REPRESSION OF THE BLOOD ENDOTHELIAL MARKER *CD146* BY THE HOMEOBOX GENE *PROX1*

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Bana anilarla da yetinebilmeyi ogreten

dedem Cemalettin Cebecioglu’na…

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Benimle daima gurur duyan annem ve babama, hayatimin her doneminde bana destek olduklari ve bana en zor anlarda bile kendime guvenimi yitirmemeyi ogrettikleri icin tesekkur ederim. Ayrica, ucak yolculugundan odu koptugu icin okyanuslari gemiyle asip gene de beni ziyarete gelmeyi dusunebilecek kadar “sinirlari asabilen” teyzeme de, bana ne kadar ozel oldugumu her firsatta hatirlatigi icin tesekkurler. Beni hicbir zaman yalniz birakmayan aksi huylu kusum Kus’a: Iyi ki varsin. Son olarak, hala var olduguna dair suphelerim olan Gizem’e ve 13 yildir bana sabirla katlanan Cetin’e dostum olduklari ve her zaman dostum kalacaklari icin tesekkur ederim.

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LIST OF ABBREVIATIONS

4A	Empty pCMV Tag 4A Vector
4A-*Prox1*	pCMV Tag 4A Vector encoding *Prox1*
Ad*HDPD*	Adenovirus encoding Homeo-Prospero Domain deleted *Prox1*
Ad*Prox1*	Adenovirus encoding *Prox1*
AIDS	Acquired Immunodeficiency Syndrome
Amp	Ampicillin
Antp	*Antennapedia*
APS	Ammonium Persulfate
BEC	Blood Endothelial Cell
CAM	Cell Adhesion molecule
COUP-TFII	Chicken Ovalbumin Upstream Promoter Transcription Factor II
CRE	cAMP response element
Cyp7a1	Cholesterol 7α-Hydroxylase gene
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethyl Pyrocarbonate
DTT	Dithiothreitol
E8	Embryonic day 8
EBM-2	Endothelial Cell Basal Medium-2
EC	Endothelial Cell
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced Green Fluorescent Protein
EGM-2	Endothelial Cell Growth Medium-2
EGM-2MV	Microvascular Endothelial Cell Growth Medium-2
FBS	Fetal Bovine Serum
Flt4	Fms-related Tyrosine Kinase Receptor-4
FOXC2	Forkhead box C2
GMC	Ganglion Mother Cell
HA	Hyaluronan
HD	Homeo Domain
HDPD	Homeo-Prospero Domain
HEK 293	Human Embryonic Kidney Cells
HepG2	Hepatocarcinoma Cells
HHV8	Human Herpes Virus 8
HLT	Hypotrichosis-Lymphedema-Telangiectasia
HNF4α	Hepatocyte Nuclear Factor 4α
HUVEC	Human Umbilical Vein Endothelial Cell
ICAM-1	Intercellular Adhesion Molecule
IgG	Immunoglobulin G

Kan	Kanamycin
KS	Kaposi's Sarcoma
LB	Luria Broth
LD	Lymphedema-Distichiasis
LEC	Lymphatic Endothelial Cell
LRH-1	Liver Receptor Homolog-1
LYVE-1	Lymphatic Vessel Endothelial Hyaluronan Receptor-1
MAPK	p38 Mitogen-activated Protein Kinase
MMP-2	Metalloproteinase-2
NF-KB	Nuclear Factor-KB
Nrp2	Neuropilin-2
ONPG	Ortho-nitrophenyl-β-galactoside
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-Triton X-100
PD	Prospero Domain
PDGFR	Platelet-derived Growth Factor Receptor
pen/strep	Penicillin/Streptomycin
Prox1	Mouse Prospero-related Homeobox gene
PROX1	Human Prospero-related Homeobox gene
PROX1	Mouse/Human Prospero-related Homeobox protein
qPCR	Quantitative Real Time Polymerase Chain Reaction
RIPA	Radioimmunoprecipitation assay
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
siRNA	Small interfering RNA
SMC	Smooth Muscle Cell
SOX18	Sex Determining Region Y-box 18
TAE	Tris-Acetate-EDTA
TBS	Tris Buffer Saline
TBST	Tris Buffer Saline Tween 20
TEMED	Tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
VEGF-C	Vascular Endothelial Growth Factor-C
VEGF-D	Vascular Endothelial Growth Factor-D
VEGFR-3	Vascular Endothelial Growth Factor Receptor-3
VSMC	Vascular Smooth Muscle Cell
WM-266-4	Human Melanoma cells
ΔHDPD	Homeo-Prospero Domain deleted

ABSTRACT

CD146 is a cell adhesion molecule that has been shown to regulate cell adhesion, migration and proliferation of different cell types. It is highly expressed in blood endothelial cells (BECs), but is only lowly expressed in lymphatic endothelial cells (LECs). The *PROX1* homeobox gene is a master regulator of lymphangiogenesis and its expression is necessary and sufficient to drive venous endothelial cells into a LEC phenotype. The highly permeable nature of the lymphatic vessels may partially derive from PROX1 mediated repression of *CD146* transcription. We hypothesize that PROX1 promotes lymphatic differentiation by repressing *CD146* transcription.

In gain of function studies, Human Umbilical Vein Endothelial Cells (HUVECs) were infected with adenoviruses encoding EGFP, wild type PROX1 (Ad*Prox1*) or a Homeo-Prospero domain deleted version of PROX1 (AdHDPD), which cannot bind DNA. In order to knockdown *PROX1*, LECs were transfected with *PROX1* specific siRNA.

When compared to EGFP infected HUVECs, Ad*Prox1* infected HUVECs had decreased *CD146* expression both at protein and mRNA levels. In contrast, AdHDPD infected HUVECs had increased levels of *CD146* expression. In support of a role for PROX1 in repressing *CD146*, *PROX1* siRNA transfected LECs express higher levels of *CD146* as compared to mock transfected LECs or LECs transfected with control siRNA.

Based on these results, we predict that *CD146* expression is kept at basal levels by an unknown repressor bound to the *CD146* promoter. By interacting with this unknown repressor, PROX1 further represses *CD146* expression. On the other hand, the DNA binding-deficient ΔHDPD version of PROX1 binds the unknown repressor and sequesters it from the *CD146* promoter, thereby relieving the repression of *CD146* expression in ECs.

Different levels of *CD146* expression between BECs and LECs might reflect the structural and functional differences between blood and lymphatic vessels. Since CD146 plays a critical role in EC adhesion, regulation of *CD146* expression in ECs might be one of the key factors regulating vessel permeability.

LITERATURE REVIEW

1. Circulatory system

1.1. Cardiovascular system

1.1.1. Function and structure

The cardiovascular system is the earliest functioning organ system in the embryo because continued development of the rest of the body is dependent on oxygen and nutrients supplied by blood vessels (Lohela et al., 2009). The cardiovascular system functions as a distribution network that circulates blood, nutrients, metabolites, signaling molecules, waste products and blood cells throughout the body (Swift & Weinstein, 2009).

The cardiovascular system consists of the heart, arteries, veins and blood. In the systemic circulation, arteries transport oxygenated blood away from the heart to the organs. In contrast, the veins return oxygen-depleted blood from the organs back to the heart. In the pulmonary circulation, arteries carries oxygen-depleted blood from the heart to the lungs and veins return oxygen-rich blood from the lungs back to the heart (dela Paz & D'Amore, 2009).

Arteries and veins are classified according to their size and location within the vasculature. The aorta, the largest artery in the body, originates from the left ventricle and branches into large arteries. These larger arteries further branch into smaller arteries and once they reach their target organs; they form the smallest arteries called arterioles. The venous circulation has a similar hierarchical structure.

Venules, the smallest veins, merge to form small veins which exit the organs and then form larger veins by increasing in size. These larger veins then converge to form the two largest veins called the venae cavae. The superior vena cava, receiving blood from the upper trunk and the inferior vena cava, receiving blood from the lower trunk, both empty into the right atrium. The last component of the blood vasculature is the capillaries which connect the arterial and venous halves of the circulatory system (dela Paz & D'Amore, 2009).

1.1.2. Blood circulation

The heart works as a pump and generates two phases called systole and diastole. Systole is the contraction of the ventricles, which increases the pressure inside the chambers to pump blood from the heart. After the ejection of the blood, aortic and pulmonary valves close. This is followed by relaxation, called diastole, which allows the filling of the chamber for the next cycle (Munoz & Sacco, 1997). Normal systolic and diastolic blood pressure values of a healthy adult human in a resting state are 120 mmHg and 80 mmHg respectively. These values are not static, but vary throughout the day and also depend on the factors such as stress, nutrition, exercise and disease (Papadopoulos & Makris, 2008).

Both arterial and venous vessel walls have several layers with different cell types. The innermost part of the wall consists of a single layer of endothelial cells (ECs), and the outermost layer consists of connective tissue. The region between these two layers contains elastin, collagen, and vascular smooth muscle cells

(VSMCs). VSMCs modify the vessel diameter by either contracting or relaxing. The thickness and arrangement of these layers can vary among the different types of blood vessels and, together with the contractile state of the smooth muscle, are responsible for the mechanical characteristics of the vessels that play an essential role in cardiovascular physiology (dela Paz & D'Amore, 2009). The vascular wall is viscoelastic, meaning that it exhibits both viscous and elastic behaviour. Viscoelastic materials resist shear flow, strain linearly when a stress is applied and return to their original state once the stress is removed. As a result of the viscoelasticity of the vessels, pulses and the pressure generated by the heart are transmitted as they travel in the vascular system (Munoz & Sacco, 1997).

Despite their common progenitors, arterial and venous ECs differ widely in their morphology and function (Figure 1). In general, veins are larger in diameter than arteries but have much thinner walls (dela Paz & D'Amore, 2009). Arteries are covered with multiple layers of smooth muscle cells (SMCs) and high amount of extracellular matrix (ECM) components required for mechanical support and elasticity respectively. On the other hand, veins have thinner and more rigid walls, because they have less SMCs and elastic fibres (Hirashima & Suda, 2006). The abundance of intercellular junctions is also different between arteries and veins. The endothelial junctions play a role in regulation of cell-cell adhesion, permeability and contact inhibition of cell growth. In arteries, tight junctions predominate in order to limit fluid exchange between the vessels and the tissues (Figure 2). In veins,

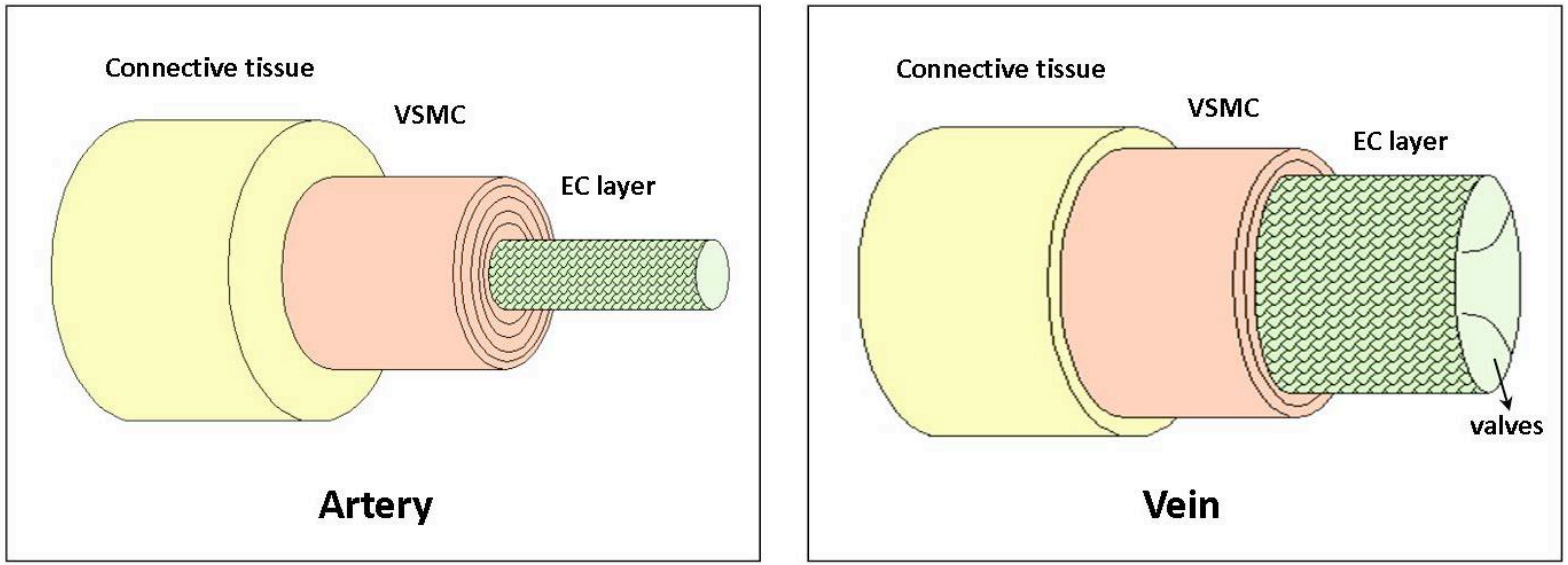


Figure 1: Structural differences between arteries and veins. In general, veins have larger diameters than arteries. For mechanical support and elasticity, arteries are covered with thick layers of vascular smooth muscle cells (VSMCs) and connective tissue, whereas these layers are thinner in veins. Valves prevent backflow of blood in veins, but these structures are absent in arteries due to their high blood flow rate when compared to venous flow. EC: Endothelial cell

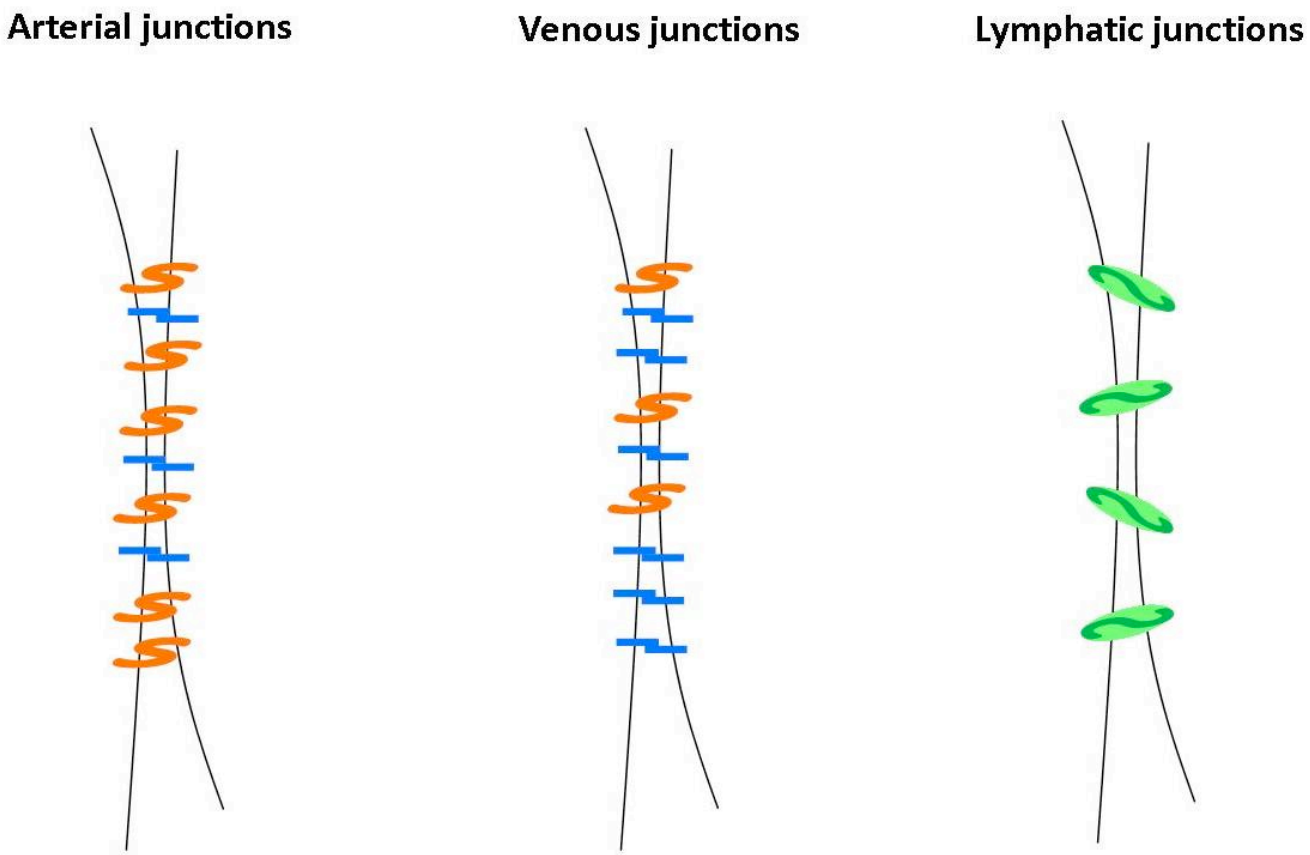


Figure 2: Representation of different endothelial cell junctions. In arteries and veins, endothelial cells are connected by tight (orange) and adherens (blue) junctions to limit fluid exchange between the vessels and the environment. When compared to arteries, there are more adherens junctions and less tight junctions in veins. In lymphatic vessels, endothelial cells are connected by loose, valve like junctions (green), allowing the entry and the drainage of fluid from the extracellular environment.

however, there are more adherens junctions than tight junctions (Dejana et al., 2009). In addition to vessel structure, arterial and venous ECs also have different morphologies. Arterial ECs, which are exposed to high shear stress, are long and narrow. Venous ECs are exposed to lower flow rates, thus they are short and wide. Due to the low flow rate in veins, there are venous valves that prevent the backflow of the blood in veins (Swift & Weinstein, 2009).

1.2. Lymphatic system

1.2.1. Function and Structure

The lymphatic system drains excess fluid from the extracellular spaces, thus maintaining the fluid homeostasis of the body (Jurisic & Detmar, 2009). Other than carrying lymph, the lymphatic system also absorbs lipids from the digestive system and transports them to the circulatory system. Lacteals are specialized lymph capillaries with fingerlike projections extend into the small intestine and absorb lipids from the intestinal tract (Jurisic & Detmar, 2009). The lymph within these capillaries, called chyle, has a white colour due to the presence of fats. In addition to these two functions, the lymphatic system can also be considered as a part of the immune system because lymphocytes and immune cells use it to circulate to and from the lymphoid organs (Oliver, 2004). Lymphatic system impairment leads to a number of diseases characterized by lymphedema, inflammation and cancer progression (Skobe & Detmar, 2000).

The lymphatic system is composed of lymph fluid, lymphatic vessels and lymphoid organs, such as bone marrow, thymus, lymph nodes, spleen and tonsils (Hong et al., 2004). Lymph capillaries are the smallest lymphatic vessels, which merge to form collecting vessels. Collecting vessels unite to form lymphatic trunks, which drain lymph from different regions of the body. The lymphatic system is connected to the blood circulation by the thoracic duct, the largest collecting lymphatic vessel that drains lymph to the blood circulation at the right and left subclavian veins (Adams & Alitalo, 2007). Blood flow into the lymphatic system is prevented by the valves present in the lymphatic ducts.

1.2.2. Lymph circulation

Unlike the blood circulation, lymph flows passively from the tissues into the lymph capillaries, aided by the contraction of the nearby muscles and blood vessels (Witte et al., 2006). Collecting vessels are coated with a SMC layer that facilitates lymph flow by their intrinsic contractile capacity and the presence of valves to prevent backflow (Tammela et al., 2005). Lymphatic capillaries are blind ended vessels that are intimately associated with the interstitium. Their ECs are connected to the connective tissue by anchoring filaments, attaching the ECs to surrounding collagen (Skobe & Detmar, 2000). Unlike the tightly joined ECs that make up the walls of blood capillaries, lymph capillary ECs loosely overlap and there are valve-like openings between adjacent ECs. When interstitial pressure increases, anchoring filaments pull the overlapped cells apart, allowing fluid to enter the lymph

capillaries. When the capillary pressure exceeds the interstitial pressure, the spaces between the ECs close, keeping the fluid inside the capillary (Witte et al., 2006).

1.3. Cardiovascular versus lymphatic system

Blood and lymphatic vessels differ in their structures, reflecting their specific functions. Vertebrates have a closed cardiovascular system, which means that the blood never leaves the vessel network. On the other hand, the lymphatic system is an open system that interacts with the environment, allowing fluid exchange between the lymphatic capillaries and the extracellular space (Oliver & Alitalo, 2005). Blood vessels are covered with a SMC layer that facilitates blood flow. Arteries have a thicker SMC layer than do veins. In the lymphatic system, collecting lymphatic vessels have a thin layer of SMC layer, but lymphatic capillaries are not covered by SMC (Adams & Alitalo, 2007). Both small blood vessels and collecting lymphatic vessels are covered with pericytes, but lymphatic vessels have no encircling pericytes. Unlike lymphatic vessels, blood vessels have a continuous basement membrane (Tammela et al., 2005). Blood vessels have tight and adherens cell-cell junctions. On the other hand, lymphatic vessels have loose, valve like junctions that allow fluid exchange with the extracellular space (Figure 2).

2. Lymphatics Diseases

2.1. Lymphedema

One of the main functions of the lymphatic vessels is to collect fluid from the extracellular space and return it to the circulatory system. Blockage of the lymphatic

vessels results in accumulation of lymph in extracellular space, leading to lymphedema (Oliver & Alitalo, 2005). Lymphedema is a pathological condition that is characterized by the regional accumulation of excessive amounts of lymph, giving rise to a disfiguring swelling of the extremities (Rockson, 2001). According to their origin, lymphedemas are classified into either primary or secondary lymphedemas. Primary lymphedema is a hereditary disorder that is caused by an intrinsic defect in lymphatic system. It can be present at birth or develop at later stages. As opposed to primary lymphedema, secondary lymphedema is an acquired disorder, which can result from any disruption of lymphatic vessel network, mostly by other diseases, infections or surgical processes (Oliver & Alitalo, 2005).

2.1.1. Primary lymphedema

A primary lymphedema, known as Milroy disease, was first described by Milroy in 1892 (Ferrell et al., 1998). To date, mutations in three different genes have been linked to inherited lymphedemas.

Hereditary lymphedema generally shows an autosomal dominant pattern of inheritance with substantial variability of both penetrance and the age of onset (Irrthum et al., 2000). Several groups have reported linkage of primary lymphedema to a region of chromosome 5q (5q34-q35) (Ferrell et al., 1998). Mutations in vascular endothelial growth factor receptor-3 (*VEGFR-3*) gene have been found to be responsible for 5q34-q35 linked lymphedema (Karkkainen et al., 2000). *VEGFR-3* is essential for the development of blood and lymphatic vessels during embryonic

development (Kato et al., 2006). Missense mutations at the VEGFR-3 tyrosine kinase domain block its function, thus inactivating the VEGFR-3 signalling pathway (Irrthum et al., 2000). Since the mutant *VEGFR-3* acts as dominant negative, mutation of one copy is sufficient to inhibit tyrosine kinase activity of the receptor (Karkkainen et al., 2001).

Lymphedema-Distichiasis (LD) is an autosomal dominant disorder with variable age at onset. It usually presents as lymphedema of the limbs and the double rows of eyelashes (distichiasis) that results in irritation of the cornea (Witte et al., 2001). LD was linked to mutations in chromosome 16, where the Forkhead box C2 (*FOXC2*) gene is located (Fang et al., 2000). *FOXC2* belongs to the forkhead family of transcription factors, whose members are involved in developmental pathways of mesenchymal tissues. *FoxC2* knockout mice display cardiovascular, craniofacial, and vertebral abnormalities, which are also present in LD (Petrova et al., 2004). Mutations in the forkhead domain activate one allele of the gene and are responsible for LD (Fang et al., 2000). In $FoxC2^{+/-}$ mice, there is a generalized hyperplasia of the lymphatic system, abnormal lymphatic drainage, retrograde lymph flow and distichiasis, which are the distinct phenotypes of most LD patients (Kriederman et al., 2003). During lymphatic system development, *FOXC2* is required for valve morphogenesis and the regulation of the interaction between LECs, pericytes and SMCs (Petrova et al., 2004). Defects in these processes could lead to impaired permeability of the lymphatic vessels and retrograde lymph flow in the

collecting vessels. Other than loss of function mutations in the forkhead domain, it has been recently shown that, missense mutations outside the forkhead domain cause a gain of function which also causes lymphedema by a dominant negative mechanism (Steensel et al., 2009). Considering that *FOXC2* is important in determining the balance between promotion and inhibition of lymphatic vessel growth, both gain and loss of function of *FOXC2* would result in a defective lymphatic system.

SRY (sex determining region Y)-box 18 (*SOX18*) belongs to the SRY-related HMG domain family of developmental transcription factors which share 50% homology in DNA binding domain with SRY (Downes & Koopman, 2001). *SOX18* is the human orthologue of the mouse *ragged* gene, which plays a role in hair and blood vessel development during embryogenesis. It is also important during lymphatic vessel development and maintenance (Francois et al., 2008). *Ragged*$^{-/-}$ mice have hair and cardiovascular anomalies and lymphedema as a result of lymphatic dysfunction (Irrthum et al., 2003). In humans, *SOX18* mutations cause both recessive and dominant hypotrichosis-lymphedema-telangiectasia (HLT) syndrome, with phenotypes similar to those observed in *Ragged*$^{-/-}$ mice. Recessive missense mutations in SOX18 HMG box region render the transcription factor unable to bind DNA, while retaining some activity (Downes et al., 2009). On the other hand, dominant nonsense and dominant frameshift mutations truncate the SOX18 protein at its transactivation domain, resulting in a dominant-negative loss of function of

SOX18 (Downes et al., 2009). Patients with HLT syndrome have lower limb lymphedema with variable penetranca, early-onset hypotrichosis with absence of eyebrows and eyelashes and visible red legion on the skin caused by the dilation of the capillaries (Irrthum et al., 2003).

2.1.2. Secondary lymphedema

Secondary lymphedema is caused by an extrinsic factor that damages the lymphatic system. Lymphatic filariasis or elephantiasis is the most common form of lymphedema worldwide, mostly seen in tropical regions. Although the disease is not fatal, it causes severe morbidity and disfiguration of limbs. It is estimated that 120 million people have been affected by this disease worldwide (World Health Organization, 2000). It is an infectious disease caused by the parasitic worms of the *Filarioidea* superfamily, which invade the lymphatic system, thus causing lymphedema (Witte et al., 2001; Rockson, 2001). In developed countries, cancer treatment is the most common cause of secondary lymphedema, which can develop any time following treatment such as lymph node removal or radiotherapy (Detmar & Hirakawa, 2002). In 1997, the National Cancer Institute in the US reported a 52% incidence of lymphedema among breast cancer patients receiving radiation therapy or lymph node dissection. Other than infections and cancer treatment, there are many other factors causing secondary lymphedema, such as surgery, tissue damage, burns, immobility and inflammation, which are all associated with the interruption of the lymphatic drainage system (Honnor, 2008).

2.2. Inflammation

Inflammation is a response of the body to injury or infection, which is characterized by pain, redness, heat and swelling due to the changes in blood vasculature (Ji, 2009). There are two stages of inflammation, acute and chronic. Acute inflammation is the activation of the innate immune system for a short period of time to protect the body against infections. Chronic inflammation on the other hand, persists for a long period of time and it is linked to various chronic illnesses such as cardiovascular, pulmonary and neurologic diseases, diabetes and cancer (Aggarwal et al., 2009).

In some cases, lymphedema can be accompanied by an inflammatory response. Under normal conditions, immune cells use lymphatic vessels to migrate to the lymph nodes to initiate adaptive immune response. In chronic lymphedema, the impairment of lymph circulation blocks immune cell trafficking, thus leading to inefficient antigen clearance and thereby promoting chronic inflammatory changes (Tabibiazar et al., 2006).

It is also known that inflammation promotes lymphangiogenesis at the inflammation site (Jurisic & Detmar, 2009). Upon binding to VEGFR1 on monocytes and macrophages, VEGF-A recruits these immune cells to the inflammation site. VEGF-A binding initiates the immune amplification cascade, leading to lymphangiogenic response by the secretion of lymphangiogenic factors VEGF-C and VEGF-D by the activated monocytes and macrophages (Cursiefen et al., 2004).

Formation of new lymphatic vessels promotes immune cell migration to the lymph nodes. Additionally, new lymphatic vessels facilitate the resolution of inflammation since they drain accumulated fluid, immune cells and inflammatory cytokines from the sites of inflammation (Cueni & Detmar, 2006). On the other hand, it has been shown that mice overexpressing *VEGF-A* are unable to downregulate induced inflammation. Excessive *VEGF-A* expression results in the proliferation of lymphatic ECs, leading to lymphatic vessel enlargement, fluid leakage and chronic inflammation (Kunstfeld et al., 2004).

