PROX1 Utilizes Distinct Mechanisms to Induce Two Key Lymphatic Growth Factor Receptors: *Vascular Endothelial Growth Factor Receptor-3* (*VEGFR-3*) and *Fibroblast Growth Factor Receptor-3* (*FGFR-3*)

by

Mehdi Eshraghi

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Department of Biochemistry and Medical Genetics
Faculty of Medicine
University of Manitoba

This is dedicated to

my beloved wife, Elham

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List of Abbreviations

Ang	Angiopoietin
ATCC	American Type Culture Collection
BEC	Blood Endothelial Cells
ChIP	Chromatin Immunoprecipitation
CNS	central nervous system
COUP-TFII	chicken ovalbumin upstream promoter transcription factor II
CYP7A1	cholesterol 7α-hydroxylase
DMEM	Dulbecco's Modified Eagles's Medium
EGF	epidermal growth factor
EMSA	electrophoretic mobility shift assay
FBS	Fetal Bovine Serum
FGF	fibroblast growth factor
FGFR-3	Fibroblast Growth Factor Receptor-3
FOXC2	*forkhead transcription factor*
GMC	ganglion mother cell
HDPD	Homeoprospero domain
HEK 293A	Human embryonic kidney cells
HMVEC	Human neonatal dermal lymphatic microvascular endothelial cells
HNF-4α	hepatocyte nuclear factor-4α
HUVECs	Human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule-1
IFN-γ	Interferon-gamma
lacZ	β-galactosidase
LEC	Lymphatic Endothelial Cells
LRH-1	Liver Receptor Homolog-1
Lyve-1	hyaluronan receptor 1
NR1	nuclear receptor box 1
NR2	nuclear receptor box 2
NRP2	Neuropilin 2
PCR	polymerase chain reaction
PlGF	placental growth factor
Prox1	prospero-related homeobox 1
PTB	phosphotyrosine binding
Q	glutamine rich domain
RTKs	receptor tyrosine kinases
SH2	Src homology 2
SLC	secondary lymphoid chemokine
VEGF-C	Vascular Endothelial Growth Factor-C
VEGF-D	Vascular Endothelial Growth Factor-D
VEGFR-3	Vascular Endothelial Growth Factor Receptor-3

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Abstract

The lymphatic vasculature is a network of unidirectional capillaries and ducts, which serve to return extracellular fluid and macromolecules to the systemic blood circulation. In addition, the lymphatic vessels have important roles in immune surveillance and fat absorption. Dysfunction of lymphatic vessels has profound physiological consequences. Insufficient lymph uptake results in lymphedema, a chronic disabling condition that currently has no cure. Lymphedemcanoccureither due to developmental defects (primary lymphedema) or due to injuries of existing lymphatic vessels (secondary lymphedema). The significance of lymphangiogenesis in tumour metastasis is demonstrated by the finding that increased lymphangiogenesis is associated with a higher rate of metastasis and poorer prognosis in cancer patients. *Prox1*, a homeoboxgene, regulates the development of the lymphatic vasculature by upregulating the expression of lymphatic endothelial markers and simultaneously repressing the expression of blood endothelial markers. To explore the mechanisms by which *Prox1* establishes lymphatic cell fate, we compared *Prox1* mediated activation oftwo key lymphatic cell surface receptors: the*Vascular Endothelial Growth Factor Receptor-3* (*VEGFR-3*) and the*Fibroblast Growth Factor Receptor-3* (*FGFR-3*) genes.

Using a combination of luciferase gene reporter assays and immunoblotting, we compared the ability of different *Prox1* constructs to activate either *VEGFR-3* or *FGFR-3* at both the mRNA and protein levels, respectively. Furthermore, we tested whether recombinant PROX1 protein was able to bind to the proximal

promoter regions of *VEGFR-3* and *FGFR-3*usingelectrophoretic mobility shift assays (EMSA).

DNA binding deficient *Prox1* versions did not activate the *FGFR-3* promoter. In contrast, these versions of *Prox1*still efficiently activated transcription of the *VEGFR-3* promoter. In agreement with our luciferase reporter gene assays, immunoblotting of HUVECs demonstrated that only infection with *wt Prox1*adenovirus increased expression of the FGFR-3 protein. Infection of HUVECs with adenoviral vectors encoding either*wt Prox1*or*HDPD*Δ *Prox1*was sufficient to induce a significant increase in VEGFR-3 protein levels. Surprisingly, our EMSA results with recombinant PROX1 demonstrated that PROX1 can bind to the promoter region of both *VEGFR-3* and *FGFR-3* genes via its DNA binding domain. We showed that PROX1 potentially binds to the promoter region of the *VEGFR-3* gene via a consensus Prospero binding site (CGCCTCGGC).

Our data demonstrates that, in endothelial cells, PROX1 utilizes distinct mechanisms to activate these two key endothelial growth factor receptors.

Introduction

Lymphatic vessels were discovered in the canine mesentery by Gasparo Aselli in 1622, at nearly the same time as the discovery of the systemic blood circulation by William Harvey.Unlike the blood vasculature, the lymphatic vasculature was little studied for centuriesdue to the difficulty in tracing the colourless lymph vessels and the lack of specific markers to detect these vessels(Witte, Jones et al. 2007; Ohtani and Ohtani 2008). Despite these challenges, scientists tried to determine the importance and functions of these vessels in the body. Duringthe 20th century, some important characteristics of lymphatic vessels, including their distribution and function,were found. But it was onlyduring the last years of the last century that specific markers for lymphatic vessels were identified and verified(Wigle and Oliver 1999). These discoveries provided new approaches to elucidate themolecular mechanisms that control lymphatic vessel growth and function(Dumont, Jussila et al. 1998; Wigle and Oliver 1999; Ohtani and Ohtani 2008). Using these tools, it was established that development of lymphatic vessels is secondary to the development of the blood vasculature(Wigle and Oliver 1999; Wigle, Harvey et al. 2002),which supported a theory proposed by Sabin in the early20thcentury (Sabin 1916). However, the field is still very young and many aspects of lymphatic development remain to be revealed. Understanding the normal development of lymphatic vessels will allow us to better understand the exact mechanisms behind pathological conditions with lymphatic vessel involvement such as lymphedema, inflammation and

cancer progression, which in turn will help us to develop improved treatment strategies.

Functions and Organization of the Lymphatic vascular system

During most physiological and pathological conditions, protein rich fluid and different immune cells extravasate from the blood circulation into the surrounding tissues(Baluk, Fuxe et al. 2007). The lymphatic vasculature is a network of unidirectional vessels, which serves several important functionalroles within the body. They act to maintain body fluid homeostasis by returning excess protein-rich extracellular fluid back to the blood circulation(Wigle and Oliver 1999). They also participate in immune surveillance by serving as conduits for activated immune cells to the regional lymph nodes(Wigle, Harvey et al. 2002).Another important function of lymphatic vessels is in the gastrointestinal system where theytransportlipids along with lipid soluble vitamins (A, D, E and K) from the intestines to the systemic blood circulation(Oliver 2004; Ohtani and Ohtani 2008).

The lymphatic vasculature consists of different levels of absorbing and conducting vessels. Overall, it can be divided into two major parts: initial lymphatic capillaries and collecting lymphatic vessels(Ohtani and Ohtani 2008).

1- Initial lymphatic capillaries

Initial lymphatic networks are non-contractile, blind-ended capillaries which lack smooth muscle cells, valves and basement membrane. The diameter of lymphatic capillaries is between 10-60 μM consisting of a monolayer of lymphatic endothelial cells, which are attached to the extracellular matrix using elastic fibers, called anchoring filaments(Swartz 2001). Lymphatic capillaries form a

tubulo-saccular network within tissues, and unlike blood capillaries, do not have fenestration, continuous basal membrane or pericytes. Instead, these vessels are covered with a continuous layer of overlapping endothelial cells called lymphatic endothelial cells (LECs)(Oliver 2004). Lymphatic endothelial cells are overlapping flat cells with loose junctions. However they are strongly attached to the surrounding intracellular collagen and elastin fibers via their anchoring filaments (Witte, Jones et al. 2007; Ohtani and Ohtani 2008).

