uptake oxLDL. In the initiating phase of atherosclerotic lesion development, CD36 binds and internalizes oxLDL into macrophages that are localized in the arterial intima of the macrovessel. Once inside macrophages, oxLDL triggers a signal cascade that upregulates expression of CD36, which then further facilitates the internalization of oxLDL. The accumulation of oxLDL pushes macrophages to differentiate into foam cells, which are specialized cells that secrete oxidants into the atherosclerotic site, thereby generate more oxLDL. The interaction of CD36 and oxLDL also stimulates cytokine secretion by macrophages present, these cytokines then recruit more immune cells to infiltrate the vascular intima, triggering an inflammatory response, which accelerates and exacerbates the atherosclerotic lesion formation. Related to the role of CD36 in atherosclerosis is its ability to promote thrombosis, or blood clot formation in vessels. CD36 expressed on platelets also binds to oxLDL and facilitates its internalization. Once inside the

platelets, oxLDL activates transcriptional modifications that initiate a hyperactive state in platelets. This hyperactivity promotes platelet aggregation and increased expression of platelet activation markers, such as P-selectin and the activated form of the fibrinogen receptor, thereby causing clot development.

Another immune-related disease which CD36 is known to mediate is malaria. CD36 expressed on ECs is known to bind with great exclusivity to *P. falciparum* erythrocyte membrane protein 1 (P*f*EMP-1), an antigenically variable receptor expressed on erythrocytes (red blood cells) infected with *P. falciparum*, the most virulent and fatal strain of the malaria parasite. Though a number of EC receptors have been shown to bind P*f*EMP-1, however under physiological blood flow conditions, CD36 accounts for 90% of the adhesion of infected erythrocytes (Udomsangpetch et al., 1996, 1997), and over 84% of P*f*EMP-1 protein variants contain domains predicted to bind CD36 (Smith et al., 2013). By facilitating the adhesion of erythrocytes to ECs, specifically in post-capillary venules, CD36 sequesters red blood into the microvascular beds of organs, causing obstruction of the microcirculation, which ultimately causes ischemia and metabolic dysfunction (Coburn & Abumrad, 2003). The expression of CD36 on macrophages however may be beneficial to the host defence against malaria, thus counteracting the detrimental effects of the CD36-P*f*EMP-1 interaction on ECs. The binding of macrophage CD36 to P*f*EMP-1 on infected erythrocytes induces a respiratory burst, this leads to the rapid release of reactive oxygen species, which lead to oxidation in the malaria parasite and its subsequent death (Ockenhouse et al., 1989). CD36 has also been shown to directly mediate the phagocytosis of infected cells, therefore participating in the immune response during a malaria infection (McGilvray et al., 2000).

An unexpected role of CD36 is in the pathogenesis of Alzheimer's disease. This omnipresent receptor is also expressed by microglia cells in the brain, and intercedes the uptake of β-amyloid (Aβ) peptides by these cells (Doens et al., 2017). The hallmark of Alzheimer's disease is the presence of Aβ aggregates, neurofibrillary tangles, and inflammation in the brain, which is believed to be caused by the CD36 mediated activation of microglial cells (Doens et al., 2017). The binding of Aβ peptides by CD36 initiates a cascade of intracellular signaling that induces internalization of Aβ and the release of proinflammatory cytokines by the glial cells.

Attributed to its function in FA metabolism, CD36 is implicated in metabolic syndromes including insulin resistance, diabetes and obesity. The mechanism behind CD36 mediated metabolic disorders is complex, and varies depending on the model organism and tissue site, but is likely always related to altered lipid metabolism. Numerous studies have demonstrated that mice and rodent models of genetic obesity or diabetes, or those fed high-fat diets, have increased expression of CD36, especially in adipocytes, relative to the respective wild-type groups (Berk et al., 1999; Berk et al., 1997; Greenwalt, Scheck, & Rhinehart-Jones, 1995; Luiken et al., 2001; Memon et al., 1999; Pelsers et al., 1999). A study on mice with a muscle specific overexpression of CD36, displayed significantly high blood glucose levels, while their insulin levels were normal during fed states, but higher than the control group while fasting (Ibrahimi et al., 1999). Conversely, blood glucose levels are reduced in CD36 knockout mice (Febbraio et al., 1999), however glucose tolerance and insulin sensitivity in these mice was dependent on the diet. On a standard show diet, CD36 deficient mice were more insulin sensitive because of increased glucose tolerance and glucose uptake in cardiac and skeletal muscle, relative to control animals (Hajri et al., 2002). However, when the knockout mice were given a high-fructose or high-fat diet insulin insensitivity and glucose intolerance was observed (Hajri et al., 2002). The decreased blood glucose levels in CD36 knockout mice has been attributed increased whole-body insulin-sensitive uptake of glucose, specifically due to increased insulin sensitivity in muscle tissue (Goudriaan et al., 2003). The liver of the CD36 deficient mice however demonstrated insulin resistance (Goudriaan et al., 2003).

In humans, observations similar to those stated above for mice and rats have been reported (Handberg et al., 2006). The blood plasma concentration of soluble CD36 (sCD36) was five-fold increased in obese individuals with type-2 diabetes, as compared to lean individuals, and two to three-fold higher as compared to non-diabetic obese subjects (Handberg et al., 2006). This study also reported a strong inverse correlation between sCD36 and insulin-stimulated glucose uptake and a direct relationship with fasting plasma glucose, fasting insulin levels, and body mass index of patients (Handberg et al., 2006). A study from 2001, on a limited number of human subjects with a CD36 deficiency reported these individuals had irregularities in plasma lipids, impaired insulin responsiveness and glucose metabolism (Miyaoka et al., 2001). However the findings of this study have not been supported by similar investigations in CD36 deficient humans, which report these individuals have no indication of insulin resistance (Furuhashi et al.,

2003; Kajihara et al., 2001; Yanai et al., 2000). The presence of a CD36 deficiency is naturally occurring in the human population, with an average prevalence of 3-11% in Asians, 5-18.5% in Africans, and less than 0.3% in Caucasians (Aitman et al., 2000; K. Lee et al., 1999; Yamamoto et al., 1990). This deficiency can be categorized into two forms based on the cell types lacking in CD36 expression. Individuals with a type I CD36 deficient phenotype lack CD36 expression on the surface of both platelets and monocytes, those with a type II phenotype lack surface expression primarily on platelets (Coburn & Abumrad, 2003). The pathophysiology of a CD36 deficiency is generally benign, with those effected appearing generally healthy. In certain cases however, defects in myocardial uptake of long-chain FAs have been described in CD36 deficient patients, and it may also be associated with some cases of cardiac hypertrophy (Kashiwagi et al., 1995; Tanaka et al., 2001).

1.4.4 CD36 AS AN ALBUMIN RECEPTOR

Just as with albumin, or any other macromolecule, the endothelium, also presents as a gatekeeper for FA transport. In order to be utilized by the tissues sites which require them, FAs must be transported through endothelial cells by transcytosis. Due to their hydrophobic structure FAs cannot traverse the endothelial barrier through paracellular means, thus require receptor mediated transport in vesicles through ECs. The mechanism of how exactly FAs are transported through the endothelium is not known, nor is the identity of the FA receptor or transporter involved. Based upon *in vivo* results from CD36 knockout mice studies (**see section 1.4.3**), FA uptake and metabolism by skeletal muscle and adipose tissue is significantly reduced, suggesting disruption in FA availability to these tissue sites. If in fact CD36 is involved in FA transport across the endothelium, then it must come into close contact with albumin, the primary carrier of FAs in the circulation. In order for CD36 to transport FAs, they must either first disassociate from albumin, or a second possibility is that CD36 mediates the internalization and transcytosis of FAs bound to albumin.. A review article from 2001 on the mechanism of long-chain FA uptake and trafficking, proposed a schematic for this process based upon the studies that were available at the time (Stremmel et al., 2001). They hypothesized that once albumin carrying FAs came into close proximity to a high affinity receptor for FAs on the cell surface, FA readily

dissociated and was transported into the cell by the receptor. They implicated CD36 to be the said high-affinity FA transporter (Stremmel et al., 2001). The authors however did not speculate whether CD36 and albumin had any direct binding or interaction during this FA transport process.

Explicit evidence for the ability of CD36 to bind albumin was presented in a study on the mechanism of renal tubular injury by advanced oxidation protein products (AOPPs), specifically of AOPP of human serum albumin (Iwao et al., 2008). The results from this showed that CD36 was at least partly involved in the uptake of AOPP-albumin in human proximal tubular cells. They showed that blocking CD36 with a neutralizing antibody prevented both endocytic uptake and degradation of AOPP-albumin. The relationship between CD36 and AOPP-albumin was shown to be reciprocal, treatment with AOPP-albumin increased CD36 expression in the proximal tubular cells (Iwao et al., 2008). Following this publication, a study in 2012 on proteinuric nephropathies, reported that CD36 was involved in the endocytosis of unmodified albumin, in proximal tubule epithelial cells of the kidneys (Baines et al., 2012). Transfection of opossums kidney proximal tubule cells with exogenous CD36, drastically enhanced both binding and uptake of albumin, and SSO treatment (inhibitor of CD36; **see section 1.4.2**) abrogated this effect. To ensure the increase in albumin uptake and binding in CD36 transfected cells was not due to megalin (**see subsection 1.3.7.2**), they blocked its function, and found it did not abrogate the increase. The researchers also showed co-localization between CD36 and fluorescently-tagged albumin at the surface of the proximal tubule cells. Based upon the minimal, but intriguing research presented in favour of the CD36 and albumin interaction, it became the receptor of focus in studying albumin transcytosis for my thesis project. I wished to shed more light on whether or not there is any correlation between these two ubiquitous proteins.

CHAPTER 2: HYPOTHESIS AND AIMS

2.1 ENDOTHELIAL CELLS FROM THE MICROVASCULATURE OF DIFFERING TISSUE BEDS SHOULD EXHIBIT DIFFERENTIAL KINETICS AND AFFINITY FOR TRANSCYTOSIS OF ALBUMIN

Until recently, most of the research on endothelial permeability has focused on paracellular leak while largely ignoring transcytosis. Though paracellular leak has been greatly studied, regulating and modifying it poses significant challenges, as enhanced paracellular permeability is key step in tissue specific inflammatory responses. Thus, it would be beneficial to study and better understand the mechanism of transcytosis, as it may be a more easily manipulated route of endothelial transport. The lack of research into transcytosis is due largely to longstanding technical limitations, in particular the inability to distinguish paracellular from transcellular permeability across an endothelial monolayer in culture. This has confounded previous attempts to understand the mechanisms of transcytosis. An ideal candidate molecule for studying the process of endothelial transcytosis is albumin, the most abundant plasma protein, which is a key determinant of oncotic force, thus is actively transported between blood vessels and the interstitial space, through endothelial cells. It is well established that albumin leakage into to the interstitium leads to edema formation during states of inflammation (Mehta, Bhattacharya, Matthay, & Malik, 2004) Albumin is also being actively explored as a drug delivery tool due to its ability to bind diverse ligands and improve their pharmacokinetics (e.g. the insulin analogue Detemir) (Kratz, 2008; Larsen et al., 2016). Understanding the mechanisms of how albumin transcytosis leaves the circulation is therefore likely to have implications for the study of tissue edema distinctly for paracellular leak, and for the development of more efficient drug delivery to tissues. The objective for my Master's thesis project was to delineate the mechanism and kinetics of transcellular transport of albumin through microvascular endothelium derived from differential tissue beds. I hypothesize that due to the vast heterogeneity in the endothelium from differencing tissue beds, it is likely that the mechanism of albumin transcytosis varies as well. To test this hypothesis, I will undertake the following two specific aims:

Aim 1: Compare the kinetics and affinity for albumin transcytosis in pulmonary and dermal derived endothelial cells

Given the propensity for edema formation in the human lung, I selected human pulmonary microvascular endothelial cells (HPMECs) as an initial model to begin studying albumin transcytosis (Mehta et al., 2004; Parker et al., 2006). Another tissue known to be susceptible to edema formation during injury and inflammation is the skin (Aukland et al., 1984; Senger et al., 1983; Wufuer et al., 2016), thus I selected human dermal microvascular endothelial cells (HDMECs) as the second endothelial cell type to study albumin transcytosis. In line with previous literature on the permeability of pulmonary endothelial cells (Mehta et al., 2004; Parker et al., 2006; Shen, Ham, & Karmiol, 1995; Tiruppathi et al., 1997), I predicted that HPMECs would exhibit increased rates of albumin transcytosis due to the contributions of both receptor-dependent and –independent modes of uptake. Whereas, due to the more localized nature of skin edema and the impervious structure of the skin, I rationalized that the dermal microvascular endothelial cells would transcytosis albumin relatively to a lesser extent.

In order to compare the kinetics of albumin transcytosis in the HPMECs and HDMECs, I will use an assay developed within my lab utilizing total internal reflection fluorescence (TIRF) microscopy that allows for the quantification of exocytosis of transcytosed ligands by single cells in a confluent endothelial cell monolayer (Armstrong et al., 2015). Specifically, I will begin by conducting a dose response assay using fluorescently-tagged albumin, and compliment this experiment with a competition assay using unlabeled serum albumin to assess the affinity of both cell types for albumin transcytosis; the measurements will be made through the TIRF assay. I will attempt to verify results obtained from the TIRF assay, using a modified version of standard transwell assay used for studying transcytosis.

Aim 2: Identifying a receptor which mediates albumin transcytosis in human microvascular endothelial cells

Internalization of a molecule forms the first step in transcytosis, thus a better understanding of how albumin is internalized will shed more light on the mechanism of transcytosis, and potentially provide a target for manipulating this process. Molecules can be

internalized into cells through receptor-independent and receptor-dependent pathways. Previous studies have reported that albumin uptake by rat lung microvascular endothelial cells mainly occurs through a receptor-independent manner, specifically by fluid-phase internalization (John et al., 2003). As a first step in identifying an endothelial receptor for albumin, I want to confirm whether albumin internalization in human lung endothelial cells also occurs by fluid phase internalization, and also test whether it is also the case for dermal endothelial cells. This will be tested by using the chemical inhibitor of micropinocytosis (fluid-phase internalization), amiloride, and the TIRF assay. The existence of a specific receptor involved in albumin internalization by endothelial cells was reported in studies from two decades ago implicated Glycoprotein 60 (Gp60), as the receptor of albumin in endothelial cells, however, no genetic sequence has been identified for this receptor, and no recent studies have been able to confirm the old data on this receptor (Schnitzer et al., 1988; Tiruppathi et al., 1997). Furthermore, no other receptor in endothelial cells for albumin has been investigated to date. There are however, several other albumin binding-proteins and receptors that have been identified in other cell types. We predict there must exist a receptor, or even multiple receptors, other than Gp60, which mediate albumin internalization in endothelial cells. The focus will be on the scavenger receptor CD36, a receptor recently shown to bind albumin in kidney epithelial cells (Baines et al., 2012), thus it may also play a role albumin uptake in endothelial cells.