2.3. Cancer

2.3.1. Metastasis

Metastasis is the spread of tumors from the primary site to distant organs. This complex process involves a variety of interactions among many different cell types in the tumor microenvironment, called tumor stroma. Other than tumor cells, tumor stroma consists of blood and lymphatic vasculature, ECM and the other stromal cells such as immune cells, fibroblasts, pericytes and mesenchymal stem cells (Joyce & Pollard, 2009).

The tumor microenvironment promotes cancer progression in several ways. In order for tumor cells to disseminate from their primary site and invade blood vessels, they need to break down ECM. This process is facilitated by stromal cells including macrophages that release proteases such as matrix metalloproteases and serine proteases (Egeblad & Werb, 2002). Cleavage of cell adhesion proteins

loosens cell-cell contacts, thus facilitating invasion of tumor cells into the surrounding tissue and vasculature. For the growth and the hematogenous dissemination of cancer cells, angiogenesis is induced by growth factors and cytokines that are released from the stromal cells (Sund & Kalluri, 2009).

The blood circulation is a hostile environment for tumor cells to survive during metastasis because of the mechanical destruction caused by shear force and immune surveillance (Joyce & Pollard, 2009). Tumor cells enhance their survival by using platelet aggregates for protection against immune cells and for reducing the disruptive effects of shear force (Nieswandt et al., 1999). These aggregations also facilitate the extravasation at a secondary tumor site by enhancing the slowing, arresting and adhesion of tumor cells. When compared to blood vessels, lymphatic vessels have passive and low-shear lymph flow that increases tumor cell survival. In addition, lymphatic vessels lack pericytes, intact basement membranes or tight junctions between adjacent ECs, which makes them favourable for tumor cells to migrate from their primary site to other tissues (Wong & Hynes, 2006). The lymphatic system is the main route for metastasis of some tumors such as breast, lung and gastrointestinal tract, which colonize lymphs nodes before dissemination to other sites (Saharinen et al., 2004). Lymph nodes serve as supportive intermediate way points where cancer cells are filtered and concentrated to form aggregates, which increases their survival (Joyce & Pollard, 2009).

In addition to a passive role, the lymphatic system is also directly involved in cancer metastasis. In several human cancers, such as melanoma and breast cancer, tumors have been found to induce lymphangiogenesis. In these tumors, increased lymphangiogenesis results in enhanced sentinel lymph node metastasis, which is the initial step of metastatic spread (Schietroma et al., 2003; Skobe et al., 2001). Once metastatic tumors reach regional lymph nodes, they continue inducing lymphangiogenesis and promote their further dissemination to distant lymph nodes and ultimately to distant organs (Hirakawa et al., 2005). These findings suggest that by inducing lymphangiogenesis, primary tumors facilitate their spread.

VEGF-C plays an important role in cancer metastasis by inducing lymphangiogenesis. Overexpression of *VEGF-C* results in LEC proliferation and vessel enlargement, thus inducing lymphatic hyperplasia, which is involved in tumor metastasis (Jeltsch et al., 1997). In many human tumors, such as colorectal cancer, lung adenocarcinoma and breast cancer, there is a strong correlation between *VEGF-C* expression levels and tumor invasiveness and aggressiveness (Akagi et al., 2000; Niki et al., 2000; Kurebayashi et al., 1999). It has been shown that, tumor lymphangiogenesis and lymph node metastasis can be suppressed either by inhibition of VEGFR-3 signalling pathway or downregulation of *VEGF-C* in many cancers (He et al., 2002; 2004; Chen et al., 2005).

In addition to VEGF-C, VEGF-D and VEGF-A have also been shown to play roles during tumor progression. By inducing the proliferation of LECs and the

formation of new lymphatic vessels within tumors, these growth factors promote the spread of tumors to lymph nodes and this effect can be blocked by antibodies against them (Stacker et al., 2001; Hirakawa et al., 2005).

2.3.2. Kaposi's sarcoma

Kaposi's sarcoma (KS) is a malignant tumor caused by human herpes virus 8 (HHV8). It is a common symptom of acquired immunodeficiency syndrome (AIDS) (Chang et al., 1994). There are 4 variants of KS sharing identical histologic appearance: 1) Classic KS, primarily affecting elderly Eastern European and Mediterranean men; 2) Endemic KS, which is common in some regions of Africa; 3) Immunosuppression or transplantation-associated KS, which is an aggressive type of KS affecting organ-transplant recipients and patients receiving immunosuppressive therapies; and 4) Epidemic or AIDS-associated KS, which is an aggressive and frequently fatal form of KS, and it is the most common AIDS-associated cancer (Antman & Chang, 2000).

KS mainly affects skin and forms lesions and tumors with spindle-like cells. Primary characteristic of KS lesions are neoangiogenesis, inflammation, and cellular proliferation (Wang et al., 2004). Lesions are often associated with lymphedema, resulting from increased defective lymphatic vessels, which also results in compromised immune surveillance and increased angiogenesis, thus promoting malignancy. At later stages, the endothelium-derived neoplastic spindle-shaped cells

form vascular channels by aggregating into sheet-like structure (Dubina & Goldenberg, 2009).

Due to the morphological similarity of the cells, and the expression of LEC markers such as VEGFR-3 and podoplanin, KS has been proposed to be neoplasm of lymphatic endothelium caused by HHV8 infection (Beckstead et al., 1985). It has been shown that BECs infected with HHV8 differentiate into LECs by upregulating LEC specific markers such as Prospero-related homeobox-1 (*PROX1*). Therefore, HHV8 infection has been suggested to induce BEC reprogramming into a more LEC phenotype (Hong et al., 2004).

3. Lymphangiogenesis

3.1. Early models for lymphatic development

Lymphatic vessels were first identified in 1622 by Gasparo Aselli. While he was dissecting a well fed dog, he observed lymphatic vessels and named them *venae albae et lacteae;* white and lacteal veins (Ambrose 2006). Despite their discovery in the early seventeenth century, the origins of the lymphatic vessels remained unknown until the twentieth century due to the lack of molecular markers specific to LECs.

In 1902, Florence Sabin proposed the first model for the origins of the lymphatic vessels. According to her model, lymphatic vessels originate from embryonic veins. She proposed that the primary lymph sacs are initially formed by budding of the ECs from the veins and lymphatic vessels further sprout from these

sacs to form the mature lymphatic vessel network in the body (Hong et al., 2004). In 1999, Sabin's theory was supported by the discovery that *Prox1* expression was required for a subpopulation of ECs to migrate from the cardinal vein to form lymph sacs in mice (Wigle & Oliver, 1999). *Prox1* knockout mice completely lack a lymphatic vasculature, suggesting that *Prox1* is required for lymphatic differentiation of vein ECs during embryonic development.

As an alternative to Sabin's model, Huntington and McClure proposed that lymphatic vessels are formed independently in the mesenchyme and later connect to the circulatory system (Oliver & Detmar, 2002). Huntington and McClure's model has been supported by the findings in chick and quail embryos. It has been shown that lymphatics of the early wings are formed not only by sprouting of the ECs from the lymph sacs, but also by recruitment of the mesenchymal lymphangioblast; LEC progenitors (Schneider et al., 1999). Another theory combines Sabin's centrifugal development theory with Huntington and McClure's centripetal development theory and supports the venous-mesenchymal origin of the lymphatic vessels. According to this theory, the central lymph vessels develop by sprouting from lymph sacs whereas the peripheral lymph vessels arise by differentiation of the mesenchymal precursors (Wiegand et al., 2008). The theory of the presence of LEC progenitors has been further supported by recent studies. Irradiated mice reconstituted with donor bone marrow cells have been shown to have bone marrow-derived circulating endothelial precursor cells in their newly formed lymphatic vessels (Religa et al., 2005). Another

study conducted on human renal transplants also shows the participation of donor-derived circulating lymphatic progenitor cells in *de novo* lymphangiogenesis (Kerjaschki et al., 2006). These studies suggest that lymphatic progenitor cells derived from circulation incorporate into the newly formed lymphatic vessels, thus contributing to lymphangiogenesis in adults.

3.2. *Prox1* and lymphatic development

The lack of specific LEC markers has made lymphatic vessels difficult to identify and study. In 1999, it was shown that during mouse early development, *Prox1* expression was required for the migration of a subpopulation of ECs in the cardinal vein to form the lymph sacs and subsequent lymphatic vasculature (Wigle & Oliver, 1999). In the nullizygous *Prox1* mice, initial budding of the ECs from the cardinal vein was not affected. However, at later stages of lymphangiogenesis, LECs migrate in a random manner instead of a more polarized migration seen in the wild-type embryos. In addition, these budding ECs lose their LEC identity and start to express BEC markers (Wigle et al., 2002). As a result, *Prox1* null mice completely lack a lymphatic system.

Prox1 gene dosage is also crucial for lymphatic development and postnatal survival of mice. Starting at E14.5, $Prox1^{+/-}$ embryos develop severe edema, which indicates the mispatterning of the lymphatic vessels (Wigle & Oliver, 1999). In most genetic backgrounds, $Prox1^{+/-}$ mice die soon after birth and have lymphatic dysfunction phenotypes such as abnormal chyle accumulation in the peritoneal and

thoracic cavity (Wigle & Oliver, 1999). Further studies show that *Prox1* haploinsufficiency causes abnormal leakage of the lymph due to the severe disruption of lymphatic vascular network, which results in early postnatal lethality (Harvey et al., 2005). Therefore, *Prox1* is required not only for acquiring the lymphatic identity of endothelial cells, but also for maintaining the formation and patterning of the lymphatic vascular network at later stages of lymphatic development. Another aspect of *Prox1* during lymphatic development is its role as a cell fate switch (Figure 3). Throughout the embryonic development, constant *Prox1* expression is necessary to maintain the LEC phenotype (Johnson et al., 2008). In the absence of *Prox1*, LECs dedifferentiate into venous ECs and lose their lymphatic characteristics. Induction of *Prox1* in venous ECs on the other hand, upregulates expression of lymphatic markers and suppresses blood endothelial phenotype (Hong et al., 2002; Petrova et al., 2002).

3.3. Lymphangiogenesis model

The circulatory system is the first functioning system in the developing embryo. It originates from mesodermal cells that give rise to endothelial precursor cells, called hemangioblasts (Vogeli et al., 2006). At around embryonic day 8 (E8.0), hemangioblasts start to form blood islands, which are composed of hematopoietic cells and ECs (Oliver, 2004). As a result of the fusion of these blood islands, the primary capillary plexus is formed in the yolk sac (Risau, 1997). *De novo* formation of these primitive vessels is called vasculogenesis. At later stages, new vessels are

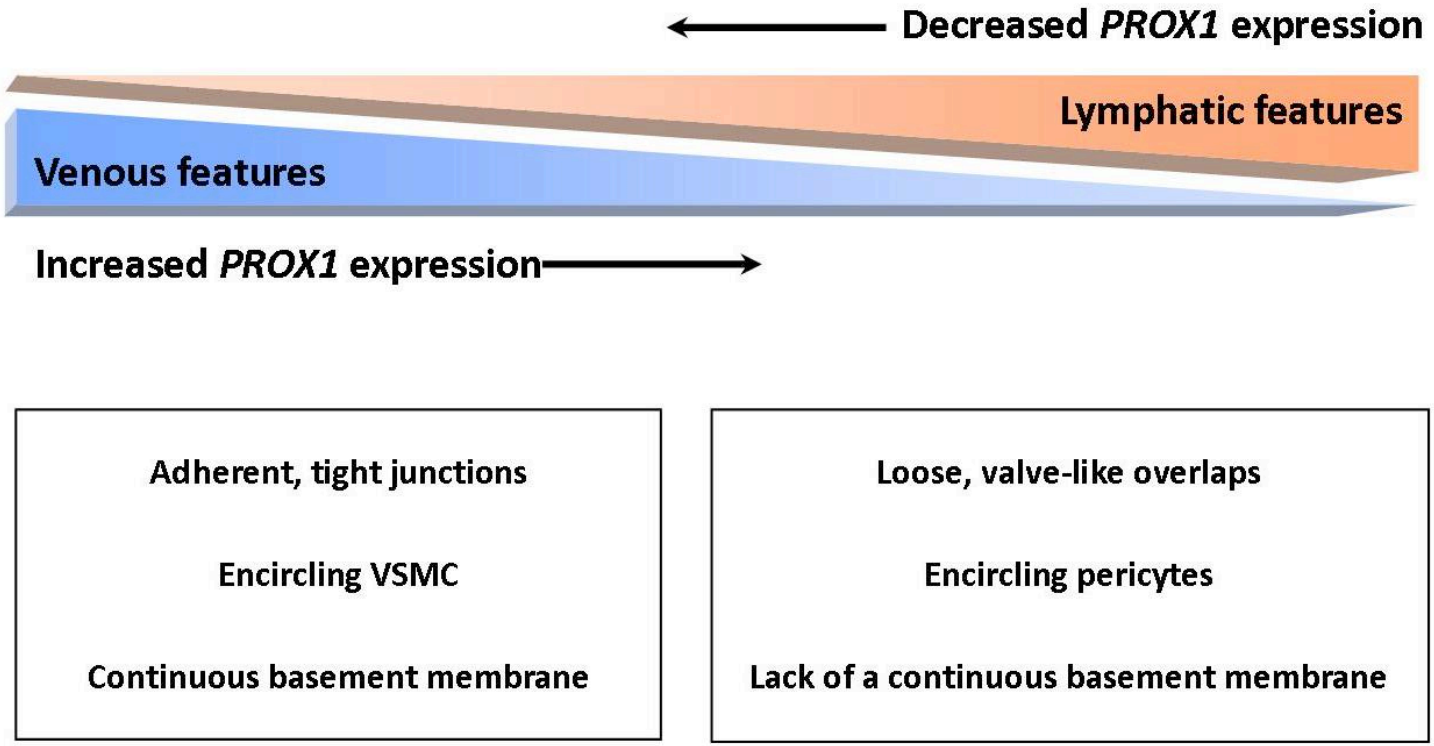


Figure 3: *PROX1* as a cell fate switch. Venous identity is a prerequisite for lymphatic fate and *PROX1* expression is required for venous ECs to adopt and maintain a lymphatic phenotype. Downregulation of *PROX1* in lymphatic endothelial cells (LECs) results in loss of lymphatic features and gain of venous characteristics. On the other hand, *PROX1* overexpression in blood endothelial cells (BECs) results in gain of lymphatic characteristics and loss of venous phenotype. Therefore, *PROX1* acts as a cell fate switch to turn on LEC phenotype and turn off BEC phenotype.

formed from pre-existing vessels by sprouting, splitting and remodelling, collectively called angiogenesis (Zetter, 1998). One of the earliest stages of angiogenesis is the differentiation of arterial and venous ECs, which depends on many transcription factors and signalling pathways including Notch and the Chicken ovalbumin upstream promoter transcription factor II (*COUP-TFII*) (You et al., 2005; Tsai et al., 2005) Once the primitive arteries and veins are formed, blood circulation is established at E9.5 (Bugge et al., 1996).

The lymphatic system originates from veins, but the presence of the veins is not sufficient for lymphangiogenesis. For venous ECs to change their fate into LECs, there must be a lymphatic-inducing signal (Oliver, 2004). During mouse development, homeobox protein PROX1 starts to be expressed in a restricted subpopulation of ECs in the anterior cardinal vein at embryonic day E9.5 (Figure 4) (Wigle & Oliver, 1999). Along with *Prox1*, the lymphatic vessel endothelial hyaluronan receptor-1 (*LYVE-1*), another early LEC marker, also starts to be expressed on this subpopulation of cells at the anterior cardinal vein and its expression on LECs remains constant throughout the lymphatic development (Wigle & Oliver, 1999).

The transcription factor that induces *Prox1* expression in vein ECs was not known for a long time (Oliver, 2004). Recently, it was found that, transcription factor SOX18 was responsible for *Prox1* induction at early stages of lymphatic development (Francois et al., 2008). At E9.0, SOX18 induces *Prox1* expression in the anterior

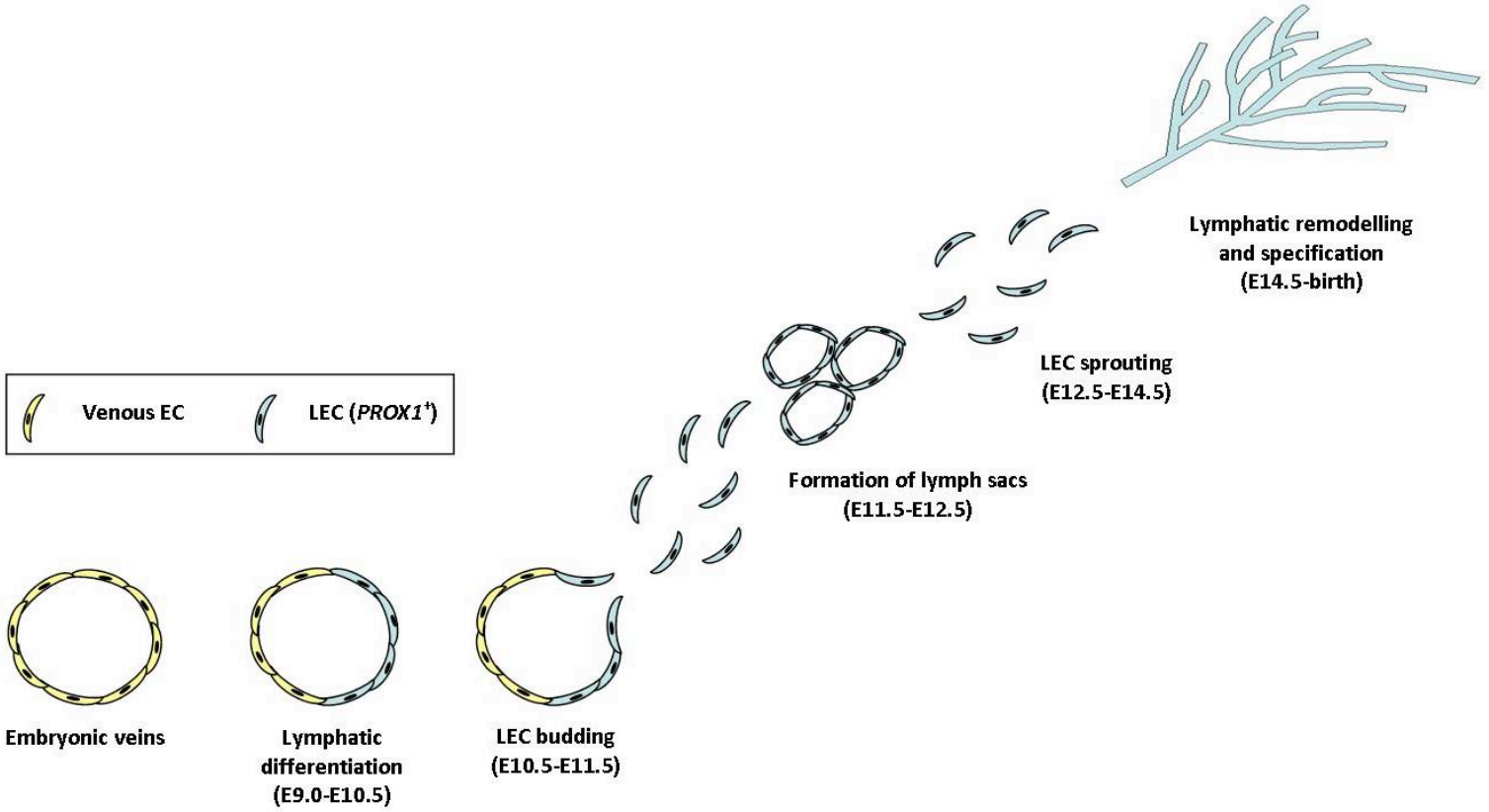


Figure 4: Lymphangiogenesis model. During mouse embryonic development, *PROX1* expression in venous ECs located in anterior cardinal veins begins at E90.0-E9.5. *PROX1* expression promotes lymphatic differentiation followed by sprouting of LECs from the cardinal vein to form primary lymph sacs by E12.5-E14.5. Lymphatic vessels are formed by the sprouting of LECs from the lymph sacs and finally, lymphatic maturation and remodelling to form the complete lymphatic network.

cardinal vein ECs, which are committed to a lymphatic fate upon *Prox1* expression (Francois et al., 2008). Therefore, in addition to its roles during cardiovascular development and cell fate determination, *Sox18* is also involved in early lymphangiogenesis (Downes & Koopman, 2001). Human hereditary lymphedema that are associated with *SOX18* mutations and lymphedema associated with mutations in *Sox18* in mouse models also emphasizes its importance during lymphatic development (Irrthum et al., 2003).

After *Prox1* is activated, lymphatic differentiation begins and another LEC marker VEGFR-3 also starts to be expressed on LECs (Wigle & Oliver, 1999). During early development, *VEGFR-3* expression is observed in both venous and lymphatic ECs. VEGFR-3 is required for remodelling of blood vascular network, as *VEGFR-3* knockout mice die at E10.5 due to defective blood vessels and cardiovascular failure (Dumont et al., 1998). As development proceeds *VEGFR-3* expression becomes restricted to LECs and its inactivation results in lymphedema (Kaipainen et al., 1995; Karkkainen et al., 2000). VEGFR-3 binds both VEGF-C and VEGF-D and promotes lymphangiogenesis. Starting from E10.5, VEGFR-3/VEGF-C signalling is necessary for lymphatic budding and sprouting of LECs from the embryonic veins. VEGF-C acts as a chemotactic factor that directs the migration of *Prox1/VEGFR-3* expressing LECs from the cardinal vein to form the primary lymph sacs at around E12.5 (Karkkainen et al., 2004).

VEGFR-3 is not the only receptor that VEGF-C binds during lymphangiogenesis. It has been shown that Neuropilin-2 (Nrp2) acts as a coreceptor for VEGF-C and VEGF-D and modulates lymphatic development by interacting with these growth factors and their receptor VEGFR-3 (Karpanen et al., 2006). *Nrp2* has been shown to be co-expressed with *VEGFR-3* in venous and lymphatic ECs at early stages of development and at later stages; its expression, along with *VEGFR-3*, is downregulated in venous ECs (Yuan et al., 2002). Blocking the interaction between Nrp2 and VERGFR-3 inhibits sprouting of lymphatic endothelial tip cells, resulting in the reduction of the number of lymphatic vessels and capillaries. Therefore, Nrp2 and VRGFR-3 are required for the sprouting of lymphatic vessels from the pre-existing lymphatic vessels (Xu et al., 2010). These findings are consistent with the previous studies showing the importance of VEGFR-3 signalling in endothelial tip cells during angiogenic sprouting and vessel formation (Tammela et al., 2008).

At around E14.5, LECs start sprouting from the primary lymph sacs to form lymphatic vessels and capillaries throughout the embryo (Oliver, 2004). Not much is known about lymphatic maturation, but it involves decreasing in LEC proliferation, assembling lymphatic capillary network and vessels, valve formation and lumen formation for lymph transport. (Oliver & Srinivasan, 2008).

4. LEC markers

To date, many molecular markers for LECs have been identified. These LEC specific markers have made differentiation between blood and lymphatic ECs

possible thus enabling a more extensive study of the lymphatic system. Some of the LEC markers are listed and described below:

4.1. Podoplanin

Podoplanin, is a type-I integral membrane glycoprotein which was first identified in normal rat kidney podocytes (Al-Rawi et al., 2005). In the adult, it is expressed in LECs but not in blood endothelial cells (BECs) (Breiteneder-Geleff et al., 1999). In mouse embryos, *Podoplanin* is first expressed at around E10.5 in both the cardinal vein ECs and PROX1-positive LECs bud from the cardinal vein. Later during development, *Podoplanin* expression becomes restricted to LECs (Schacht et al., 2003).

The function of Podoplanin in LECs is not known. In contrast to *Prox1* knockout mice, which completely lack lymphatic vasculature (Wigle and Oliver, 1999), *Podoplanin*$^{-/-}$ mice develop a lymphatic system; however, it is defective in lymphatic vascular organization and function. In these mice, lymphatic malformations cause impaired lymph circulation, thus leading to lymphedema and dilation of lymphatic vessels (Schacht et al., 2003). Therefore, Podoplanin may be involved in regulation of lymphatic vessel permeability and integrity at the later stages of lymphatic development. In addition to the lymphatic abnormalities, *Podoplanin*$^{-/-}$ mice have defects in alveolar airspace formation and they die immediately after birth, due to respiratory failure (Ramirez et al., 2003)

4.2. LYVE-1

LYVE-1 is a type I integral membrane glycoprotein that binds to hyaluronan (HA) which is a large mucopolysaccharide present in the ECM (Banerji et al., 1999). HA interacts with a wide range of proteins, so it is involved in a variety of cellular processes such as inflammation, adhesion and matrix stability (Laurent & Fraser, 1992). HA turnover involves its release from the ECM into the lymph, degradation in lymph nodes, and removal via the lymphatic vessels (Jackson, 2009). Under physiological conditions, the levels of HA and its degradation products are maintained at very low levels. Since the lymphatic vasculature is involved in HA turnover, HA homeostasis is regulated by the lymphatic system (Jackson, 2003).

4.3. VEGFR-3

VEGFR-3, also known as Fms-related tyrosine kinase receptor-4 (Flt4), is a member of class III tyrosine kinase subfamily that also includes VEGFR-1 and VEGFR-2 (Galland et al., 1992). Members of this subfamily have seven immunoglobin related domains on the extracellular region and an intracellular domain homologous to the platelet-derived growth factor receptor (PDGFR) subfamily. There are two alternative splice forms of VEGFR-3 differing in their cytoplasmic domains and they are suggested to have different signaling properties (Sleeman et al., 2001). To date, there are two known ligands for VEGFR-3: VEGF-C and VEGF-D. During embryonic development, the VEGF-C,D/VEGFR-3 pathway is required for both angiogenesis and lymphangiogenesis (Kato et al., 2006).

VEGFR-3 plays an essential role in the development of cardiovascular system. During early mouse embryogenesis, targeted inactivation of *VEGFR-3* results in defective blood vessel development. *VEGFR-3* knockout mouse embryos had normal vessel formation, but the vessels had defective lumens with abnormal organization, leading to accumulation of fluid in the pericardial cavity and they die at E9.5 due to cardiovascular failure (Dumont et al., 1998). At later stages of development, expression of *VEGFR-3* becomes restricted to lymphatic vessels (Kaipainen et al., 1995). In the adult mouse, blocking VEGFR-3 activity using specific antibodies inhibits VEGF-C enhanced lymphangiogenesis, but has no effect on either angiogenesis or the pre-existing lymphatic vessels (Pytowski et al., 2005). Heterozygous missense mutations in *VEGFR-3* have also been linked to lymphedema due to the inactivated VEGFR-3 signalling pathway. (Irrthum et al., 2000).

During early lymphangiogenesis, *VEGF-C* is expressed in the regions towards which LECs migrate from the anterior cardinal vein to form the lymph sacs (Kukk et al., 1996). In other words, VEGF-C signalling forms a concentration gradient that LECs use to migrate and form new lymphatic vessels. In $Vegfc^{-/-}$ mice, vein ECs differentiate into LECs, but fail sprout to form lymphatic vessels. As a result, $Vegfc^{-/-}$ mice die before birth due to the accumulation of lymph in tissues (Karkkainen et al., 2004). It has also been observed that $Vegfc^{+/-}$ mice have lymphedema and hypoplastic cutaneous lymphatic vasculature due to defective lymphatic

development. These findings indicate that both copies of *VEGF-C* genes are essential for the proper lymphatic vessel formation (Karkkainen et al., 2004).

As opposed to *VEGFR-3* and *VEGF-C* knockout mice, *VEGF-D* knockout mice displayed only a subtle decrease in the abundance of lymphatic vessels (Baldwin et al., 2005). The lack of profound lymphatic defects in *Vegfd*$^{-/-}$ mice suggests that VEGF-D is virtually dispensable for lymphatic development. Another possibility is that VEGF-C and VEGF-D can compensate each other during lymphatic development, since it has been shown that *VEGF-D* overexpression rescued the lymphatic phenotype of the *Vegfc*$^{-/-}$ mice (Haiko et al., 2008).

5. Homeobox Genes

5.1. Function and Structure

Homeotic genes are involved in differentiation pathways that determine segment formation during early development. Many studies have shown that the homeotic mutations in *Drosophila* lead to transformation of the body parts into another, such as generation of an extra pair of legs, transformation of wings into halteres (Postlethwait & Schneiderman, 1969; Morata, 1975). The Homeobox is a 180 bp DNA sequence which was first identified in homeotic genes in *Drosophila* (McGinnis et al., 1984). Homeobox genes are transcriptional regulators that play fundamental roles during embryonic development by acting on their target genes. They are important for many aspects of development such as cell fate

determination, pattern formation and cellular differentiation (Kornberg 1993; Gehring 1994).