It was commonly believed that the endothelium of lymphatic capillaries have imperfect intercellular junctions. Recently Baluk *et al.* showed that lymphatic endothelial cells at this part of the lymphatic vasculature have overlapping loose flaps which act like primitive valves and provide unidirectional fluid flow from the interstitial space into the lymphatic capillaries. They also showed thatthe initial lymphatic capillaries of the mouse tracheal mucosa have unusual, discontinuous, but perfect endothelial junctions. By using high resolution confocal microscopic imaging, they were able to show that these junctions are organized in a form of button-like structures rather than the zipper-like junctions (zippers) present in the blood capillary endothelium. They also showed that the elements of both adherens junctions and tight junctions exist in these structures. Finally, they suggested that regions between the buttonstructures are places where the extracellular fluid enters the lymphatic capillaries (Baluk, Fuxe et al. 2007).

2- Collecting lymphatic vessels.

Collecting lymphatic vessels are situated downstream ofthe lymphatic capillaries and conduct the absorbed fluid and cells via regional and central

lymph nodes, larger collecting lymphatic vessels, and finally lymphatic trunks and ducts (e.g. thoracic duct) back into the systemic blood circulation (Ohtani and Ohtani 2008). The diameter of these lymphatic vessels isbetween 1 to 10 mm (Swartz 2001). Collecting lymphatic vessels consist of functional and anatomical units called lymphangions. Each lymphangion includes a valve and its adjacent segment of lymphatic tube extending to the next valve. Lymph fluid moves along collecting lymphatic vessels by contraction of lymphangions. Fluid is propelled by extrinsic and intrinsic driving forces such as negative internal hydrostatic pressures due to the expansion of the chest and diaphragm movementsor positive external pressures due to limb movement and muscle contractions(Ohtani and Ohtani 2008). All of the collected lymph fluid passes through lymph nodes by afferent and efferent collecting lymphatic vessels (pre- and post-nodal vessels). Lymph fluid is filtered and diluted in lymph nodes. Efferent collecting lymphatic vessels carry filtered lymph to the lymph trunks. Lymphatic trunks join to the lymphatic ducts which in turn joinsto the central main veins and re-circulate lymph fluid within the systemic blood circulation (Swartz 2001).

Avascular tissues (e.g. skin epidermis, nail , lens and cartilage) normally lack lymphatic vessels. In addition, these vessels are not seen in CNS organssuch as the retina and brain(Andrade and Jacomo 2007).

The lymphatic system also contains lymphoid organs including lymph nodes, spleen, thymus, tonsils and Peyer's patchesof the intestines. Lymphoid organs along with lymphatic vessels are a key componentcomponents of the body's

immune system (Oliver 2004).

Development of the Lymphatic Vasculature

During embryogenesis, hemangioangioblasts (blood endothelial precursor cells) originate from the mesoderm layer, differentiate into endothelial cellsand formthecells theprimary embryonic vessels(Douville and Wigle 2007). This process ,which happens only in embryonic life, is called “Vasculogenesis”. During the rest of fetal life and post-natal life, these primary networks of vessels grow, sprout and remodel to form the mature blood vasculature (“Angiogenesis”)(Douville and Wigle 2007).

The origin of lymphatic vessels was unknown until the 20th century. Through her seminal studies on pig embryos in 1902, Florence Sabin was the first scientist to show that primary lymphatic sacs originate from the central embryonic veins and it is from these sacs that lymphatic capillaries sprout and spread centrifugally to form the mature lymphatic vasculature(Sabin 1916). With progress in molecular biology, it was shown that many blood vascular markers are also expressed by lymphatic vessels, supporting the hypothesis that lymphatics originate from the blood vasculature (Kaipainen, Korhonen et al. 1995; Wigle and Oliver 1999; Karkkainen and Petrova 2000; Wigle, Harvey et al. 2002).

In an alternative model presented by Huntington and McClure (1910) it was proposed that primary lymph sacs originate from mesenchyme independently and then the lymphatic vasculature connects to systemic blood circulation during its development. However, this model is less supported by experimental evidence in mammalianfrom studies(Oliver 2004).

Embryonic development of the lymphatic vasculature is initiated by differentiation of lymphatic endothelial cells (LECs) from blood endothelial cells (BECs). This process starts when a population of endothelial cells residing in the anterior wall of cardinal vein become committed toward a lymphatic phenotype and express high levels of lymphatic vessel endothelial *hyaluronan receptor 1* (*Lyve-1*) (Figure 1A) (Wigle and Oliver 1999; Wigle, Harvey et al. 2002). However, the*Lyve-1* knockout mouse apparently has normal lymphatic function, indicating that *Lyve-1* is not required for lymphatic development(Oliver 2004). The next step of lymphatic differentiation is marked by expression of the homeobox gene*Prox1* in a subgroup of *Lyve-1* expressing “committed lymphatic endothelial cells” (Figure 1B). The expression of *Prox1* in endothelial cells disseminates caudally along embryonic central veins such asthe posterior cardinal vein and the peri-mesonephric veins(Wigle and Oliver 1999).

After this stage, endothelial cells expressing PROX1 migrate from the veins (Figure 1C) and gather to form the lymph sacs through the anteroposterior embryonic axis. The migration of LECs is an important indicator of lymphatic differentiation. In addition to migration, *Prox1* expressing endothelial cells undergo a phenotypic switch from BECtoward LEC, which is marked by suppression of BEC markers and the upregulation of LEC markers(Wigle, Harvey et al. 2002). During later stages of development, lymphatic vessels sprout from primary lymph sacs and spread all over the body forming the lymphatic vasculature network (Figure 1D).However the maturation and patterning of lymphatic vessels are only completed after birth (Wigle and Oliver 1999).

Molecular Aspects of lymphatic vessels development

The discovery of novel molecular markers hasfacilitatedthestudy of the stepwise development of the lymphatic vasculature. In this regard four different stages of lymphatic vasculature development are recognized(Wigle, Harvey et al. 2002):

1- Lymphatic endothelial cell (LEC) competence

Competence is a stage of development in which cells attain the capability to respond to an initiating developmental signal. It is an intrinsic feature that does not rely on the surrounding tissues(Oliver and Srinivasan 2008). As Sabin's observations revealed, lymphatic vessels originate from pre-existing embryonic veins(Sabin 1916). Almost a century later it was shown that endothelial cells located in anterior cardinal vein (E 9.0-9.5 in mouse) express *Lyve-1* (a lymphatic marker) and demonstrate competence towards the lymphatic phenotype(Wigle and Oliver 1999).

2- Lymphatic endothelial cell bias

The "bias" stage is dependent on surrounding microenvironments which induce an ability in competent cells to differentiate toward a specific phenotype(Oliver and Srinivasan 2008). It was shown that in the mouse embryo (E9.5) a subpopulation of *Lyve1* expressing endothelial cells in the anterior cardinal vein express the transcription factor *Prox1* (*prospero-related homeobox 1*). Expression of *Prox1*is limited to one side of the vein. It is believed that *Prox1* expressing endothelial cells represent the LEC progenitors. Later these lymphatic progenitors leave the venous wall in a specific direction.