CHAPTER 3: CD36 MEDIATES ALBUMIN TRANSCYTOSIS BY DERMAL BUT NOT LUNG MICROVASCULAR ENDOTHELIAL CELLS – ROLE IN FATTY ACID DELIVERY

3.1 Introduction

Albumin is the most abundant protein in the circulation and is also found in large amounts in lymph. Its molecular radius (Tojo & Kinugasa, 2012) precludes its passage between adjacent cells of an intact endothelial monolayer, which limits paracellular diffusion to molecules less than 3-5 nm in size (Bundgaard, 1984; Michel & Curry, 1999). However, the detection of albumin in interstitial and lymphatic fluid (Aukland et al., 1984; Rutili & Arfors, Karl, 1977) – to as much as 40-60% of the level in plasma - indicates that the protein is nonetheless capable of leaving the lumen of the microvasculature, even in the absence of inflammation. Pioneering work by Palade and colleagues established that albumin injected into the circulation of animals was later detected in *intra*cellular capillary endothelial vesicles (Millici et al., 1987); in some cases, these vesicles appeared to be releasing the albumin into the interstitium while it was never observed crossing inter-endothelial junctions. This vesicular transport process, termed transcytosis, is mediated by caveolae; importantly, knockdown or deficiency of caveolin-1 – which ablates caveolae - prevents albumin transcytosis (Miyawaki-Shimizu et al., 2006; Schubert et al., 2001). Thus, under physiological conditions, transcytosis of albumin is thought to be the dominant route for albumin transport out of the circulation (Millici et al., 1987).

To date however, little is known about its regulation; this is attributable in part to technical difficulties in its study. In particular, when measuring endothelial permeability by cultured cells it has been challenging to distinguish the contribution of paracellular leakage from *bona fide* transcytosis (Armstrong et al., 2012). Most work has focused on the lung endothelium, which has been reported to bind to albumin via the gp60 receptor in caveolae (Schnitzer et al., 1988; Tiruppathi et al., 1997); however, this low-affinity binding contributes only modestly to transcytosis in the lung, which is instead dominated by fluid phase uptake (John et al., 2003).

Despite its description approximately 30 years ago, no molecular reagents for gp60 appear to be commercially available and it is possible that alternative receptors for albumin may exist on the endothelium, particularly outside of the lung. In fact, essentially nothing is known about albumin transcytosis in non-pulmonary tissues including the skin. Given the marked heterogeneity of endothelial cells supplying different organs (Aird, 2007a, 2007b), it is plausible that the mechanisms of albumin transcytosis are distinct between different tissues. There is now growing interest in delineating the mechanisms of albumin kinetics in the circulation given its potential utility as a drug carrier (Larsen et al., 2016).

Another reason for the paucity of research on albumin transcytosis by endothelial cells is likely due to uncertainty as to its physiological importance; its purpose remains obscure. While mice deficient in caveolin-1 possess no caveolae and exhibit decreased endothelial internalization of albumin, these animals also display compensatory endothelial paracellular leakage (Schubert et al., 2002). Thus it is not possible using this model to draw conclusions about the physiological function of albumin egress from the microcirculation by transcytosis. Given its numerous binding sites for fatty acids (Berde et al., 1979), one possibility is that albumin transcytosis is important for the regulated traffic of circulating fatty acids to downstream tissues. It is noteworthy that deficiency of albumin is associated with elevated circulating cholesterol and phospholipid levels (Baldo et al., 1983; Cormode et al., 1975).

To elucidate the mechanisms of endothelial transcytosis, we recently devised a single-cell assay that uses total internal reflection fluorescence (TIRF) microscopy and automated image analysis using a MATLAB script (Armstrong et al., 2015; Kuebler et al., 2016) TIRF uses an evanescent wave generated by total internal reflection to illuminate just the proximal 100 nm or so of the cell; thus, we can selectively image the basal membrane of a live endothelial cell with minimal confounding from the overlying cytoplasm and apical surface. Briefly, confluent endothelial monolayers are exposed to a fluorophore-tagged ligand at the apical cell surface while the basal membrane of the cell is imaged by TIRF. Cytoplasmic vesicles undergoing exocytosis with the basal membrane are directly visualized and quantified. This approach is not affected by paracellular gaps between endothelial cells in the monolayer (Armstrong et al., 2015) and is quantitative, thereby being well suited to mechanistic studies. Using this and

complementary *in vitro* and *in vivo* methods, we now systematically compare the transcytosis of albumin by primary human dermal and lung microvascular endothelial cells.

For the first time, we identify CD36 as a receptor capable of mediating albumin transcytosis by the skin but not the lung endothelium. In keeping with these findings, mice deficient in endothelial CD36 exhibit decreased basal vascular permeability to albumin in the skin but not in the lung. Furthermore, these mice exhibit decreased amounts of subcutaneous adipose tissue despite having similar circulating fatty acid levels and similar total body weight. These findings suggest that CD36 mediates endothelial transcytosis of albumin in the skin where its purpose is to regulate fatty acid delivery.

3.2 **Methods**

Cell Culture

Primary human microvascular endothelial cells of dermal origin (HDMECs) were a obtained from the lab of Dr. May Ho at the University of Calgary. The cells were isolated from discarded human foreskin as previously described (Gillrie et al., 2007) and were used throughout the study. Primary human microvascular pulmonary endothelial cells (HPMECs) were purchased from Lonza (HMVEC-L, CC-2527, Basel, Switzerland). All endothelial cells were cultured in EGM-2^{TM} BulletKit^{TM} medium (Lonza, Switzerland), grown in 37°C at 5% CO_2, and were used from passages 3 to 8. CHO cells were a gift from the lab of Dr. Gregory Fairn (Toronto), and were cultured in DMEM/HAM'S F-12 50/50 mix with L-Glutamine (MULTICELL, Wisent Bioproducts), supplemented with 10% fetal bovine serum and penicillin/streptomycin. The cells were sub-cultured every 5-7 days using trypsin, and the medium was changed every 2–3 days.

Total Internal Reflection Fluorescence (TIRF) Assay

Total internal reflection fluorescence (TIRF) microscopy images were acquired on an Olympus cell TIRF Motorized Multicolor TIRF module mounted on an Olympus IX81 microscope (Olympus, Hamburg, Germany). Samples were imaged using a ×150/1.45 objective with 491 nm excitation lasers and Volocity acquisition software. Unless otherwise indicated, the penetration depth was set at 110 nm. For each cell, 150 TIRF images were acquired at a frame rate of 10 per second for a constant duration of 15 s, generating a video for every cell imaged. At least 10 randomly selected cells were imaged in each experimental replicate. Endothelial cells were sub-cultured and seeded on 25 mm glass coverslips until confluency was reached (24-48 hrs post-seeding), and placed in serum-free EGM-2 media 2 hrs prior to every albumin TIRF assay. The coverslip to be imaged was placed inside an Attofluor® cell chamber (Life technologies, Catalog no. A7816). Alexa-Fluor 488 (AF488) albumin (ThermoFisher Scientific, A13100) was added to the cells which were then incubated at 4°C for 10 min to allow apical membrane-binding (Betzer et al., 2015). Cells were rinsed twice in cold PBS(+) to remove unbound albumin and fresh warm media (HEPES-buffered RPMI, i.e. HPMI) was added, along with nuclear stain NucBlue® Live ReadyProbes® (Molecular Probes, Oregon, United States).

The chamber was placed immediately on a heated (37°C) stage, and TIRF images of the basal membrane (to detect exocytosis of the apically-applied ligand) were taken after 2 min of equilibration. Images of randomly selected cells were taken every 1 minute, from 2 to 15 minutes post addition of warm HPMI. For the dose-response curves, AF488-Albumin was dissolved in RPMI to concentrations of 5-50 μg/mL, and added to coverslips of confluent HPMECs and HDMECs followed by analysis by TIRF. For the competition assay, 100-fold excess (by mass, i.e. 1000 μg/mL) and 200-fold excess (2000 μg/mL) of fatty acid-free bovine serum albumin (BSA) (Sigma, Cat. No. A7030) was dissolved in RPMI with 10 μg/mL of AF488-Albumin, this solution was then added to cells followed by analysis by TIRF.

Inhibitor Pre-treatments

To inhibit fluid phase internalization by macropinocytosis, serum-starved HPMECs and HDMECs were treated with 1 mM of amiloride hydrochloride hydrate (Sigma, Cat. No. A7410) for 20 minutes at 37°C prior to TIRF (Koivusalo et al., 2010). A 100 mM stock of amiloride in DMSO was diluted in serum-free EGM-2 media to a final concentration of 1 mM. Since amiloride is insoluble in aqueous solution, the media was heated at 45°C to ensure dissolving of the drug, before addition to the cells. Due to the reversibility of amiloride's effects, it was added during the membrane-binding step of AF488 albumin and to the warm media for imaging. To block CD36, 400 μM sulfo-N-succinimidyl oleate (SSO; Sigma) was applied to HPMECs and HDMECs (Kuda et al., 2013). Following 2 hrs of serum starvation, HPMECs and HDMECs were treated with SSO for 30 min at 37°C, immediately following which albumin transcytosis was measured by TIRF.

Quantification of TIRF Videos

Blinded and automated quantification of the transcytotic events was performed using a tracking algorithm for MATLAB as previously reported (Armstrong et al., 2015; Azizi et al., 2015). Briefly, the scripts correct the image for noise and local background using a Gaussian filter. Then putative vesicles are identified based on size (9–36 pixels^2, XY dimension, 73.5 nm/pixel), aspect ratio (>0.2; the ratio of the minor axis to the major axis) and intensity (threshold of 10% above mean image intensity). The tracking algorithm then tracks each moving vesicle based on a maximum-probability assessment of how closely those potential tracks

resemble free and super-diffusive Brownian diffusion. The resulting tracks are analyzed for the duration of the vesicle being stationary in the TIRF field (vesicle docking), the speed of vesicular movement and the degrees to which the particles' movements deviates from free Brownian diffusion (γ). Vesicles undergoing fusion with the plasma membrane (exocytosis) are identified as those having a γ significantly less than that of an equivalent model population undergoing Brownian diffusion, typically $0 < \gamma < 0.873$, and which undergo a decrease in fluorescence signal over the last two time points of their tracks equivalent to a drop of at least 2.5 standard deviations of vesicular intensity over the entire period the vesicle has been tracked. After disappearance, tracks are monitored for two additional frames to ensure they do not return.

Transwell Transcytosis Assay and ELISA

Endothelial cells were seeded on 0.1% gelatin-coated 0.4-μm-pore polyester transwells (Costar, Corning, NY) and grown until confluency for 2 days. Only monolayers that appeared healthy by phase-contrast microscopy and had a transendothelial electrical resistance (TEER; measured using Endohm-12 from World Precision Instruments, Sarasota, FL) of >19 Ωcm^2 were used for permeability experiments. Cells were changed to serum free media for 2 hrs, following which 50 and 300 μg/mL of biotin-tagged albumin (biotin-BSA; Thermo Scientific, Rockford, IL) dissolved in serum-free EGM-2 media was added to the upper chamber followed by incubation for 10 mins at 4°C to allow membrane-binding. Unbound albumin was removed by washing the cells twice then fresh serum-free EGM-2 media was added to both the upper and lower chambers of the transwell system followed by incubation at 37°C for 4 hrs. Aliquots from the lower chamber were taken for measurement of biotin-BSA by enzyme-linked immunosorbent assay (ELISA). For the ELISA, 100 μL of media from the lower chamber and 50 μL of 0.5 μg/mL of horseradish peroxidase–conjugated sheep anti-BSA (Immunology Consultants Laboratory, Inc., Newberg, OR) were incubated in 96-well plates coated with streptavidin (Thermo Scientific) at room temperature for 2 hours with shaking. After washing with buffer (25 mmol/L Tris, pH 7.2, 150 mmol/L NaCl, 0.05% Tween-20, 0.1% gelatin), 50 μL of 3,3',5,5'-tetramentylbenzidine (Thermo Scientific) was added to the well and incubated at room temperature for 15 minutes before adding 50 μL of 2 mol/L sulfuric acid. Quantification of BSA was determined by measuring absorbance at 450 nm with a ThermoMax Microplate Reader

(Molecular Devices) after verification with a standard curve using known amounts of biotin-BSA.

Albumin Internalization Assay

CHO cells transfected with wild-type GFP-tagged CD36 or the GFP-vector alone were incubated with 20 μg/mL of AF-555 albumin for 10 min at 37°C, following which cells were washed twice with PBS(+), and immediately fixed using 4% paraformaldehyde (PFA). After neutralization with glycine, coverslips were mounted on glass slides using mounting medium (Dako) supplemented with 1 ug/mL DAPI (Sigma-Aldrich). Coverslips were imaged using the spinning disk microscope by spinning disk confocal microscopy (Quorum Diskovery/Nipkow, Hamamatsu ImagEM X2 EM-CCD camera, at 63x objective, numerical aperture 1.47) using the 488 laser for GFP, and 561 nm laser for AF-555 Albumin, and acquiring z-stacks at 0.3 μm intervals; random fields of transfected cells were imaged keeping the microscope settings constant. Albumin internalization was quantified in the merged z-stacks using the puncta analysis tool in the ImageJ software (NIH); this value was normalized to the total number of GFP-transfected cells in the image.

CD36 Knockdown and mutant constructs

Depletion of CD36 in both HPMECs and HDMECs was carried out by electroporation using the Neon^{TM} Transfection System (Invitrogen by Life Technologies, Thermo Fisher Scientific Inc), and CD36 siRNA (FlexiTube GeneSolution GS948 for CD36, Cat. No. 1027416) from Qiagen (Valencia, CA, USA). The HPMECs were transfected at 1650 V for 20 ms, and with 1 pulse, the HDMECs were transfected at 1200 V for 40 ms, and with 1 pulse. TIRF experiments and cell lysis for western blotting and qPCR were performed 48 h post-transfection. To overexpress CD36 plasmids, CHO cells were transfected with WT-GFP-CD36 or indicated GFP-tagged mutants (or GFP alone) using Lipofectamine 3000 (ThermoFisher Scientific); experiments were performed 48 h later.

GFP-CD36 mutants were generated by ligation-independent cloning using the In-Fusion HD Eco Dry Cloning kit from Clontech (#639689) and verified by sequencing. All mutants were confirmed to retain targeting to the plasma membrane by fluorescent microscopy. Transfected cells were exposed to AF-555 albumin for 10 minutes, rinsed, fixed and then imaged.

Transfection efficiency, level of expression and uptake of AF-555 albumin by transfected cells was recorded and analyzed by ImageXpress Micro XLS & MetaXpress 6 (Molecular Devices) counting over 30 cells per condition in each experiment.