The vertebrate homeobox gene family can be divided into two subfamilies: 1) the clustered homeobox genes, also known as Hox genes or class I homeobox genes and 2) the non-clustered homeobox genes, also known as divergent homeobox genes (Nunes et al., 2003). Hox genes control the formation of various regions along the main body axis. The order of Hox genes on the chromosome correlates with their expression pattern on the body axis of vertebrate embryos (Duboule & Dolle, 1989). In mice and humans, there are at least 39 HOX genes organized into four HOX loci. Each cluster is localized to different chromosomes and they contain 9 to 11 genes (Lemons & McGinnis, 2006). Non-clustered homeobox genes are scattered throughout the genome and can be further organized into families based on their sequence similarities. Some of these families include *distalless (Dlx), paired (Pax) and engrailed (En),* which are named after their homologs in *Drosophila* (Mark et al., 1996).

Antennapedia (Antp) is a Hox gene that was first discovered in *Drosophila. Antp* gain-of-function mutants results in transformation of antennae to legs, whereas loss-of-function leads to antennae formation instead of the second legs (Gibson 1999, Gehring et al., 2009). Antp was used as model to elucidate the general structure of homeobox proteins. The Antp homeodomain consists of 3 α-helices forming a globular structure, a flexible N-terminal arm and a flexible fourth helix.

Helix II and helix III form a helix-turn-helix motif which is almost identical to the helix-turn-helix motif found in many prokaryotic gene regulatory proteins (Laughon & Scott, 1984). Helix III, also known as recognition helix, contacts with DNA in the major groove. In addition to the recognition helix, the N-terminal arm also contacts the DNA in the minor groove. Homeodomain DNA binding is highly specific and conserved among species (Gehring, 1992; Affolter et al., 1990; Gehring 1993).

5.2. Prospero

The Prospero/Prox1 family is an evolutionary conserved class of atypical homeodomain proteins, which have been identified in many animals such as *Caenorhabditis elegans, Xenopus laevis* (African clawed frog), *Danio rerio* (Zebra fish), chicken, mouse and human (Ryter et al., 2002). Atypical homeodomains are structurally homologous to typical *Antennapedia* class homeodomain proteins, but their homeodomains differ at amino acid sequence level both among themselves and when compared to typical homeodomain proteins (Hassan et al., 1997).

Prospero contains 4 functional regions (Figure 5): 1) An asymmetric localization domain that targets Prospero to basal cortex during neuroblast division (Spana & Doe, 1995); 2) A nuclear localization signal that enables Prospero to enter the nucleus; 3) A Prospero domain (PD) that is conserved among the Prospero family proteins; and 4) A Homeo domain (HD), another conserved region located at C-terminal. The Prospero and Homeo domains, form a single structural unit called the Homeo-Prospero domain (HDPD), which is required for sequence specific DNA

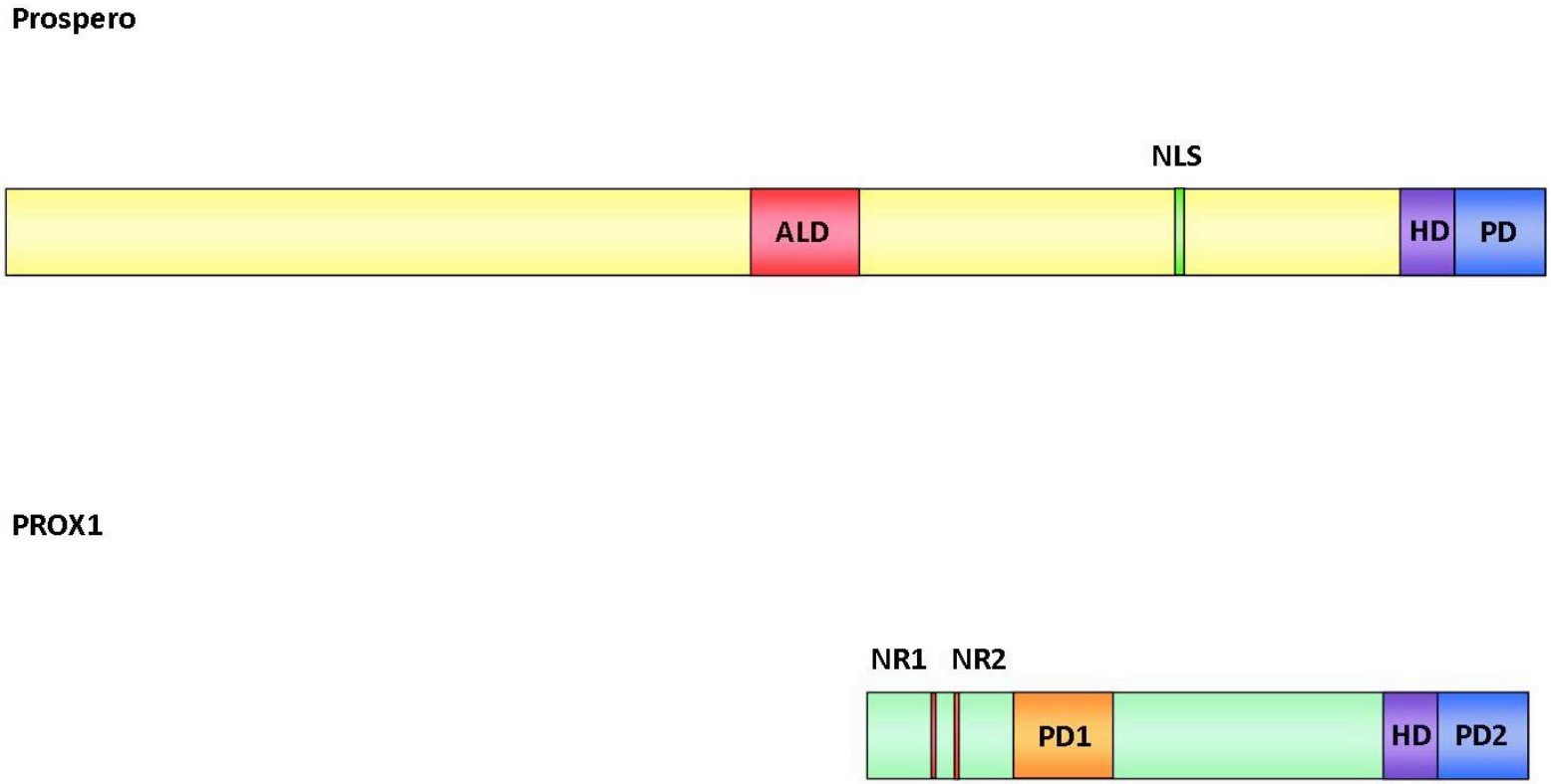


Figure 5: Prospero and PROX1 protein structure and their functional domains. The asymmetric localization domain (ALD), nuclear localization signal (NLS), Homeo domain (HD) and Prospero domain (PD) are shown on Prospero. On PROX1, 2 nuclear receptor boxes (NR1 and NR2) and 2 regions homologous to the prospero domain, Prospero domain 1 (PD1)-Homeo domain (HD) and Prospero domain 2 (PD2) are shown.

binding and transcriptional activation (Ryter et al., 2002). It recognizes the consensus sequence C A/t c/t N N C T/c, which is different than that of the typical homeodomain binding motif (Hassan et al., 1997). At its N-terminal end, HD has a nuclear export signal that mediates transport of Prospero from the nucleus via the exportin pathway to the cytosol. The PD has been shown to mask this nuclear export signal and enable Prospero to be retained in nucleus. The masking effect of PD is important in regulating subcellular distribution of Prospero during neuroblast differentiation (Demidenko et al., 2001).

Depending on the cellular context, Prospero can either activate or repress downstream target genes by acting as a transcription factor. It can also function as a cofactor by interacting with other transcription factors at its N-terminal region (Hassan et al., 1997).

During *Drosophila* nervous system development, neuroblasts divide unequally to give rise to 2 different cells: one large cell with self regeneration capacity and one small cell called the Ganglion mother cell (GMC) that further divides into two terminally differentiated neuron or glial cells (Choksi et al., 2006). Prospero is first expressed in neuroblasts, where it is associated with the basal cortex. During asymmetric cell division, Prospero is selectively partitioned to the GMCs, where it is localized to the nuclei (Choksi et al., 2006). Prospero is a critical regulator for the developmental transition of mitotically active cells to terminal differentiation. In GMCs, Prospero arrests cell division by inhibiting the expression of

regulators of cell cycle such as Cyclin A and Cyclin E (Li and Vaessin, 2000). In addition to cell cycle arrest, Prospero also represses neuroblast genes and activates genes required for terminal differentiation, thus controlling the transition from self renewal to differentiation of neural cells (Choksi et al., 2006).

5.3. *Prox1*

Prox1, the vertebrate ortholog of the *Drosophila* gene *prospero*, was first cloned in 1993 (Oliver et al., 1993). Orthologs of *prospero* have been identified in many different organisms including *C. elegans* (Burglin, 1994), zebrafish (Glasgow & Tomarev, 1998), chicken (Tomarev et al., 1996), mouse (Oliver et al., 1993) and human (Zinovieva et al, 1996). In humans, *PROX1* is mapped to chromosome 1q32.2-q32.3 and it consists of 5 exons and 4 introns encoding a protein with a mass of approximately 83 kDa (Figure 5) (Zinovieva et al, 1996). PROX1 is a conserved protein, which is 98% identical between mouse and human. The Homeo/prospero domain is identical between the two species (Tomarev et al., 1996).

In addition to lymphatic vessels, *Prox1* is expressed in many other tissues including retina, lens, pancreas and liver (Oliver et al, 1993; Tomarev et al, 1996; Dudas et al, 2004; Wang et al, 2005). In the retina, PROX1 promotes retinal progenitor cells to exit cell cycle and to differentiate into amacrine and horizontal cells (Dyer et al, 2003). During lens development, *Prox1* is expressed in differentiating lens fibres and promotes cell cycle exit by upregulating the cyclin-dependent kinase inhibitors CDKN1B (p27) and CDKN1C (p57) in these cells (Wigle et

al, 1999). In the lens, PROX1 also regulates *γ* and *βB1 crystallin* expression (Lengler et al, 2001; Cui et al, 2004; Chen et al, 2008). *Prox1* is one of the earliest markers in the developing liver and the pancreas. It is highly expressed in the endoderm which gives rise to the liver and the pancreas (Burke & Oliver, 2002). In the pancreas, PROX1 controls the size and the morphogenesis of the pancreas. It has been shown that *Prox1* null mice have 63% smaller and morphologically defective pancreas when compared to wild type mice (Wang et al, 2005). In the liver, PROX1 is required for hepatocyte migration and proliferation as *Prox1* knockout mice have a 70% reduced liver size (Sosa-Pineda et al, 2000). PROX1 increases hepatocyte proliferation by direct repression of liver receptor homolog-1 (*LRH-1*) (Kamiya et al, 2008). In addition, by co-repressing *LRH-1* and hepatocyte nuclear factor 4α (*HNF4α*) function in liver, PROX1 suppresses expression of cholesterol 7α-hydroxylase (*Cyp7a1*) gene that is involved in bile acid and cholesterol homeostasis (Song et al, 2006; Qin et al, 2004).

5.4. *Prox2*

Prox2, a recently cloned vertebrate homolog of the *Drosophila* homeodomain protein *prospero*, is evolutionary conserved in rat, mouse and human (Pistocchi et al., 2008). In mice, it is expressed in cranial ganglia during embryogenesis, adult eyes and testes (Nishijima & Ohtoshi, 2006). Unlike *Prox1*$^{-/-}$ mice, *Prox2*$^{-/-}$ mice develop normally, showing no obvious defects. Therefore, *Prox2* is dispensable for embryonic development, postnatal survival, growth and fertility (Pistocchi et al., 2008).

Although PROX2 has been shown to be transcriptionally active, its function is not known yet. PROX2 was found to be able to interact with other transcriptional factors, which implies that it might act as a cofactor (Nishijima & Ohtoshi, 2006).

6. Cell fate determination

The circulatory system originates from mesodermal cells, known as hemangioblasts, which give rise to both hematopoietic and endothelial cells (Vogeli et al., 2006). At earlier stages of vascular development, hemangioblasts aggregate to form blood islands, which are later fused to form the primitive vessels in the yolk sac and the embryo (Risau, 1997). VEGF signalling is known to be required for the differentiation of hemangioblasts into endothelial precursor cells (Eichmann et al., 1997). From these endothelial precursor cells, arterial and venous ECs differentiate depending on many transcription factors and signalling pathways (Adams & Alitalo, 2007).

6.1. Arterial-venous differentiation

From the earliest stages of angiogenesis, ECs are genetically programmed to differentiate into either arterial or venous ECs (dela Paz & D'Amore, 2009). Dynamic forces such as flow rate, shear forces and blood pressure have been shown to have the capacity to program the specification of arteries and veins during development. However, other studies have demonstrated the importance of genetic factors and the different identities of these cells from very early stages (Swift & Weinstein, 2009).

Ephrin-B2 is an Eph family transmembrane ligand that is expressed in arterial ECs, but not in venous ECs. On the other hand, *Eph-B4*, a receptor for Eph-B2 is a venous EC marker that is not expressed in arterial ECs (Wang et al., 1998). They are the earliest genes that are differentially expressed in arterial and venous ECs, even before the initiation of blood flow (Swift & Weinstein, 2009). *Eph-B2* knockout mice undergo normal vasculogenesis, but display defects in remodelling of both arteries and veins during angiogenesis (Wang et al., 1998). *Eph-B4* knockout mice have the same phenotype with the *Eph-B2* knockout mice, indicating that the complementary expression of the Eph-B2 ligand and its Eph-B4 receptor in arterial and venous ECs is required for proper remodelling and the separation of these two different vascular networks (Gerety et al., 1999).

COUP-TFII is an orphan nuclear receptor in the steroid/thyroid hormone receptor superfamily. By interacting with other transcription factors, it regulates many developmental processes such as angiogenesis and heart development (Lee et al, 2009). Inactivation of *COUP-TFII* in ECs results in embryonic lethality due to a variety of vascular defects such as hemorrhage and dilated thin vessels (Yamazaki et al., 2009). In the vasculature, *COUP-TFII* is expressed in vein ECs but is absent in arterial ECs (Tsai et al., 2005).

Venous-arterial differentiation is dependent on Notch signalling (Tsai et al., 2005). In arterial ECs, Notch signalling activates the expression of arterial markers, leading to arterial differentiation (You et al., 2005). Disruption of the Notch signalling

pathway results in embryonic lethality due to vascular defects and haemorrhage (Tsai et al., 2005). Venous identity has been thought to be the default outcome due to the lack of Notch signalling pathway. Recently, it was found that, COUP-TFII was required for venous differentiation (Tsai et al., 2005). In vein ECs, COUP-TFII suppresses Neuropilin-1, thus inhibiting Notch signalling. Without Notch signalling, venous markers are expressed and vein identity is maintained (You et al., 2005). Therefore, vein identity is not a default state, but is acquired by *COUP-TFII* expression (Tsai et al., 2005). Furthermore, disruption of *COUP-TFII* in ECs results in arterial differentiation of vein ECs via the activation of Notch signalling (Yamazaki et al., 2009). Mutant vein ECs start to express arterial markers and acquire arterial characteristics upon activation of Notch signalling (Tsai et al., 2005).

6.2. Venous-lymphatic differentiation

For the establishment of LEC fate, venous EC identity is a prerequisite. (Oliver & Srinivasan, 2008). For venous ECs to adopt lymphatic phenotype, *Prox1* expression is required. During early lymphangiogenesis, the transcription factor Sox18 induces *Prox1* expression at around E9.0 (Francois et al., 2008). However, LEC identity is reversible and for its maintenance, it is necessary to have constant *Prox1* expression in LECs. In this context, Prox1 functions as a “binary switch” to turn on the LEC phenotype and to turn off the BEC phenotype (Johnson et al., 2008).

Prox1 knockdown is sufficient to dedifferentiate LECs into BECs; they lose the expression of LEC specific markers and gradually start to express BEC markers

(Johnson et al., 2008). Downregulation of *Prox1 in vivo* results in severe lymphatic vasculature defects. *Prox1* downregulated lymphatic vessels acquire blood vessel characteristics; they circulate blood, make aberrant connections to the blood vasculature, are covered with pericyte-like cells and loose button-like junctions (Bixel & Adams, 2008). On the other hand, induction of *PROX1* expression in BECs is sufficient to induce a lymphatic fate. Gain of function studies have shown that, *PROX1* induction in BECs results in upregulation of lymphatic endothelial markers and downregulation of blood endothelial markers (Hong et al., 2002; Petrova et al., 2002).

In addition to the venous differentiation, *COUP-TFII* is also important for lymphatic identity. By acting as a coregulator, COUP-TFII modulates transcription of many target genes (Lee et al., 2009). PROX1 is one of the interacting partners of COUP-TFII and this interaction plays essential role in determining lymphatic fate (Yamazaki et al., 2009). Starting from E11.5, when LECs start budding from the cardinal vein, PROX1 and COUP-TFII are temporally and spatially co-expressed in LECs throughout development (Yamazaki et al., 2009). COUP-TFII and PROX1 physically interact and form a stable complex to control expression of several LEC specific genes such as *VEGFR-3* and *FGFR-3*, thus maintaining the lymphatic phenotype (Lee et al., 2009). COUP-TFII regulates venous identity, while PROX1 controls the differentiation of LECs from venous ECs. Taken together, the interaction between COUP-TFII and PROX1 during lymphatic differentiation strongly supports

the hypothesis that venous origin is a prerequisite for LEC specification (Srinivasan et al., 2007).

7. *CD146*

Cell adhesion molecules (CAMs) are located on the cell surface and are involved in cell adhesion process by binding to other cells or the ECM. They also function as transmembrane receptors that transfer extracellular signals into the cells, thus playing roles in many physiological processes (Mintz-Weber & Johnson, 2000). Changes in their expression profile could mediate angiogenesis, tumor cell migration and metastasis, which are processes in which cell adhesion play important roles (Yan et al., 2003). Deregulated expression of CAMs was observed during the progression of many tumours (Behrens et al., 2009; Hofmann et al., 1991).

CD146, also named MUC18 or MCAM, is a CAM that belongs to the immunoglobulin superfamily (Lehmann et al., 1987). It is a highly glycosylated 113-119 kDa transmembrane protein with five immunoglobulin–like extracellular domains, one transmembrane domain and a cytoplasmic tail, which has potential recognition sites for protein kinases (Figure 6, Yan et al., 2003). Besides its function as an adhesive protein, CD146 serves as a signalling molecule involved in focal adhesion assembly via the phosphorylation of p59fyn, p125FAK, and paxillin. Therefore, CD146 is not only a cell adhesion protein, but is also a signalling molecule involved in cell-cell contacts (Yan et al., 2003).

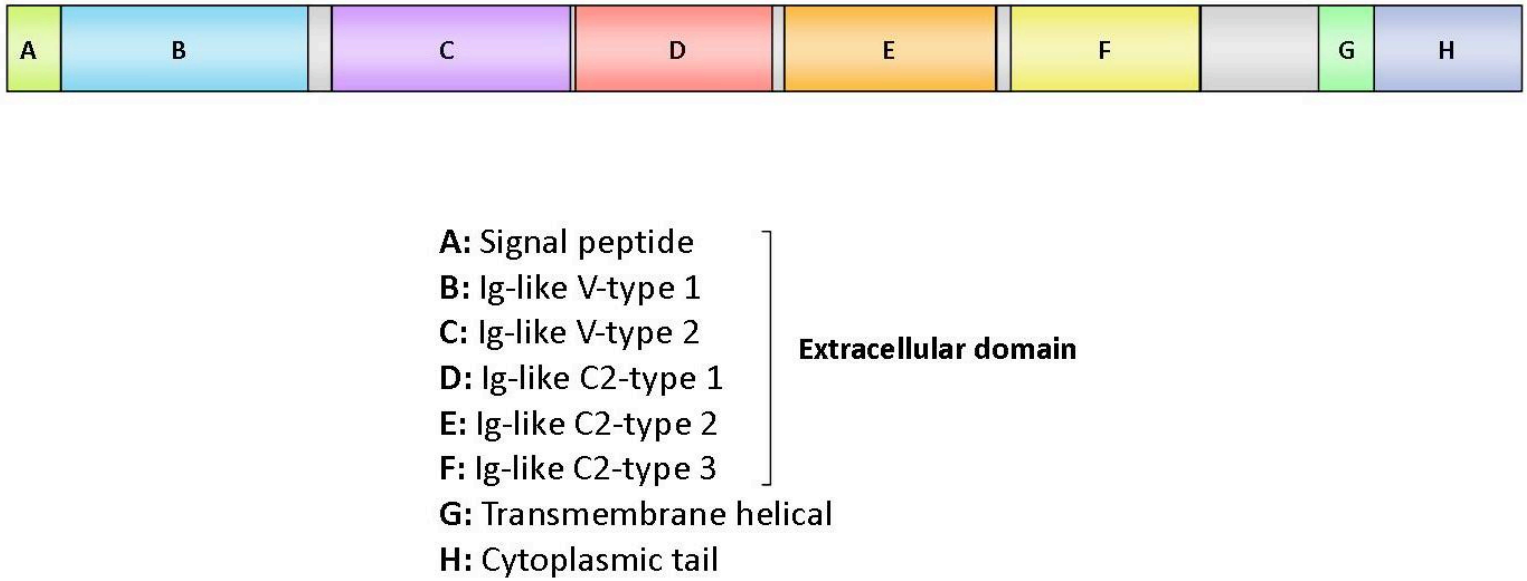


Figure 6: Representation of CD146 protein structure. CD146 is a 646 amino acid-long transmembrane glycoprotein belonging the immunoglobulin (Ig) superfamily. The extracellular part of CD146 consists of a signal peptide (A) and five Ig-like domains, forming α-pleated sheets. CD146 has a single transmembrane helix, attaching the cell surface protein to the cellular membrane. Inside the cell, CD146 has a short cytoplasmic tail possessing potential recognition sites for several protein kinases involved in signal transduction.

CD146 was first identified as a marker of melanoma progression since it is not expressed in normal melanocytes, and has been shown to play important roles during melanoma progression and metastasis (Kang et al., 2006). CD146 expression increases cell adhesion, tumor invasiveness and metalloproteinase-2 (*MMP-2*) expression in melanoma cells. It also activates the Akt pathway, resulting in inhibition of apoptosis, thus increasing survival of melanomas (Ouhtit et al., 2009). In melanoma cells, *CD146* has been shown to be regulated by Sp1, AP-2 and cAMP response element (CRE), which all have potential binding sites in the *CD146* promoter. The AP-2 transcription factor has been shown to repress *CD146* promoter activity, whereas Sp1 and CRE are important for activating *CD146* promoter (Mintz-Weber & Johnson, 2000).

CD146 has also been shown to enhance the ability of non-metastatic prostate cancer cells to metastasize to distant organs from the prostate gland. There is a correlation between the progression of prostate cancer and *CD146* expression (Wu et al., 2001).

Although CD146 promotes the progression of several tumors, including melanoma and prostate, it appears to have an inhibitory effect on breast tumor progression. During breast cancer development, *CD146* expression is lost. This finding suggests that decreased *CD146* expression might be involved in breast carcinoma development, by allowing breast cancer cells to detach and thereby

facilitate their migration. As a result, CD146 might be considered as a tumor suppressor in breast cancer (Shih et al., 1997).

Other than melanoma cells, *CD146* is also expressed in SMCs, activated T lymphocytes, bone marrow and in vascular ECs. It is one of the most widely used EC markers to isolate circulating ECs from peripheral blood (Ouhtit et al., 2009). In contrast to BECs, LECs have been shown not to express *CD146* either *in vivo* or *in vitro* (Amatschek et al., 2007).

During angiogenesis, ECs migrate to the perivascular space and proliferate. New capillaries are formed by the adhesion of these cells each other and to the ECM (Kang et al., 2006). This process is regulated by CAMs and ECM proteins. In ECs, CD146 has been found to play important roles in cellular adhesion, migration and proliferation, which are critical processes in angiogenesis (Bu et al., 2006). In order to elucidate its role during angiogenesis, variety of blocking antibodies against CD146 protein were used in most of the functional studies. In ECs, mAb AA98, one of the CD146 antibodies, has been shown to inhibit phosphorylation of p38 mitogen-activated protein kinase (MAPK), suppress nuclear factor-KB (NF-KB) activation and downregulate *MMP-9* and intercellular adhesion molecule (*ICAM-1*) expression (Bu et al., 2006). Therefore, NF-KB is important for CD146 function in EC migration, angiogenesis, and tumor metastasis. Since early angiogenic processes such as proliferation and migration of ECs are required for tumor angiogenesis, CD146 might

also be involved in increasing microvessel density in tumor area, thus tumour progression (Yan et al., 2003).

Even though the role of CD146 during angiogenesis is extensively studied, its regulation during lymphangiogenesis is unclear. As mentioned earlier, LECs differentiate from venous ECs during development of the lymphatic system (Oliver, 2004). However, it is not clear how *CD146* expressing BECs lose their *CD146* expression during lymphatic differentiation.

RATIONALE

Prox1 is a prospero related homeobox gene, which is essential for determining and maintaining the lymphatic endothelial cell fate. *Prox1* expression is restricted to a subpopulation of ECs in embryonic veins and its expression promotes the differentiation of venous ECs into LECs (Wigle & Oliver, 1999). During lymphangiogenesis, *Prox1* expression is required for the polarized migration of LECs from the anterior cardinal vein (Wigle et al., 2002). In the absence of *Prox1* expression, LECs lose their polarized migration and migrate in a random manner. In addition to the defects in budding of LEC progenitors from the cardinal vein, LECs in *Prox1* null mice lose their lymphatic identity and start inappropriately expressing BEC markers (Wigle et al., 2002). Consequently, *Prox1* null mice fail to develop a lymphatic system.

The dosage of *Prox1* expression is critical for lymphatic development and maintenance. *Prox1* heterozygous embryos develop mispatterned lymphatic vessels, thus resulting in severe edema (Wigle & Oliver, 1999). At early postnatal stages, *Prox1* haploinsufficiency is often lethal due to severe disruption of lymphatic vessels, resulting in abnormal leakage of the lymph (Wigle & Oliver, 1999). Therefore, *Prox1* is required for both acquiring and the maintaining lymphatic identity.

Although *PROX1* expression leads to lymphatic differentiation of venous ECs, LEC identity is reversible. *PROX1* expression is required not only during lymphangiogenesis but also throughout adulthood to maintain the LEC phenotype

(Johnson et al., 2008). Constant *PROX1* expression preserves the LEC phenotype and represses the BEC phenotype. Loss of *PROX1* function leads lymphatic vessels to acquire a blood vessel phenotype which results in severe lymphatic vasculature defects (Bixel & Adams, 2008). *PROX1* gain of function on the other hand, upregulates expression of lymphatic markers in blood vessels, thus suppressing the blood endothelial phenotype (Hong et al., 2002; Petrova et al., 2002).

CD146 was originally identified as being a marker for melanoma progression and metastasis (Shih et al., 1997). In addition to being expressed by melanoma cells, *CD146* is also expressed in blood ECs (Bardin et al., 1996). Since CD146 is a cell adhesion protein (Lehmann et al., 1989), it is not surprising that CD146 has been shown to be involved in the regulation of EC adhesion and migration (Yan et al., 2003). In addition, CD146 is known to induce EC proliferation *in vitro*. Since these processes are critical for angiogenesis, CD146 has been predicted to be an important regulator of blood vessel growth.

CD146 has been identified as a specific molecular marker of BECs both *in vivo* and *in vitro* (Ouhtit et al., 2009). In contrast to BECs, LECs has been shown not to express *CD146* either *in vivo* or *in vitro* (Amatschek et al., 2007). This specific expression pattern makes *CD146* a candidate marker to study the loss of BEC phenotype during lymphatic differentiation.

The different phenotypes of blood and lymphatic vessels are at least partially due to their different transcriptomes. Different sets of transcription factors are required

for the maintenance of the BEC and LEC phenotypes (Amatschek et al., 2007). One of the most important differences between blood and lymphatic vessels is their degree of permeability. Blood vessels have tight and adherens cell-cell junctions, which limit fluid exchange between the blood vessels and the surrounding tissues (Dejana et al., 2009). When compared to the tightly joined ECs in blood vessels, lymphatic vessels have loose overlapping ECs and valve-like openings between adjacent ECs (Tammela et al., 2005). These characteristics make lymphatic vessels highly permeable, allowing for fluid exchange with the environment (Oliver & Alitalo, 2005). This permeability also has important roles in cancer metastasis by promoting tumor cell migration via the lymphatic system as blood circulation is a more hostile environment for tumor cells due to the mechanical stress and immune surveillance (Joyce & Pollard, 2009).