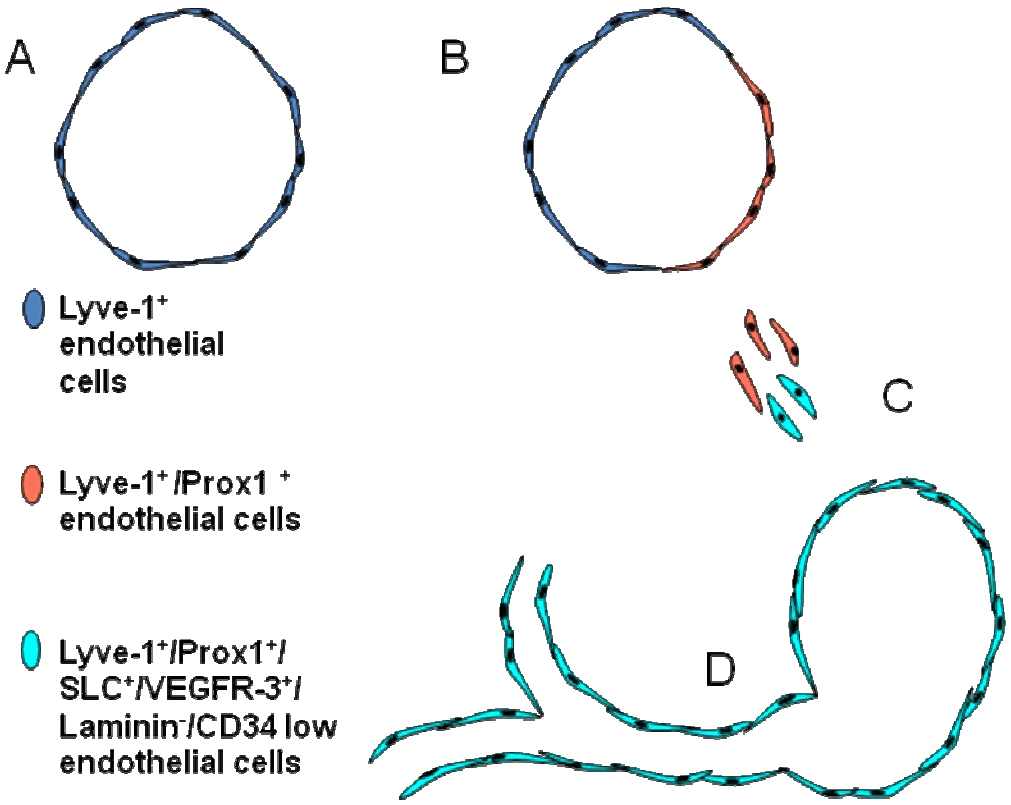

Figure 1- Development of lymphatic vessels in the mouse.
A) At E9, endothelial cells lining the cardinal vein express *Lyve-1*. B) Polarized *Prox1* expression in a subpopulation of *Lyve-1* positive endothelial cells leads to their commitment towards a lymphatic phenotype. C) *Lyve-1+/Prox1+* cells migrate from the venous wall and form primary lymphatic sacs. D) Lymphatic endothelial cells now express markers of fully differentiated lymphatic endothelial cells. They sprout from primary lymph sacs and spread all over the body to form the lymphatic vasculature (Wigle, Harvey et al. 2002) .

This polarized migration of progenitors also indicates that LEC bias is induced by factors released from surrounding tissues(Wigle and Oliver 1999).In the *VEGF-C*null mouse, committed endothelial cells fail to migrate, showing that the migration of LECs is induced and guided by specific signals from surrounding mesenchymal tissues.

The *Prox1*null mouse is completely devoid of lymphatic vessels. Microscopic examination of the *Prox1* null mouse showed that *Lyve-1* expressing cells bud from anterior cardinal vein but do not acquire a lymphatic phenotype. On the other hand they retaina blood endothelium phenotype(Wigle, Harvey et al. 2002). In other experimentsit was shown that over-expression of *Prox1* in cultured BECwas sufficient to induce the lymphatic phenotype(Hong, Harvey et al. 2002; Petrova, Makinen et al. 2002). In other words, it can be concluded that expression of *Prox1* is necessary and sufficient to bias competent venous endothelial cells toward a lymphatic endothelium cell fate(Wigle, Harvey et al. 2002).

3- Lymphatic endothelial cell specification

In order to form a specific tissue, biased cells should respond to the specific environmental signals by expressing specific receptors (markers). In fact bias and specification are the first steps toward differentiation(Oliver and Srinivasan 2008). In the lymphatic vasculature,this stage is identified by phenotypic changes specific for lymphatic endothelial cells in budding cells from the venous wall. After expression of *Prox1* in LEC progenitors, these cells expressdefinitivedefinitive lymphatic vasculature markers and receptors (e.g. VEGFR-3, Neuropilin 2 and

podoplanin), at the same time the expression of blood endothelial markers in these cells is downregulated (e.g. CD34 and VEGR-2).In the mouse, budding continues until E12.5, when the primary sacs are formed (Wigle and Oliver 1999; Wigle, Harvey et al. 2002). Expression of *secondary lymphoid chemokine* (*SLC*) in *Prox1* positive cellsat E12.5 is an important sign of LEC differentiation (Figure 1D). At this time expression ofofBEC specific markers has almost completely disappeared from LECs. *VEGFR-3* is highly expressed by LECs but also expressed at low levels in BECs. All of these changes confirm the specification of lymphatic endothelial cells at this point(Wigle and Oliver 1999).

4- Lymphatic vessel differentiation and maturation

Lymphatic vessels bud and sprout fromthe theprimary lymph sacs and spread centrifugally through the body. At E14.5-E15 in the mouse embryo, lymphatic vesselsvessels have spread all over the body. Later andduringthe final stages of lymphatic vessels maturation, markers like desmoplakin and the β-chemokine receptor D6are expressed by LECs(Wigle, Harvey et al. 2002).

Prospero Related Homeobox Gene 1 (*Prox1*)

History

Proliferation and transformation of undifferentiated progenitor cells toward mature and differentiated cells are very complicated processes that are controlled by a network of regulatory genes. Traditionally, due to its simplicity, developmental scientists studied these genes in lower species like worms (e.g. *C. Elegans*) and insects (e.g. *Drosophila Melanogaster*). With the progress of molecular biology, it was discovered that many of these genes areevolutionaryare

evolutionarily conserved in higher species (i.e. orthologous genes) and have crucial roles in their growth and development(Oliver, Sosa-Pineda et al. 1993).

Prospero, a divergent homeobox gene in *Drosophila*, is expressed in different tissues (CNS, lens and midgut) during embryonic life and has an important role in differentiation of neurons in the developing CNS(Oliver, Sosa-Pineda et al. 1993). *Prospero* is expressed in neuronal precursor cells of neuroblasts. During division of neuroblasts, its product Prospero, is asymmetrically distributed to one of the daughter cells and switches the fateof the recipient cell toward that of a ganglion mother cell (GMC). In GMCs, Prosperolocalizes to the nucleus and as a potent transcription factor alters the transcription profile of the cell to initiate the generation of differentiated neural or glial cells(Doe, Chu-LaGraff et al. 1991; Spana and Doe 1995).

To find a possible orthologue for *Prospero* in mammals, Oliver*et al.* studied the mouse embryo. Using a degenerate polymerase chain reaction (PCR) approach, theycloned and sequenced*Prox1*, anan orthologue of *Prospero* in the mouse. They further studied the expression pattern of *Prox1*in *Prox1*the mouse embryo and it was found that it has a similar expression pattern to its orthologue in *Drosophila*: subventricular zone of the CNS, lens, pancreas, liver, heart and in the skeletal muscles(Oliver, Sosa-Pineda et al. 1993).