Macropinocytosis Assay

The macropinocytosis assay was modified from (Canton et al., 2016). Briefly, HDMECs and HPMECs plated on 25-mm coverslips were serum-starved for 2 hrs, following which they were treated with 1 mM Amiloride for 20 mins in serum-free EGM-2. Cells were then incubated with 100 μg/mL of tetramethylrhodamine-conjugated 70 kDa dextran in serum-free media at 37°C for 15 min, and then washed twice with PBS(+), and imaged immediately after addition of the nuclear stain NucBlue. Random fields of cells were then imaged by spinning disk confocal microscopy (Quorum Diskovery/Nipkow, Hamamatsu ImagEM X2 EM-CCD camera, at 63x objective, numerical aperture 1.47) using a 561 nm laser, and acquiring z-stacks at 0.3 μm intervals. Macropinosomes across all z-stacks were then counted using the puncta analysis tool in the ImageJ software (NIH), with the filter for size set to an area of 10-100 pixels^2 and circularity to 0.2-1.0. This value was normalized to the total number of nuclei in the image. Microscope settings were kept constant between conditions.

Western Blot

Cell lysates were prepared with SDS lysis buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 10 mM DTT). Equal amounts of protein were run on an SDS-PAGE using 8% polyacrylamide gels at 120 volts; proteins were transferred to nitrocellulose membranes (110 volts for 70 mins) and blocked for 1 hour with 5% milk in TBS-T. Membranes were incubated with the following primary antibodies overnight at 4°C: anti-CD36 (Santa Cruz Biotechnology: sc-7309) and anti-β-actin (Santa Cruz: sc-47778). The following day membranes were washed in TBS-T, and incubated with anti-rabbit, and anti-mouse horseradish peroxidase–conjugated secondary antibodies, respectively, at a 1/10,000 dilution for 1 hour, washed, and then visualized by enhanced chemiluminescence (Amersham), and imaged using the ChemiDoc™ Imaging System (Bio-Rad), and quantified using the ImageLab software (Bio-Rad).

qPCR

cDNA synthesis was carried out using the High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions. For each sample, RNA was reverse-transcribed using T-Gradient Thermoblock (Biometra) according to the manufacturer's directions. Q-PCR was conducted using Power SYBR Green PCR Master Mix (Applied Biosystems). cDNA was denatured at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds then 60°C for 1 minute. Q-PCR was performed with the ABI Prism 7900HT (Applied Biosystems), and the data were analyzed with SDS software v2.1 (Applied Biosystems) and Microsoft Excel 2003 (Microsoft). Relative gene expression was compared using the comparative C_T method using 18S as the reference. Primer sets used for this study are as follows: CD36: (Q-PCR) Forward 5'-TCTTTCCTGCAGCCCAATG-3'; Reverse 5'-AGCCTCTGTTCCAACTGATAGTGA-3'; 18s rRNA: (Q-PCR) Forward 5'-GATGGAAAATACAGCCAGGTCCTA-3' and Reverse 5'-TTCTTCAGTCGCTCCAGGTCTT-3'.

Mice

Male mice with an endothelial cell specific deletion of CD36 (Fl/FlCD36 Tie2eCre+; EC-CD36KO) were generated by crossing CD36 floxed mice on a C57BL6 background with C57BL6 mice expressing Cre driven by the Tie2 5'promoter and first intron enhancer element. Tie2eCre is distinct from Tie2Cre and is restricted to endothelium(Yu et al., 2014). Mice were genotyped by ear clippings and PCR using the RedExtract-N-Amp Tissue PCR Kit (Sigma, XNAT 100RXN), and primers for floxed CD36: FL1(For): 5'-CCA CAC TGT ATG GGG AAA GTT TCA GG-3', FL3(Rev): 5'-CGC CCT ATC TAG TTT CTC CAC CC-3', and the primers for Tie2Cre: (Tie2::Cre-FWD): 5'-CCC TGT GCT CAG ACA GAA ATG AGA (Tie2 FOR), (Tie2::Cre-REV): 5'-CGC ATA ACC AGT GAA ACA GCA TTG C (CRE REV) (Braren et al., 2006). Age and weight-matched male C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and used for controls. Mice were housed in the St. Michael's Hospital Vivarium on a standard light:dark cycle and were given free access to food and water. Experimental procedures were conducted in accordance with the St. Michael's Hospital Animal Care Committee guidelines and subject to approved animal protocols (ACC721/772 and ACC670).

Wet-to-Dry Ratio and Miles Assay

To measure basal vascular permeability, a modified Miles Assay was performed. After anesthesia with inhaled isoflurane and shaving of the dorsal hair, mice were injected by tail vein with 50 to 150 uL of 1% Evan's blue bye solution in 0.9% NaCl. 24 hours later, mice were sacrificed and the circulatory system was flushed with 10 mL cold PBS by instillation through the left ventricle. Both lobes of the lung was removed, patted dry, and then weighed for determination of wet weight. The dorsal skin of the mice was removed by gentle dissection and immediately weighed. The skin and lung tissues were dried for 24 hours and then weighed for the dry weight. The dried skin and lung tissue were then placed in formamide at 50°C for 72 hours to extract Evan's blue dye. The absorbances at 620 (A620) and 740 nm were measured in the extractions. Dye content was calculated by correcting A620 for heme and converted to µg/mL Evan's blue dye by comparing to a standard curve, and normalized to tissue weight and corrected for the amount of dye injected.

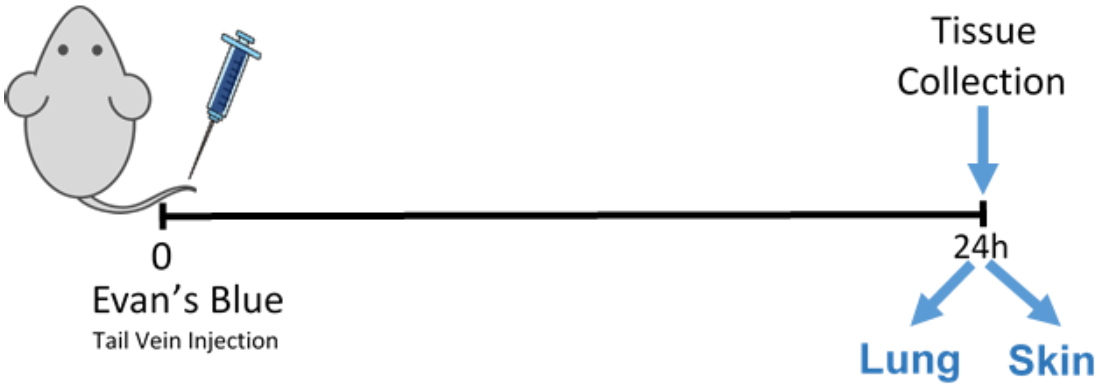


Figure 3.1 Modified miles assay experimental time-line

Immunohistochemistry

Skin samples were dissected from 5 standardized regions of the mouse dorsum (age-matched mice, 10-22 weeks old) and embedded in either paraffin wax or Optimal Cutting Tissue compound (OCT, Fisher Healthcare™). For paraffin embedding, the skin sections were fixed in 10% formalin for 2-4 days, following which they were processed and embedded in paraffin wax. For OCT embedding, fresh skin samples were snap frozen in liquid nitrogen, and immediately

placed in OCT compound, frozen and stored at -80°C until cut. The paraffin and OCT blocks of skin tissue were cut in serial 5 μm thick sections, mounted on glass slides and stained with hematoxylin and eosin.

Quantification of Subcutaneous Fat Layer Thickness

The H&E stained sections were imaged using the Nikon Upright E800 Microscope at 4X magnification, and images were acquired and analyzed using the NIS-elements software. Blinded quantification for the thickness of the subcutaneous fat layer was done using the measurement tool on the NIS-element software; 10 randomly selected, evenly distributed vertical measurements were taken across the entirety of every skin section. The 10 measurements were averaged to obtain the mean thickness of the skin sections, and this number was normalized to the area of the skin section.

Blood chemistry analysis

Animals (age-matched, 16-20 weeks old) were fasted for 12 hours before blood was sampled; serum and plasma were sent to The Centre for Phenogenomics (Toronto, Ontario), for analysis of total cholesterol, HDL, triglycerides, LDL, glucose and albumin. The measurement for blood plasma fatty acid levels was done using the NEFA-HR(2) Free Fatty Acid detection kit (Wako Diagnostics) in accordance with the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Prism 5.0; GraphPad Software Inc., La Jolla, CA, USA). Student's t-tests and one sample t-tests (GraphPad, La Jolla, CA, USA) were used to determine the significance of raw or normalized data (respectively) from two groups. A one-way ANOVA and *post-hoc* t-tests were conducted for experiment with three groups or more. All experiments were performed at least three times on different batches of cells; data are presented as mean ± SEM. At least ten cells were imaged per group in each experiment.

3.3 **Results**

Albumin transcytosis is saturable in dermal but not lung microvascular endothelial cells

Using TIRF, we compared transcytosis by primary human dermal and lung microvascular endothelial cells of Alexa 488-conjugated albumin added to the apical surface. Dermal endothelial cells exhibited a plateau in transcytosis events when the dose of fluorescent albumin was increased, while lung endothelial cells performed transcytosis at an increasing rate (Figure 1A). To validate this observation by a second method, we grew endothelial cells to confluency on transwells (Boyden chambers). Monolayers were allowed to bind biotinylated albumin in the cold, preventing internalization (Vida & Emr, 1995), then unbound ligand was rinsed away. This binding and rinsing step decreases any contribution from paracellular diffusion of unbound albumin (Armstrong et al., 2012). The cells were then returned to 37ºC and flux of biotinylated albumin across the monolayer was measured by ELISA. As with the TIRF assay, dermal endothelial cells exhibited a plateau in permeability to albumin which was not observed with the lung endothelial cells (Figure 1B). Consistent with the saturability data, competition with excess unlabelled and defatted albumin significantly attenuated transcytosis by dermal but not lung microvascular endothelium (Figure 1C). Thus, transcytosis of albumin by dermal and lung microvascular endothelial cells displays different kinetics, demonstrating saturability in the skin but not in the lung.

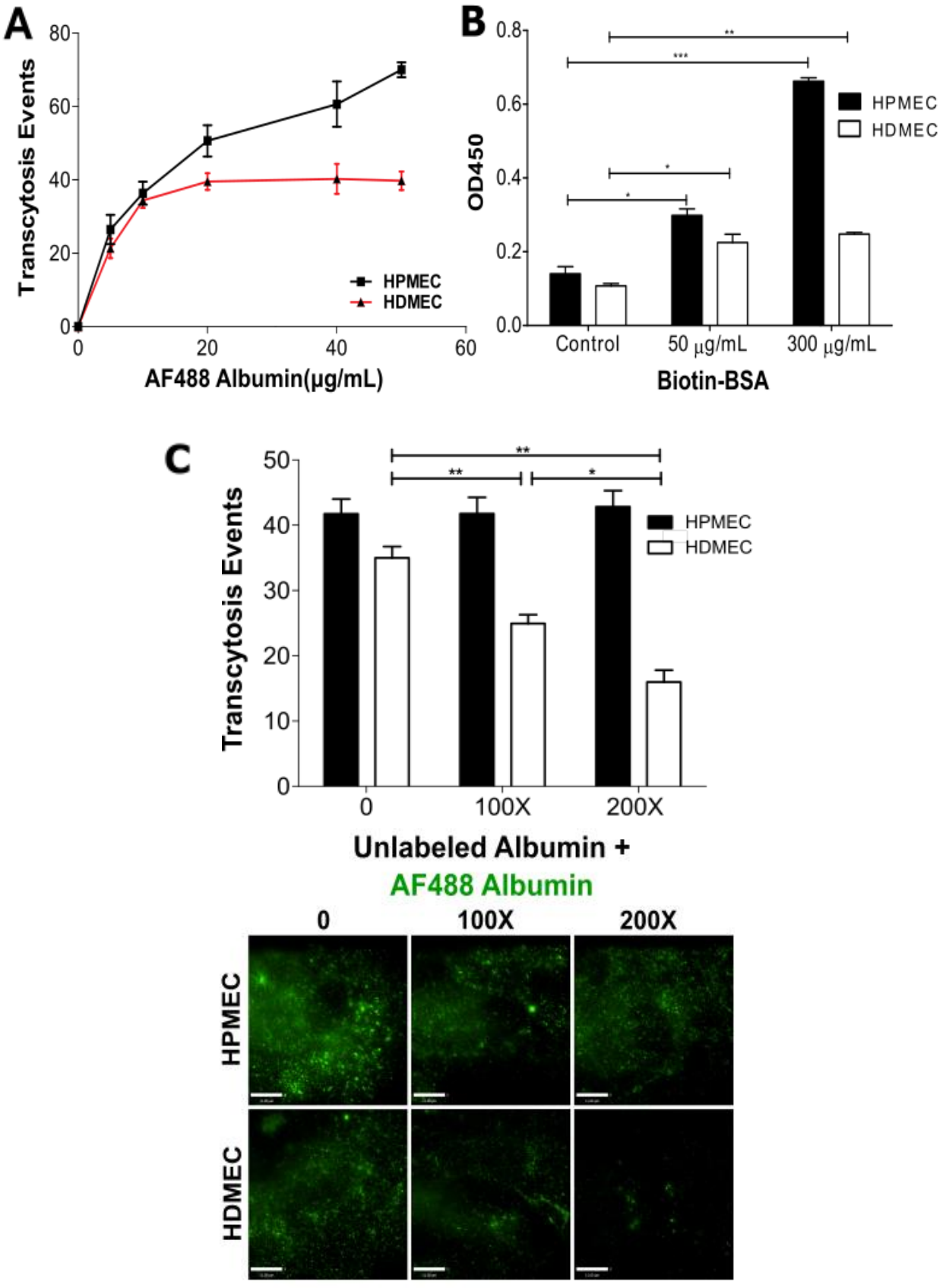

A
Transcytosis Events
AF488 Albumin(µg/mL)
HPMEC
HDMEC
B
OD450
Biotin-BSA
Control
50 µg/mL
300 µg/mL
HPMEC
HDMEC
C
Transcytosis Events
Unlabeled Albumin +
AF488 Albumin
0
100X
200X
HPMEC
HDMEC

Figure 3.2. Albumin transcytosis is saturable in dermal but not lung microvascular endothelial cells. (A) Dose responsiveness of human pulmonary microvascular endothelial cells (HPMECs; lung) and human dermal microvascular endothelial cells (HDMECs, skin) to Alexa-Fluor 488 (AF488)-Albumin transcytosis determined by TIRF microscopy (n=5 for each concentration). **(B)** Quantification albumin transcytosis by modified transwell assay using biotin-BSA and quantified by ELISA (n=3). **(C)** Competition of AF-488 albumin (10 μg/mL) transcytosis by 100-fold and 200-fold excess unlabelled and defatted BSA, representative stills shown on the right (n=3); size bar is 11 μm. 10-15 single cells were imaged for each n in **(A)** and **(C)**. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0001$.