PROX1 is a cell fate switch gene that is sufficient to convert BECs into LECs during lymphatic development. Since permeability is a characteristic of lymphatic vessels, we propose that PROX1 downregulates the expression of the BEC-specific cell adhesion molecule CD146.

HYPOTHESIS

We hypothesize that PROX1 promotes lymphatic differentiation by repressing *CD146* transcription (Figure 7).

OBJECTIVES

Our main objective was to elucidate the mechanism and the function of PROX1 regulation of *CD146* in endothelial cells. The specific aims of this project were:

- To determine whether PROX1 represses *CD146* expression
- To demonstrate if the HDPD deleted version of PROX1 (DNA binding deficient) acts as a dominant negative molecule with respect to *CD146* expression

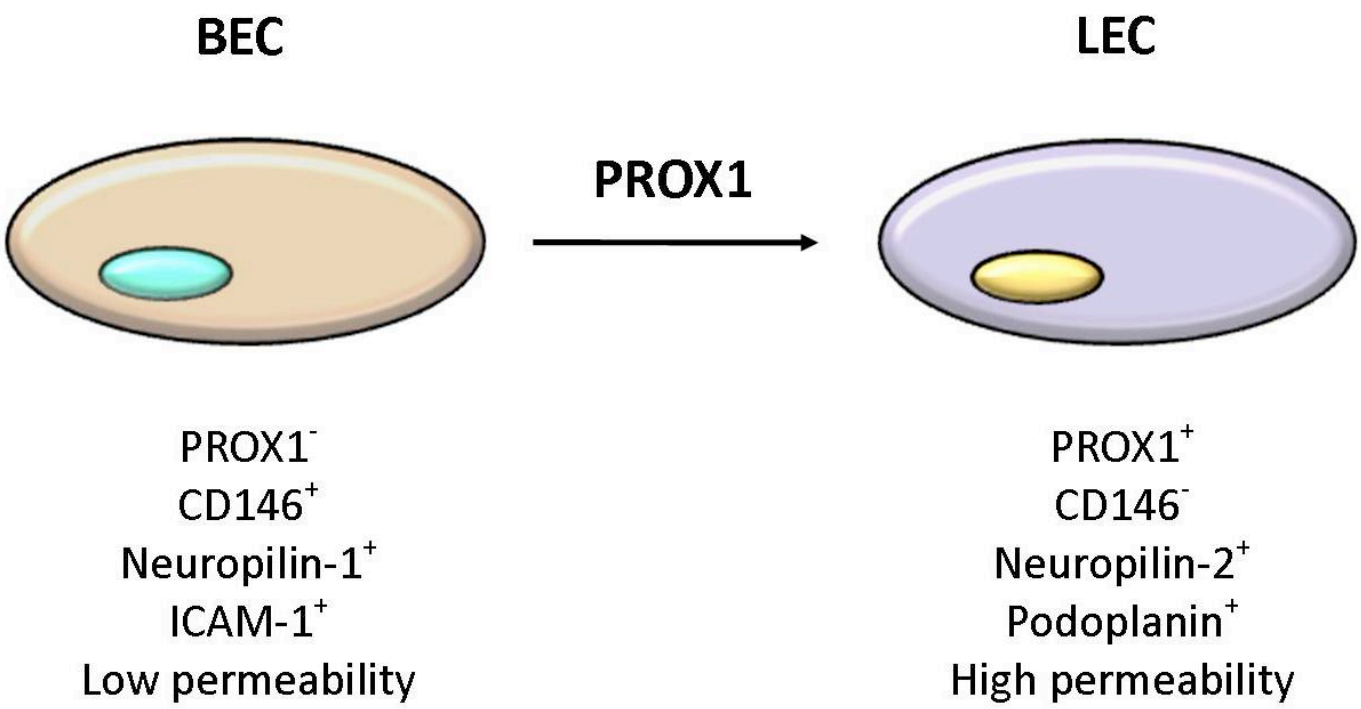


Figure 7: PROX1 promotes lymphatic differentiation by acting as a co-repressor of *CD146* transcription. PROX1 acts as a cell fate switch that converts blood endothelial cells (BECs) into lymphatic endothelial cells (LECs). CD146 is a BEC marker that has been shown to regulate endothelial cell adhesion and migration. When compared to blood vessels, lymphatic vessels are highly permeable. Therefore, we propose that PROX1 suppresses the expression of the BEC-specific cell adhesion molecule CD146 in LECs.

MATERIALS AND METHODS

1. Cell Culturing

Human Umbilical Vein Endothelial Cells (HUVEC, Clonetics, Walkersville, MD) were grown in Endothelial Cell Growth Medium-2 (EGM-2**,** Clonetics, Walkersville, MD). EGM-2 contains endothelial cell basal medium-2 (EBM-2) with growth factors, cytokines and supplements (Table 1). Neonatal Human Dermal Lymphatic Microvascular Endothelial Cells (LEC, Clonetics, Walkersville, MD) were grown in Microvascular Endothelial Cell Growth Medium-2 (EGM-2MV, Clonetics, Walkersville, MD). EGM-2MV contains EBM-2 with growth factors, cytokines and supplements (Table 1). Human Embryonic Kidney Cells (HEK 293, ATCC, Manassas, VA) were grown in HyClone DMEM/High Glucose (Thermo Scientific, Logan, UT) supplemented with 5% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin (pen/strep). Human Melanoma cells (WM-266-4, ATCC, Manassas, VA) were grown in HyClone MEM/EBSS (Thermo Scientific, Logan, UT) supplemented with 10% FBS and 1% pen/strep. Human Hepatocarcinoma Cells (HepG2, ATCC, Manassas, VA) were grown in HyClone DMEM/High Glucose (Thermo Scientific, Logan, UT) supplemented with 10% FBS and 1% pen/strep. All cells were grown at 37°C and 5% CO_2 in a humidified incubator (Thermo Scientific, Logan, UT).

2. Adenoviral Infections

For *Prox1* gain-of-function experiments, $5x10^5$ HUVECs were infected with different doses of adenoviruses encoding Enhanced Green Fluorescent Protein

(EGFP, as infection control), FLAG tagged wild type *Prox1* or FLAG tagged HDPD deleted *Prox1* (ΔHDPD) and incubated at 37°C and 5% CO_2 for 48 hours. The adenovirus encoding EGFP was a gift from Dr. Grant Pierce (University of Manitoba). Adenoviruses encoding *Prox1* and ΔHDPD were constructed previously in our laboratory using the pAdEasy system (QBiogene, Solon, OH) according to manufacturer's instructions. Cells were harvested for protein or RNA 48 hours post infection.

3. Quantitative Real-Time PCR

Total RNA was isolated from either HUVECs or LECs using an RNeasy Plus Mini kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. The annealing temperatures for each primer set were optimized prior to the generation of the standard curves. 63°C was determined to be the optimum annealing temperature for all primer sets. PCR efficiency and the optimal RNA amount required for each primer set were initially determined by generating standard curves using serial RNA dilutions and different concentrations of primers. The primers used are listed in Table 2. Real-Time reaction mixes were prepared using a 1-step SYBR Green qRT-PCR kit (Quanta Biosciences, Gaithersburg, MD). Real-Time amplification and analyses were performed on a iQ5 real-time PCR machine (BioRad, Hercules, CA). The thermocycler conditions used are listed in Table 3. Data analysis was performed using the $2^{-\Delta\Delta ct}$ method (Livak & Schmittgen, 2001) and expression levels were normalized to β-Actin which expression is not altered by PROX1.

In order to visualize Real-Time PCR products, 6X Orange Loading Dye (Fermentas, Glen Burnie, MD) was added to PCR samples before gel electrophoresis. 2 % agarose gels were prepared using 1X TAE buffer (40 mM Tris, 20 mM Acetic Acid, 1mM EDTA) and SYBR Safe (0.05%, Invitrogen, Carlsbad, CA) which was added to visualize DNA. Gel electrophoresis was performed in 1X TAE running buffer at 150 V for 30 minutes. The Gene Ruler 100 bp plus (Fermentas, Glen Burnie, MD) DNA ladder was used to estimate the size of the PCR products. DNA fragments were visualized under UV illumination using a Gel Doc XR (BioRad, Hercules, CA).

For sequence verification, amplified DNA was run on a 2% agarose gel, excised and purified using a QIAquick Gel Extraction kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. Isolated DNA was cloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA) and transformed into chemically competent DH5α-T1^{R} *Escherichia coli* (*E.coli)* using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Transformed bacteria were then spread onto Luria Broth (LB) + Kanamycin (Kan) plates and incubated overnight at 37°C. Isolated colonies were picked from the plates into 5 ml LB + Kan and shaken overnight at 37°C. In order to purify the plasmid DNA, a PureLink Quick Plasmid Miniprep kit (Invitrogen, Carlsbad, CA) was used according to the manufacturer's instructions. Purified plasmid DNA was sent for sequencing (University of Calgary) using the T_3 and T_7 sequencing primers listed in Table 4.

4. Western blotting

Cells were lysed with new RIPA buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1% NaDeoxycholate, 0.1% SDS, pH=7.4) with protease inhibitors (Protease inhibitor cocktail tablet, Roche, Mannheim, Germany). Protein concentrations were measured using the DC protein assay (BioRad, Hercules, CA; Lowry, 1951). We electrophoresed 20 µg protein samples in an SDS-PAGE gel (Resolving gel: 8% acrylamide:bis (37.5:1), 375 mM Tris pH=8.8, 0.1% SDS, 0.1 % APS, 0.1 % TEMED; Stacking gel: 4% acrylamide:bis (37.5:1), 130 mM Tris pH=6.8, 0.1% SDS, 0.1 % APS, 0.1 % TEMED) at 150 V for 70 minutes in 1x running buffer (25 mM Tris, 170 mM Glycine, 3.5 mM SDS, pH=8.3). The gel was then transferred onto a nitrocellulose membrane (BioRad, Hercules, CA) with 1x transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol, 0.01 % SDS) at 30 V at 4°C overnight. Membranes were blocked with 5% skim milk powder in Tris buffer saline (TBS, 50 mM Tris, 140 mM NaCl, 2.7 mM KCl, pH=8) for one hour at room temperature.

In order to detect PROX1, we used a rabbit anti-PROX1 polyclonal antibody (1:1000 dilution, Upstate, Temecula, CA) recognizing the amino terminal of the protein (amino acids 196-215) which is identical between mouse and human. The membrane was incubated with a rabbit anti-PROX1 polyclonal antibody for one hour at room temperature, followed by incubation with a goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate (1:2500 dilution, Molecular Probes, Eugene, OR)

for 30 minutes at room temperature. Between antibody treatments, blots were washed twice with TBST (0.5% Tween in TBS) for 15 minutes.

To detect CD146, proteins were transferred to a nitrocellulose membrane as described above and incubated with a mouse anti-CD146 monoclonal antibody (1:1000 dilution, Abcam, Cambridge, MA) for one hour at room temperature, followed by incubation with a goat anti-mouse IgG (H+L) horseradish peroxidase conjugate (1:2500 dilution, Molecular Probes, Eugene, OR) for 30 minutes at room temperature.

For FLAG detection, proteins were transferred to a nitrocellulose membrane as described above and incubated with a mouse anti-FLAG monoclonal antibody (1:5000 dilution, Sigma, St. Louis, MO) for 30 minutes at room temperature, followed by incubation with a goat anti-mouse IgG (H+L) horseradish peroxidase conjugate (1:2500 dilution, Molecular Probes, Eugene, OR) for 30 minutes at room temperature. Between antibody treatments, blots were washed twice with TBST for 15 minutes.

β-Actin was used as loading controls. In order to detect Actin, the membrane was treated with a rabbit anti-Actin polyclonal antibody (1:2000 dilution, Sigma, St. Louis, MO) for 30 minutes at room temperature, followed by incubation with a goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate (1:2500 dilution) for 30 minutes at room temperature. Between antibody treatments, blots were washed twice with TBST for 15 minutes.

Horseradish peroxidase was detected with a Western Blotting Luminol Reagent (Santa Cruz, Santa Cruz, CA). Fluor S Max Multi Imager (BioRad, Hercules, CA) was used for protein visualization. Alternatively, proteins were visualized using X-Ray film (CL-X Posure Film, Thermo Scientific, Logan, UT) developed by a film processor (Konica SRX-101A). X-Ray films were scanned using a densitometer (GS-800, BioRad, Hercules, CA) and protein quantity was determined using Quantity One 4.3.1 software (BioRad, Hercules, CA) by subtracting the pixel intensity of the background from the pixel intensity of the band (Adjusted volume). For relative quantification of the proteins, the test bands were normalized to actin bands.

5. Cloning

5.1. *Prox1* Wild-Type expression constructs

In our laboratory, the full length mouse *Prox1* cDNA (clone 6490801, Invitrogen, Carlsbad, CA) was previously cloned into the SacII/XhoI sites of pCMV-Tag 4A (Stratagene, La Jolla, CA), in frame with the carboxyl terminal FLAG tag encoded by this vector (Bocangel, 2006).

5.2. *CD146* promoter constructs

HUVEC genomic DNA was isolated using the QIAmp DNA mini kit (Qiagen, Germantown, MD) according to the manufacturer's instructions and it was used as a template to amplify a 3.2 Kb sequence upstream of the *CD146* transcriptional start site using the FailSafe PCR System (Epicentre, Madison, WI) as suggested by the manufacturer (Figure 8). *CD146* promoter primers are listed in Table 5. In order to

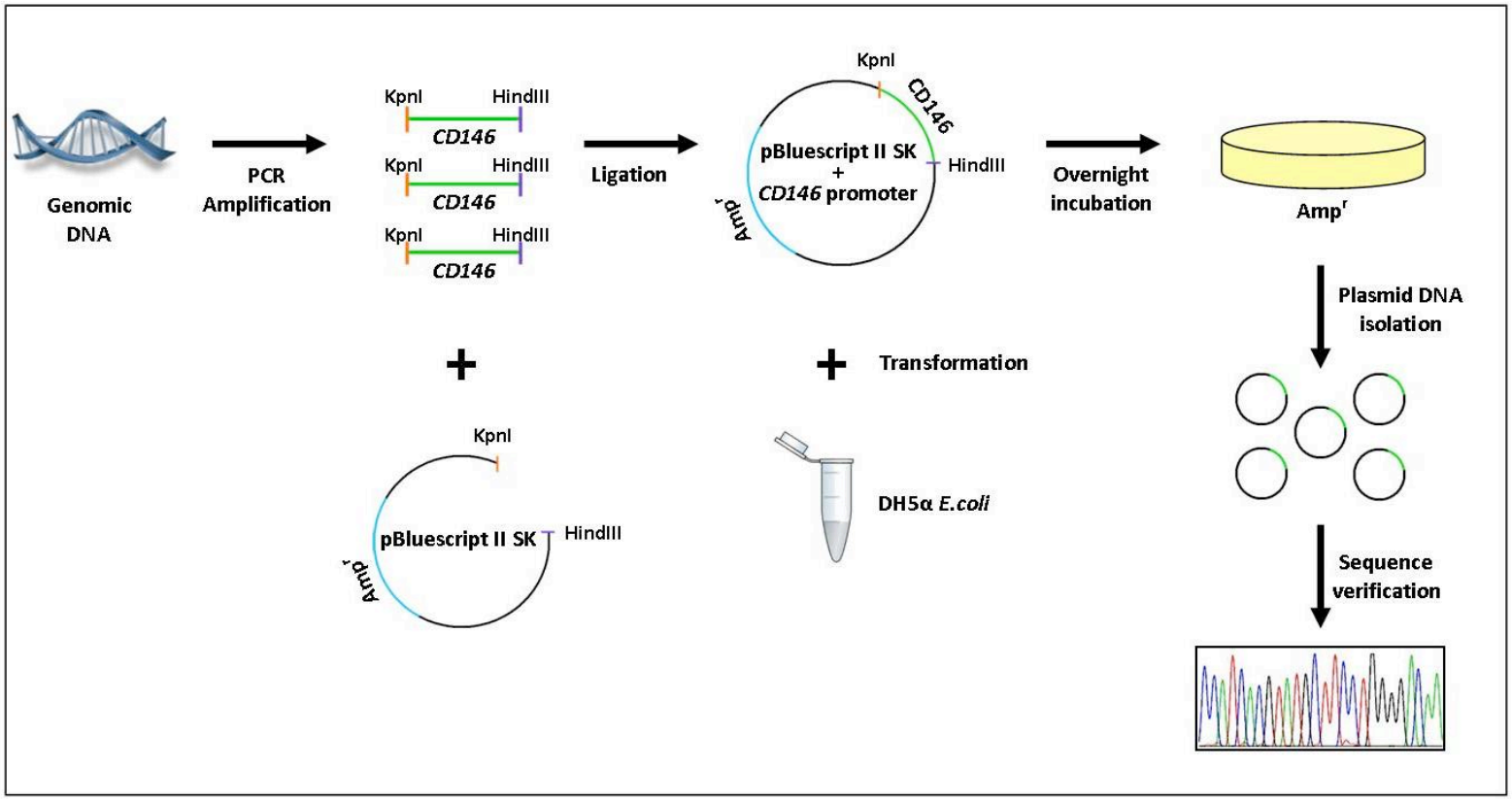


Figure 8: Cloning of the human *CD146* promoter. The 3.2 Kb long *CD146* promoter was PCR amplified from Human Umbilical Vein Endothelial Cell (HUVECs) genomic DNA using primers with KpnI and HindIII restriction sites. Amplified products were digested with KpnI and HindIII and then ligated into pBluescript II SK vector which was transformed into DH5α *E.coli* for further amplification of the plasmid. DH5α *E.coli* were grown in Amp^r agar plates and plasmid DNA was isolated from the bacteria for sequence verification. Amp^r: ampicillin resistance.

allow for directional cloning, KpnI and HindIII restriction enzyme sites were added to the 5' and 3' primer respectively. After PCR amplification, DNA was ethanol precipitated (250 µl 100% ethanol, 10 µl 3M NaOAc, 2 µl glycogen for 100 µl digested DNA) at -80°C for 20 minutes. Amplified DNA and the pBluescript II SK (+) vector (Stratagene, La Jolla, CA) were digested with the restriction enzyme HindIII at 37°C for 3 hours. After the digestion, HindIII was heat killed at 65°C for 20 minutes. HindIII digested DNA and the vector were ethanol precipitated at -80°C for 20 minutes. Once precipitated DNA and the vector were dried at 37°C and resuspended in diethyl pyrocarbonate (DEPC) treated water, KpnI digestion was performed using the same protocol for HindIII digestion. DNA and the

vector, digested with both HindIII and KpnI, were run on 0.8% agarose gel overnight at 30V in 1X TAE running buffer. The bands corresponding to the digested vector and the DNA were excised and purified using the QIAquick Gel Extraction kit according to the manufacturer's instructions. The 3.2 Kb *CD146* insert was ligated into the HindIII/KpnI site of the pBluescript II SK (+) vector using T4 ligase (Invitrogen, Carlsbad, CA) incubated overnight at room temperature. After T4 ligase was heat killed at 65°C for 10 minutes, the pBluescript II SK (+) vector with 3.2 Kb *CD146* promoter as insert was transformed into chemically competent DH5α *E.coli.* Transformed bacteria were then spread onto LB + Ampicillin (Amp) plates and incubated overnight at 37°C. Isolated colonies were transferred into 5 ml LB + Amp and shaken overnight at 37°C. In order to purify plasmid DNA, a PureLink Quick

Plasmid Miniprep kit was used according to the manufacturer's instructions. Purified plasmid DNA was sent for sequencing (University of Calgary) using the T_3 and T_7 sequencing primers listed in Table 4. After the sequence was verified, the 3.2 Kb *CD146* insert was excised with HindIII and KpnI restriction enzymes and then cloned into the HindIII/KpnI site of the pGL3-Basic Luciferase Reporter Vector (Figure 9, Promega, Madison, WI) as described above.

6. Transient Transfections

We plated 15 x 10^4 HEK 293 cells onto polystyrene 6-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) and incubated at 37°C and 5% CO_2 for 48 hours before transfection. For WM-266-4 cells, 30 x 10^4 cells were plated and incubated for 24 hours prior to transfection. Media was replaced with OptiMEM (Invitrogen, Carlsbad, CA) supplemented with 10% calf serum (Sigma, St. Louis, MO). Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used as transfection reagent according to the manufacturer's instructions. Lipofectamine 2000 (µl) / DNA (µg) ratio of 2.5 was used for all transfections. The required amount of Lipofectamine 2000 (see the ratio above) was diluted in 250 µl OptiMEM (Invitrogen, Carlsbad, CA) and incubated for 5 minutes at room temperature. At the same time, the different DNA samples were diluted in 250 µl OptiMEM (Invitrogen, Carlsbad, CA). HEK 293 cells were transfected with 1 µg pCMV Tag 4A vector encoding *Prox1* (or empty pCMV Tag 4A vector as control), 1 µg pGL3-Basic Luciferase reporter vector encoding the 3.2 Kb *CD146* promoter and 1 µg β-galactosidase expression plasmid pcDNA3-

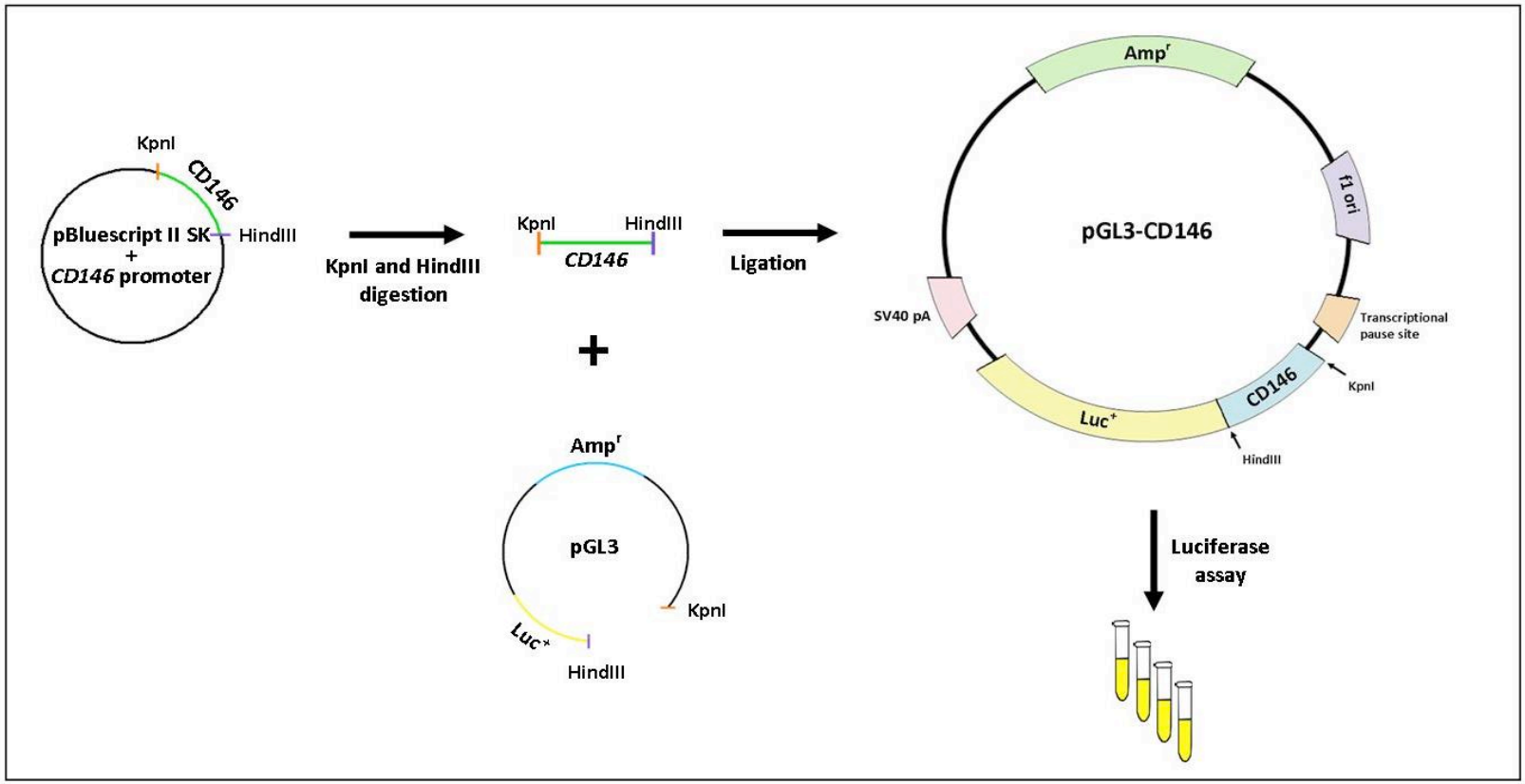


Figure 9: Cloning of the *CD146* promoter into the pGL3-Basic Luciferase Vector. 3.2 Kb long *CD146* promoter was excised from the pBlueScript II SK vector and cloned into the KpnI/HindIII sites of pGL3-Basic Luciferase vector. Amp^r: ampicillin resistance, f1 ori: f1 origin of replication, Luc^+: firefly luciferase, SV40 pA: Simian Virus 40 polyA signal.

LacZ (gift from Dr. Mesaeli, University of Manitoba). WM-66-4 cells were transfected with 1.5 µg pCMV Tag 4A vector encoding *Prox1* (or empty pCMV Tag 4A vector as control), 1.5 µg pGL3-Basic Luciferase reporter vector encoding 3.2 Kb CD146 promoter and 1 µg β-galactosidase expression plasmid pcDNA3-*LacZ*. After 5 minutes of incubation, DNA and Lipofectamine 2000 mixtures were combined together and incubated for 20 minutes at room temperature. The resulting mixture was then added to the cells dropwise and cells were incubated at 37°C and 5% CO_2. 4 hours after transfection, OptiMEM with 10% calf serum was replaced with HyClone DMEM/High Glucose (Thermo Scientific, Logan, UT) supplemented with 5% FBS and 1% pen/strep for HEK 293 cells and HyClone MEM/EBSS (Thermo Scientific, Logan, UT) supplemented with 10% FBS and 1% pen/strep for WM-266-4 cells. 48 hours after transfection, cells were harvested with NP40 lysis buffer (10% NP40, 1M Tris pH=7.8) for luciferase assay.

7. Luciferase Assay

For Luciferase assay, 20 µl of cell lysates were aliquoted into duplicate tubes and 100 µl Luciferase assay buffer (20 mM Tricine, 1.07 mM $MgCO_3$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 mM coenzyme A, 470 mM Luciferin, 530 mM ATP) was used per tube. Luciferase activity was measured using a Luminometer (Lumat LB 9507, Berthold Technologies, Oak Ridge, TN). For β-galactosidase assay, 20 µl cell lysates were aliquoted into duplicate wells in a polystyrene 96-well plate (Becton Dickinson, Franklin Lakes, NJ). We added 70 µl ddH_2O and 30 µl ONPG (Sigma, St.

Louis, MO) onto the wells. After incubating for an hour at 37°C, OD (415 nm) values were measured using a microplate reader (MRX Revelation, Dynex Technologies, Chantilly, VA).

Luciferase values were divided by β-galactosidase values in order to normalize Luciferase activity for differences in transfection efficiency of each sample. Fold activation values for each sample were obtained by dividing the normalized Luciferase activity values of the samples by the average normalized Luciferase activity value of the control sample.

8. siRNA Transfections

Twenty four hours before siRNA transfection, $3x10^5$ LECs were plated in 6-well plates. Prior to transfection, fresh LEC media (EGM-2MV) was added into the plates. DharmaFECT1 (Dharmacon, Lafayette, CO) was used as transfection reagent according to the manufacturer's instructions. 4 µl DharmaFECT1 for each plate was mixed with 100 µl EBM-2 and incubated at room temperature for 5 minutes. At the same time, 2 µM siRNA aliquots (2 µl 100 µM stock siRNA in 98 µl DEPC water) were prepared on a RNase free benchtop and mixed with 200 µl EBM-2. After 5 minutes, DharmaFECT1 and siRNA mixtures were combined together and incubated for 20 minutes at room temperature. The final mixture was added to the cells dropwise and cells were incubated at 37°C and 5% CO_2 for either 48 or 72 hours. Afterwards, the cells were harvested for Western blotting or Real-time PCR analysis. ON-TARGETplus Non-Targeting Pool (Dharmacon, Lafayette, CO) was used as control

siRNA and ON-TARGETplus SMARTpool siRNA *PROX1* (Dharmacon, Lafayette, CO) was used as the test siRNA. Mock transfected samples (transfection reagent with no siRNA added) were used as transfection control.