Zinovieva *et al.* found that in the human, the*Prox1* gene is localized on chromosome 1 (1q32.2–q32.3). They alsoalsofound that the gene spans a 42 kb region in the human genome and is composed of 5 exons. The *Prox1* cDNA consists of 2924 nucleotides including an open reading frame of 2208 nucleotides

which encodes a predicted product of 736 amino acids with a mass of 83.2 kDa(Zinovieva, Duncan et al. 1996).Comparing the *Prox1*gene in different species revealed that it is highly conserved among vertebrates and also with its orthologue, *Prospero*, in *Drosophila*(Hassan, Li et al. 1997; Glasgow and Tomarev 1998).

Prox2 is arecently cloned member of prospero-related homeobox genes in mammals. It shares most of its functional domains with *Prox1*. However Nishijima *et al*. reported that the *Prox2* null mouse shows no obvious defects and, in contrast to *Prox1*,isdispensable for embryonic development(Nishijima and Ohtoshi 2006).

Structure of PROX1

As mentioned above the protein encoded by *Prox1* consists of 737 amino acids. So far, several functional domains have been identified in PROX1 (Figure 2A): 1) two nuclear receptor (NR1 and NR2) boxes at the amino terminal of PROX1,

2) one glutamine (Q) rich domain,

3) one Homeodomain (HD) and

4) one Prospero- domain(PD). 5) one nuclear localization signal

AsAsin Prospero, the Homeo- and Prospero- domains are located at the carboxyl terminal of the PROX1 protein (Figure 2A)(Hassan, Li et al. 1997; Yousef and Matthews 2005). It was shown that the carboxyl-terminal of Prospero (comprisedof Homeo- and Prospero- domains) regulates its nuclear localization and DNA-binding characteristics. This region of the protein is very well conserved among different species in all Prospero related homeodomain proteins (Hassan,

Li et al. 1997; Demidenko, Badenhorst et al. 2001).

Prox1 is a divergent homeobox gene. Members of the homeobox gene family play important roles in development by determination of the body plan and cell fate. The homeobox encodes the homeodomain, a functional protein domain of 60 amino acids. This domain is strongly conserved among different species. Homeodomain transcription factorsbind to regulatory sequences in their target genes and regulate their transcription. This binding is between the homeodomain of the transcription factor and a consensus *cis* element of “TAAT/ATTA” in the double stranded DNA(Gehring, Qian et al. 1994). However, some members of homeobox genes show variations in amino acids sequencesof their homeodomains. Chu-Lagraff*et al.* reported that the sequence of the homeodomain of PROX1 is divergent from classic homeodomains due to substitution of some amino acids in this domain. This resulted in unique structural and functional characteristicsof the homeodomain in PROX1 (Chu-Lagraff, Wright et al. 1991).

In addition,PROX1contains another DNA binding domain, theProspero-domain. It is shown that the homeo and Prospero- domains are integrated and together form a continuous single DNA binding domain in PROX1 (termed the Homeoprosperodomain; HDPD) (Yousef and Matthews 2005). This domain consists of six α helices with an approximate length of 160 amino acids. It is suggested that PROX1 binds directly to the major groove of DNA double helix through the third helix of its HDPD (Figure 2B). The Prospero- domain also forms

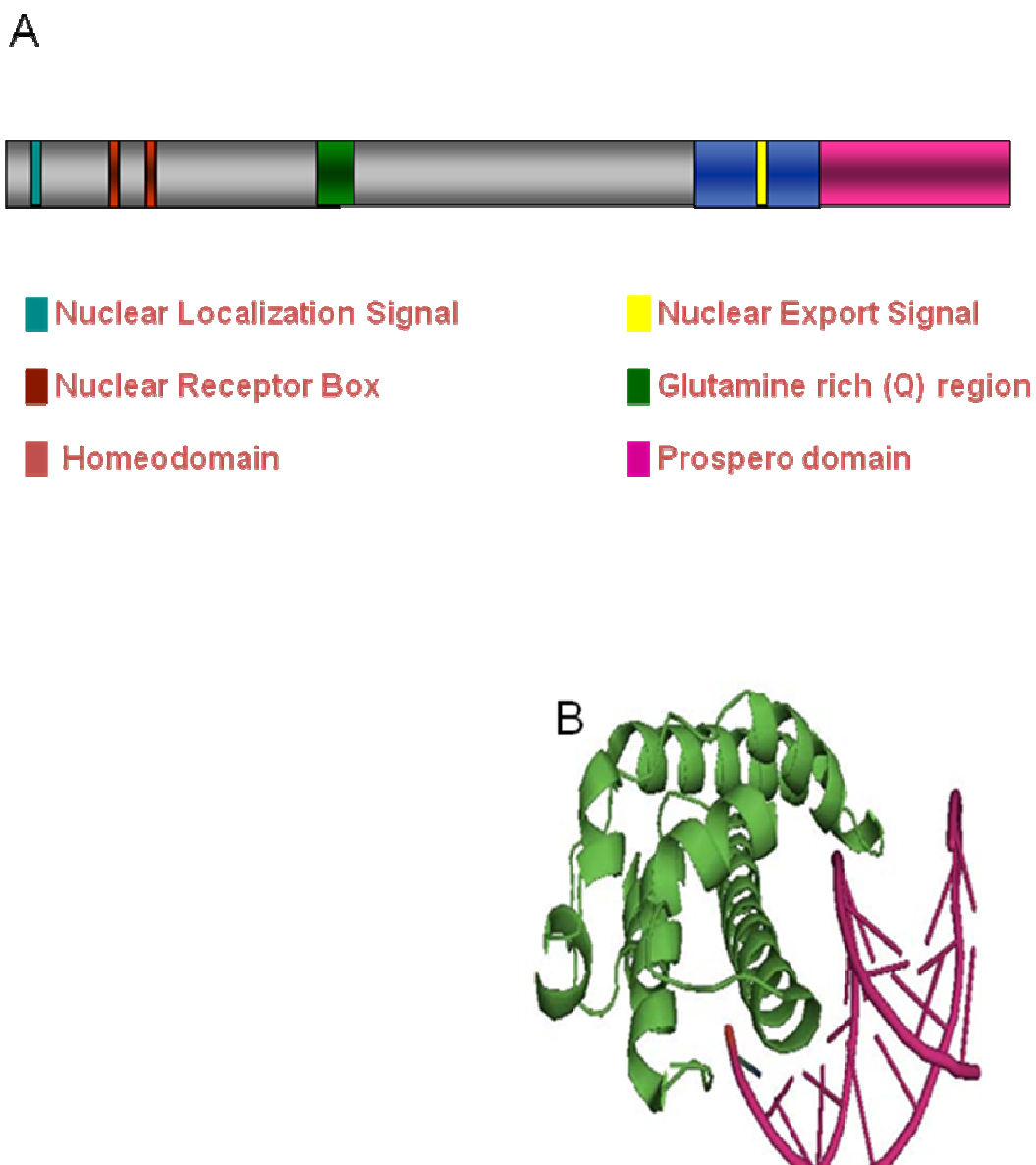

Figure 2- Structure of PROX1.

A) Schematic PROX1 structure showing known functional domain.

B) Homeoprospero domain interacts with DNA through its 3rd helix.

(Hassan, Li et al. 1997; Yousef and Matthews 2005)

a finger like structure (i.e. PD finger) which contacts the double helix DNA. This contact results in a different alignment in the double helix DNA from other homeodomain proteins(Yousef and Matthews 2005). The consequence of such variations in the DNA binding domain is reflected in the variety of DNA *cis*elements targeted by PROX1(Chen, Taube et al. 2008).

Recently Nishijima *et al.*reported an amino terminal conserved prospero domain in PROX1 and PROX2 thatthey named PD1 and thusthe C-terminal prospero domain is now known as PD2. The function of the PD1 domain is still unknown (Nishijima and Ohtoshi 2006). Both Prospero and PROX1 bind to a common DNA consensus sequence; A A/T C/T N N C T/C(Hassan, Li et al. 1997; Cui, Tomarev et al. 2004).However, Nishijima *et al.* reported that PROX2 does not bind to this consensus sequence(Nishijima and Ohtoshi 2006).