CD36 mediates albumin transcytosis by dermal microvascular endothelial cells

The saturability and competition data suggested the presence of a receptor on dermal endothelial cells capable of mediating albumin transcytosis. The scavenger receptor CD36 is expressed on capillary endothelial cells of the skin (Davis et al., 2013), although its expression in the lung endothelium is controversial (Lisanti et al., 1994; Shen et al., 1995). Given reports that it can bind albumin in epithelial cells (Baines et al., 2012), we considered that it might contribute to albumin transcytosis. Dermal and lung microvascular endothelial cells expressed CD36 in whole cell lysates, while the receptor was absent from CHO cells (Figure 2A). Inducing its expression by transient transfection in CHO cells was sufficient to increase albumin internalization well over two-fold. Knockdown of CD36 by siRNA in the dermal endothelial cells depleted the protein by almost 80% and reduced albumin transcytosis by almost 40%, relative to scramble siRNA (sc Sc) (Figure 2B). In contrast, a 55% reduction in CD36 in the lung endothelial cells had no effect on albumin transcytosis. Thus, CD36 is both necessary and sufficient for albumin internalization and transcytosis.

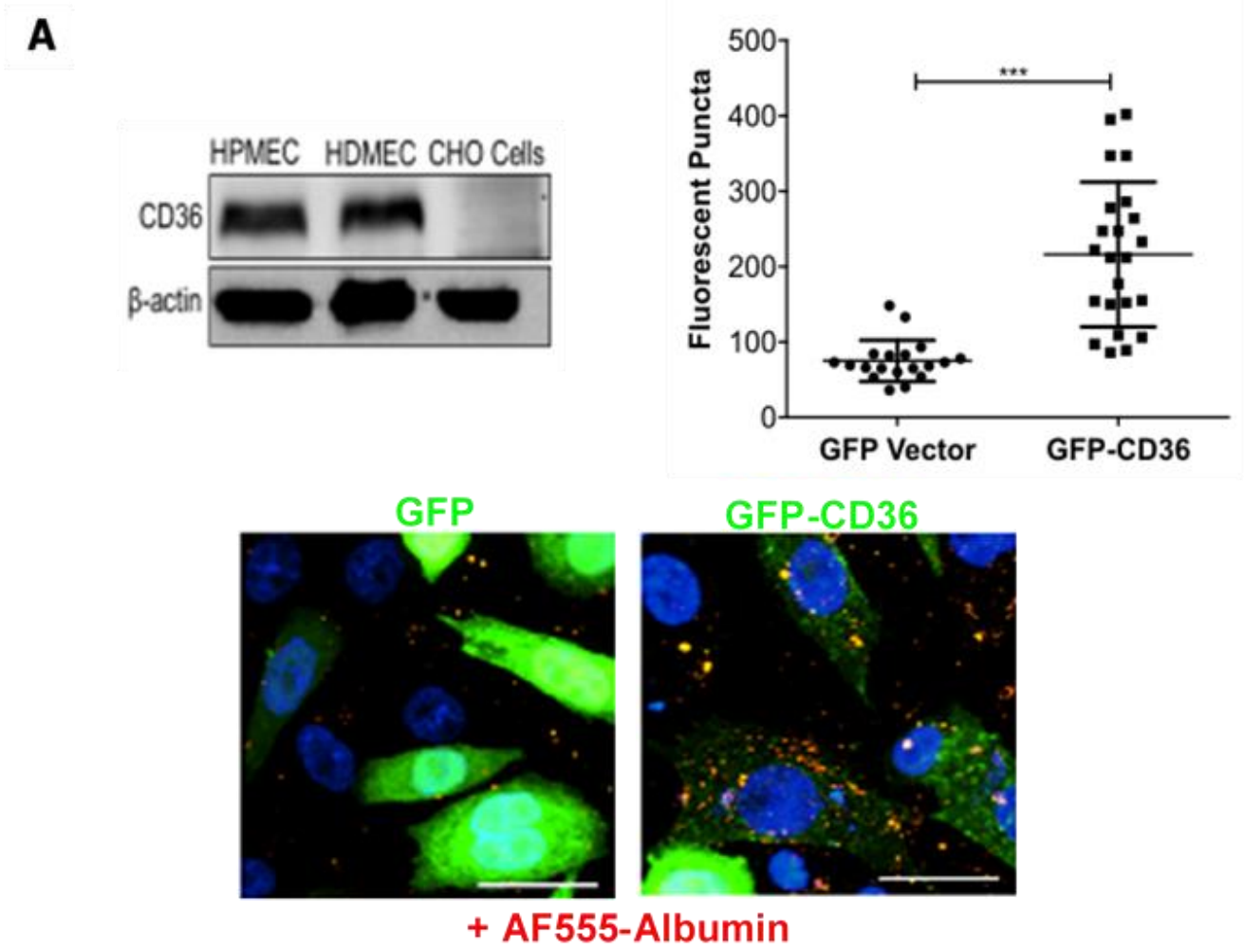
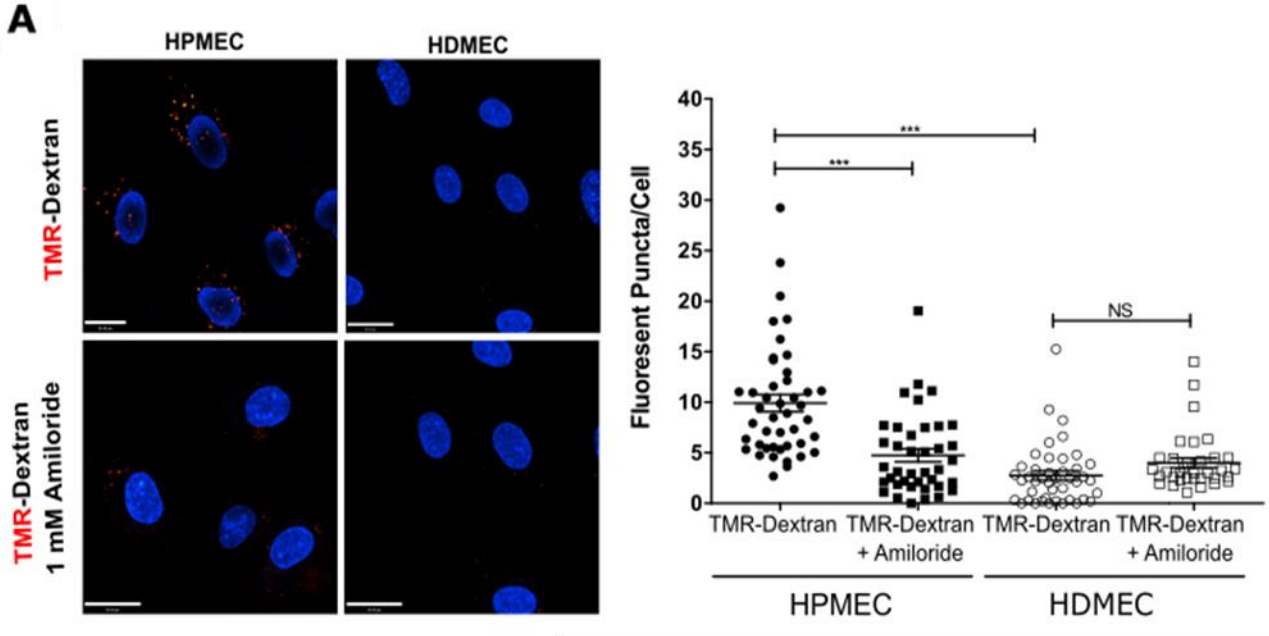

A
HPMEC
HDMEC
CHO Cells
CD36
β-actin
Fluorescent Puncta
500
400
300
200
100
0
***
GFP Vector
GFP-CD36
GFP
GFP-CD36
+ AF555-Albumin
B
HDMEC
HPMEC
Relative Expression
Transcytosis Events
2.0
1.5
1.0
0.5
0.0
50
40
30
20
10
0
*
***
NS
si Sc
si CD36
CD36
β -Actin

Figure 3.3. CD36 mediates albumin transcytosis by dermal microvascular endothelial cells. **(A)** Western blot showing expression of CD36 (88 kD) in HDMECs (skin) and HPMECs (lung) but its absence in CHO cells (left panel). Transfection with GFP-CD36 significantly increases internalization of Alexa 555-conjugated (AF555) albumin (middle and right panels); n=3; 8 randomly acquired images for each n; size bar is 20 μm. **(B)** Knockdown of CD36 expression by siRNA significantly reduces Alexa 488-conjugated (AF488) albumin transcytosis in HDMECs, **(C)** but not HPMECs. Knodown was assessed using western blot analysis and qPCR (data not shown) (n=3; 10-15 cells were imaged for each n). *$p < 0.05$; ***$p < 0.0001$; NS = Not significant.

Pinocytosis contributes to albumin transcytosis in lung but not dermal microvascular cells

It was intriguing that while both skin and lung microvascular endothelial cells express abundant amounts of CD36, the receptor appears to perform albumin transcytosis only in the skin. We reasoned that a high rate of constitutive pinocytosis by lung endothelial cells might outweigh the contribution of plasmalemmal CD36 to albumin internalization. To test this notion, we incubated skin and lung endothelial cells with 70 kDa rhodamine-conjugated dextran to measure macropinocytosis (Canton et al., 2016). As a control, we incubated the cells with amiloride, which inhibits macropinocytosis by lowering the submembranous pH. Lung endothelial cells internalized several-fold more dextran than skin endothelial cells, and this was significantly attenuated by amiloride (Figure 3A). Accordingly, while amiloride significantly inhibited albumin transcytosis by lung endothelial cells, it had no effect on the dermal microvascular endothelium (Figure 3B). Thus, it is likely that a high basal rate of macropinocytosis obviates a meaningful contribution of CD36 to albumin transcytosis in the lung.


A
HPMEC
HDMEC
TMR-Dextran
TMR-Dextran
1 mM Amiloride
Fluoresent Puncta/Cell
40
35
30
25
20
15
10
5
0
***
***
NS
TMR-Dextran
TMR-Dextran
+ Amiloride
TMR-Dextran
TMR-Dextran
+ Amiloride
HPMEC
HDMEC

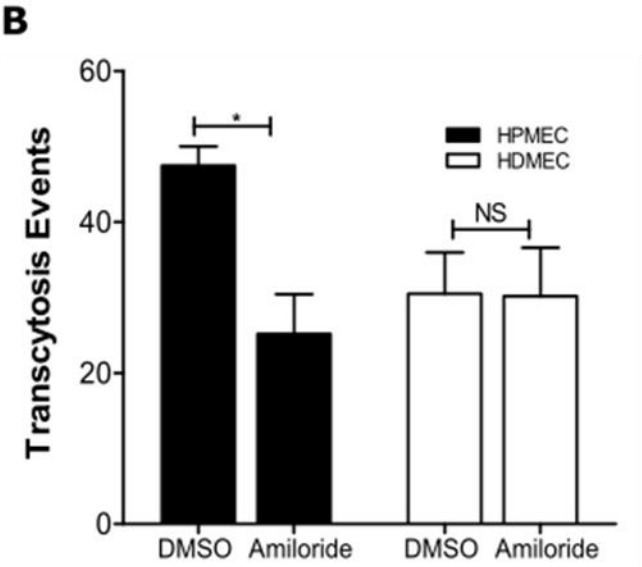

B
Transcytosis Events
60
40
20
0
*
NS
HPMEC
HDMEC
DMSO
Amiloride
DMSO
Amiloride

Figure 3.4. Pinocytosis contributes to albumin transcytosis in lung but not dermal microvascular cells. (A) HPMECs (lung) perform more macropinocytosis relative to HDMECs (skin) determined by internalization of 70 kDa tetramethylrhodamine(TMR)-dextran (red); amiloride is a known inhibitor of macropinocytosis (n=3); nuclei are stained with NucBlue (blue). Scatterplot shows the quantification of TMR-dextran internalization (punctae) normalized to the number of cells per field (n=3; 10 randomly-acquired fields for each n). **(B)** Inhibition of macropinocytosis by amiloride attenuates AF488-albumin transcytosis in HPMECs but not HDMECs, as measured by TIRF; DMSO is the solvent control (n=3; 10 single cells were imaged for each n). *$p < 0.05$; ***$p < 0.0001$; NS = Not significant.

SSO and CD36 mutants define a putative binding region for albumin

Sulfo-N-succinimidyl oleate (SSO) binds irreversibly to lysine 164 in the extracellular loop of CD36, inhibiting its binding to long-chain fatty acids and oxidized LDL (Kuda et al., 2013). Incubation with SSO for 30 minutes significantly attenuated albumin transcytosis by dermal microvascular endothelial cells but had no effect on the lung endothelial cells (Figure 4A). These data suggested that the binding site for albumin was likely overlapping with that reported for long-chain fatty acids and other ligands. To test this by a more specific approach, we generated GFP-tagged CD36 mutants spanning the ligand-binding extracellular domain of the protein and verified that none of the mutations interfere with delivery of the receptor to the plasma membrane (not shown). We next transfected the mutant CD36 constructs into CHO cells, taking advantage of the lack of endogenous receptor; the cellular internalization of Alexa-555-conjugated albumin by transfected cells was then compared. Mutations in the alpha-helical bundle near the apex of the protein (Neculai et al., 2013) (amino acid residues 153, 154, 156, 166) significantly decreased albumin uptake relative to the wild-type receptor (Figure 4B), despite their unimpaired localization to the plasma membrane (Figure 4C). In contrast, a mutation just outside of the putative ligand-binding region (amino acid residue 78) had no effect on albumin internalization.