9. Immunocytochemistry

For siRNA transfections and comparison of BECs versus LECs, 3 x 10^5 cells were plated onto collagen coated coverslips and either left non-treated for comparison of BECs versus LECs or transfected with siRNA as explained above. Cells were fixed with 4% paraformaldehyde (EMD, Gibbstown, NJ) and washed with PBS-T (0.3% Triton X-100 in PBS). Cells were then blocked with 5% Goat serum (Sigma, St. Louis, MO) in PBS-T overnight at 4°C. In order to detect PROX1, we used a rabbit anti-PROX1 polyclonal antibody (1:250 dilution, Chemicon, Billerica, MA), a synthetic peptide recognizing the carboxyl terminal of mouse PROX1, which also reacts with human. After blocking, cells were treated with the rabbit anti-PROX1 polyclonal antibody for one hour at room temperature, followed by Alexa Fluor 488 goat anti-rabbit IgG (H+L) (1:200 dilution, Molecular Probes, Eugene, OR) incubation for one hour at room temperature. Between antibody treatments, coverslips were washed twice with PBS-T for 15 minutes. After the antibody treatments, coverslips were mounted onto glass microscope slides (VWR, Mississauga, ON) using SlowFade Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, Carlsbad, CA).

For HUVEC dose response experiments, 1.5 x 10^5 HUVECs were plated onto collagen coated coverslips and infected with adenoviruses as explained above. Cells

were fixed with 4% paraformaldehyde and washed with PBS-T. Cells were then blocked with 5% Goat serum in PBS-T overnight at 4°C. In order to detect FLAG tagged exogenous PROX1, we treated cells with a mouse anti-FLAG monoclonal antibody (1:1000 dilution, Sigma, St. Louis, MO) for one hour at room temperature, followed by Alexa Fluor 488 goat anti-mouse IgG (H+L) (1:400 dilution, Molecular Probes, Eugene, OR) incubation for one hour at room temperature. Between antibody treatments, coverslips were washed twice with PBS-T for 15 minutes. After the antibody treatments, nuclei were stained with propidium iodide (1:100 dilution, Invitrogen, Carlsbad, CA) for 3 hours at room temperature. After propidium iodide stainint, coverslips were washed twice with PBS-T for 15 minutes and mounted onto glass microscope slides (VWR, Mississauga, ON) using FluorSave reagent (Calbiochem, Gibbstown, NJ).

For all experiments, slides were visualized with a fluorescence microscope (Axioscop 2 mot plus, Zeiss, Thornwood, NY) and 5 pictures for each slide were taken at 40x magnification with an AxioCam digital camera (Zeiss, Thornwood, NY) and AxioVision Rel.4.6 software.

10. Statistical Analysis

Statistical analysis was performed using the data analysis and graphing software Origin 8. Statistical significance of results was determined by the analysis of variance (ANOVA) test. P values of <0.05 were considered statistically significant.

Table 1: List of growth factors, cytokines and supplements in EGM-2 and EGM-2MV

Human Epidermal Growth Factor (hEGF)
Hydrocortisone
Gentamicin-Amphotericin B (GA-1000)
Vascular Endothelial Growth Factor (VEGF)
Human fibroblast Growth Factor basic (hFGF-B)
Insulin-like Growth Factor (R3-IGF-1)
Ascorbic Acid
Heparin (only in EGM-2)
Fetal Bovine Serum (FBS)

Table 2: Real-time PCR primers

Name	**Sequence**
Human *PROX1* Fwd	CGTGTGTAAAGTCTCTATTAGC
Human *PROX1* Rev	CACTGTCTGTAGAGTTGTGC
Human *CD146* Fwd	TATTTCCTCTATAAGAAGGGCAAGC
Human *CD146* Rev	CTTCTGGGAGCTTATCTGACTTAAC
Human β-Actin Fwd	AGGCCAACCGCGAGAAGATG
Human β-Actin Rev	CAGAGGCGTACAGGGATAGCAC

Table 3: The thermocycler conditions for Real-time PCR

Step	**Cycles**	**Temperature**	**Duration**
I. Initial denaturation	1	50°C	10 minutes
II. Denaturation	1	95°C	5 minutes
III. Amplification **a)** Denaturation **b)** Annealing (Data collection, real time analysis)	30	 95°C 63°C	 10 seconds 30 seconds
I**V.** Denaturation	1	95°C	1 minute
V. Annealing	1	55°C	1 minute
VI. Melt curve data collection	81	55°C (+0.5°C each cycle)	10 seconds

Table 4: Sequencing primers

Name	**Sequence**
T_3	AATTAACCCTCACTAAAGGGA
T_7	CTAATACGACTCACTATAGGGC

Table 5: 3.2 Kb CD146 promoter primers

Name	**Sequence**
CD146 3.2 kb promoter Fwd	GCGGTACCTCACTCCTTCAGTATAATCTTCTCCTACTC
CD146 3.2 kb promoter Rev	GCAAGCTTATACTCTAGAATCCCGGCTGCAAACT

Orange: KpnI restriction enzyme site

Purple: HindIII restriction enzyme site

RESULTS

1. Differences in *PROX1* and *CD146* expression between BECs and LECs

1.1 *PROX1* is a specific LEC marker

Prox1 is a specific marker for lymphatic endothelium as it is highly expressed in LECs, but not in BECs (Wigle & Oliver, 1999). We wanted to verify PROX1 expression in the primary endothelial cell types used in our *in vivo* system by Western Blotting. We used Human Umbilical Vein Endothelial Cells (HUVECs) as our source of BECs and Human Dermal Lymphatic Microvascular Endothelial Cells as our source of LECs in our experiments. We also used Human Hepatocarcinoma cells (HepG2) as a positive control for PROX1 expression (Dudas et al., 2004). A 83 kDa PROX1 band was detected in HepG2 cells and LECs, but not in BECs (Figure 10A). Similar results were obtained with immunocytochemistry. We detected nuclear PROX1 expression in LECs, but not in HUVECs (Figure 11). Consequently, we confirmed previous studies by showing that PROX1 is a specific marker of LECs *in vitro*.

1.2 *CD146* expression is lower in LECs than in BECs

CD146 has been shown to be a BEC marker both *in vivo* and *in vitro*. In contrast to BECs, LECs do not express *CD146* (Amatschek et al., 2007). In order to compare CD146 protein levels in BECs and LECs, we performed Western Blotting using a mouse anti-CD146 monoclonal antibody. In contrast to previous studies, we

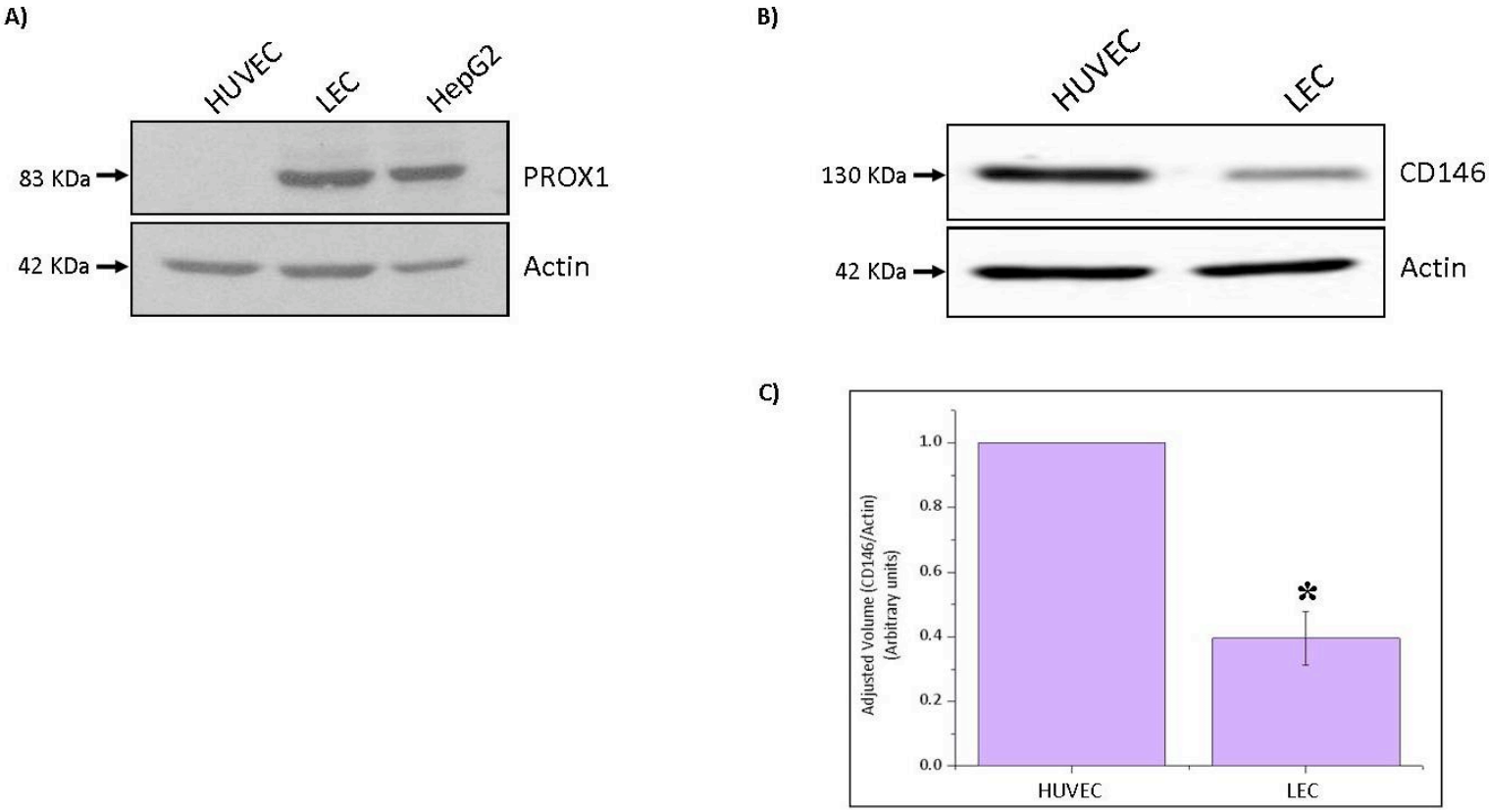


Figure 10: Comparison of PROX1 and CD146 expression in primary Human Venous and Lymphatic Endothelial Cells. **A)** A representative Western Blot comparing PROX1 protein expression in Human Umbilical Vein Endothelial Cells (HUVECs), Neonatal Human Dermal Lymphatic Microvascular Endothelial Cells (LECs) and Hepatocarcinoma cells (HepG2, positive control) is shown. Actin was used as a loading control. PROX1 expression was detected in both LECs and HepG2 cells, but not in HUVECs. **B)** A representative Western blot comparing CD146 protein expression in HUVECs and LECs. **C)** Quantification of CD146 protein levels from B are shown. (n=6, $p=2.66 \times 10^{-5}$. Error bars represent standard error).

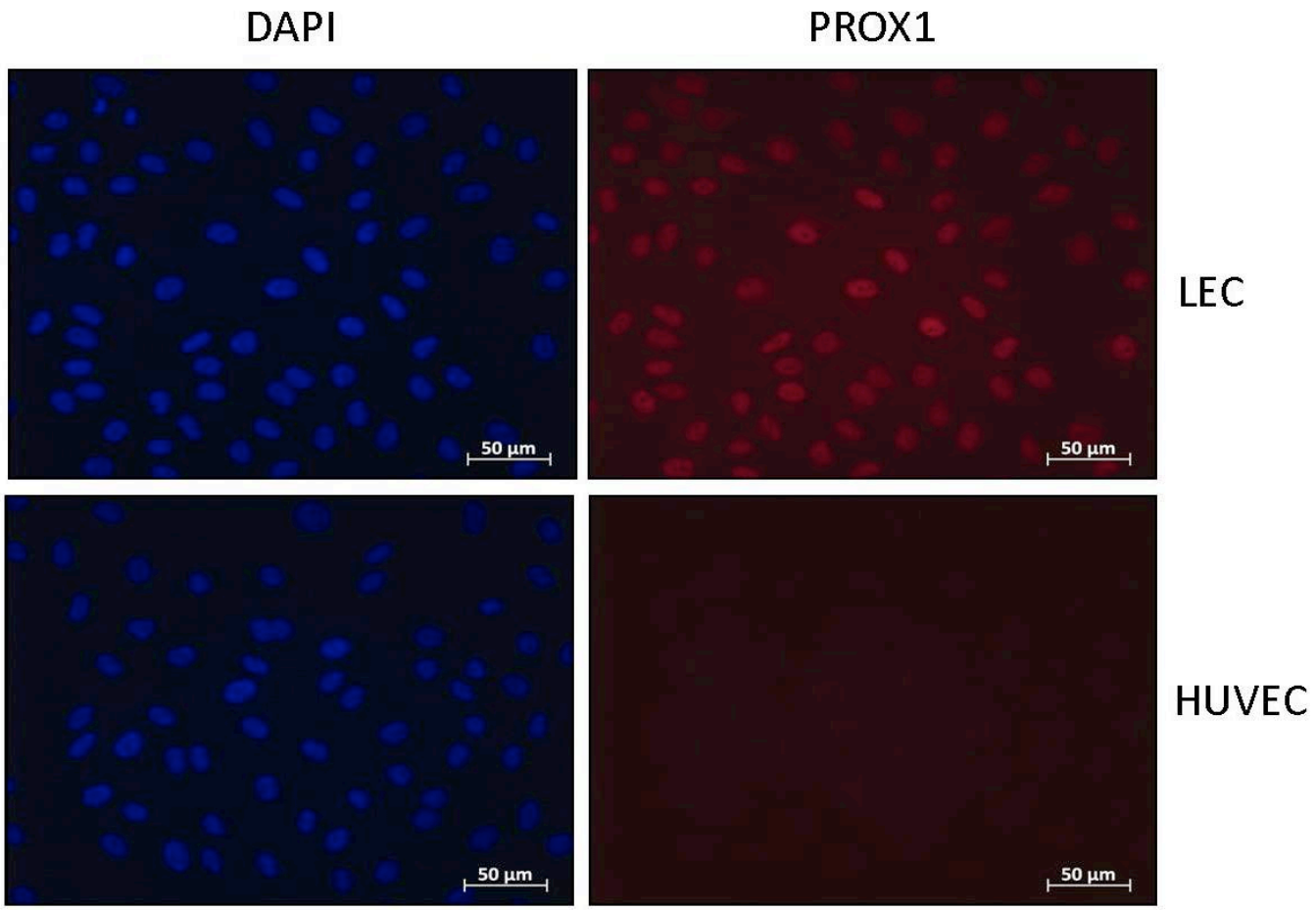


Figure 11: PROX1 expression and subcellular localization in Neonatal Human Dermal Lymphatic Microvascular Endothelial Cells (LECs) and Human Umbilical Vein Endothelial Cells (HUVECs). PROX1 (Red) was detected exclusively in the nuclei of LECs but not in HUVECs. DNA was stained with 4',6-diamidino-2-phenylindole (DAPI; Blue). 40X magnification was used. Scale bar = 50 µm.

detected CD146 expression in LECs at the protein level (Figure 10C). However, it was significantly less expressed in LECs as compared to BECs ($p=2.66x10^{-5}$).

1.3 The effect of *in vitro* culture on PROX1 and CD146 expression in ECs

Since it is known that ECs lose their lineage-specific properties when they are isolated from their microenvironment and cultured *in vitro* (Lacorre et al., 2004), we wanted to check levels of PROX1 and CD146 expression at different passages of LECs (Figure 12). When we performed Western blotting using the same PROX1 and CD146 antibodies described above, we observed decreased PROX1 expression with increased passage numbers. On the other hand, increased CD146 expression was observed with increased passage numbers.

Therefore, we concluded that PROX1 expression in LECs gradually decreases with extended *in vitro* culture. In contrast, LECs express higher levels of CD146 as they are passaged *in vitro*. Accordingly, we used LECs with a passage number lower than 6 for all our experiments in order to better model the *in vivo* situation.

2. The effect of *Prox1* gain of function on *CD146* expression in ECs

In order to understand the effect of ectopic *Prox1* expression on CD146 protein levels in endothelial cells, HUVECs were infected with adenoviruses encoding either wild type *Prox1* (Ad*Prox1*), or *EGFP* (Ad*EGFP*) as a control for infection. As compared to the uninfected HUVECs, Ad*Prox1* infected HUVECs expressed significantly lower levels of CD146 protein at both 200 MOI ($p=0.0123$) and 400 MOI ($p=0.00515$) as

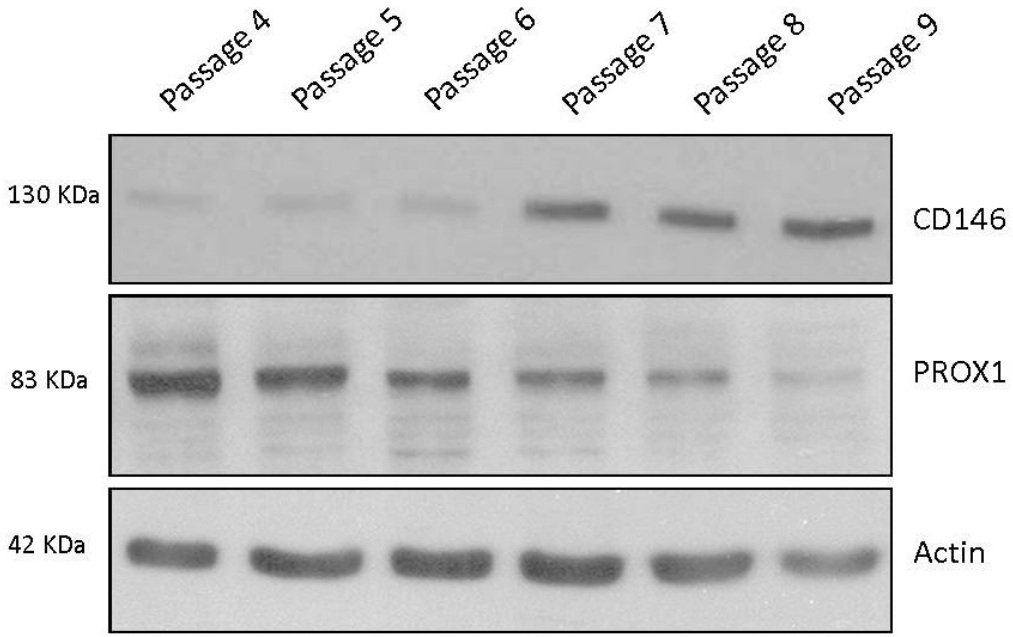


Figure 12: The effect of *in vitro* culture on PROX1 and CD146 expression in LECs. A representative Western Blot comparing PROX1 and CD146 protein expression levels in LECs at different passages is shown. Actin was used as a loading control. With increased passaging, decreased PROX1 protein expression was observed. On the other hand, CD146 protein expression increased with increasing passage number. (n=1)

detected by Western Blotting (Figure 13A). EGFP expression on the other hand, had no effect on CD146 protein levels.

As the next step, we wanted to see whether a DNA binding deficient version of *Prox1* (Δ*HDPD*) had a similar effect on CD146 expression in endothelial cells. HUVECs were infected with adenoviruses encoding either Δ*HDPD* (Ad*HDPD*) or Ad*EGFP*, as an infection control. In contrast to wild type *Prox1, HDPD* overexpression surprisingly resulted in significantly increased CD146 protein levels in HUVECs, when compared to both uninfected ($p=0.0208$) and Ad*EGFP* infected HUVECs ($p=9.89x10^{-4}$, Figure 13B).

In order to determine whether ectopic expression of *Prox1* or HDPD altered CD146 protein levels in a dose dependent manner, we used different multiplicities of infection (10, 50, 100 and 200 MOI). HUVECs infected with Ad*EGFP* at 200 MOI were used as the infection control. When compared to the uninfected HUVECs, Ad*Prox1* infected HUVECs had significantly decreased CD146 protein expression at 50 MOI ($p=0.00293$), 100 MOI ($p=4.15x10^{-4}$ and 200 MOI ($p=6.59x10^{-6}$, but not at 10 MOI (Figure 14). At higher MOIs, Ad*Prox1* infection had greater effects on decreasing CD146 expression, but the trend was not significant.

Opposite results were obtained with Ad*HDPD* infections. With increasing MOI of Ad*HDPD*, CD146 protein expression was gradually increased (Figure 15). However, the only significant increase was at 200 MOI when compared to the uninfected controls ($p=0.0729$). At lower MOI levels, the increase in CD146 protein levels was

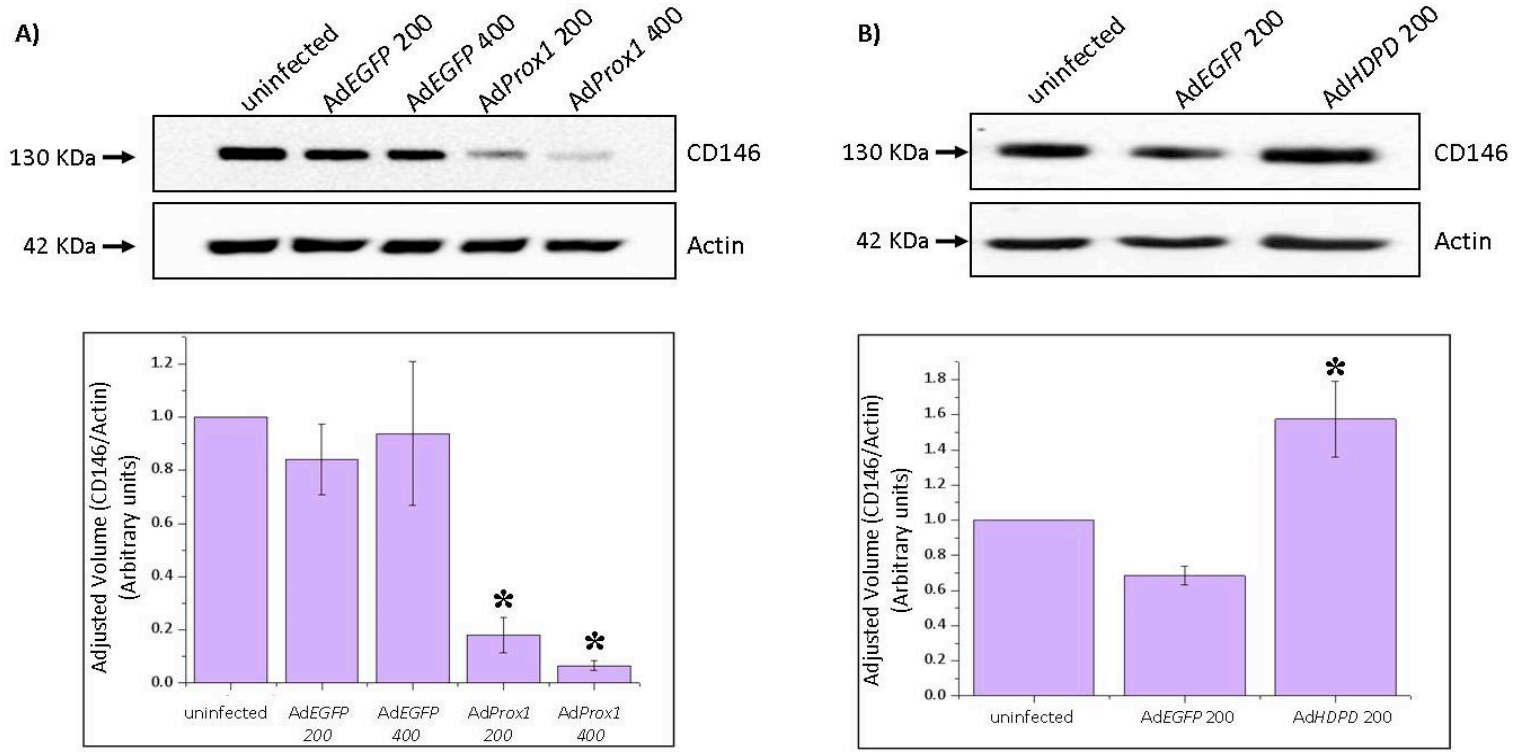
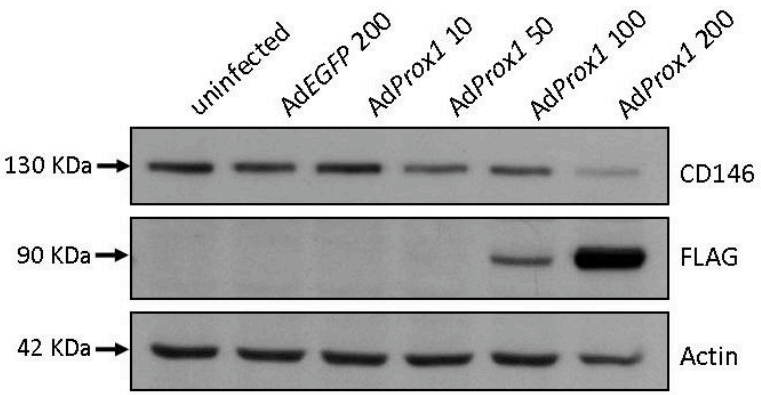


Figure 13: The effect of *Prox1* gain of function on CD146 expression in HUVECs. A) A representative Western Blot showing CD146 protein levels in HUVECs infected with adenoviruses encoding wild-type *Prox1* (Ad*Prox1*) at 200 and 400 Multiplicity of Infection (MOI) for 48 hours. Ad*EGFP* was used as an infection control. Actin was used as a loading control. When compared to uninfected HUVECs, Ad*Prox1* infected HUVECs had lower levels of CD146 expression at both 200 MOI (p=0.01228) and 400 MOI (p=0.00515) (n=3, error bars represent standard error) **B)** A representative Western Blot showing CD146 protein levels in HUVECs infected with adenoviruses encoding HDPD deleted *Prox1* (Ad*HDPD*) at 200 MOI for 48 hours. 400 MOI was not used in future studies due to increased cell death following PROX1 expression (data not shown) When compared to uninfected HUVECs, Ad*HDPD* infected HUVECs have higher levels of CD146 expression (n=5, p=0.02079, error bars represent standard error).

A)



B)

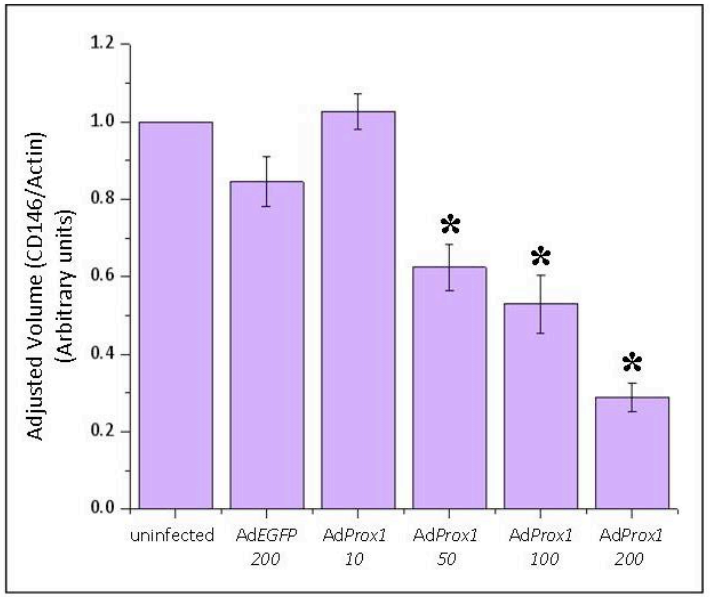
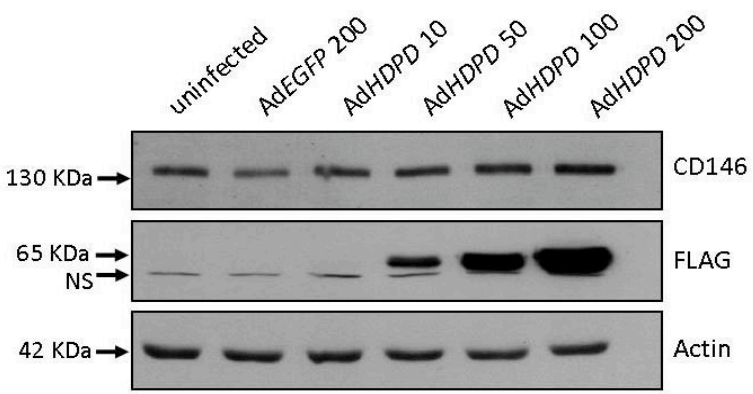


Figure 14: HUVECs overexpressing different doses of *Prox1* have decreased CD146 protein expression. A) A representative Western Blot showing CD146 and PROX1 protein levels in HUVECs infected with adenoviruses encoding *Prox1* (Ad*Prox1*) at 10,50,100 and 200 multiplicity of infection (MOI) for 48 hours. Exogenous PROX1 protein levels were detected using an anti FLAG monoclonal antibody. Actin was used as a loading control. **B)** Quantification of CD146 protein levels are shown. When compared to uninfected HUVECs, Ad*Prox1* infected HUVECs at 50 MOI ($p=0.00293$), 100 MOI ($p=4.15\times10^{-4}$) and 200 MOI ($p= 6.59\times10^{-6}$) have lower levels of CD146 expression (n=3, error bars represent standard error).