N-terminal nuclear receptor boxes have important roles in PROX1 function and its interaction with other transcription factors. It was shown that PROX1 binds to LRH-1 through its NR1 box andco-represses the transcription of the *cholesterol 7α-hydroxylase* (*CYP7A1*) gene(Qin, Gao et al. 2004; Steffensen, Holter et al. 2004).

Functionsof *Prox1*

Studying the developing chick retina, Belecky-Adams *et al.* proposed that *Prox1* (along with 2 other homeobox genes *Pax6* and *Chx10*) determinines the fate of retinal precursor cells (Belecky-Adams, Tomarev et al. 1997). Torii *et al.* also suggested a crucial role for *Prox1* in the differentiation of neural stem cells in the developing central nervous system in the mouse and rat embryo. Using the

immortalized cell line MNS-70 which has many neural stem cell characteristics, they could show that expression of *Prox1* in these cells correlates with neuronal differentiation(Torii, Matsuzaki et al. 1999).

Observations on the *Prox1*null mouse embryo revealed its crucial role in development of several different organs in mammals. In 1999 Wigle *et al.* observed that inactivation of *Prox1* gene in mouse embryo is lethal and that *Prox1*null mouse embryos die at mid-gestation (E14.5 – E15). These embryos show defects in several organs including lens, liver, pancreas and lymphatic vasculature. In the lens, there is a defect in terminal fiber differentiation and elongation(Wigle, Chowdhury et al. 1999). There is a deregulation of the cell cycle in lens fiber cells; these cells show inappropriate proliferation andincreased increasedapoptosis and several factors relating to cell cycle and apoptosis were dysregulated (e.g. *Cdkn1b* and *Cdkn1c*)(Wigle, Chowdhury et al. 1999).

*Prox1*null mouse embryos also have abnormalities in the development of liver and pancreas. In the liver of*Prox1*nullmouse embryos,there is abnormal proliferation and defective migration of hepatocytes but their differentiation is unaffected(Sosa-Pineda, Wigle et al. 2000). It was also shown that *Prox1* has a role in morphogenesis and cell fate in hepatocytes. Papoutsi *et al.*have reported a profound alteration on the transcriptional profile of met-murine hepatocytes (MMH) after the forced expression of *Prox1*. They concluded that *Prox1* acts as a multifunctional regulator of liver morphogenesis(Papoutsi, Dudas et al. 2007). In the pancreas of*Prox1*null mice there is abnormal epithelial morphology and formation of islet cell precursors. *Prox1* in the pancreas has a role in the

differentiation of multipotent pancreatic progenitors toward islet cell precursors(Wang, Kilic et al. 2005).

Risebro *et al.* showed that *Prox1* is expressed in the embryonic heart and plays an important role in heart development. They conditionally knocked out *Prox1* in cardiomyocytes of the mouse embryo. The conditional null animals died after birth. The authors observed that the sarcomeres are disrupted and unorganized in the hearts of these animals(Risebro, Searles et al. 2009).

In order to determine the fate of progenitor cells, prospero-related homeoproteins (both Prospero and PROX1) regulate cell cycle events; depending onthe cellular context, they either promote or pause cell division(Wigle, Chowdhury et al. 1999; Sosa-Pineda, Wigle et al. 2000). In lens fiber cells, inactivation of *Prox1* resulted in downregulation of CDKN1C which is a cell cycle inhibitor(Wigle, Chowdhury et al. 1999). On the other hand, overexpression of *Prox1* in endothelial cells increased the expression of Cyclin E1 and Cyclin E2, which resulted in promotion of the cell cycle. However, this effect of *Prox1* was not specific for endothelial cells and was also observed in non-endothelial cells(Petrova, Makinen et al. 2002). There are also reports that expression of *Prox1* in colon cancercells is associated with increased dysplasia in colon epithelium and cancer progression(Petrova, Nykanen et al. 2008).

Lymphatic Vasculature and *Prox1*

For a long time, the main problem in studying lymphatics was the inability to discriminate between lymphatic vessels and blood vessels. This distinction was based on histological characteristics and no reliable markers for lymphatic

vessels were available. Using heterozygous *Prox1* mice, Wigle *et al*. reported that *Prox1* is expressed by lymphatic endothelial cells(Wigle and Oliver 1999). This observation was also confirmed in a xenograft mouse tumour model. Lymphatic vessels adjacent or within the tumour co-expressed*Prox1* with other lymphatic markers(Wigle, Harvey et al. 2002). Studying human tissues, Wilting *et al.* also showed that *Prox1* is a reliable marker for human lymphatic endothelial cells (LECs). They studied several human embryonic, normal adult and malignant tissues and compared the expression of *Prox1* to other vascular markers such as VEGFR-3, CD31, CD34 and PAL-E(Wilting, Papoutsi et al. 2002). They observed that *Prox1* is absolutely excluded from human blood endothelial cells, but expressed by all human lymphatic endothelial cells(Wilting, Papoutsi et al. 2002).

Indeed,data from analysis of the *Prox1* knockout mouse was the most important evidence to date which supported Sabin's theory suggesting that lymphatic vessels originate from central embryonic veins(Sabin 1916).One of the most striking findings in the *Prox1* knockout mouse is the total arrest of lymphatic vessel development. In other words, these transgenic animals are completely devoid oflymphatic vasculature(Wigle and Oliver 1999). Severe edema develops in both heterozygous and homozygous animals at E14.5. Heterozygous animals developan apparently normal lymphatic capillary network. However, the lymphatic vasculature is completely absent in the homozygous*Prox1*null mouse (die at E14.5 –E15). On the other hand, the cardiovascular system develops completely normally in these animals at this stage(Wigle and Oliver 1999). It seems that expression of *Prox1* in a subgroup of embryonic venous endothelial cells

(presumably bipotent progenitors of lymphatic endothelial cells) commits them toward a lymphatic phenotype(Wigle, Harvey et al. 2002). Heterozygous animals die at the age of P2 – P3 due to insufficiency of the lymphatic system within their gastrointestinal system which indicates the haploinsufficiency effect of *Prox1* in development(Wigle and Oliver 1999).

In addition, the insertionof an in-frame β-galactosidase (lacZ) in mouse *Prox1*gene wasan excellent tool with which to follow the expression of *Prox1* during embryonic development(Wigle and Oliver 1999). *Prox1* expression in endothelial cells starts at E9.5 in the mouse embryo in the wall of anterior cardinal vein. *Prox1* expression is polarized and limited to the lateral venous margin. *Prox1* expressing endothelial cells start to bud off from the venous wall, migrating dorsoanteriorly, which suggests the presence of a specific guidance signaling system(Wigle and Oliver 1999).

Using a combinatorial set of different vascular markers, Wigle *et al.* confirmed the vascular origin of *Prox1* expressing cells(Wigle and Oliver 1999). After budding from the venous wall, *Prox1* expressing endothelial cells gather in the jugular and perimesonephric regions to form the lymphatic sacs. At E14.5 in the mouse embryo, lymphatic vessels start to grow from lymph sacs centrifugally toward the periphery of the embryo. This experiment also confirmed that *Prox1* is a specific marker for the lymphatic vasculature(Wigle and Oliver 1999). They further investigated the fate of the sprouted endothelial cells in the*Prox1* null mouse embryo. In the *Prox1*null mouse embryo, endothelial cells bud and sprout from the venous wall at E10.5, but theirsprouting is not polarized and the

sprouting terminates prematurely at E11.5-E12.5.As a result, no lymphatic sacs or vesselsdevelop. Using antibodies against LEC markers and BEC markers, they found that none of the sprouted endothelial cells in the homozygous mutant animal expressed LEC markers,but instead inappropriately expressed BEC markers(Wigle, Harvey et al. 2002).