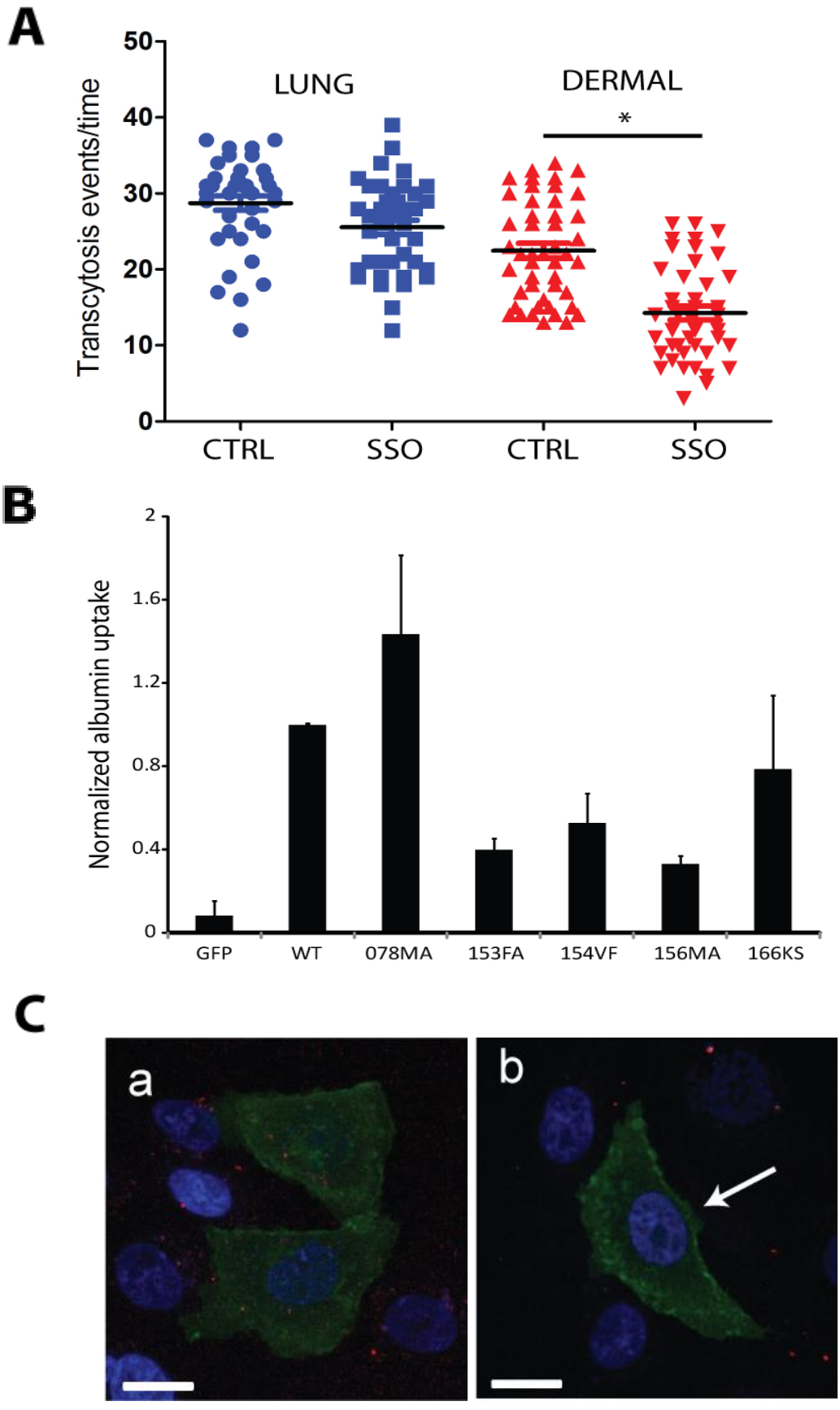
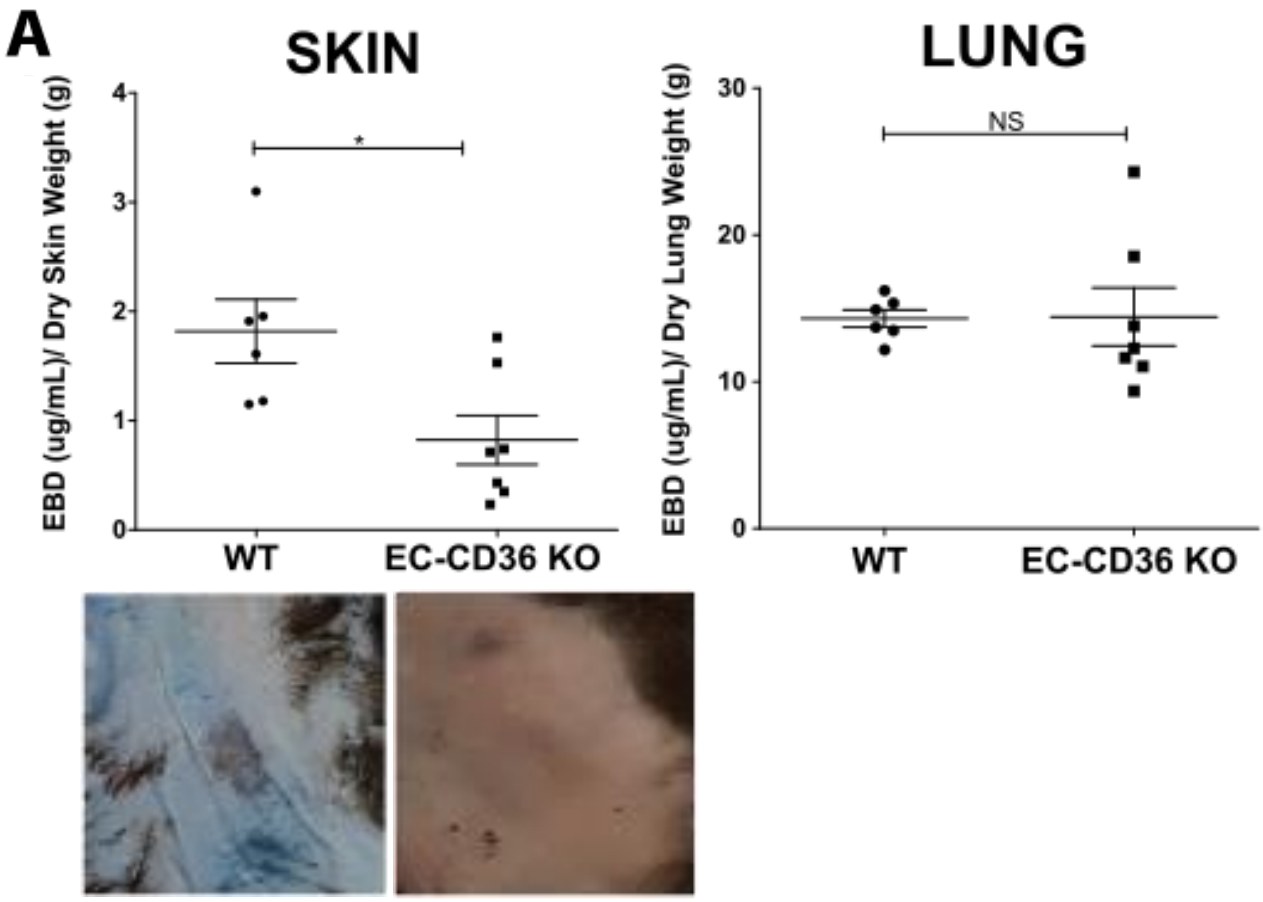

A
Transcytosis events/time
50
40
30
20
10
0
LUNG
DERMAL
*
CTRL
SSO
CTRL
SSO
B
Normalized albumin uptake
2
1.6
1.2
0.8
0.4
0
GFP
WT
078MA
153FA
154VF
156MA
166KS
C
a
b

Figure 3.5. SSO and CD36 mutants define a region important for albumin internalization. **(A)** HPMEC (lung) and HDMEC (dermal) were incubated with Sulfo-N-succinimidyl oleate (SSO, 400 μM) or solvent control for 30 minutes prior to measurement of albumin transcytosis by TIRF; data are from three independent experiments with each point representing one TIRF video, *$p < 0.05$; **(B)** CHO cells were transfected with GFP vector alone or GFP-tagged wild-type (WT) or mutant CD36 constructs (mutated amino acid residue as indicated on the x-axis) and allowed to internalize Alexa-Fluor 555-conjugated albumin for 10 minutes. Internalization of albumin was quantified and corrected for transfection efficiency and level of expression; n = 4-7 independent experiments per construct with > 30 transfected cells counted per experiment. Data are normalized to WT CD36-GFP and are presented as mean and SEM; $p < 0.01$ by one-way ANOVA; **(C)** Representative images of CHO cells transfected with WT CD36-GFP (***a***) or the 156MA (***b***) mutant; note persistent targeting of the CD36 mutant construct to the plasma membrane (white arrow) and relative paucity of internalized albumin (red). Scale bar is 15 μm.

Endothelial-specific loss of CD36 leads to decreased albumin permeability and tissue edema in the skin

To determine whether our in vitro findings could be replicated in vivo, we obtained mice deficient in CD36 specifically in the endothelium (EC-CD36KO) and performed a modified Miles assay. In this procedure, Evans Blue dye is injected into the circulation where it binds tightly to albumin. Extravasation of Evans Blue-bound albumin is often used as an indicator for increased endothelial leakage in response to an inflammatory stimulus (Patterson et al., 1992). By injecting Evans Blue into healthy wild-type and EC-CD36KO mice – and without administering any inflammatory mediator – we measured the flux of albumin out of the circulation under basal conditions. In this setting, transcytosis is thought to account for the majority of albumin efflux from the blood (Millici et al., 1987). We observed significantly less Evans Blue accumulation in the skin of EC-CD36KO mice compared to wild-type; importantly, this difference was not observed in the lungs (Figure 5A). As albumin is the main contributor of oncotic force and edema formation, we then measured the wet-to-dry ratio for the skin and lungs of the animals. While the wet-to-dry ratio in lungs was the same between knockout and wild-type mice, it was significantly lower in the skin of the EC-CD36KO animals (Figure 5B). Thus, CD36 contributes to albumin transcytosis across the endothelium both in vitro and in vivo, in mice.


A
SKIN
EBD (ug/mL)/ Dry Skin Weight (g)
4
3
2
1
0
*
WT
EC-CD36 KO
LUNG
EBD (ug/mL)/ Dry Lung Weight (g)
30
20
10
0
NS
WT
EC-CD36 KO

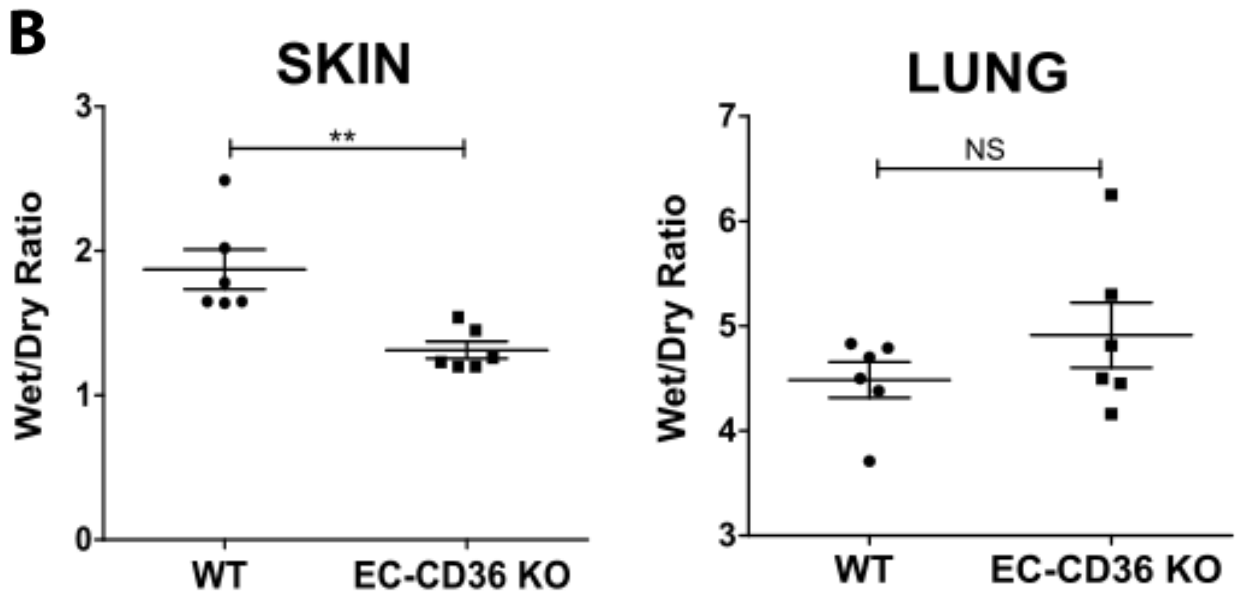

B
SKIN
Wet/Dry Ratio
3
2
1
0
**
WT
EC-CD36 KO
LUNG
Wet/Dry Ratio
7
6
5
4
3
NS
WT
EC-CD36 KO

Figure 3.6. Endothelial-specific loss of CD36 leads to decreased albumin permeability and tissue edema in the skin. (A) Fl/FlCD36 Tie2-Cre+ (EC-CD36 KO) and age-matched C57BL6 WT mice were injected intravenously with 1% Evans Blue dye (EBD); 24 hours later, the accumulation of dye in the skin (left) and lungs (right) was compared. Dye is normalized to dry weight of tissue (n=6 for WT mice, n=7 for EC-CD36 KO mice); photographs display representative images of shaved dorsal skin region of mice 24 hours after EBD. **(B)** The basal tissue fluid content was determined by calculating the wet-dry ratio; note the lower ratio in the skin but not the lungs of the EC-CD36 KO mice relative to WT (n=6 for WT mice, n=6 for EC-CD36 KO mice). *$p < 0.05$; **$p < 0.005$; NS = Not significant. Each point represents one animal.

Endothelial-specific loss of CD36 leads to decreased fat deposition in the skin

Given that albumin possesses multiple binding sites for fatty acids (Curry, Mandelkow, Brick, & Franks, 1998), we hypothesized that albumin transcytosis might play an important and as-of-yet undescribed role in fatty acid metabolism. Blinded pathological examination of the skin of age-matched mice revealed a significantly thinner subcutaneous fat layer in the knockout mice (Figure 6A), despite indistinguishable circulating fatty acid and albumin levels (Figure 6B-C). The difference in subcutaneous fat thickness was not due to a lower total body weight, as both groups of animals displayed similar body weights and weight gain over time (Figure 6D). Circulating triglycerides tended to be lower in the KO mice but this was not statistically significant; LDL and HDL levels were unchanged (Figure 6E). Thus, deficiency in albumin transcytosis is associated with decreased deposition of subcutaneous fat despite similar circulating fatty acid levels. This suggests a role for albumin transcytosis in the traffic of circulating fatty acids to downstream tissues such as the skin.