A)



B)

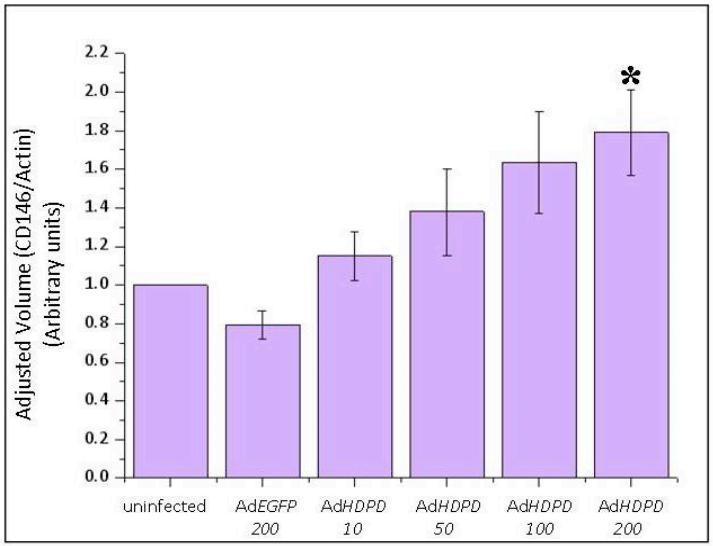


Figure 15: HUVECs overexpressing different doses of ΔHDPD form of *Prox1* have increased CD146 protein expression. **A)** A representative Western Blot showing CD146 and PROX1 protein levels in HUVECs infected with adenoviruses encoding HDPD deleted *Prox1* (Ad*HDPD*) at 10,50,100 and 200 multiplicity of infection (MOI) for 48 hours. PROX1 protein levels were detected using an anti-FLAG monoclonal antibody. Actin was used as a loading control (NS: Non-Specific). **B)** Quantification of CD146 protein levels are shown. When compared to Ad*EGFP* infected HUVECs (200 MOI), Ad*HDPD* infected HUVECs at 200 MOI have higher CD146 expression (n=3, p=0.0184, error bars represent standard error).

not significant when compared to the Ad*EGFP* infected HUVECs. Therefore, we did not observe a clear dose dependent effect of Ad*HDPD* infection on CD146 protein expression.

Both wild type and ΔHDPD version of PROX1 in our adenoviral constructs were tagged with FLAG at their carboxyl termini. Therefore, we performed Western Blotting with an anti-FLAG monoclonal antibody to detect exogenous PROX1 expression (Figure 14A). PROX1 expression was not detected at 10 and 50 MOI. However at 50 MOI, we observed a significant decrease in CD146 expression. On the other hand, HDPD expression was readily detectable starting at 50 MOI (Figure 15A). HDPD protein levels were consistently higher than for PROX1 at identical MOI.

We also performed immunocytochemistry using the FLAG antibody in order to visualize exogenously expressed PROX1 proteins. In HUVECs that were infected with either Ad*Prox1* or Ad*HDPD*, protein expression was reproducibly increased with increasing MOI (Figure 16, 17). Consistent with our Western blot results, we observed a higher percentage of FLAG-positive nuclei when the HUVECs were infected with Ad*HDPD* than Ad*Prox1*. This could be due to the differences in protein stability or the protein expression between the wild type and ΔHDPD PROX1. Another difference between the wild type and ΔHDPD PROX1 is their sub-nuclear distribution (Figure 18). Wild type PROX1 was concentrated at small and abundant speckles within the nuclei, whereas the nuclear speckles in which ΔHDPD was concentrated, were larger and fewer in number.

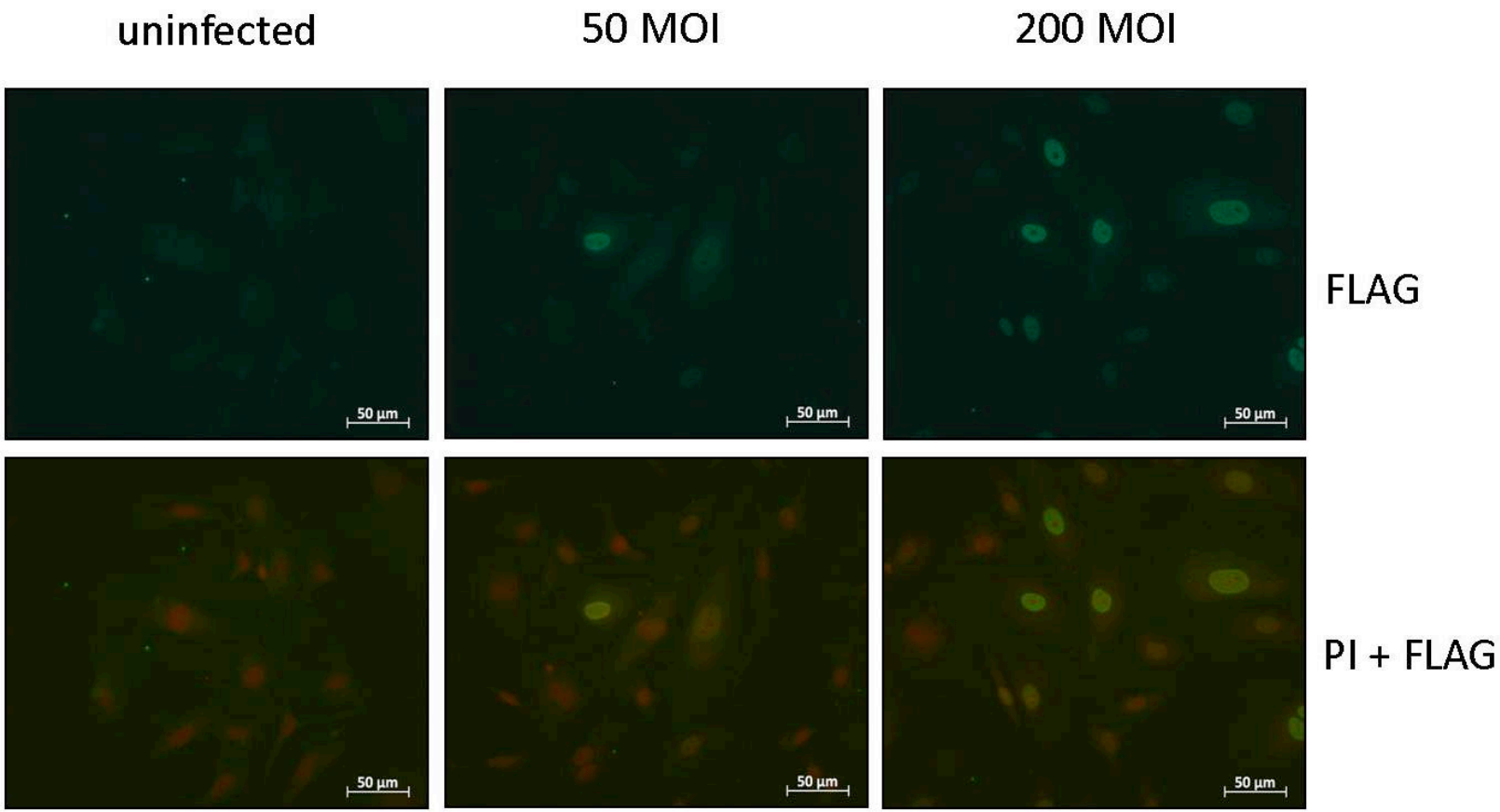


Figure 16: PROX1 expression and subcellular localization in Ad*Prox1* infected HUVECs. Immunocytochemistry for PROX1 in HUVECs infected with adenoviruses encoding *Prox1* at 10, 50, 100 and 200 multiplicity of infections (MOI) for 48 hours. Uninfected HUVECs were used as a control. PROX1 expression increased with increasing MOI level. Nuclei are shown in red (Propidium Iodide; PI), PROX1 is shown in green. 40x magnification was used. Scale bar = 50 µm.

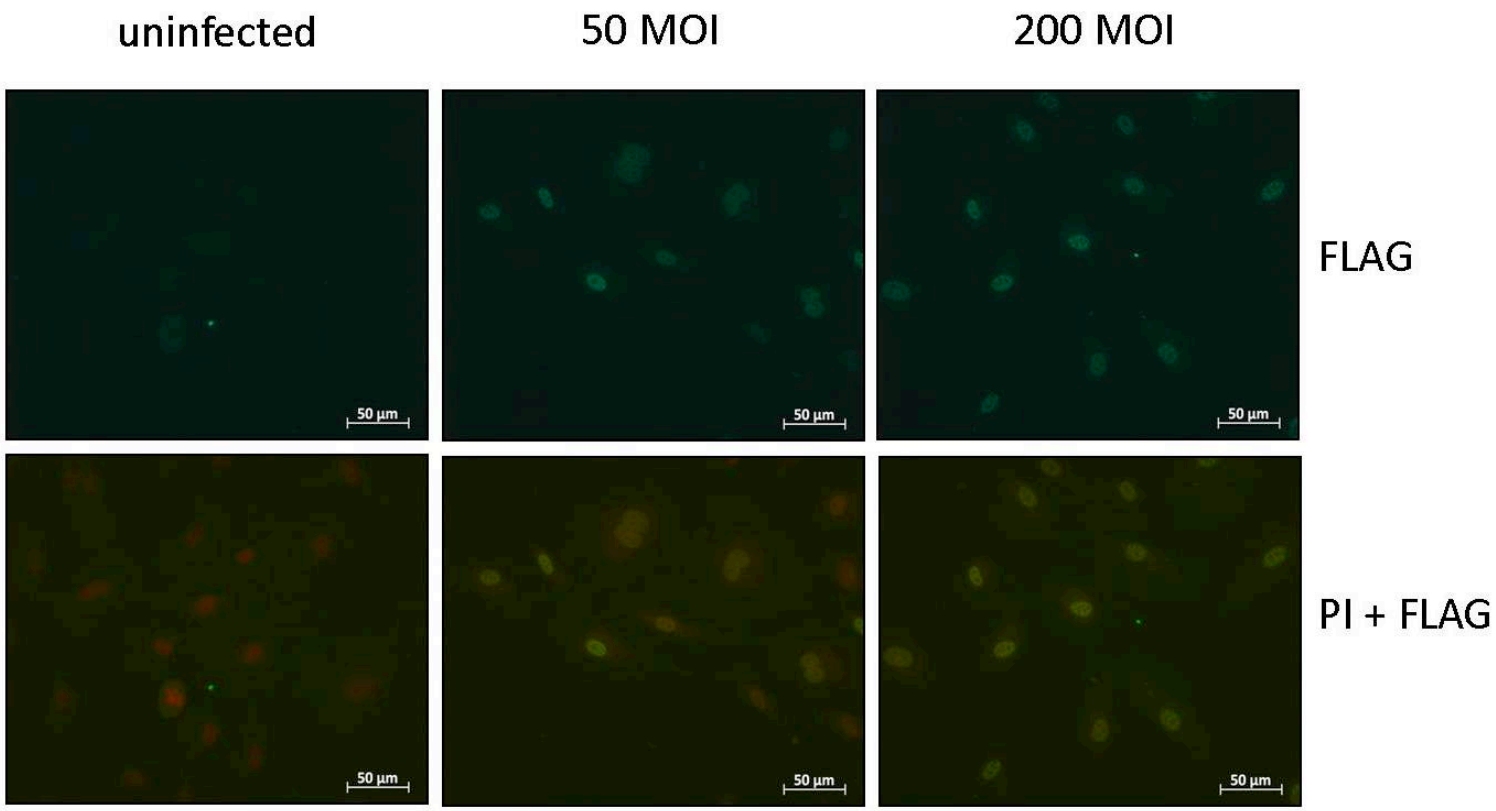


Figure 17: PROX1 expression and subcellular localization in Ad*HDPD* infected HUVECs. Immunochemistry for PROX1 in HUVECs infected with adenoviruses encoding HDPD deleted *Prox1* (Ad*HDPD*) at 10, 50, 100 and 200 multiplicity of infections (MOI) for 48 hours. Uninfected HUVECs were used as a control. PROX1 expression increases with increasing MOI level. Nuclei are shown in red (Propidium Iodide; PI), PROX1 is shown in green. 40x magnification was used. Scale bar = 50 µm.

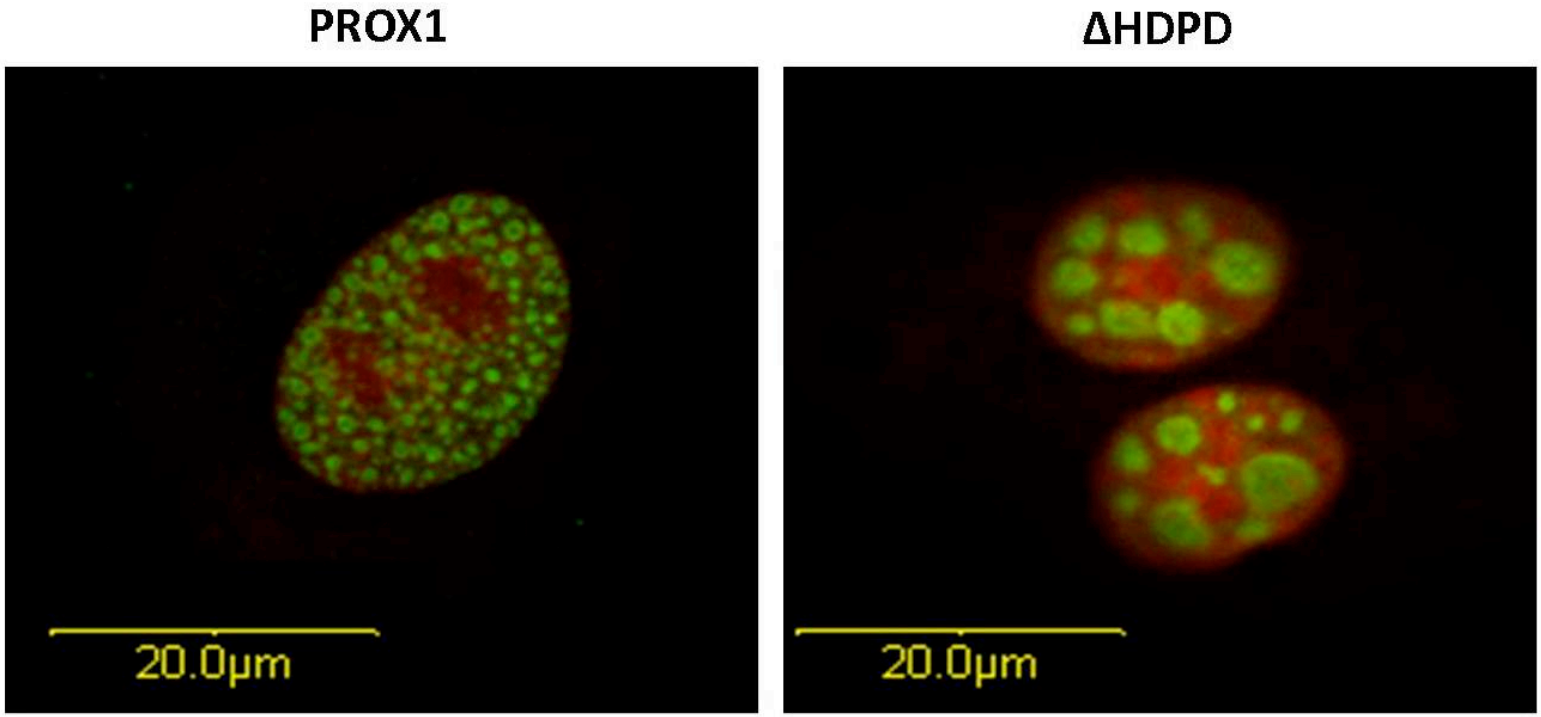


Figure 18: Subcellular localization of wild type and ΔHDPD version of PROX1 in HUVECs. Enlarged representative immunocytochemistry images for the nuclear distribution of PROX1 in HUVECs infected with adenoviruses encoding wild type or HDPD deleted version of *Prox1* at 200 multiplicity of infection (MOI) for 48 hours. Wild type PROX1 was concentrated in small and abundant speckles, whereas ΔHDPD PROX1 was concentrated in larger and fewer speckles within the nuclei. Nuclei are shown in red (Propidium Iodide; PI), PROX1 is shown in green. Scale bar = 20 µm.

In addition to the protein expression, we wanted to confirm changes in *CD146* expression at the mRNA level as well. We used identical infection conditions to infect HUVECs with Ad*EGFP*, Ad*Prox1* and Ad*HDPD* at 200 MOI. After isolating total RNA, we performed qPCR using primers specific to *CD146*. When compared to Ad*EGFP* infected controls, Ad*Prox1* infected HUVECs had a significantly lower level of *CD146* expression ($p=3.58x10^{-4}$, Figure 19). However, there was no significant difference between uninfected and Ad*Prox1* infected HUVECs. On the other hand, Ad*HDPD* infected HUVECs had significantly higher level of *CD146* expression when compared to both uninfected ($p=8.53x10^{-8}$) and Ad*EGFP* infected controls ($p=6.87x10^{-5}$).

We repeated the Western Blotting and qPCR experiments using LECs with the same adenoviral infection conditions and obtained results similar to what we showed with HUVECs. At the protein level, Ad*Prox1* infection of LECs resulted in decreased CD146 expression, when compared to uninfected LECs (p=0.0444, Figure 20A). Ad*HDPD* infected LECs on the other hand, had increased levels of CD146 expression only when compared to Ad*Prox1* infected LECs ($p=8.14x10^{-4}$). At the mRNA level, Ad*Prox1* infection of LECs did not decrease *CD146* expression, whereas Ad*HDPD* infected LECs had significantly higher levels of *CD146* expression when compared to both uninfected (p=0.0108) and Ad*EGFP* infected controls (p=0.0368, Figure 20B). Therefore, in contrast to the wild type PROX1, the ΔHDPD version of PROX1 increases *CD146* expression at the protein and mRNA levels, thereby acting as a dominant negative molecule in blood and lymphatic endothelial cells.

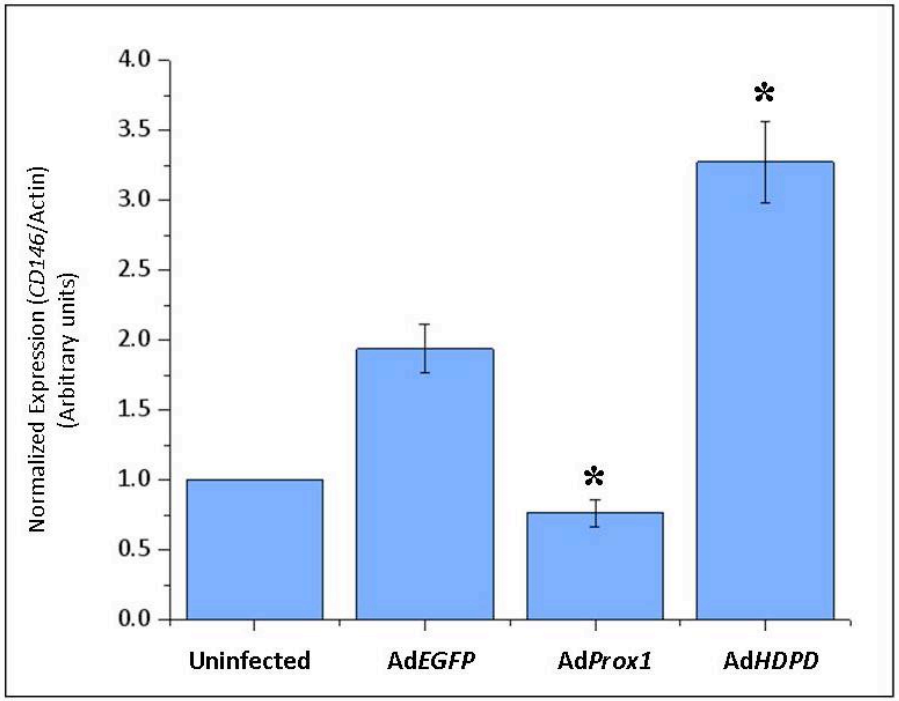


Figure 19: *Prox1* and ΔHDPD overexpression in HUVECs have opposing effects on *CD146* expression at the transcript level. Quantification of qPCR results showing *CD146* mRNA levels in HUVECs infected with adenoviruses encoding *Prox1* (Ad*Prox1*) and HDPD deleted *Prox1* (Ad*HDPD*) at 200 MOI for 48 hours. Ad*EGFP* was used as infection control. When compared to Ad*EGFP* infected HUVECs, Ad*Prox1* infected HUVECs have lower ($p=3.58\times10^{-4}$) and Ad*HDPD* infected HUVECs have higher ($p=6.87\times10^{-5}$) levels of *CD146* expression (n=4, error bars represent standard error).

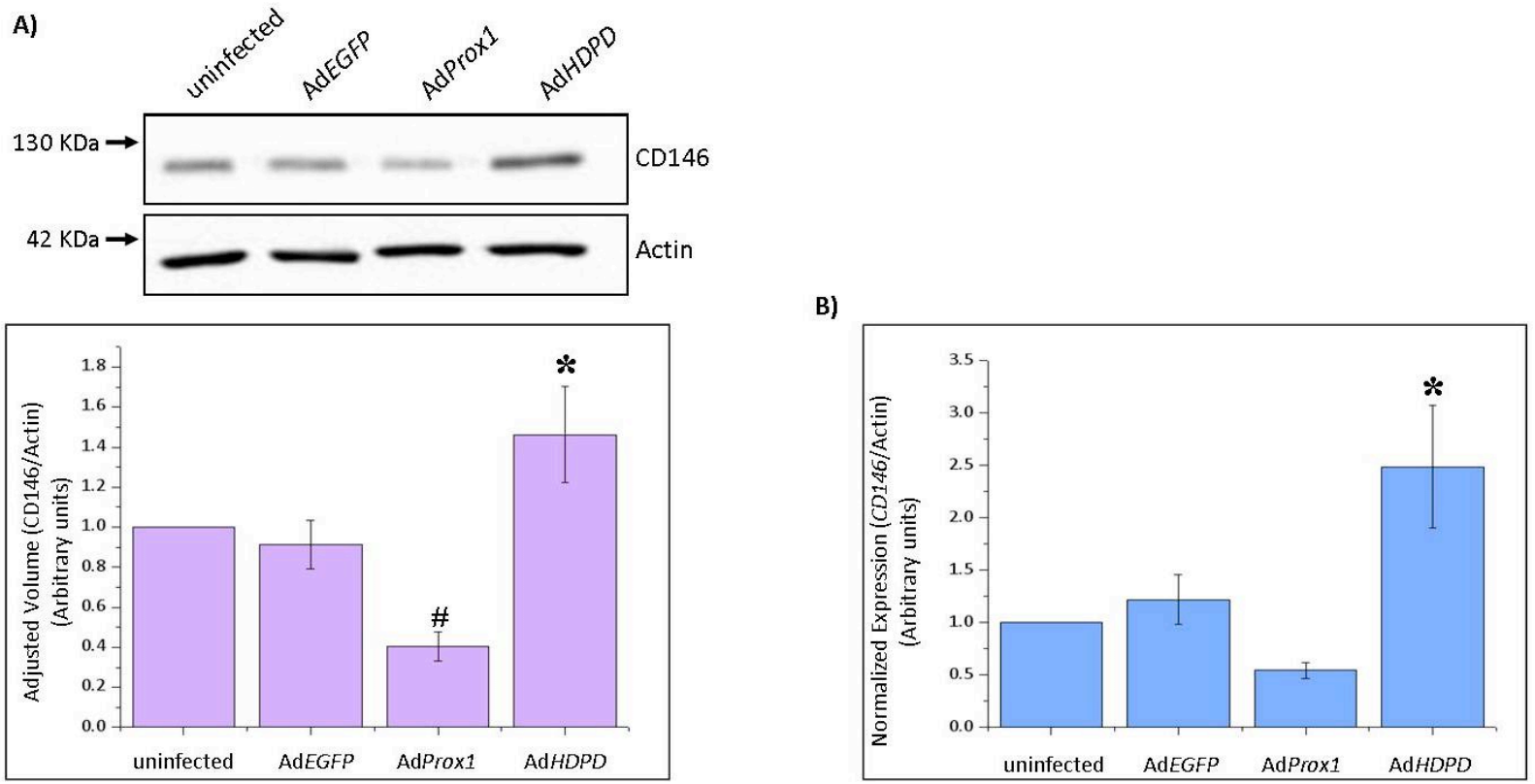


Figure 20: The effect of *Prox1* gain of function on *CD146* expression in Lymphatic Endothelial Cells (LECs). A) A representative Western Blot showing CD146 protein levels in LECs infected with adenoviruses encoding *Prox1* (Ad*Prox1*) and HDPD deleted *Prox1* (Ad*HDPD*) at 200 MOI for 48 hours. Ad*EGFP* was used as an infection control. Actin was used as a loading control. When compared to uninfected HUVECs, HUVECs infected with Ad*Prox1* (#) had a lower level of CD146 expression ($p=0.0444$). Ad*HDPD* infected LECs (*) had a higher level of CD146 expression when compared to Ad*Prox1* infected LECs ($p=8.14\times10^{-4}$) ($n=4$, error bars represent standard error). **B)** qPCR results showing *CD146* mRNA levels in LECs infected with Ad*Prox1* and Ad*HDPD* at 200 MOI. When compared to uninfected LECs, Ad*HDPD* infected LECs have a higher level of *CD146* expression ($n=5$, $p=0.011$, error bars represent standard error).

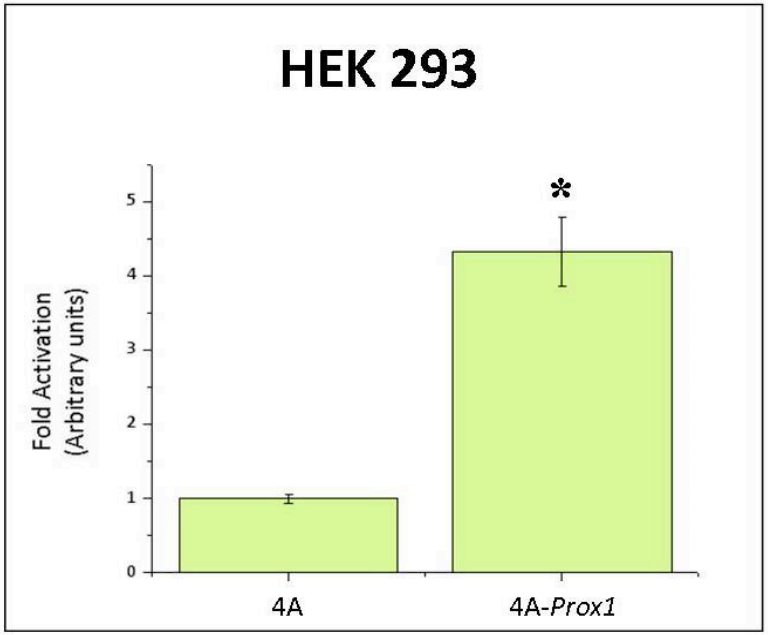
3. The Effect of PROX1 on the *CD146* promoter

In order to determine whether the effects of *Prox1* gain of function on *CD146* expression is due to PROX1 binding to the *CD146* promoter, we cloned a 3.2 Kb upstream region of *CD146* from HUVEC genomic DNA into the pGL3 reporter vector. We first used Human Embryonic Kidney (HEK 293) cells, which do not express *CD146* or *PROX1*, to test the effect of PROX1 on *CD146* promoter activity. When compared to the empty pCMV-Tag 4A vector (4A), 4A-*Prox1* activated the *CD146* promoter >4 fold in HEK 293 cells ($p=3.68x10^{-4}$, Figure 21A). In order to determine the effect of PROX1 on *CD146* promoter in a cell line expressing endogenous *CD146*, we performed the same Luciferase assay using Human Melanoma cells (WM-266-4), which express *CD146*. Similar to our findings with HEK 293, 4A-*Prox1* activated *CD146* promoter ~2.5 fold when compared to the empty vector (4A) in WM-266-4 cells (p=0.0415, Figure 21B).

4. *PROX1* is required to repress *CD146* expression in LECs

We showed that *Prox1* is sufficient to decrease *CD146* expression in both LECs and BECs. As the next step, we hypothesized that loss of *PROX1* function would result in increased *CD146* expression in LECs. To test our hypothesis, we used a pool of *PROX1* specific siRNA targeting four different regions of the *PROX1* mRNA. For transfection control, we incubated cells with transfection reagent alone with no added siRNA (mock transfected) transfected the cells with a non-targeting siRNA pool (control).