VEGFR-3 is expressed in the blood vasculature at early stages of development, but as the embryo grows *VEGFR-3* expression becomes limited to the lymphatic vasculature. *VEGFR-3* null mice die before the development of lymphatic vessels due to extensive malformations in systemic blood vessels(Karkkainen and Petrova 2000).At E10.5,*VEGFR-3* is highly expressed in *Prox1* expressing budding endothelial cells. At E12.5, no *Prox1* positive cellsare detected in the cardinal vein and expression of *VEGFR-3* in blood endothelial cell is lowits(Wigle, Harvey et al. 2002).

Expression of other lymphatic markers like SLC in the*Prox1+/Lyve1+/VEGFR-3+* endothelial cells indicates that the phenotype of these cells has now switched towards the LEC phenotype. In contrast, *Prox1* negative endothelial cells express neither *VEGFR-3* nor *SLC*, suggesting that *Prox1* has a role in the lymphatic phenotype switch in endothelial cells. This hypothesis was further confirmed by the fact that in the*Prox1*$^{-/-}$ mouse embryo the expression of *VEGFR-3* in budding endothelial cells is very low and SLC expression is not detected(Wigle, Harvey et al. 2002).

Using microarray technology, Petrova *et al.* reported that the expression of about 300 genes differs between lymphatic endothelial cells (LECs) and blood

endothelial cells (BECs)(Petrova, Makinen et al. 2002). The largest differences in expressionwere observed in the expression of pro-inflammatory cytokines, cell junction and cytoskeleton proteins(Petrova, Makinen et al. 2002). They also reported that infection of human blood endothelial cells by Ad*Prox1*was able to increase the expression of many LEC-specific genes like *VEGFR-3*, *Cdkn1c*and *desmoplakin I/II.* In contrast, overexpression of *Prox1* in blood endothelial cells suppressed many BEC specific genes such as*STAT6*, *neuropilin-1* (NRP-1) and *integrin α5*(Petrova, Makinen et al. 2002). These findings are compatible with *in vivo* studies of the lymphatic vasculature(Wigle, Harvey et al. 2002). Examining different cell types they alsoshowed that over-expresssion of *Prox1* is able to induce lymphatic specific markers (such as*VEGFR-3*) in endothelial cells but not in other cell types(Petrova, Makinen et al. 2002). The authors concluded that *Prox1* is able to change the default phenotype of primary blood endothelial cells toward that of a lymphatic endothelial cell(Petrova, Makinen et al. 2002).

In very similar experiments, Hong *et al.*reported that the forced expression of *Prox1* in BECs is sufficient to induce two important lymphatic markers; podoplanin and VEGFR-3. They also observed that the expression of some BEC specific markers such as intercellular adhesion molecule-1 (ICAM-1), neuropilin-1, VEGFR-2 and VEGF-C were strongly repressed(Hong, Harvey et al. 2002).Further *in vivo* examination of mouse skin confirmed the co-expression of *Prox1* with other lymphatic markers in LECs. *Prox1* positive LECs also co-expressed*VEGFR-3* and *podoplanin*. In contrast, none of the *Prox1* positive cells in mouse skin expressed BEC specific markers such as neuropilin-1 and ICAM-1

(Hong, Harvey et al. 2002).

Generating a conditional *Prox1* knockout, Johnson *et al.*recently reported interesting findings when they down-regulated *Prox1*at different developmental stages(Johnson, Dillard et al. 2008). At early stages of lymphatic development, down-regulation of *Prox1* resulted in skin edema and the formation of blood-filled superficial vessels, which closely resembles the distribution of dermal lymphatic vessels. They repeated the experiment at later stages of lymphatic development until postnatal day 5. At these stages, the lymphatic vasculature is fully developed, yet they observed blood-filled lymphatic vessels both in the skin and viscera. They examined lymphatic vessels at a molecular level and observed that except for*Lyve-1*expression, expression of all the other lymphatic markers is suppressed in LECs of *Prox1*conditional null animals. In contrast, these LECs expressed blood endothelial markers such asENDOGLIN and CD34. They also found aberrant connections between the systemic blood circulation and lymphatic vessels. Using siRNA technology, they also observed that the lymphatic phenotype is reversible in culturedLECs. They concluded that continued expression of *Prox1* is necessary for the maintenance of the lymphatic phenotype even in the mature and differentiated lymphatic vessels*in in vivo*(Johnson, Dillard et al. 2008).

Gene Regulation by PROX1

Despite the fact that *Prox1* is a homeobox gene (i.e. has a homeoprospero DNA binding domain) and has a crucial role in development of a variety organs and tissues, little evidence of its direct interaction with DNA has been provided in

mammalian cells. However several researchers have reported the indirect binding of PROX1 to its target genes through other transcription factors and its role as a co-activator (e.g. CBP/p300)(Chen, Dowhan et al. 2002) or co-repressor (e.g. LRH-1)(Qin, Gao et al. 2004).

So far, it is reported that PROX1 interacts with four nuclear receptors: LRH-1, HNF4α, SF-1/ff1b and COUP-TFII. All of these proteins interact with PROX1 through a motif (LLLRLP) in the first nuclear receptor box domain (NR1) of PROX1 (Qin, Gao et al. 2004; Song, Li et al. 2006; Yamazaki, Yoshimatsu et al. 2009). In hepatocytes, PROX1 is a co-repressor for several genes (*cholesterol 7-alpha hydroxylase* and *small heterodimer partner*) by interacting with nuclear receptors like Liver Receptor Homolog-1 (LRH-1) and hepatocyte nuclear factor-4α (HNF-4α). Using a yeast two-hybrid assay, Qin *et al.* showed that PROX1 interacts with FTF/LRH-1. Further investigation revealed that PROX1 indeed is a co-repressor of LRH-1 in hepatocytes(Qin, Gao et al. 2004).This interaction is also via a LRKLL motif in theNR1 domain of PROX1. However, gel shift assays showed that Prox1 interferes with binding of hLRH-1 to the promoter of its target gene *Cholesterol 7-α-hydroxylase (CYP7A1)*(Qin, Gao et al. 2004).Song *et al.*also found that in human hepatocytes, PROX1 interacts with HNF-4α and acts as a co-repressor for the *cholesterol 7α-hydroxylase* (*CYP7A1*) gene(Song, Li et al. 2006). In contrast to LRH-1, PROX1 does not block the binding of HNF-4α to the CYP7A1 promoter, but represses its transcriptional activity by interfering with its co-activation with PGC-1α. The N-terminal of PROX1 interacts with the AF2 domain of HNF-4α.Specifically,the nuclear receptor box 1 (NR1) of PROX1

(which contains the LRKLL sequence) is crucial for this interaction. This is in accordance with the fact that co-regulators of nuclear receptors often interact with them via a consensus sequence of LXXLL(Song, Li et al. 2006).

Inthe blood vasculature,the chicken ovalbumin upstream promoter transcription factor II (COUP-TFII), an orphan nuclear receptor, is expressed invenous endothelial cells, but not in arterial endothelial cells, and is important for the maintenance of venous identity. COUP-TFII is expressed in LECs and interacts with PROX1. Lee *et al.* showed that this interaction is important for regulation of some PROX1 target genes such as*FGFR-3*(Yamazaki, Yoshimatsu et al. 2009).

Wang *et al.* reported that PROX1 acts as a co-repressor for the*Interferon-gamma* (*IFN-γ*) gene. They showed that in T cells PROX1 interacts directly with the nuclear receptor *peroxisome proliferator-activated receptor gamma* (*PPARγ*) and induces the down-regulation of *IFN-γ* in these cells(Wang, Zhu et al. 2008).