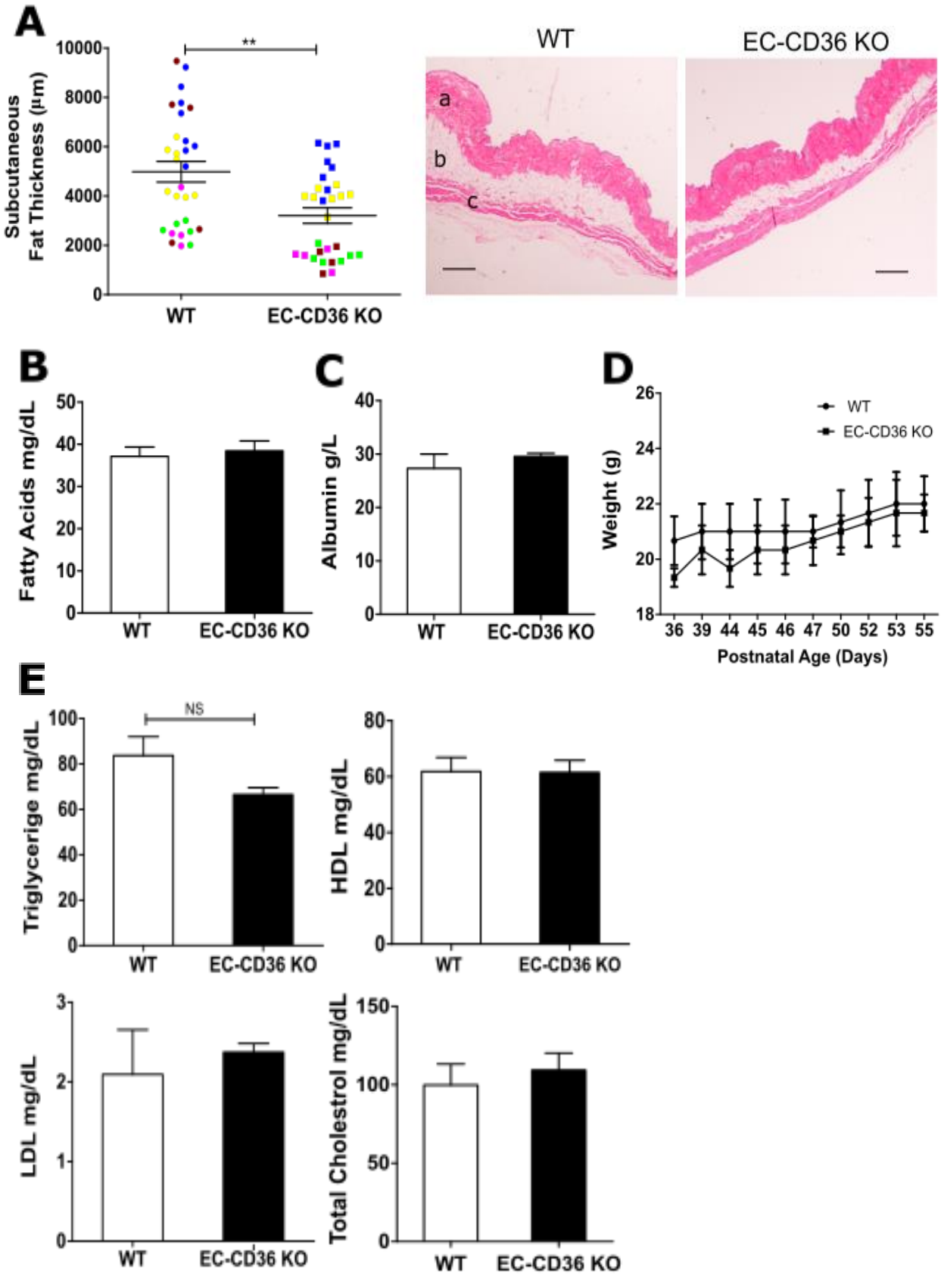

A
Subcutaneous Fat Thickness (µm)
10000
8000
6000
4000
2000
0
**
WT
EC-CD36 KO
WT
EC-CD36 KO
a
b
c
B
Fatty Acids mg/dL
50
40
30
20
10
0
WT
EC-CD36 KO
C
Albumin g/L
40
30
20
10
0
WT
EC-CD36 KO
D
Weight (g)
26
24
22
20
18
WT
EC-CD36 KO
36 39 44 45 46 47 50 52 53 55
Postnatal Age (Days)
E
Triglycerige mg/dL
100
80
60
40
20
0
NS
WT
EC-CD36 KO
HDL mg/dL
80
60
40
20
0
WT
EC-CD36 KO
LDL mg/dL
3
2
1
0
WT
EC-CD36 KO
Total Cholestrol mg/dL
150
100
50
0
WT
EC-CD36 KO

Figure 3.7. Endothelial-specific loss of CD36 leads to decreased fat deposition in the skin. **(A)** H&E-stained dorsal skin sections (5 μm) from EC-CD36 KO and age/weight-matched C57BL6 mice were measured for thickness of the subcutaneous fat layer; a=epidermis, **b=**subcutaneous fat, c=muscle; data reflect sections from 5 mice in each group, colors represent age-matched mice (Blue = 22 weeks, Yellow = 20 weeks, Green, Pink and Red = 12 weeks. Size bar is 1000 μm. **(B-C)** Circulating free fatty acid levels and serum albumin in EC-CD36 KO and WT mice. **(D)** Total body weight in EC-CD36 KO and WT mice measured over two weeks (n=3 for each). **(E)** Plasma triglycerides, HDL, LDL, and total cholesterol levels in EC-CD36 KO and WT mice (n=3 per group); $^{**}p < 0.005$; NS = Not significant.

3.4 Discussion

The regulation and the purpose of albumin transcytosis by the endothelium has remained a largely unanswered question in vascular biology. Serial electron microscopic (EM) studies in animals have demonstrated that intracellular vesicles transport albumin from the vascular lumen to the interstitial space, but while elegant, this approach is not well suited to addressing mechanistic questions. Work-to-date has reported that a receptor named gp60 mediates low-affinity binding of albumin by the lung endothelium (Tiruppathi et al., 1997), but also showed that most transcytosis of albumin in this organ is performed via fluid-phase uptake (John et al., 2003). Almost nothing is known about the mechanisms of albumin transcytosis in other organs. Organ-specific mechanisms of endothelial permeability are not without precedent (Aird, 2007a, 2007b); endothelial cells from different tissue beds are extremely heterogeneous, with the same receptor exhibiting different downstream signaling in different vascular beds (Fung et al., 2017; Saddar et al., 2010).

Using two independent approaches, we report for the first time that albumin transcytosis by human dermal microvascular endothelial cells is saturable, suggesting a receptor-mediated process. In contrast (and as reported by others (John et al., 2003)), albumin transcytosis by lung microvascular endothelial cells is not saturable and is dominated by fluid-phase uptake. We extend these observations to report a novel role for the scavenger receptor CD36 in albumin transcytosis. The receptor is expressed in high levels at the endothelial membrane and its transfection into CHO cells - which lack endogenous CD36 - is sufficient to increase albumin uptake. Depletion or pharmacologic inhibition of CD36 in dermal (but not lung) microvascular endothelial cells significantly attenuates albumin transcytosis. Finally, mice deficient in CD36 in endothelial cells exhibit reduced accumulation of Evans Blue in the skin, confirming a reduction in basal albumin traffic.

CD36 is a transmembrane protein composed of a single extracellular loop with two relatively short cytoplasmic tails (Rac, 2007). It is expressed on numerous cell types, including phagocytes, myocytes and capillary endothelial cells (Febbraio et al., 2001); for the purposes of our study, it is interesting to note that it is absent from dermal lymphatics (Hawighorst et al., 2002). Several putative ligands have been described, including collagen, oxidized LDL (Febbraio

et al., 2001) and long-chain fatty acids (Ibrahimi & Abumrad, 2002). We now add albumin to this list; the specificity of the interaction is supported by the observation that *defatted* albumin significantly attenuated albumin transcytosis, effectively excluding confounding by fatty acids. While kidney epithelial cells have been reported to bind to albumin via CD36 (Baines et al., 2012), to our knowledge, this is the first description of it mediating endothelial albumin transcytosis.

The CD36 inhibitor SSO binds irreversibly to lysine 164 (Kuda et al., 2013) in the extracellular loop in a region that has been reported to interact with long-chain fatty acids (Baillie et al., 1996). Our data using both SSO and mutants of CD36 with single codon substitutions in the apical helical domain (Neculai et al., 2013) suggest that this region is also important for binding or for internalizing albumin. Almost nothing is known about post-receptor signaling during albumin transcytosis. Binding of oxidized LDL to CD36 initiates Src-kinase dependent signaling with effects on MAP kinase activation and focal adhesion kinase (Silverstein et al., 2010); whether albumin binding and transcytosis by CD36 requires a similar or a distinct signal transduction cascade remains to be elucidated.

The binding of CD36 to long-chain fatty acids has been a focus of much research, with mutations in the receptor being associated with defects in fatty acid utilization by adipose tissue, the myocardium and other tissues (Coburn et al., 2000). CD36 has been postulated to bind to fatty acids at the cell surface, facilitating their integration into the cell by a "flip-flop" mechanism. However, non-esterified fatty acids cannot circulate freely in plasma and are bound to albumin (Peters, 1977). As albumin possesses up to 5 binding sites for medium or long-chain fatty acids (Curry et al., 1998), rather than simply binding fatty acids at the cell surface, CD36 and albumin likely serve to shuttle fatty acids out of the vasculature in a regulated fashion. Our observation that mice selectively deficient in endothelial CD36 exhibit reduced subcutaneous fat is therefore intriguing, given that circulating lipid levels were comparable to control animals and both groups displayed similar total body weights. This would be consistent with a defect in lipid transport or metabolism at the interface between the endothelium and the skin. Consistent with this hypothesis, analbuminemic rats exhibit marked hypercholesterolemia (Nagase et al., 1979) while patients congenitally deficient in albumin display elevated cholesterol and serum phospholipid concentrations (Baldo et al., 1983; Cormode et al., 1975) which return to normal

transiently following intravenous albumin infusions. While deficiency of or mutations in CD36 have not previously been linked to a phenotype in the skin (Lin & Khnykin, 2014), previous studies have examined the effect of global deficiency of CD36 rather than endothelial-cell specific deletion; this may have confounded the phenotype.

Lastly, our data also suggest that additional receptors are likely to contribute to albumin transcytosis. Several candidates exist, although little is known about their tissue distribution or downstream signaling (Merlot et al., 2014). The development of a quantitative and sensitive assay for albumin transcytosis in primary endothelial cells using TIRF microscopy (Armstrong et al., 2015; Batchu et al., 2016; Kuebler et al., 2016) may facilitate their study. There is growing interest in using albumin as a drug carrier (Merlot et al., 2014), taking advantage of its abundance in the circulation and its long half-life. It is evident that understanding the mechanisms of albumin transcytosis in different tissues is likely to be critical to the success of this approach.

In conclusion, we report a novel role for the CD36 receptor in mediating albumin transcytosis across human dermal microvascular endothelial cells. In mice, loss of endothelial CD36 is associated with decreased dermal fat and diminished albumin traffic out of the circulation, suggesting a potential function for albumin transcytosis in fatty acid metabolism.

CHAPTER 4: CONCLUSION, GENERAL DISCUSSION, AND FUTURE DIRECTIONS

4.1 General Discussion and Future Directions

Despite the vast abundance of albumin in the blood and the essential physiological functions it serves, the mechanism surrounding its transport in and out of circulation under basal physiological conditions remains poorly understood. Albumin has long been known to serve two essential physiological functions: one as regulator of fluid balance between capillary beds and the interstitium, and as a transporter of fatty acids in the blood (Peters, 1995). However, why a complex intracellular process has evolved to remove it from the circulation and deposit it in the interstitium remains unexplained. More recently, the transport capacity and stability of albumin in circulation is being harnessed by pharmaceutical manufacturers to carry drug compounds to treatment sites in the body (Larsen et al., 2016). In order to reach its target tissues, whether for its endogenous function or its bioengineered role, albumin must first exit the circulation and enter the intestinal space. Microvascular endothelial cells serve as the primary barrier for macromolecules in the blood, including albumin, to accessing the underlying tissue. While under disrupted physiological states, the endothelium sustains damage, allowing fluid and albumin to freely seep into the interstitium through paracellular gap formation between adjacent endothelial cells. Under basal physiological conditions however, the gaps between neighbouring endothelial cells are far too narrow to allow for albumin to pass. Instead, albumin is actively internalized into vesicles by endothelial cells and transcytosed through to the opposing end of the cell, and released into the interstitial space (Palade, 1960; Tuma & Hubbard, 2003). Though it is widely accepted that basal albumin transport out of blood vessels occurs through transcytosis, there is very little research on how the process of transcytosis occurs, and what key proteins are involved (Bern et al., 2015). Early studies implicated Gp60, as a potential receptor for albumin in endothelial cells, but some have also suggested fluid phase internalization as a method of internalization. Without clarity on how albumin is taken up, we cannot accurately understand the mechanism of transcytosis (Bern et al., 2015). Moreover, to date no study has taken into account the heterogeneity of ECs across different vascular beds, a factor which would likely add variability in the mechanism by which albumin is transcytosed.

4.1.1 BENEFITS AND LIMITATIONS OF A TIRF MICROSCOPY BASED ASSAY FOR STUDYING TRANSCYTOSIS

The mechanistic study of albumin transcytosis is impeded by technical limitations associated with distinguishing transcytosis from its counterpart, paracellular transport. Our lab has developed a novel assay using the powerful imaging technique of TIRF microscopy, to visualize in real-time the basal membrane exocytosis of ligands that have been internalized at the apical cell membrane (Armstrong et al., 2015). This methodology allows for the study of transcytosis at a single cell level, thereby minimizing the confounding effect of any paracellular leak due to damage in the endothelial cell monolayer. Damage to EC monolayers is highly prevalent and almost unavoidable during *in vitro* studies, especially those involving chemical treatments and transfection of cell culture. Using this valuable imaging technique I have shown that indeed the mechanism of albumin transcytosis varies depending on the tissue bed from which the endothelial cells originate. The data presented above strongly suggests that human lung ECs internalize albumin through fluid phase internalization, and thereby transcytose albumin to a much greater degree than human skin ECs. Human skin ECs instead uptake and transcytosis albumin more restrictively through a receptor, with our data implicating CD36. No other study to date has identified CD36 as a receptor for albumin in endothelial cells, so in this present study I have attempted to diligently validate its capacity to internalize albumin using both *in vitro* and *in vivo* methods.

One limitation of the TIRF microscopy based transcytosis assay is that the concentration of fluorescently-tagged albumin that is allowable through this technique is well below the physiological concentration of albumin, approximately 1000-fold lower. The extremely high physiological concentrations of HSA (35-50 g/L), make replicating these concentrations during sensitive *in vitro* studies very challenging, whether through microscopy based techniques or through standard transwell based assays. The use of the transwell assay in Figure 1B partially addresses the issue associated with the limited range in albumin concentrations allowable by the TIRF assay. It is important to highlight that I used a modified transwell assay; by allowing for membrane binding and rinsing of the biotin-tagged-albumin, the likelihood of measuring paracellular leak is minimized. The modified transwell assay allowed us to test albumin concentrations six-fold greater than those allowable by TIRF microscopy. Another potential