A)



B)

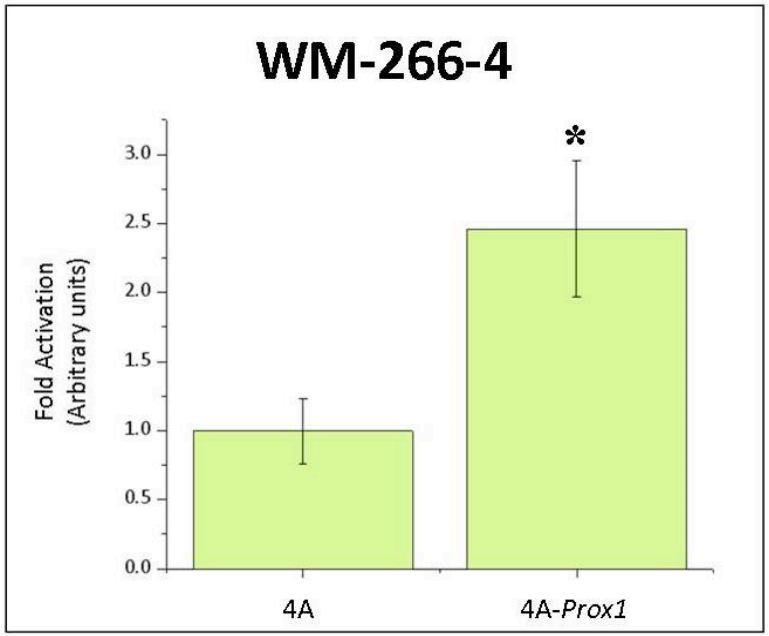


Figure 21: PROX1 activates the human *CD146* promoter in Human Embryonic Kidney (HEK 293) and Human Melanoma (WM-266-4) cells. Both HEK 293 and WM-266-4 cells were transfected with pCMV Tag 4A vector encoding *Prox1* (4A-*Prox1*), pGL3-Basic Luciferase reporter vector encoding the 3.2 Kb human *CD146* promoter and β-galactosidase expression plasmid pcDNA3-LacZ. Empty pCMV Tag 4A vector (4A) was used as control. Transfection efficiencies were normalized using β-galactosidase expression. **A)** When compared to the 4A transfected HEK 293 cells, 4A-*Prox1* transfected HEK 293 cells had >4 fold increased *CD146* promoter activation (n=4, $p=3.68x10^{-4}$, error bars represent standard error). **B)** In WM-266-4 cells, 4A-*Prox1* transfection resulted in ~2.5 fold activation of the *CD146* promoter when compared to the 4A vector transfection (n=3, p=0.0415, error bars represent standard error).

We first wanted to confirm the efficient and specific siRNA mediated knockdown of *PROX1* in LECs. We harvested cells 48 hours after siRNA transfection and performed Western Blotting using a rabbit anti-PROX1 polyclonal antibody to detect PROX1 in LECs (Figure 22A). *PROX1* siRNA transfected LECs had a 93% reduction in PROX1 expression, when compared to both mock transfected ($p=0.00167$) and control siRNA transfected cells ($p=8.25 \times 10^{-4}$). Using immunocytochemistry, we also demonstrated that mock transfected and control siRNA transfected LECs expressed abundant amounts of PROX1 (Figure 23). On the other hand, *PROX1* siRNA transfected LECs had almost no detectable PROX1 expression.

After confirming that *PROX1* siRNA strongly knocked down *PROX1* in LECs, we used a mouse anti-CD146 monoclonal antibody to determine whether CD146 expression was increased (Figure 24A). 48 after siRNA transfection, *PROX1* siRNA transfected LECs had a slight, but significant increase in CD146 protein levels when compared to both mock transfected ($p=0.0326$) and control siRNA transfected LECs ($p=0.0260$).

Before quantifying the changes in *CD146* mRNA levels by qPCR, we wanted to confirm that *PROX1* was being efficiently knocked down at the mRNA level. 48 hours after transfection, we isolated total RNA and performed qPCR using primers specific to *PROX1*. As opposed to the 93% reduction of PROX1 expression at protein level, we observed only a 64% decrease at the mRNA level, when compared to mock transfected LECs ($p=1.79 \times 10^{-4}$, Figure 22B). However, after running the PCR products

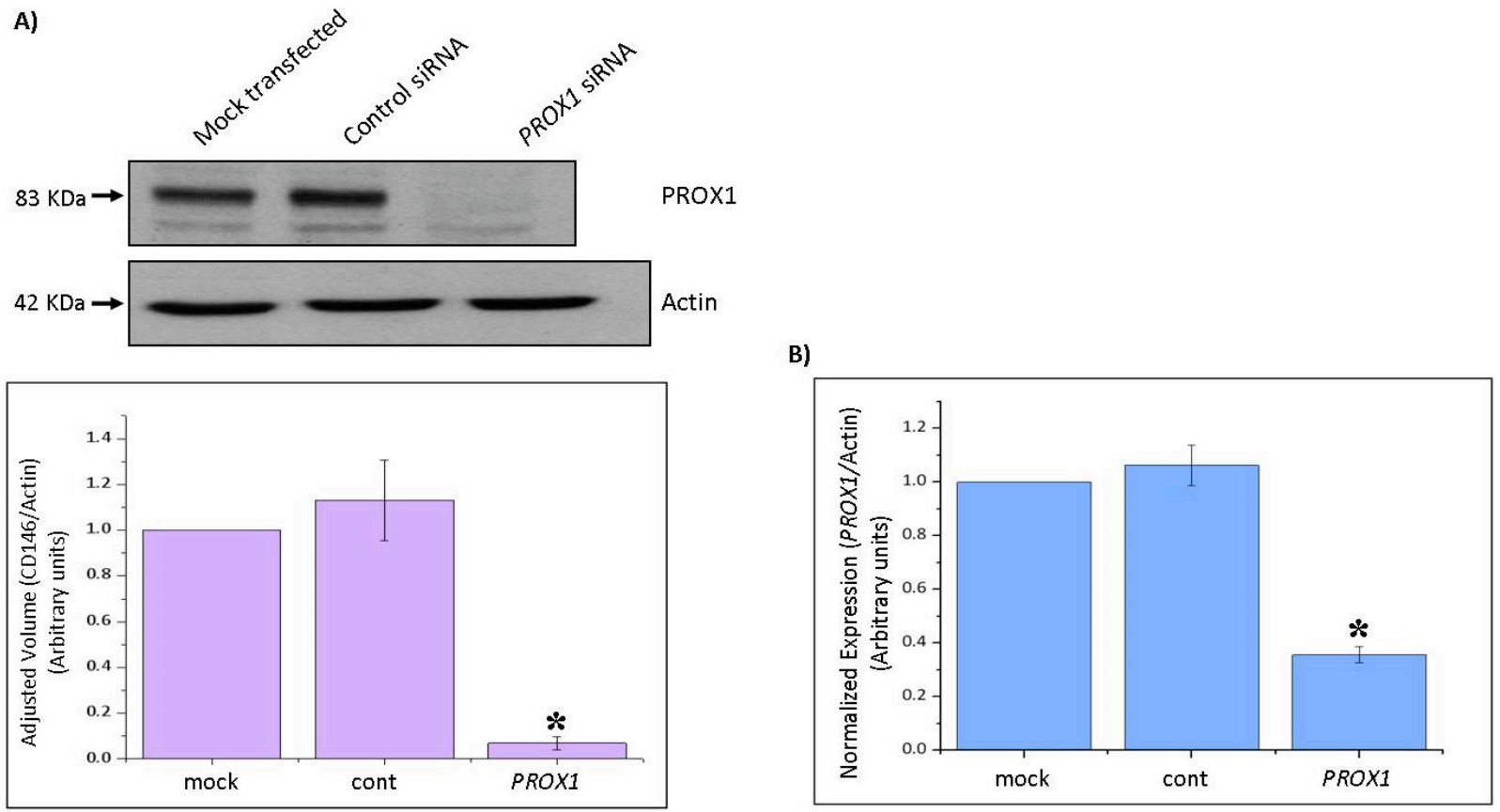


Figure 22: 48 hour-*PROX1* siRNA transfection effectively knocked down *PROX1* expression in LECs. A) A representative Western Blot showing PROX1 protein levels in LECs transfected with non-targeting control (cont), a pool of 4 different *PROX1* siRNA (*PROX1*) or no siRNA (mock). Actin was used as a loading control. *PROX1* siRNA resulted in 93% reduction in *PROX1* expression (n=3, p=0.00167, error bars represent standard error). **B)** Quantification of qPCR results showing *PROX1* mRNA levels in LECs transfected with control and *PROX1* siRNA. *PROX1* siRNA transfection resulted only in an apparent 64% reduction in *PROX1* mRNA levels (n=3, $p=1.79 \times 10^{-4}$, error bars represent standard error).

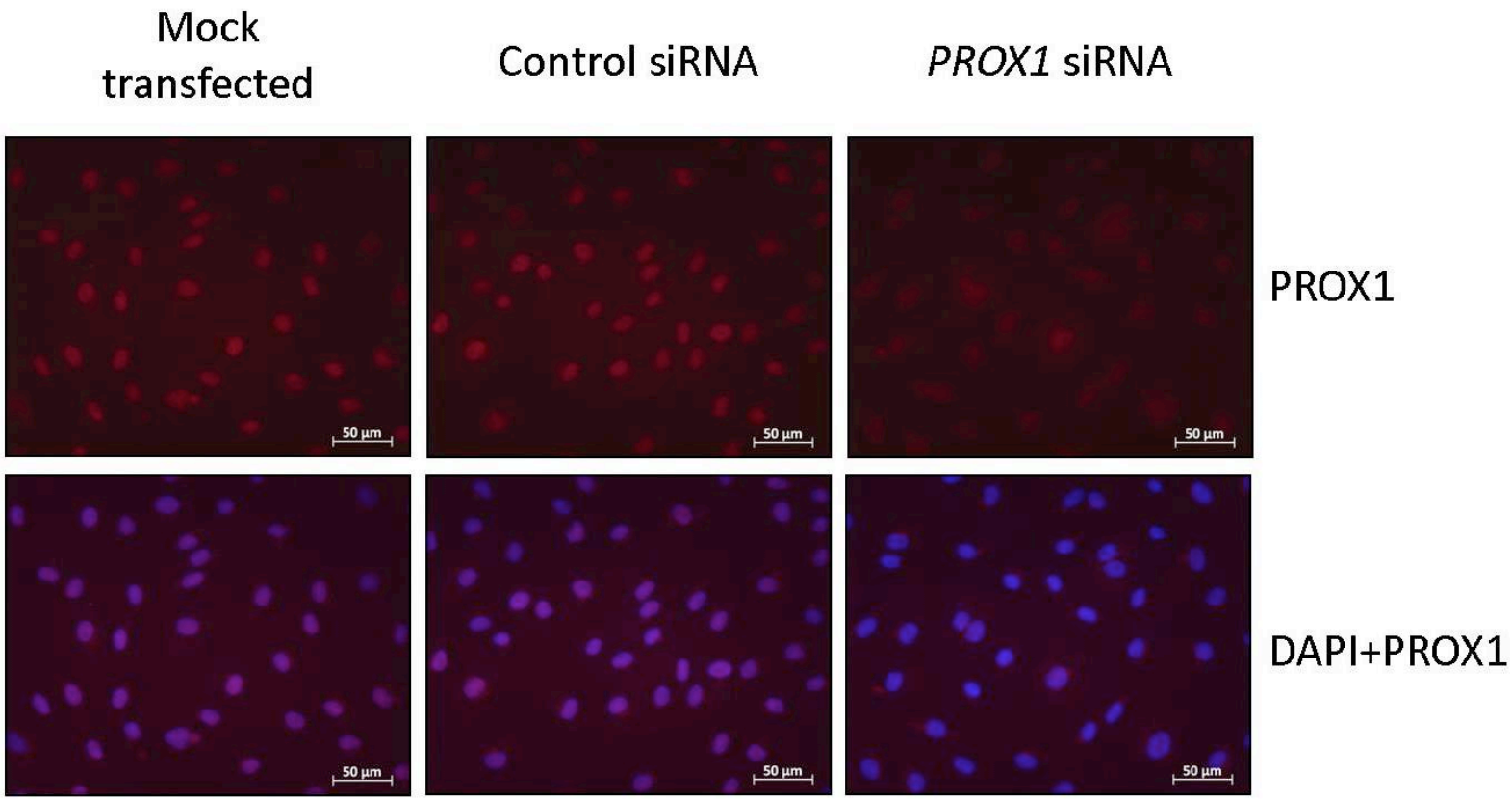


Figure 23: PROX1 was efficiently knocked down 48 hours *after PROX1* siRNA transfection. Immunocytochemistry for PROX1 in LECs. LECs transfected with non-targeting control (cont) and a pool of 4 different *PROX1* siRNA (*PROX1*). Mock transfection was used as a transfection control. PROX1 is readily detected in the nuclei of mock transfected and control siRNA transfected cells, but not in *PROX1* siRNA transfected cells. Nuclei are shown in blue (DAPI), PROX1 is shown in red. 40x magnification was used. Scale bar = 50 µm.

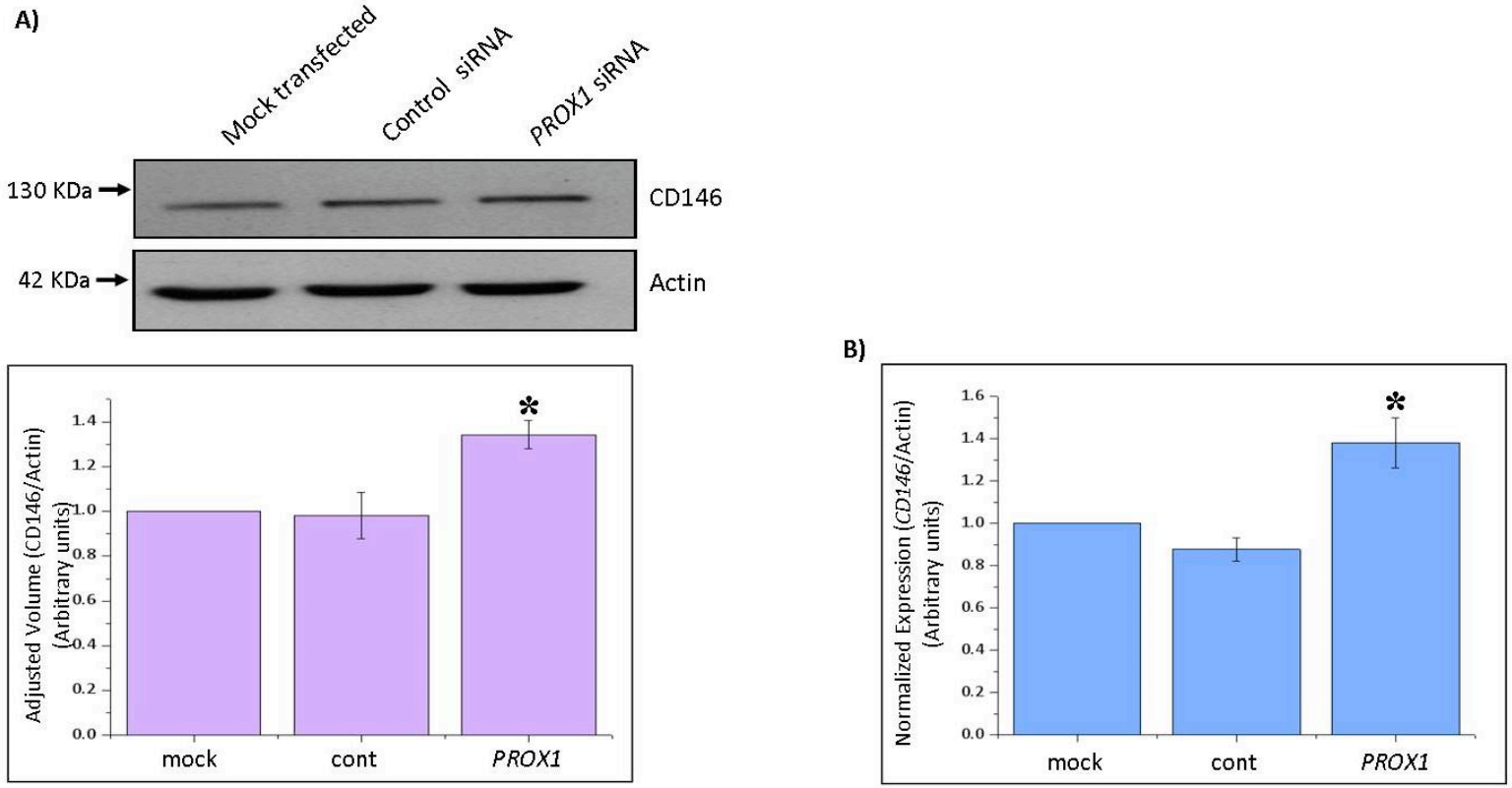


Figure 24: 48 hour-*PROX1* siRNA transfection increased *CD146* expression in LECs. A) A representative Western Blot showing CD146 protein levels in LECs transfected with non-targeting control (cont) and a pool of 4 different *PROX1* siRNA (Prox1). Mock transfection was used as transfection control. Actin was used as loading control. When compared to mock transfected LECs, *PROX1* siRNA transfected LECs had a significantly increased level of CD146 expression (n=3, p=0.0326, error bars represent standard error). **B)** Quantification of qPCR results showing *CD146* mRNA levels in LECs transfected with control and *PROX1* siRNA. At the mRNA level, *PROX1* siRNA transfected LECs had a significantly increased level of *CD146* expression, when compared to mock transfected LECs (n=3, p=0.0271, error bars represent standard error).

on agarose gel, we detected large amounts of *PROX1* amplicons in mock transfected and control siRNA transfected LECs, but very low amounts in *PROX1* siRNA LECs after 48 and 72 hours transfection (Figure 27A). When we checked the melting curve for the *PROX1* siRNA transfected LECs, we observed a second peak, which has a lower Tm than the *PROX1* amplicon (Figure 27B). The second peak corresponds to a primer dimer artifact, which frequently occurs when there are trace amounts of template. With the quantification method we use, primer dimers are problematic, because we measure total DNA synthesis. Therefore, the Ct value reflects not only the amplification of the correct target, but any other amplified sequences, such as primer dimers as well. When we calculated the area under each curve, we found that only 17% of the total area corresponded to the *PROX1* amplicon. Consequently, the actual reduction in *PROX1* expression was approximately 94%. However, this is only a rough estimation since the amplification curve reached a plateau after 30 cycles and the exact percentage of the real target amplification cannot be measured once it passes the linear range.

To quantify *CD146* mRNA expression, we isolated total RNA 48 hours after transfection and performed qCPR using primers specific to *CD146*. At the mRNA level, we observed ~1.4 fold *CD146* expression increase when compared to mock transfected LECs, which is higher than we observed at the protein level ($p=0.0271$, Figure 24B). We next increased the time following transfection from 48 hours to 72

hours in order to determine whether the extent of *PROX1* knockdown of CD146 increased with time.

After following the same protocol for siRNA knockdown as described above, we performed Western Blotting and qPCR 72 hours post transfection in order to check *PROX1* protein and mRNA levels. At the protein level, we observed a 96% reduction in PROX1 expression when compared to both mock transfected (p=0.00122) and control siRNA transfected controls (p=0.00227, Figure 25A). Therefore, PROX1 remained effectively knocked down even after 72 hours. As we observed at 48 hours, we detected a primer dimer artifact while measuring *PROX1* knock down at mRNA level at 72 hours. We only observed a 60% decrease in *PROX1* mRNA levels in *PROX1* siRNA transfected LECs, when compared to mock transfected LECs ($p=3.12x10^{-4}$, Figure 25B). By calculating the area under each curve, we estimated that 17% of the total area corresponded to the *PROX1* amplicons. This means that the actual reduction in *PROX1* expression was approximately 93%.

To measure *CD146* expression, we performed Western Blotting and qPCR 72 hours post transfection using the same protocol in order to check *CD146* protein and mRNA levels respectively. At the protein level, we observed a >1.3 fold increase in *CD146* expression in *PROX1* siRNA transfected LECs when compared to mock transfected (p=0.0270) and control siRNA transfected controls (p=0.0334, Figure 26). The increase in CD146 mRNA levels upon *PROX1* siRNA transfection was similar to that of protein levels when compared to both mock transfected (p=0.00301) and

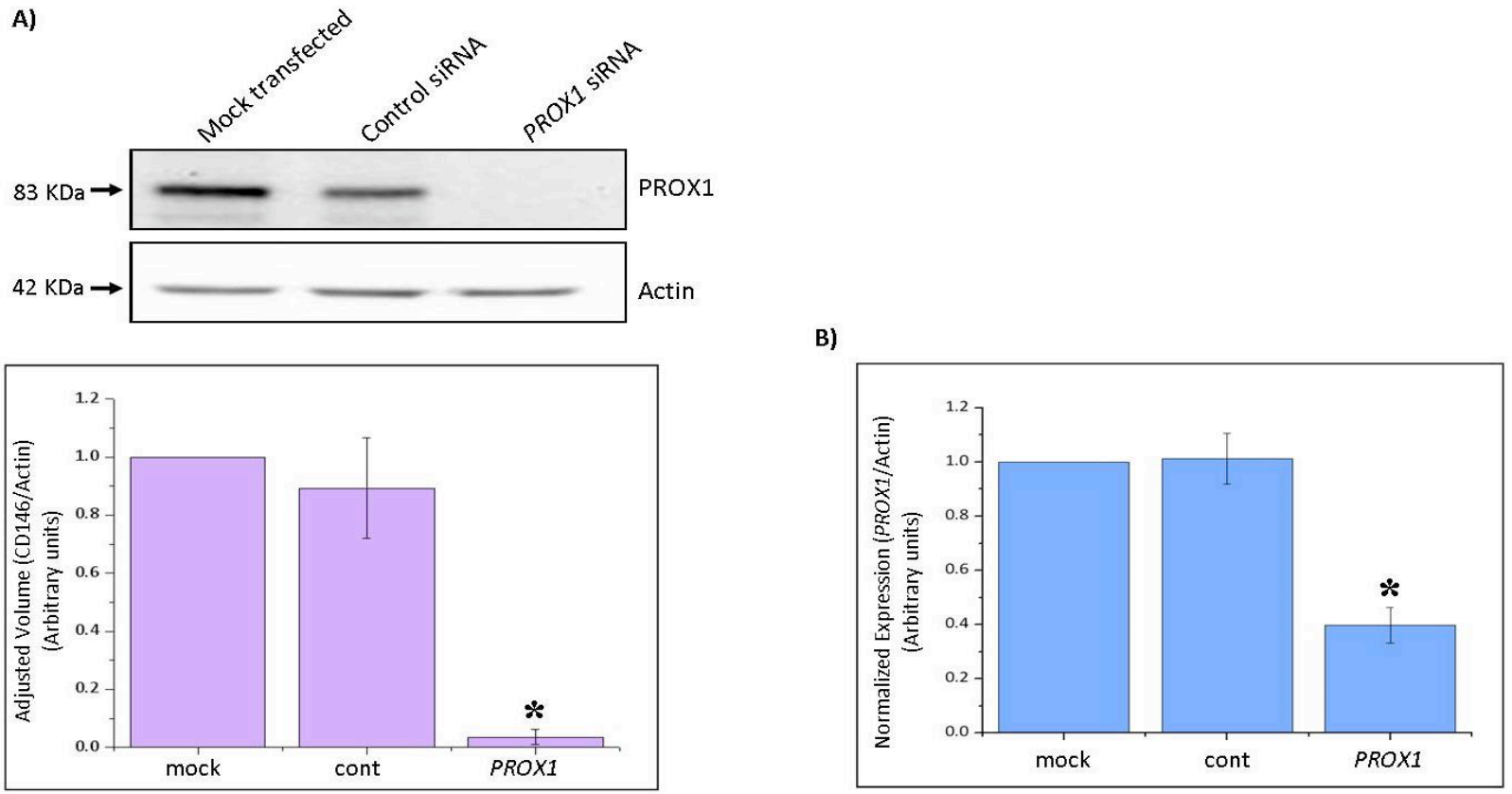


Figure 25: *PROX1* expression remained knocked down 72 hours after siRNA transfection in LECs. A) A representative Western Blot showing PROX1 protein levels in LECs transfected with non-targeting control (cont) and a pool of 4 different *PROX1* siRNA (*PROX1*). Mock transfection and control siRNA were used as transfection controls. Actin was used as a loading control. *PROX1* siRNA resulted in 96% reduction in PROX1 expression levels (n=4, p=0.00122, error bars represent standard error). **B)** qPCR quantification showing *PROX1* mRNA levels in LECs transfected with control and *PROX1* siRNA. *PROX1* siRNA transfection resulted only in an apparent 60% reduction in *PROX1* expression levels (n=3, $p=3.12 \times 10^{-4}$, error bars represent standard error).

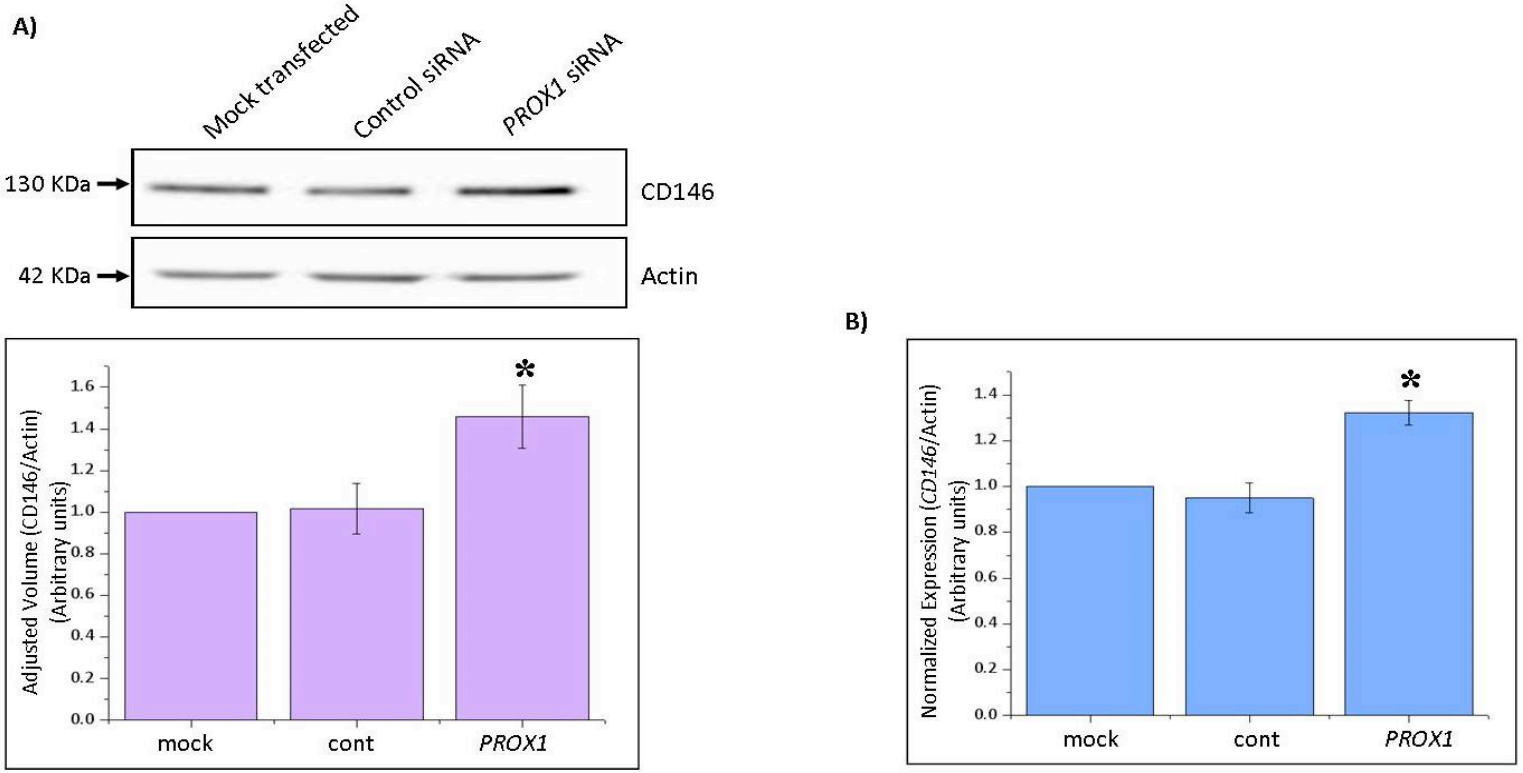


Figure 26: *PROX1* siRNA transfected LECs had increased levels of *CD146* expression 72 hours after transfection. **A)** A representative Western Blot showing CD146 protein levels in LECs transfected with non-targeting control (cont) and a pool of 4 different *PROX1* siRNA (Prox1). Mock transfection was used as a transfection control. Actin was used as a loading control. When compared to mock transfected LECs, *PROX1* siRNA transfected LECs have higher CD146 expression levels (n=6, p=0.0269, error bars represent standard error). **B)** qPCR quantification showing *CD146* mRNA levels in LECs transfected with control and *PROX1* siRNA. At the mRNA level, *PROX1* siRNA transfected LECs had increased levels of *CD146* expression when compared to mock transfected LECs, (n=4, p=0.00301, error bars represent standard error).