Shan *et al.*reported that the PROX1 is modified post-translationally by sumoylation within its repression domain. They observed that sumoylation of PROX1 downregulates its transcriptional co-repressor activity and interferes with PROX1 interaction with HDAC3(Shan, Wang et al. 2008). Pan*et al.* showed that sumoylation of PROX1 regulates its function; inhibition of PROX1 sumoylation, by inducing mutation in its sumoylation site in PROX1 (Lysine 556), inhibited the ability of PROX1 to induce VEGFR-3 expression in endothelial cells(Pan, Chang et al. 2009).

Transgenicanimal studies have shown that *Prox1* is required for the

expression of *γ-Crystallin* in lens fiber cells. Using luciferase gene reporter assays, Chen *et al.*showed that *Prox1* is able to activate *βB2-* and *γF- Crystallin* promoters in monkey kidney cells (COS-1)(Chen, Dowhan et al. 2002). Lengler *et al.*also showed that PROX1 is able to activate the*γF- Crystallin* promoter which contains a PROX1 binding site(Lengler, Krausz et al. 2001).

In the lens, expression of *Prox1* along with *Maf* and *Sox* is responsible for the differentiation of lens fiber cells and expression of *crystallin*geneswithin these cells(Ogino and Yasuda 2000). Co-transfection of *Prox1* with *c-Maf* is shown to have a synergistic effect in the activation of the*βB2-Crystallin*promoter. Since the reporter construct lacked any PROX1 binding sites, Chen *et al.* proposed that PROX1 acts as a co-activator in the case of *βB2-Crystallin*the *βB2- Crystallin* promoter. In addition CBP/P300 was able to co-activate the *βB2-Crystallin* promoter with *c-Maf*, but not *Prox1*. However,*Prox1* enhanced this co-activation.This effect was independent of binding of PROX1 to DNA. They further showed that PROX1 directly binds to the full length CBP/P300 protein. Finally the authors suggested that PROX1 is indirectly recruited to the promoter region of the*βB2- Crystallin* gene by binding to CBP/p300(Chen, Dowhan et al. 2002). It was also shown that in the inner ear sensory epithelial cells, PROX1 interacts with Atoh1 and Gfi1 and suppresses their transcriptional activity(Kirjavainen, Sulg et al. 2008).

As mentioned above, elongation of lens fiber cells is arrested and the expression of *CrygD* is down-regulatedin *Prox1* null mice (Wigle, Chowdhury et al. 1999).Lengler *et al*. demonstrated that PROX1 is able to activate the *Cryge/f*

promoter by binding to a PROX1 response element located -151 to -174 bp within the*Cryge/f* promoter. However, in the *Prox1* null mouse only the expression of *CrygD* is down-regulated(Lengler, Krausz et al. 2001).

Vascular Endothelial Growth Factor-3 (*VEGFR-3*)

Endothelial cells are key elements in the growth of new blood vessels (angiogenesis) and maintaining the integrity of the whole vascular system(Karkkainen and Petrova 2000). These cells are dependent on severalkey growth factor signaling pathways, which induce their effects in endothelial cells through receptor tyrosine kinases (RTKs). Growth signaling in endothelial cells comprises both vascular-specific and non-specific growth factors(Otrock, Makarem et al. 2007). Two main elements of vascular non-specific growth factors for endothelial cells are fibroblast growth factor (FGF) and epidermal growth factor (EGF) signaling.Vascular endothelial growth factor (VEGF) and angiopoietin (Ang) signaling pathways representthe two major vascular specific receptor tyrosine kinase signaling pathways. So far,six members of VEGF ligands have been identified: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PlGF)(Otrock, Makarem et al. 2007). Vascular endothelial growth factor receptors (VEGFRs) are identified by their distinct extracellular regions, which contain seven immunoglobulin (Ig) homology domains. There are 3 known receptors for the VEGF ligands including VEGFR-1 (flt-1), VEGFR-2 (KDR/flk-1), and VEGFR-3 (FLT4). The fifth Ig homology domain of the extracellular part of VEGFR-3 is cleaved post-translationally and the remaining extracellular parts remain linked by two disulfide bonds(Roskoski

2008). The intracellular domain of VEGF receptors contains a protein-tyrosine kinase domain. Upon binding of ligand (e.g. VEGF) to the extracellular domain of receptor, VEGFR is dimerized leading to auto/transphosphorylation of the intracellular domain (Figure 3A), generating docking sites for Src homology 2 (SH2) and phosphotyrosine binding (PTB) containing proteins. These events activate a set of canonical and non-canonical signaling pathways within the cells, which result in cell survival, proliferation and migration(Figure 3B and 3C) (Roskoski 2008).

Several *in vivo* and *in vitro* studies have shown that VEGF-C/VEGFR-3 signaling is the main VEGF signaling pathway within LECs and is sufficient for lymphangiogenesis(Makinen, Veikkola et al. 2001; Veikkola, Jussila et al. 2001). Indeed, the maintenance of *VEGFR-3*expression in endothelial cells byPROX1 is one of the key eventseventsin lymphatic vessel development(Wigle, Harvey et al. 2002).

VEGFR-3 was first cloned from human erythroleukemia cells and placental cDNA libraries by its homology to *fms* (a protooncogene). In humans, the*VEGFR-3* gene is located on chromosome 5 (5q33-q35) and is composed of 31 exons. Alternative splicing of exons 30a and 30b produces two VEGFR-3 isoforms.

During early embryogenesis *VEGFR-3* has a crucial role in development of the blood vascular system.The *VEGFR-3* null mouse is not viable due to several vascular abnormalities. Inthe mouse, *VEGFR-3* is first expressed at embryonic day 8.5 (E8.5) in endothelial cells of the venous system. Later on, its expression is restricted to lymphatic vessels(Dumont, Jussila et al. 1998).