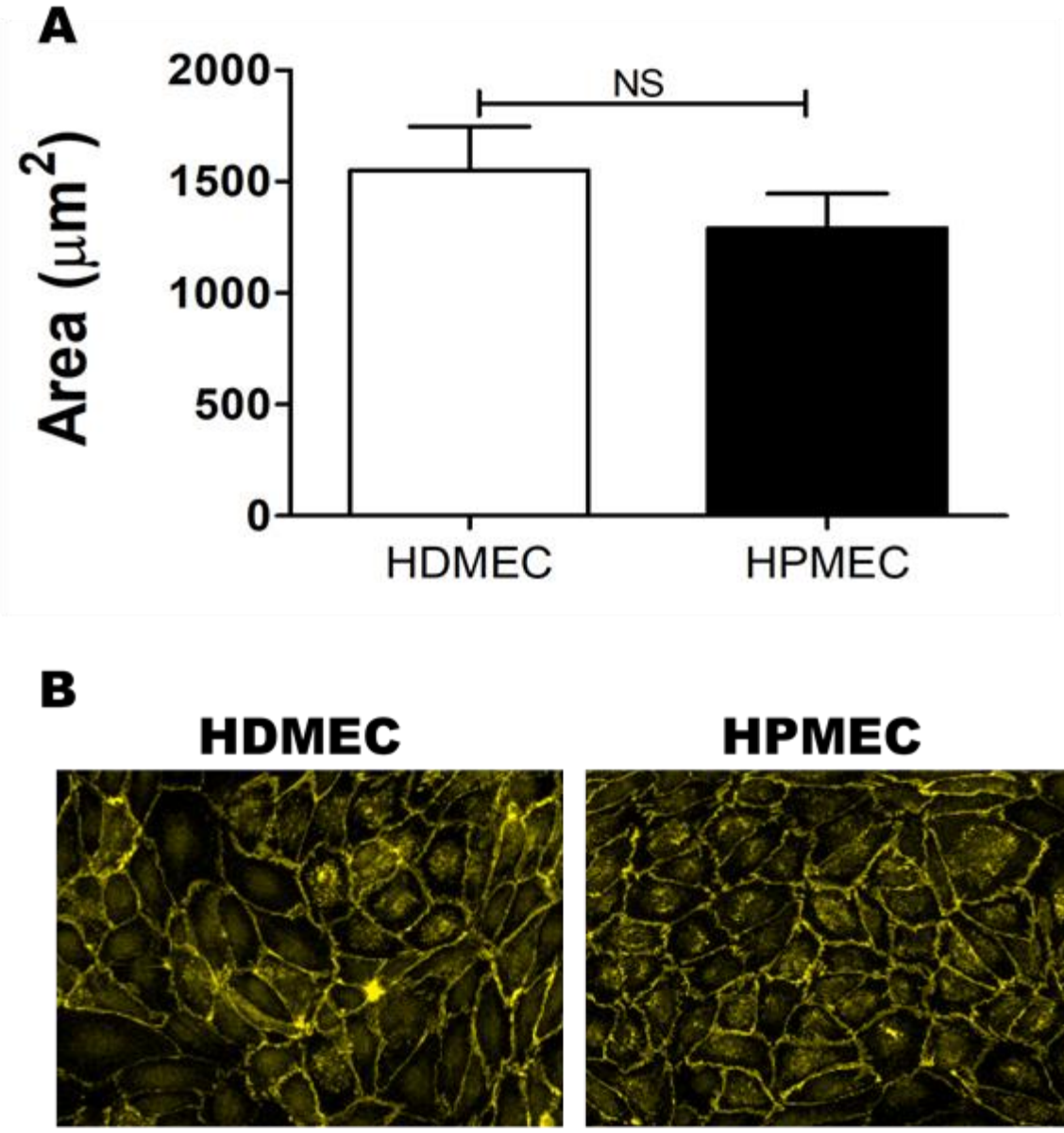
confounding factor in the TIRF microscopy assay is the use of fluorescently tagged albumin, which has been shown to possess different physiochemical properties as compared to its unlabeled counterpart (Bingaman et al., 2003; Reeves et al., 2012). Labelling of albumin has been shown to alter its molecular weight, relative molecular charge and isoelectric point, which in turn may affect its affinity, rate of internalization and transport through the microvascular endothelium (Bingaman et al., 2003). Different fluorophores are known to possess different levels of stability, phototoxicity, and photobleaching. Most microvascular research in the past has relied on fluorescein isothiocyanate (FITC) for fluorescent labelling due to its low cost and wide availability (Bingaman et al., 2003; Reeves et al., 2012). However, a study comparing the efficacy of different fluorescent probes as labels for BSA has reported that FITC labelling can alter the physiochemical properties of BSA, while also being significantly more susceptible to photobleaching (Bingaman et al., 2003). Due to the automated quantification of the algorithm, the photobleaching of a fluorophore can potentially be confused as true transcytosis events, leading to inaccuracies in interpreting the results. For this reason, in this study we were careful in selecting the type of fluorescent label on albumin, and opted to use Alexa-Fluor tagged albumin for the TIRF assay. Alexa-fluor labels are generally thought to be much more photostable, brighter in fluorescence and experience less photobleaching (Mahmoudian et al., 2011; Panchuk-Voloshina et al., 1999). Furthermore, in order to ensure that the MATLAB algorithm was successfully able to distinguish true transcytosis events from the photobleaching of fluorophores in the quantification of TIRF videos, I tested the effect of varying the laser powers during the experiment. By increasing the laser power, I increased the bleaching of the fluorescent albumin signal; however this did not alter the frequency of transcytosis events detected by the algorithm (data not shown).

4.1.2 ACCOUNTING FOR ENDOTHELIUM HETEROGENTIY AND MORPHOLOGICAL VARIABILITY *IN VIVO*

Despite being unable to test physiologically relevant concentrations of albumin, the *in vitro* findings of this study begin to shed light on endothelial heterogeneity in the mechanism of albumin transcytosis. With extensive amount of literature showing morphological variations in endothelial cells across vascular tree, I wanted to ensure that saturability of HDMECs as

compared to HPMECs is not simply due to the former cell type being significantly smaller in size than the latter. By measuring the surface area of both HPMECs and HDMECs it was verified that observed difference in the saturability of albumin transcytosis could not be attributed to morphological differences in the endothelial cell size (**Supplementary Figure 1**).

In addition to irregularities in endothelial cell morphology, there are other physiological variables that must be taken into consideration when interpreting *in vitro* results. These physiological variables include the effects of laminar shear stress from blood, and the presence of a charged glycocalyx.Some studies have shown that the composition of the endothelial glycocalyx layer is significantly altered when ECs are isolated from the vessels and grown in culture (F. E. Curry & Adamson, 2012; Kolářová et al., 2014). The likely explanation for the change in the glycocalyx structure is a lack of shear stress force on the surface of ECs under *in vitro* conditions. The shear stress of fluid flowing over ECs is known shown to modify the glycocalyx architecture and thickness, and even modify the cellular cytoskeleton (Bai & Wang, 2012; F. E. Curry & Adamson, 2012; Pahakis et al., 2007; Potter et al., 2009; Potter & Damiano, 2008; Yao et al., 2007). Under *in vivo* conditions ECs are exposed to the constant forces of flowing blood on their apical surface, which has been shown to act as a mechanical stimuli that affects intracellular signaling within ECs thereby altering their phenotype and activity (Li, Haga, & Chien, 2005). Studies evaluating this phenomenon have found that both the strength and duration of shear stress exposure affect EC physiology differently (Li et al., 2005). In my study all *in vitro* experimentation was done under static conditions, thus we do not know the effect of shear stress on the kinetics of albumin transcytosis. For future experiments, it may be interesting to expose cells in culture to shear stress in biological flow chambers, and perform the TIRF assay . Certain disadvantages of *in vitro* studies cannot be overcome, such as changes in the glycocalyx, and the absence of pericytes and connective tissues that surround the basal membrane of ECs (Aird, 2007a, 2007b). Moreover, primary ECs experience general phenotypic drift when grown in culture. For example it has been shown that number of caveolae present in ECs decreases with increasing passaging of cells in cell culture (Stan, 2005).



Supplemental Figure 1. There is no apparent difference in the size of HPMECs and HDMECs. (A) The average surface area of skin and lung ECs grown in a confluent monolayer as measure by ImageXpress Micro system. **(B)** Confluent monolayer of HDMECs and HPMECs stained for VE-cadherin to outline the plasma membrane and measurements were taken of the cell surface area using ImageXpress Micro system, (Data is mean ± SEM; ns = no significance; n=3)

4.1.3 VALIDATION OF CD36 AS A RECEPTOR FOR ALBUMIN AND FUTURE INVESTIGATIONS

The *in vivo* work done in this study on endothelial-specific CD36 knockout mice allows us to validate some of the *in vitro* findings in a physiologically relevant model. Using this line of knockout mice I have been able to show that CD36 is involved in albumin transport across the endothelium across the skin microvasculature, but not in the lungs. Interpretations of both the modified miles assay and the wet-to-dry ratio measurement also appear to support the *in vitro* kinetics data for albumin transcytosis. The lungs evidently have a significantly greater basal wet-to-dry ratio as compared to the skin (Figure 3.6), suggesting that there may be tighter regulation of and/or generally less albumin transport through the skin endothelium in comparison to the lungs. This falls in line with the dose response and competition assay results seen in isolated HPMECs and HDMECs through TIRF microscopy.

The idea that albumin transcytosis is a receptor mediated process was first proposed by Ghitescu *et al*., in 1986, when they showed that the binding of gold-labeled albumin to the endothelial cell surface was saturable and competable(Ghitescu et al., 1986). Future studies used similar kinetic data to indentify gp60 as the receptor for albumin in ECs (John et al., 2003; Schnitzer, 1992; Tiruppathi et al., 1996), although to date the relevance of this supposed receptor has not been studied in the lung or skin microvasculature. Thus I also predicted that the tendency of albumin transcytosis to be saturated and be competed by unlabeled albumin in skin ECs suggests the requirement of a receptor, in contrast to the lung microvascular endothelium. There are many studies in lung microvascular endothelial cells from animals such as bovine, mouse, and rat, that have demonstrated albumin endocytosis occurs in fluid-phase. To date however no study has tested the significance of macropinocytosis in human pulmonary ECs (John et al., 2003; Li et al., 2013; Minshall et al., 2000, 2002; Vogel et al., 2001). Due to the obvious differences in animal and human cell models, it is essential to validate the findings from these earlier studies in human lungs ECs (Poussin et al., 2014; Rhrissorrakrai et al., 2015). In this present study we are able to validate the predeminance of macropinocytosis in albumin internalization in primary human lung microvascular ECs. I was able to show this both through the competition assay, and by using an established inhibitor of macropinocytosis, amiloride. The ability of amiloride to inhibit albumin transcytosis in HPMECs but not in HDMECs is consistent

with the observation that cells' rates of macropinocytosis vary greatly between cell types depending on the metabolic needs and functional role of the cell (Kerr & Teasdale, 2009; Swanson & Watts, 1995). Through a standard macropinocytosis assay I have shown that the proclivity for fluid-phase internalization varies even between ECs derived from different tissue beds. As would be predicted, lung ECs displayed a significantly greater macropinocytosis index. Thus, even if CD36 mediates albumin internalization in lung ECs, fluid-phase internalization likely overpowers the limited amounts of albumin internalized through a receptor-mediated route. The higher rate of fluid-phase transport through pulmonary ECs and into the lung interstitium may help to explain the higher wet-to-dry ratio of lung tissue as compared to the skin.

Studying the receptor mediated internalization of albumin in skin ECs proved to be significantly more challenging then proving the fluid-phase route of albumin internalization in lung ECs. Initial attempts to overexpress and knockdown CD36 in ECs proved to be difficult. Transfection of lung and dermal ECs with wild-type CD36 tagged with GFP proved to be toxic to the cells with a transfection period greater than 12 hours (data not shown). Transfection periods less than 12 hours were insufficient to induce protein expression. It is possible the overexpression of CD36, even transiently, increases fatty acid transport into ECs such that it can lead to lipid toxicity. Accumulation of excess fatty acids in the cells can lead to a metabolic burden, and promote the generation of harmful lipid metabolites such as toxic ceramides(Goldberg & Bornfeldt, 2013). This is why CHO cells proved to be essential in determining that CD36 expression is sufficient to enhance albumin uptake. CHO cells have been used extensively in studies on CD36 function, due to their lack of endogenous CD36, they make ideal models for overexpression experiments (Eyre et al., 2008; Ohgami et al., 2001). The most direct proof of the requirement of CD36 for albumin uptake comes from loss of function experiments, including knockdown using siRNA and inhibition through SSO treatment. Though in this present study I have presented strong evidence for CD36-mediated albumin uptake and transcytosis in skin ECs, a few control experiments are necessary in order to support this finding conclusively.

Firstly, it would be useful to compare the cell-surface expression and distribution of CD36 on HDMECs and HPMECs. This can be done through immunostaining permeabilized and

non-permeabilized ECs, and by staining the cells with an antibody targeted to the extracellular domain of CD36. A greater expression of CD36 on the surface of HDMECs, rather than in intracellular stores could explain the greater involvement of receptor mediated albumin internalization. It would also be beneficial to determine whether CD36 and albumin co-localize within the same cellular region in both HDMECs and HPMECs, whether on the cell membrane or intracellularly. Likewise, immunostaining for CD36 and determining co-localization with albumin is also essential for skin and lung microvessels and whole tissue from CD36 knockout mice and compare it to samples from wild-type controls. This can be done by either isolating ECs from lung and skin tissue samples by standard microbead pulldown technique targeted to ECs, alternatively it can be done in intact microvessels. Experimenting on the microvasculature *in vivo* and *ex vivo* has long been considered technically challenging, due to difficulties in visualizing, isolating and handling these microscopic vessels, especially those of mice. However, recent advances in intravital microscopy have allowed researchers to begin visualizing biological processes at the molecular level in the organs and microcirculation of live animals (Masedunskas et al., 2014). A recent study by Schiessl *et al*. used intravital microscopy to measure albumin transcytosis through podocytes and into the urinary space (Schiessl et al., 2016). They injected fluorescently labelled albumin into circulation of rats through either the right jugular or femoral vein, along with a dye to stain for podocytes and endocytic vesicles. Simultaneously they imaged the exposed kidney of the animal using an extremely high powered and specialized confocal fluorescence microscope, and observed co-localization of the albumin in podocytes and intracellularly in endocytic vesicles (Schiessl et al., 2016). Another study used intravital microscopy to study the extravasation of fluorescently labelled albumin from post-capillary venules in the cremaster muscle of wild-type mice, as well as Cav-1 knockout mice (Chen et al., 2012).

As demonstrated by the publications mentioned above, future experimentation using intravital microscopy can be extremely beneficial and readily applicable to further enhance our present study. Through this microscopy technique we can inject live mice with fluorescent antibodies for CD36 and albumin, and image the lung and skin tissues to observe for interactions *in vivo*. The extent of co-localization and rate of transcytosis between wildtype and knockout mice can also be compared through this method. It would also be interesting and prudent to immunostain the lung and skin tissue in the CD36 knockout mice for albumin deposition in order

to compare whether dysfunction in endothelial of transport of albumin affects its storage in the underlying tissue bed. Staining for albumin in organs can be done both in live animals by intravital imaging or in isolated tissue samples by standard immunohistochemistry. Intravital microscopy could also be used to simply monitor the rate and kinetics of fluorescent-albumin extravasation from the microvessels into lung or skin interstitium in live animals. This could allow for the study of this process under real physiological conditions. It also allows us to overcome the limitation of sub-physiological concentrations of albumin used in *in vitro* transcytosis assays, such as transwells and TIRF.

Another logical avenue to further this research would be investigate whether CD36 serves as receptor for albumin in ECs from other tissue beds. As mentioned in the above the heart, skeletal muscle and adipose tissue are major consumers for fatty acids delivered by albumin (**see subsection 1.3.3**). This it is possible, even likely, that CD36 may play a role in albumin uptake and transcytosis in the endothelial cells from the microvasculature of these organs. Knockdown and inhibition of CD36 in ccoronary, skeletal muscle, and adipose endothelial cells is an easy first step to begin addressing this question. This can be preceeded by *in vivo* experimentation in CD36 knockout mice to look at albumin transport in these other organs. The brain endothelium may be another interesting and physiologically relevant site which can be investigated for CD36 mediated-albumin transport. The interaction between CD36 and albumin may serve to explain the mechanism of fatty-acid transport across the blood-brain-barrier, and may be beneficial for albumin-based drug delivery to brain. Another logical site to evaluate for a CD36 and albumin interaction is the renal endothelium, due to the kidneys' role in the filtration, reabsorption and excretion of albumin. Since the ability of CD36 to bind albumin was first observed in the epithelial cells of the proximal convoluted tubule where it facilitates its reabsorption, it is conceivable that it may be expressed on renal endothelial cells, and help in facilitating transport between capillaries and the kidneys.