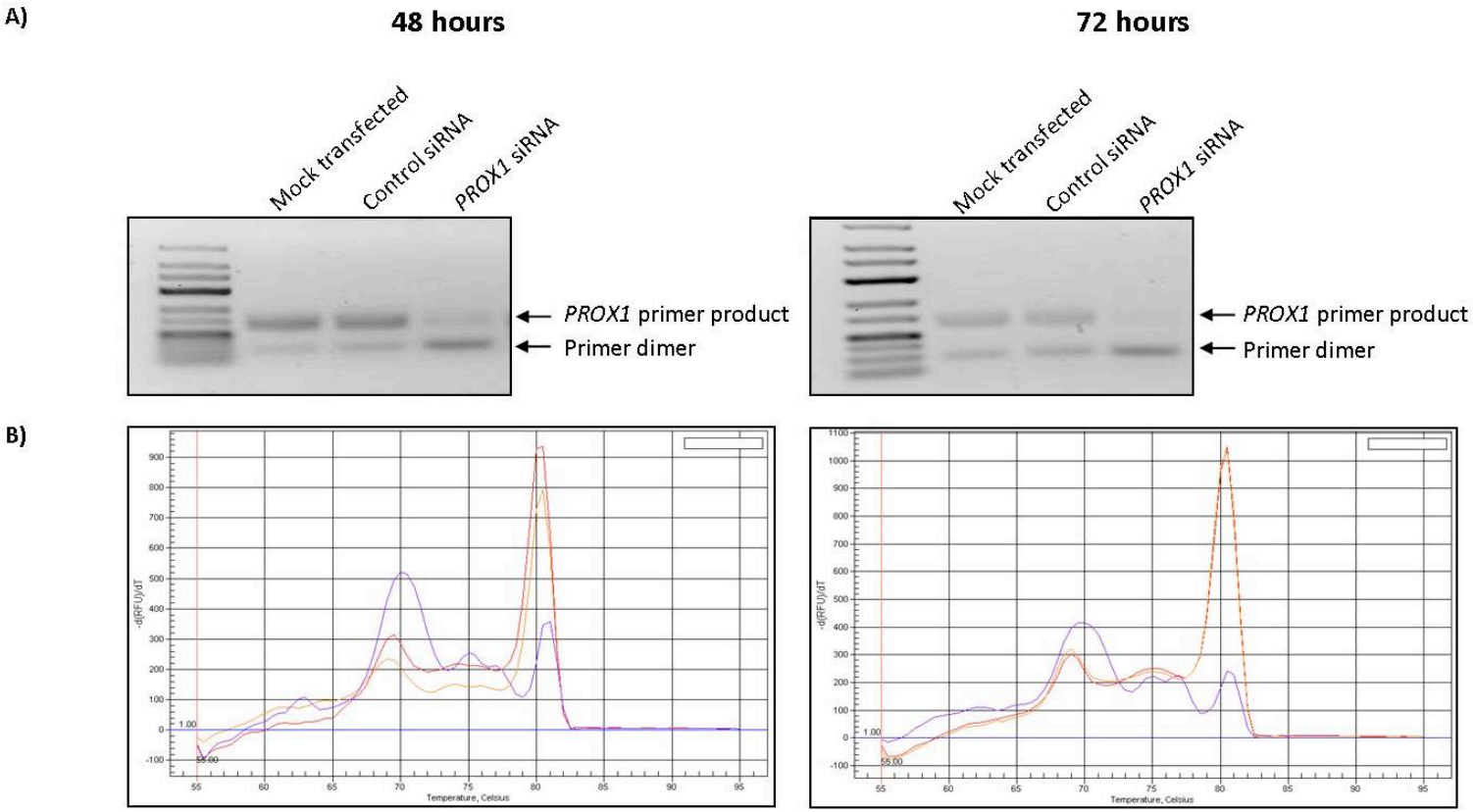


Figure 27: *PROX1* siRNA knockdown efficiently decreases *PROX1* mRNA expression in LECs. A) Representative gel electrophoresis pictures showing *PROX1* expression levels in LECs transfected with non-targeting control (cont) and a pool of 4 different *PROX1* siRNA (*PROX1*) for 48 and 72 hours. Mock transfection (mock) was used as transfection control. *PROX1* siRNA resulted in decreased *PROX1* expression at the mRNA level. **B)** Representative qPCR melting curves showing peaks corresponding to primer dimers and the *PROX1* primer products at 48 hours (left) and 72 hours (right). mock: red, cont: orange, *PROX1*: purple. Primer dimer peak is around 70°C and *PROX1* primer product peak is around 80°C.

control siRNA transfected LECs (p=0.00114). Therefore, increased *CD146* expression in *PROX1* siRNA transfected LECs persists even 72 hours after transfection at both the mRNA and protein levels. From these results, we concluded that *PROX1* is necessary for *CD146* repression in LECs, as loss of *PROX1* function increases *CD146* expression.

DISCUSSION

1. *PROX1* and *CD146* expression in BECs and LECs *in vitro*

Prox1 is one of the most reliable markers for identifying lymphatic endothelial cells (LECs) in development and adulthood (Wigle et al., 2002). During lymphangiogenesis, *Prox1* expression is crucial for the differentiation of venous ECs into LECs and the migration of these LECs from the anterior cardinal vein (Wigle et al., 2002). In addition to its roles during embryonic development, continuous *Prox1* expression is required to maintain the lymphatic phenotype during adulthood. We have confirmed previous studies by showing that *PROX1* is abundantly expressed in Neonatal Human Dermal Lymphatic Microvascular Endothelial Cells. On the other hand, Human Umbilical Vein Endothelial cells (HUVECs), which are a widely used primary blood endothelial cell type, do not express *PROX1*.

CD146 as an endothelial specific cell adhesion molecule (Bardin et al., 1996), modulates angiogenesis via the regulation of EC migration, adhesion and proliferation (Yan et al., 2003). On the other hand, CD146 expression and its importance in the lymphatic system have not been studied. Recently, it was shown that CD146 expression is restricted to BECs as LECs do not express CD146 either *in vivo* or *in vitro* (Amatschek et al., 2007). In our study, we showed that both BECs and LECs express CD146, but its expression in LECs is lower than in BECs. The difference in CD146 expression levels between BECs and LECs can be explained by the fact that

these two types of ECs have different structural and functional characteristics, as well as different transcriptional repertoires (Amatschek et al., 2007).

As opposed to the previous study, which showed that LECs do not express CD146 *in vitro* (Amatschek et al., 2007), we consistently observed CD146 expression in LECs at the protein and mRNA levels. Amatschek's group used Dermal Microvascular Endothelial Cells obtained from the skin isolated from healthy adult females undergoing breast reduction and abdominoplasty. The LECs we used on the other hand are isolated from foreskins of neonatal males. Therefore, the differences in the source of the LECs and the gender might affect the EC phenotype (Lacorre et al., 2004). Additionally, culturing conditions might also affect the endothelial gene expression profile. After isolation, Amatschek's group seeded LECs on fibronectin coated plates and cultured cells in supplemented EC growth medium without hydrocortisone. Our EC growth medium on the other hand, supplemented with hydrocortisone and we did not use fibronectin coated plates for our LEC culture. Therefore, the differences in CD146 expression between these two studies can also be explained by the different culturing conditions and supplements in the growth media.

2. *In vitro* culture affects *PROX1* and *CD146* expression in ECs

Primary cells are used as representative models of their *in vivo* counterparts. By mimicking the original tissue environment, primary cell cultures generate physiologically relevant data. However, there is increasing evidence that the

microenvironment plays a crucial role in maintaining specific cell phenotypes (Lacorre et al., 2004, Amatschek et al., 2007). ECs have been shown to interact with their microenvironment through soluble factors, cell-cell contacts or physical properties such as blood pressure and flow. These interactions are important for developing and preserving tissue specific EC phenotypes (Stolz & Jacobson, 1991). Therefore, *in vitro* culture may not be fully adequate to mimic the microenvironment necessary to preserve the *in vivo* EC phenotypes. It has been shown that ECs lose their tissue-specific properties and have altered gene expression profiles when isolated from their natural microenvironment and cultured *in vitro* (Lacorre et al., 2004). Thus, when compared to their *in vivo* states, the differences between BECs and LECs decrease dramatically and they display a higher degree of similarity *in vitro* (Amatschek et al., 2007).

When we monitored PROX1 and CD146 levels in LECs by Western Blotting, we observed decreased PROX1 expression with increasing passage number. On the other hand, CD146 expression increased with increasing passage number. Therefore, even though these primary LECs initially represent their *in vivo* states, they lose their lymphatic phenotype when they are cultured *in vitro*, as demonstrated by gradually increased CD146 expression and decreased PROX1 expression with increasing passaging *in vitro*. Therefore, we used LECs only up to passage 6 for our experiments, in order to better represent the lymphatic phenotype as much as possible.

3. *Prox1* overexpression downregulates CD146 levels in BECs

PROX1 is a key transctiption factor which plays role in the determination and the maintenance of the lymphatic fate. It has been shown to upregulate lymphatic endothelial markers such as Podoplanin and Neuropilin-2 and downregulate blood endothelial markers such as Neuropilin-1 and ICAM-1 and (Hong et al., 2002; Petrova et al., 2002). Since CD146 has been shown to be a blood endothelial specific marker, we examined whether *Prox1* overexpression in BECs downregulated CD146 expression. We observed that HUVECs infected with adenoviruses encoding wild type *Prox1* (Ad*Prox1*) have decreased CD146 expression when compared to uninfected HUVECs. Since *Prox1* expression is sufficient to drive venous ECs into a lymphatic phenotype (Petrova et al., 2002), it was predicted that *Prox1* gain of function downregulates the blood endothelial marker CD146. We observed decreased *CD146* expression in HUVECs infected with Ad*Prox1* at both the protein and mRNA levels. Therefore, we concluded that the PROX1 regulation of *CD146* expression is at the transcriptional level. Other studies have previously shown the *Prox1* mediated downregulation of other blood endothelial markers such as VEGFR-2 and Connexin-43 (Hong et al., 2002). Our study is the first to demonstrate that PROX1 represses *CD146* expression in endothelial cells.

CD146 is a blood endothelial specific cell adhesion molecule that regulates EC migration, adhesion and proliferation during angiogenesis (Yan et al., 2003). Due to the regulatory role of CD146 in cell adhesion, differences in CD146 expression levels

might reflect the structural and functional differences between the blood and lymphatic vessels. As opposed to the closed cardiovascular system, lymphatic system allows for fluid exchange between the extracellular space and the lumen of the lymphatic capillaries (Adams & Alitalo, 2007). Interactions between the environment and the lymphatic system are facilitated by the loosely attached LECs and the valve like junctions between them, thus making the lymphatic vessels highly permeable (Baluk et al., 2007). Blood vessels on the other hand, are not as permeable since they have a continuous basement membrane and adherens cell-cell junctions between adjacent BECs (Dejana et al., 2009). Therefore, EC expression of CD146 might be one of the key factors regulating vessel permeability.

4. *PROX1* is required for CD146 repression in LECs

PROX1 expression has been shown to be required for maintenance of the lymphatic phenotype during embryonic and postnatal stages (Johnson et al., 2008). Downregulation of *PROX1* is sufficient to reprogram LECs into BECs by altering their gene expression profile (Bixel & Adams, 2008). Loss of *PROX1* expression in LECs results in downregulation of LEC markers such as Podoplanin and Secondary Lymphocyte Chemokine and upregulation of BEC markers such as Endoglin and CD34 (Johnson et al., 2008). Therefore, PROX1 is not only required to specify lymphatic fate, but also to maintain the LEC phenotype.

In order to knockdown *PROX1* in LECs, we used a pool of 4 different *PROX1* specific siRNAs targeting different regions of the *PROX1* mRNA. siRNA technology is

widely used for sequence specific gene knockdown in cultured cells (Pei & Tuschl, 2006). We confirmed efficient *PROX1* knockdown by showing that *PROX1* expression is downregulated up to 96% at both the protein and mRNA levels. We also achieved long-term knockdown of *PROX1* as it was effectively downregulated up to 72 hours after siRNA transfection. One of the most common drawbacks of siRNA knockdown is the off-target effects such as activating interferon response leading to non-specific protein synthesis inhibition and RNA degradation (Scherer & Rossi, 2003). We excluded non-sequence specific effects of *PROX1* siRNA as non-targeting control siRNA pool had no effect on *CD146* expression when compared to mock transfected LECs. Therefore, we achieved efficient and specific *PROX1* knockdown using siRNA transfection in primary LECs.

After siRNA knockdown of *PROX1* in LECs, we consistently observed increased expression of *CD146* at both the protein and mRNA levels when compared to mock transfected and control siRNA transfected LECs. Accordingly, we conclude that PROX1 is necessary to repress CD146 expression. Similar decreases in the protein and mRNA levels of *CD146* after siRNA transfections show that PROX1 likely represses *CD146* expression at the transcriptional level.

Based on our findings in *PROX1* gain of function and loss of function studies, we speculate that PROX1 is both sufficient and necessary for transcriptional repression of *CD146* regulation in endothelial cells.

5. PROX1 activation of *CD146* promoter *in vitro*

PROX1 has been shown to regulate the expression of many target genes by either directly of indirectly binding to their promoters (Shin et al., 2006; Qin et al., 2004). In order to support our hypothesis, we wanted to determine whether PROX1 regulates *CD146* promoter activity using a reporter gene assay, which measures the activity of the target promoter via expression of a reporter gene (Luciferaase) upon transcriptional activation or repression. We first used Human Embryonic Kidney cells (HEK 293) for our experiment, because this cell line has previously shown to be transfected readily and is commonly used in Luciferase assays (Shin et al., 2006).

When compared to the empty pCMV-Tag 4A vector (4A) transfected HEK 293 cells, we unexpectedly observed increased *CD146* promoter activity in HEK 293 cells transfected with *Prox1* expression vector. The conflict between the results of our previous experiments and Luciferase assays could be due to the cell type specific regulation of *CD146* expression. HEK 293 cells do not express *CD146*, so the regulation of *CD146* in HEK 293 cells might be different when compared to the cells that express endogenous *CD146*. Therefore, we used the Human Melanoma cell line (WM-266-4), which are known to express *CD146* (Qiu & Wang, 2008) for our Luciferase assays. Surprisingly, we obtained similar results with these cells. Additionally, when we compared the basal *CD146* promoter activity levels, we observed higher CD146 promoter activity in HEK 293 cells than WM-266-4 cells.

The Luciferase assay results we obtained were opposed to our previous findings of PROX1 regulation of endogenous *CD146* expression. At both the mRNA and protein levels, we showed a significant decrease in CD146 expression when we infected both BECs and LECs with Ad*Prox1*. In Luciferase activity assays on the other hand, we showed increased activity of *CD146* promoter upon *Prox1* transfection. We can exclude the possibility of poor transfection efficiency, since we simultaneously performed β-galactosidase assays to normalize for transfection efficiencies and confirmed efficient transfection of both cell types. The conflicting results could be due to the cell type specific factors that play roles during *CD146* regulation. Since we are determining the effect of PROX1 on *CD146* expression in endothelial cells, the non-endothelial cell systems may not be good models as they do not express endothelial specific genes. Therefore, endothelial-specific factors, such as COUP-TFII, may be required for PROX1 repression of *CD146* expression. In a previous study, 1 Kb of the *CD146* 5' region was used as the putative *CD146* promoter. This CD146 promoter was expressed in both *CD146* negative and *CD146* positive cell types, indicating that *CD146* expression does not depend solely on the transcription factors that bind to this proximal *CD146* promoter (Mintz-Weber & Johnson, 2000). Therefore, other than PROX1, there might be additional factors regulating CD146 expression in our *in vitro* model. Another possibility is that the sequence we used as the *CD146* promoter may not contain the sites important for its regulation by PROX1, as it is not known where and how PROX1 binds to the *CD146* promoter.

Lastly, *in vitro* reporter assays are not able to completely mimic the *in vivo* state due to the lack of epigenetic factors such as chromatin structure and post transcriptional modifications. As our *CD146* promoter construct was cloned into the episomal pGL3-Basic Luciferase reporter vector, it is likely that the availability and structure of the promoter would be different at the endogenous locus in ECs.

6. ΔHDPD version of PROX1 acts as a dominant negative molecule

Homeo-Prospero domain (HDPD) is a very well conserved region of PROX1 that is required for sequence specific DNA binding (Ryter et al., 2002). It recognizes an atypical consensus sequence that is different than the binding motif of typical homeodomain proteins (Hassan et al., 1997). HDPD is also important for the subcellular localization of PROX1. The Homeodomain (HD) has a nuclear export signal mediating the transport of PROX1 from the nucleus, whereas the Prospero domain (PD) masks this nuclear export signal, thus enabling PROX1 retention in the nucleus (Demidenko et al., 2001). In our immunocytochemistry experiments, we established that both wild type and HDPD deleted version of PROX1 are localized in nucleus. However, we consistently observed a different nuclear distribution of wild type and HDPD deleted PROX1. Wild type PROX1 has a speckled distribution within the nuclei, where the spots with high levels of PROX1 are small and abundant. HDPD deleted PROX1 on the other hand, is concentrated at fewer but larger speckles within the nuclei.

Since HDPD is required for specific DNA binding, deletion of HDPD renders PROX1 unable to bind DNA (Ryter et al., 2002). We wanted to observe the effect of DNA binding deficient PROX1 (ΔHDPD) on *CD146* expression in ECs. In contrast to wild type *Prox1* overexpression, ΔHDPD *Prox1* overexpression increased CD146 levels in HUVECs. We obtained similar results in LECs; *CD146* expression was increased after infecting LECs with adenoviruses encoding ΔHDPD *Prox1.* Therefore, we speculate that ΔHDPD acts as a dominant negative molecule, acting antagonistically to the role of wild type PROX1, thus increasing *CD146* expression in both BECs and LECs. This is the first study reporting that ΔHDPD functions as a dominant negative.

7. PROX1 mediated repression of *CD146* expression in ECs

Based on these results, we propose a model for PROX1 regulation of *CD146* expression in endothelial cells (Figure 28). According to our model, *CD146* expression is kept at basal levels by an unknown repressor that is bound to the *CD146* promoter in BECs, which do not express *PROX1*. However, in *PROX1* expressing LECs, PROX1 binds to the *CD146* promoter and further represses *CD146* expression by interacting with the unknown repressor bound to the *CD146* promoter. As opposed to wild-type PROX1, ΔHDPD PROX1 is unable to bind to DNA, but still retains its protein-interacting moiety. Therefore, the DNA binding-deficient ΔHDPD interacts with the unknown repressor and sequesters it from the *CD146* promoter, thereby relieving the repression of CD146 expression in ECs.

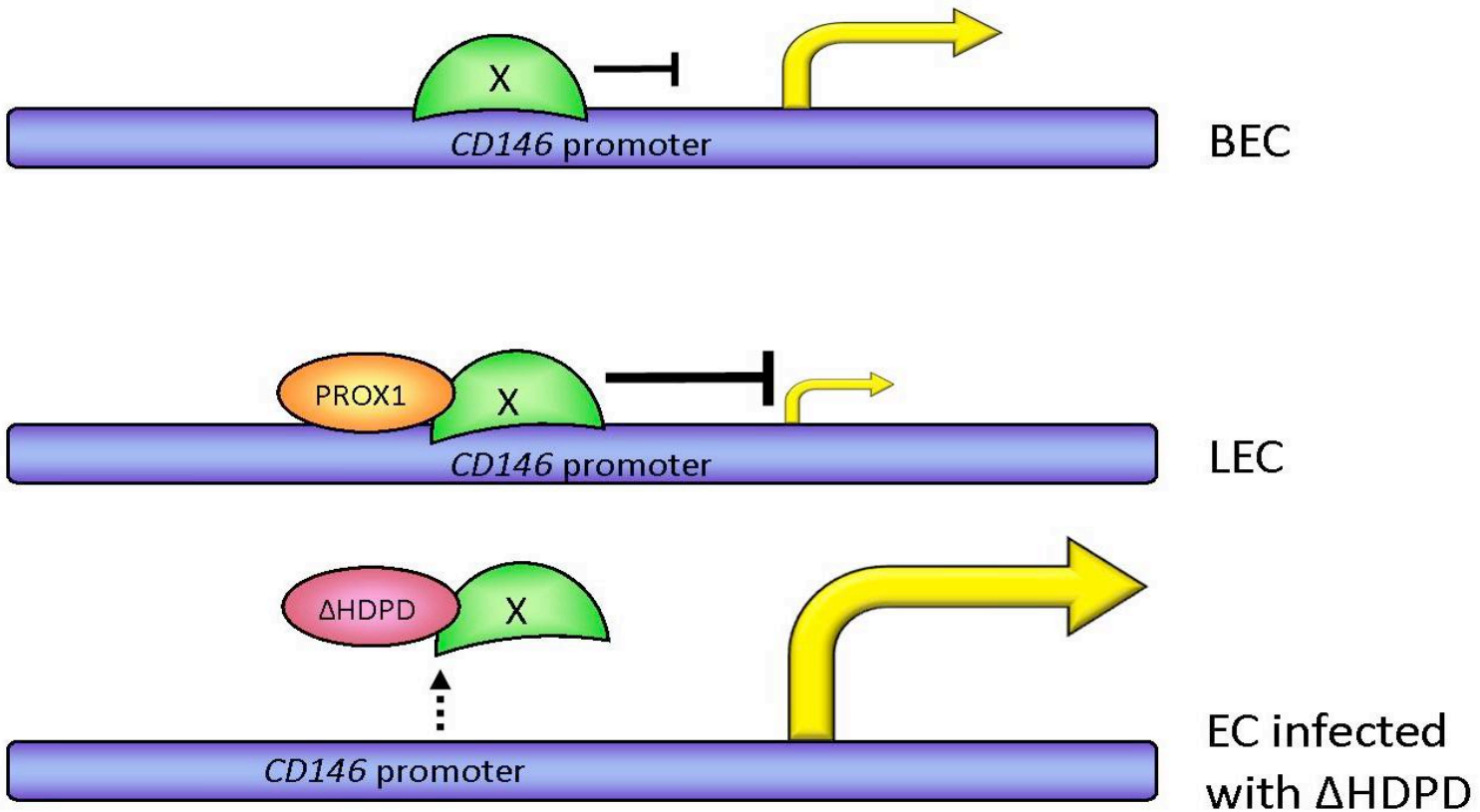


Figure 28: PROX1 regulation of *CD146* expression in endothelial cells. According to our model, *CD146* expression is kept at basal levels by an unknown repressor (X) bound to the *CD146* promoter in BECs. In LECs however, PROX1 further represses *CD146* expression by interacting with this unknown repressor. On the other hand, the DNA binding-deficient ΔHDPD version of PROX1 binds the unknown repressor and sequesters it from the *CD146* promoter, thereby relieving the repression of *CD146* expression in ECs. (⊣ : repression, ⌐→ : activation)

In addition to its roles during venous differentiation, *COUP-TFII* is also important for the maintenance of the lymphatic identity (Tsai et al., 2005). By interacting with PROX1, COUP-TFII regulates expression of lymphatic specific genes, thus playing essential role in determining lymphatic fate (Yamazaki et al., 2009). Recently, it was shown that *COUP-TFII* deletion in mice results in loss of LEC identity and gain of BEC identity (Lin et al., 2010). Therefore, we speculate that COUP-TFII is a possible candidate for the unknown repressor bound to the *CD146* promoter in endothelial cells.

An alternative to our model is that PROX1 may interact with other proteins bound to the *CD146* promoter, as PROX1 has been shown to interact with other transcription factors such as nuclear receptors, SOX18 and COUP-TFII (Song et al, 2006; Qin et al, 2004; Downes & Koopman, 2001; Yamazaki et al., 2009). Another explanation for our findings is that PROX1 may regulate the expression of another protein, which is responsible for the regulation of *CD146* expression.

CONCLUSIONS

- *CD146* is expressed in both LECs and BECs, but its expression in LECs is lower than in BECs.
- *In vitro* culture results in increased CD146 expression and decreased PROX1 expression in LECs. Therefore, LECs lose their lymphatic phenotype when they are cultured *in vitro*.
- Ectopic expression of wild type PROX1 in BECs and LECs results in decreased *CD146* expression at both the mRNA and protein levels. Therefore, PROX1 is sufficient to repress CD146 expression.
- The Homeo-Prospero domain deleted (ΔHDPD) version of PROX1 increases *CD146* expression in blood and lymphatic endothelial cells.
- The ΔHDPD version of PROX1 acts as a dominant negative molecule with an antagonist effect on regulation of *CD146* expression in LECs
- Both wild type and ΔHDPD PROX1 are localized to the nucleus. However, wild type PROX1 is concentrated in small and abundant speckles, whereas ΔHDPD PROX1 is concentrated in larger and fewer speckles.
- Wild type PROX1 activates 3.2 Kb long upstream sequence of *CD146* gene in two different non-endothelial cell lines.
- Loss of *PROX1* function in LECs results in increased *CD146* expression. Therefore, *PROX1* is necessary to repress *CD146* expression in LECs.

FUTURE DIRECTIONS

PROX1 has been shown to regulate gene expression, either directly or indirectly (Shin et al., 2006; Qin et al., 2004). In our proposed model, we predict that PROX1 represses CD146 expression by directly interacting with the *CD146* promoter in ECs. Chromatin immunoprecipitation (ChIP) will be used to verify PROX1 binding to the endogenous *CD146* promoter *in vitro* and *in vivo*. However, ChIP assay does not distinguish between direct and indirect protein-DNA interactions. Therefore, we will use electrophoretic mobility shift assay (EMSA) in order to confirm whether PROX1 binds directly or indirectly to the *CD146* promoter. Promoter deletion assays will help us to determine which regions of the *CD146* promoter are important for the PROX1 regulation. Alternatively, DNAse I footprinting, can be used to identify where PROX1 binds on the *CD146* promoter.

In order to further elucidate the mechanism of *CD146* repression by PROX1, we will determine which conserved functional domains are required for PROX1 repression of *CD146*. Investigating the function of the Homeodomain and the Prospero domain will help us to determine how ΔHDPD PROX1 acts as a dominant negative on the *CD146* promoter in ECs. PROX1 also has two nuclear receptor (NR) boxes, which are critical for protein-protein interactions. PROX1 has been shown to interact with NRs such as LRH-1 and HNF4α via these NR boxes and act as a corepressor on their promoters (Song et al., 2006; Qin et al., 2004).

Our next step for understanding the mode of *CD146* repression will be the through the identification of the unknown repressor(s) of the *CD146* promoter in ECs. SOX18 and COUP-TFII are strong candidates as both transcription factors have been shown to interact with PROX1 to establish and maintain lymphatic identity (Song et al, 2006; Qin et al, 2004; Downes & Koopman, 2001; Yamazaki et al., 2009). As a general approach, protein complexes that bind to the *CD146* promoter can be purified using affinity purification and then characterized by mass spectroscopy. After characterization of any unknown *CD146* repressors, we will confirm their role in PROX1 mediated *CD146* repression using siRNA knockdown of the repressor proteins. Interaction of PROX1 and the *CD146* repressor will be confirmed by ChIP-reChIP assay, which determines the colocalization of interacting proteins on a specific DNA sequence *in vivo*. Alternative to ChIP-reChIP, co-immunoprecipitation (Co-IP) can also be used to confirm direct protein interaction.

CD146 is important for endothelial cell-cell contacts (Yan et al., 2003). We predict that PROX1 repression of *CD146* leads to a reduction in cell adhesion thus increased permeability. We will use a cell permeability assay to detect the effects of varying PROX1 and CD146 levels on inter-EC permeability. In addition to permeability, EC migration is also regulated by CD146 (Bu et al., 2006). To test whether *CD146* repression by PROX1 affects EC migration, wound healing assays will be performed using ECs. The last functional assay we will perform is determining the effect of PROX1 downregulation of *CD146* on EC proliferation. Since *CD146* expression

increases EC proliferation (Yan et al., 2003), we predict that PROX1 repression of *CD146* expression will result in decreased proliferation of ECs.

These studies will help us to fully understand the mechanism of PROX1 mediated repression of *CD146* expression in endothelial cells. By elucidating the mechanisms of lymphatic differentiation and changes in cell permeability, we will be able to better understand the role of the lymphatic system during normal and pathological conditions and also provide novel therapeutic targets for cancers and diseases related to the lymphatic system.

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