Lastly, an obvious and important next step in further validating the role of CD36 as an albumin receptor in the skin is to determine the role of lymphatic clearance of albumin in the skin and lungs, especially in the case of CD36 knockout mice. Variabilities in the rates of lymphatic clearance serves as a potential cofounding factor in interpreting the results of the Miles Assay. The knockout of CD36 may also effect albumin transport in lymphatic ECs.

However, it is not likely that it directly modulates lymphatic clearance, as two independent studies have reported that lymphatic endothelial cells do not express CD36 (Cursiefen et al., 2011; Hawighorst et al., 2002). The study by Hawighorst *et al*. showed specifically that CD36 was absent in the skin lymphatic vessels (Hawighorst et al., 2002). Nonetheless, an absence of CD36 in the blood microvessels could indirectly lead to lymphatic dysfunction. If the lymphatic clearance rate in the skin of CD36 knockout mice tends to greater than that of the lung, then it could explain the reduced dye accumulation relative to wild-type mice. Intravital microscopy would also serve useful in this case, by allowing for the measurement of fluorescent albumin disappearance rates from the skin and lung of the CD36 knockout mice and observe its accumulation into the lymphatic vessels. A study from 2013 by Kilarski *et al*., developed an intravital microscopy based technique to study the dynamic behaviour of immune cells, the role of microcirculation, and the lymphatic vessel drainage, using mouse ear skin as a model (Kilarski et al., 2013). This technique can be readily applied to studying albumin transport between blood vessels, interstitial spaces and lymphatic vessels in many different skin locations.

4.1.4 CHARACTERIZING DOWNSTREAM SIGNALING IN CD36 MEDIATED ALBUMIN INTERNALIZATION

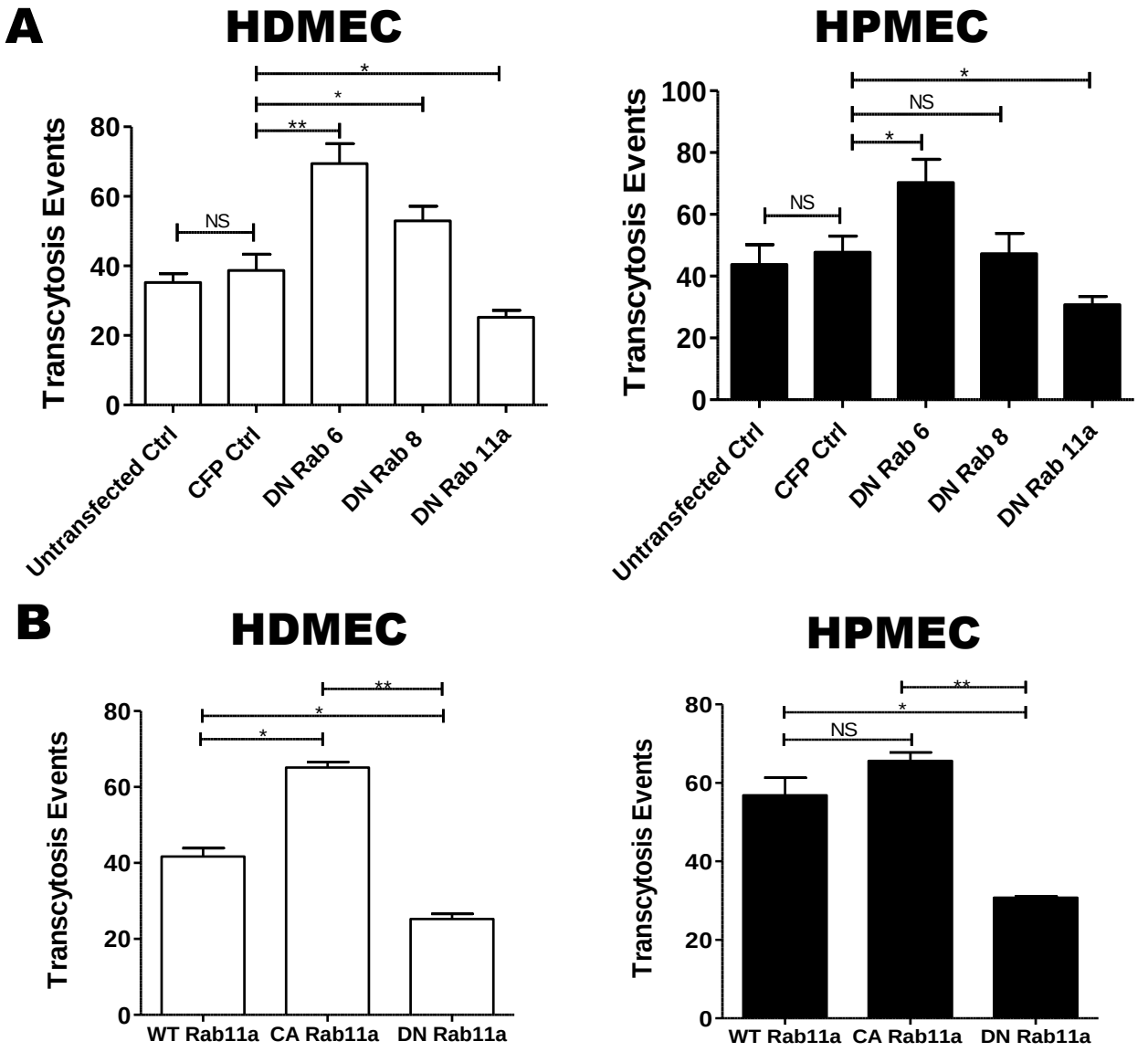
In this study I have shown that CD36 is likely involved in albumin uptake by dermal ECs, and this is represents the initiating step of transcytosis, and determines how much albumin is exocytosed. We also have data showing the potential binding site of albumin on the CD36 structure (**see Figure 3.5**). A logical primary step in further characterizing this interaction would be to evaluate the signaling cascade that triggers the internalization and transcytosis of the bound albumin. In spite of the multiple signaling pathways activated by CD36, there is a gross lack of understanding in how this receptor with a small intracellular domain, no intrinsic kinase or phosphatase activity, no known intracellular scaffolding domains, and no direct link to GTPases, transduces the variety of signals it receives. Regardless of the ligand binding which triggers the signal cascade, common mediators in transduction of signals from CD36 have been identified through pharmacological and genetic experiments. Specific to endothelial CD36 signaling pathway the proteins involved are the Src family kinases, tyrosine kinase Fyn, the mitogen-activated protein kinases (MAPKs) p38 and c-Jun N-terminal kinase (JNK), and caspase-3

(Jiménez et al., 2000). The family of Src kinases serve a common protein mediator in the downstream signaling of both CD36 and the initiation of caveolae mediated endocytosis (Shajahan, Timblin, et al., 2004; Tiruppathi et al., 1997). The initial studies implicating gp60 as the receptor for albumin showed that upon activation the receptor was associated with Src and Fyn, in addition to other proteins (Minshall et al., 2000; Tiruppathi et al., 1996). The activity of these kinases triggers a phosphorylation cascade which leads to internalization of albumin into the caveolae, and the subsequent fission (Minshall et al., 2000; Tiruppathi et al., 1997).

Due to the overlapping pathways between caveolae mediated transcytosis and CD36 signaling, it would be reasonable to begin mechanistic characterization by looking at the role of Src kinases, Fyn and Lyn. Initial experiments could involve co-precipitation of CD36 with these kinases, following albumin binding and comparing the levels of phosphorylated versus total Src and Fyn. An additional simple set of experiments could be the chemical inhibition for any of the above mentioned mediators on ECs and conduct a TIRF assay to determine the effects on albumin transcytosis. Similarly, using chemical inhibition or knockdown of the Src, Fyn or any other protein downstream to CD36, in CHO cells overexpressing CD36, and seeing if and how albumin uptake is altered. For example by treating CHO cells expressing CD36 with Dyngo4a, a known inhibitor of dynamin (Mccluskey et al., 2013), and measuring fluorescent albumin uptake the direct role of dynamin can be identified in albumin uptake. A similar experiment has been published previously by our lab in a study on the role of dynamin and Rac in the co-regulation of transcellular and paracellular transport in microvascular ECs (Armstrong et al., 2012). Additionally, very little is known in regards to how CD36 signaling affects cytoskeletal reorganization necessary for trafficking internalized ligands throughout the cell. It is well-known that that the motor proteins myosin, kinesin and dynein, and the remodeling of actin microfilaments and microtubules are required for the transcytosis process (Mehta & Malik, 2006), though their exact interactions and functions in albumin uptake, trafficking and exocytosis are not well elucidated. The involvement of these cytoskeletal proteins can be investigated by treating ECs with inhibitors of microfilament and microtubule polymerization and measuring albumin transcytosis and uptake.

Another potential downstream mediator of transcellular transport that has great potential to be involved with albumin transport are the ubiquitous Rab family of proteins. Due to the

numerous isoforms of these GTPases, and with many identified as being expressed in ECs, we selected a few members of this family of proteins as a sensible starting point. I have preliminary data showing the involvement of Rabs 6, 8 and 11 in albumin transcytosis in lung and skin ECs (**Supplementary Figure 2**). Rab6 has been implicated in regulating endosome trafficking through the Golgi apparatus. Rab8 is involved with vesicle trafficking from the Golgi or Endoplasmic reticulum to the plasma membrane exocytosis, and Rab11 is known to regulate slow endocytic recycling (Hutagalung & Novick, 2011). By transfecting HPMECs and HDMECs with dominant negative (DN) mutant plasmids (generously gifted by Dr. John Brumell) tagged with cyan fluorescent protein (CFP), I have shown that defective Rab6 and Rab 8 activity increases albumin transcytosis in HDMECs, whereas disrupted Rab11a function leads to a decrease. The same was true for HPMECs, with the exception of Rab8, a mutation in which appears to have no effect on albumin transport. I also show that a constitutively active (CA) mutant of Rab11 enhances albumin transcytosis in HDMEs, while having no effect in HPMECs. It would be worthwhile to further examine the involvement of Rabs in albumin transport as it may represent an alternative method by which this process can be manipulated.



Supplemental Figure 2. Rab proteins are involved in albumin transcytosis. (A) Quantification of albumin transcytosis via the TIRF assay on cell transfected with CFP-tagged dominant negative (DN) Rab 6, 8, and 11 plasmids; statistical analysis conducted against cells transfected with the CFP vector. **(B)** Quantification of albumin transcytosis via the TIRF assay on cell transfected with CFP-tagged wild-type (WT), constitutively active (CA), and dominant negative (DN) Rab11 plasmids; statistical analysis conducted against cells transfected with the WT Rab11. HDMECs data in white graphs and HPMECs in black (Data is mean ± SEM; *p < 0.05; **p < 0.005; ***p < 0.0001; NS = Not significant; n=3 for all graphs). The Rab plasmids were a generous gift by Dr. John Burmell.

4.1.5 EVALUATING OTHER POTENTIAL RECEPTORS FOR ALBUMIN IN ENDOTHELIAL CELLS

In order to determine the identity of the receptor for albumin in skin ECs, we used a candidate-based approach and relied on previous literature and deductive reasoning. I conducted some preliminary experiments with candidates in addition to CD36, including megalin-cubilin and FcRn. Due to challenges in overexpressing and downregulating these other candidates in primarily endothelial cells, I had to abandon them due to time constraints. With advancements in transfection systems and the common use of genome editing technique such as CRISPR/Cas9, it is possible in prospective investigations on albumin transport to evaluate the FcRn or the megalin-cubilin complex. By successfully knocking-down or overexpressing these proteins in ECs, and conducting the albumin TIRF microscopy assay, it can be quickly identified whether they serve a direct role in albumin transcytosis. Moreover, with greater access to mass spectroscopy, it can be accurately determined whether gp60 is in fact the receptor for albumin on endothelial cells. Using the pull-down techniques initially used to identify gp60 as the albumin binding protein, gp60 can be isolated and its structure characterized. Using improvements in proteomics and sequencing technology it be possible to determine what gene regulates gp60 expression if it is an actual protein.

4.2 Conclusion

Between the late 1970's into the early 2000's, research on albumin transport through the endothelium was abundant, with many scientific groups publishing multiple studies on this topic. Over the last two decades however there has been gradual decline in studies published on albumin transport, a trend reflected largely by literature referenced in the first chapter of this thesis. Perhaps the decline in research into the mechanism of this fundamental physiological process is reflective of a growing lack of interest, or fear, in the medical science community of studying purely molecular mechanisms, without immediate clinical applications. It must be remembered however that many of the great scientific discoveries and medical breakthroughs came from genuine interest and curiosity of the unknown. The notion that albumin serves vital

physiological functions is irrefutable in the field of medical science, however how albumin serves its functions has not been as actively explored. Despite abundant studies substantiating transcytosis as the dominant route of albumin transport during steady-state physiological conditions, the paracellular transport of albumin continues to receive a greater focus, likely due to its importance under inflammatory conditions. However, being able to regulate transcytosis even with the occurrence of paracellular leak, can potentially assist in mitigating some of the excessive albumin transport. Considering albumin's direct interaction with endothelial cells in circulation it is surprising that to date little to no research has been conducted on whether there is heterogeneity in the kinetics and propensity for albumin transcytosis across endothelial beds. Even more alarming is the steadfast acceptance by researchers of gp60 as the endothelial receptor for albumin, despite no genetic evidence of such a protein even existing. Unlike gp60, CD36 is a well-studied protein, known to be involved in numerous physiological responses, and to bind a variety of ligands. Thus it is not at all surprising it serves a role in transcytosis of albumin across endothelial cells.

Clearly, there is still much work to be done on characterizing the mechanism of albumin transcytosis across the heterogeneous microvascular endothelium throughout the body. A better understanding of the mechanism of albumin's interactions with cells and other proteins in order to fulfill its osmotic and/or transport roles will be essential in modulating these functions. Mechanistic insight is essential especially in the context of albumin's transport of fatty acids, which can potentially be beneficial in treating metabolic diseases. Being able to regulate albumin transcytosis in an organ specific-manner will also be advantageous to designing albumin-associated drugs. Above all, through the findings presented above I hope to have added some knowledge to the field of cell and molecular biology, and potentially better the understanding of this process for others interested in studying it in the future.

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APPENDIX: CONTRIBUTIONS

Unless otherwise indicated, all experiments were performed by Hira Raheel.

Michael Sugiyama assisted with lung and skin isolations for **Figure 3.6**.

Siavash Ghaffari contributed to experiments in **Figure 3.3(B)**, and

Siavash Ghaffari, Changsen Wang and Derek Auyeung executed experiments in **Figure 3.5**.

Victoria Mintsopoulos assisted with isolation of blood samples, and measurement of mouse body weight in **Figure 3.7**.

Yun Hye Kim conducted fatty acid measurement in **Figure 3.7**.

Hira Raheel and Warren Lee were responsible for designing the experiments and directing the project.