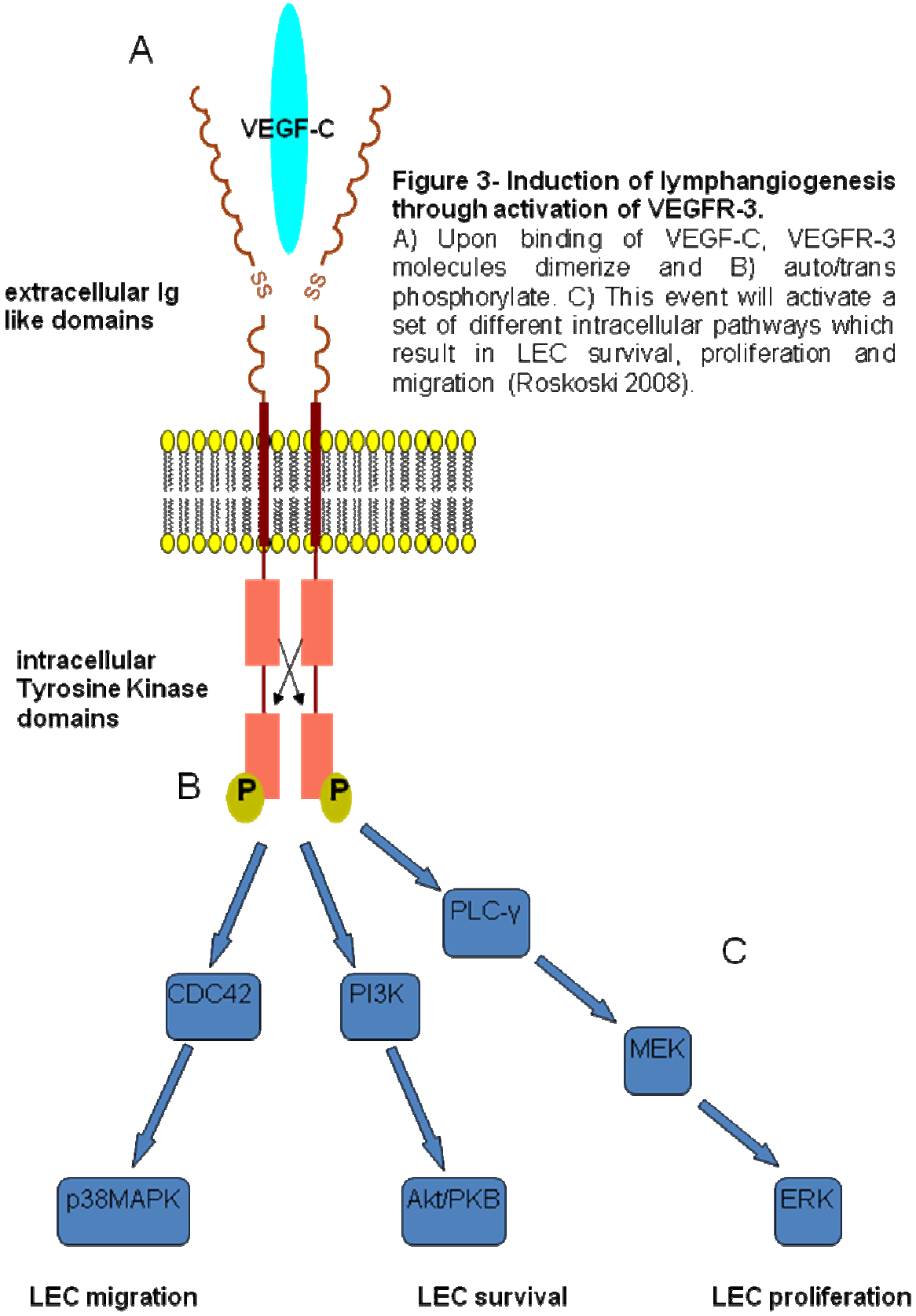

Figure 3- Induction of lymphangiogenesis through activation of VEGFR-3.
A) Upon binding of VEGF-C, VEGFR-3 molecules dimerize and B) auto/trans phosphorylate. C) This event will activate a set of different intracellular pathways which result in LEC survival, proliferation and migration (Roskoski 2008).

VEGFR-3 in adults is expressed nearly exclusivelyin lymphatic vessels(Dumont, Jussila et al. 1998).*VEGFR-3* is also expressed in adults in angiogenic sprouts during physiologic angiogenesis (e.g. wound healing); inhibition of VEGFR-3 signaling in these situations decreases normal angiogenesis and interrupts capillary network formation(Tammela, Zarkada et al. 2008). *VEGFR-3* is also expressed in BECs in pathologic conditions such as malignancies. Indeed, VEGFR-3 is so abundant in tumour vessels that can be considered as a marker for tumour angiogenesis. VEGF-C/VEGFR-3 has been shown to be important for tumour development and metastasis. Inhibition of VEGFR-3 is shown to decrease tumour lymphangiogenesis and lymph node metastasis. Thus,several researchers have proposed the inhibition of VEGF-C/VEGFR-3 signaling as a potential new approach for cancer treatment(Otrock, Makarem et al. 2007).

The human *VEGFR-3* promoter is a TATA-less promoter with a highly GC-rich proximal sequence and shows many similarities to the mouse *VEGFR-3* promoter(Iljin, Karkkainen et al. 2001). Ilgin *et al.*compared the promoter regions of the human and mouse *VEGFR-3* genes. They found two conserved regions between them with 70% identity (HR1 and HR2). Both HR1 and HR2 contain several putative transcription factor binding sites. HR2 is proximal to transcription start site and contains elements similar to other promoters lacking a TATA box(Iljin, Karkkainen et al. 2001).Using different endothelial and non-endothelial cultured cells, they showed that the *VEGFR-3* promoter has an endothelial specific activity with the highest amount of activity within the proximal 900 bp

promoter. They also showed that a 3.6 kb *VEGFR-3* promoterin a transgenic reporter mouse shows lymphatic specific activity(Iljin, Karkkainen et al. 2001).

Mutations of *VEGFR-3* result in developmental abnormalities in lymphatic vessels and lymphedema(Makinen, Veikkola et al. 2001). The classic clinical condition due to mutations in the *VEGFR-3* gene is called Milroy's disease; affected patients present with bilateral lower limb edema (Brice, Child et al. 2005).

VEGF-C and VEGF-D are the main ligands for VEGFR-3. In the mouse embryo, *VEGF-C* is expressed in tissues adjacent to cells that express*VEGFR-3*(Veikkola, Jussila et al. 2001).In one study it was shown that VEGF-C mediated activation of VEGFR-3 is sufficient to protect cultured LECs against starvation-induced apoptosis(Makinen, Veikkola et al. 2001).Overexpression of *VEGF-C*also increases lymphangiogenesis in the skin of the mouse(Makinen, Veikkola et al. 2001). Over-expression of *VEGF-C* also increases lymphangiogenesis in the skin of the mouse (Makinen, Veikkola et al. 2001). *VEGF-C* null mice do not develop lymphatic vessels and die of edema. Their phenotype resembles that of the Prox1 null mice at the same embryonic age. Under Prox1 positive endothelial cells fail to migrate from their original sitessites in the venous wall of the VEGF-C null mice. Even loss of one of the VEGF-C alleles leads to lymphedema and cutaneous lymphatic hypoplasia. (Karkkainen, Haiko et al. 2004).

Fibroblast Growth Factor Receptor-3 (*FGFR-3*)

Fibroblast growth factors (FGFs) and their receptors (including 23 different FGFs and four different FGF receptors) form one of the most important signaling

pathways in the body. They playimportant roles in normal development and physiology.FGFs are potent mitogens and are involved in cell proliferation and survival(Ornitz and Marie 2002). The role of fibroblast growth factor (FGF) signaling in angiogenesis is very well documented. It is reported that in endothelial cells, FGFs induce proliferation, differentiation and migration(Javerzat, Auguste et al. 2002). FGF signaling also contributes to some malignant transformations(Javerzat, Auguste et al. 2002).

FGF receptors are another family of receptor tyrosine kinases (RTKs) with three immunoglobulin (Ig) domains within their extracellular domains. The binding of FGFRs to their ligands requires a heparin-binding step. Different isoforms of protein exist due to alternative splicing, especially three within the third Ig-like domain (isoforms IIIa, IIIb and IIIc)(Vajo, Francomano et al. 2000). The ligands (i.e. FGFs) and receptors (i.e. FGFRs) are usually expressed by adjacent tissues. This phenomenon plays an important role in morphogenesis and organogenesis during embryonic life(Ornitz and Marie 2002).

The *FGFR-3* gene is located on chromosome 4 (4p16.3) in humans and has 19 exons. The *FGFR-3* promoter lacks any TATA or CAAT boxes, but several putative *cis* elements have been identified within it(Perez-Castro, Wilson et al. 1995). The*FGFR-3* promoter is very similar between human and mouse in its very proximal part (~100 bp 5` flanking region); this region is important for the regulation of *FGFR-3* transcription(Perez-Castro, Wilson et al. 1997). McEwen *et al.* showed that enhancer elements for SP1 family transcription factors exist within the proximal promoter of FGFR-3 gene and also in the first intron of the

gene(McEwen and Ornitz 1998).

During development *FGFR-3* is highly expressed in the central nervous system (CNS), bones and cartilages(McEwen and Ornitz 1998). It is also expressed in other cell types including lung alveolar cells (Weinstein, Xu et al. 1998) and LECs(Shin, Min et al. 2006). The main ligands for FGFR-3 are FGF-1, -2, -4,-8 and -9(Ornitz and Marie 2002; Chang, Garcia-Cardena et al. 2004). Mutations in the *FGFR-3* gene are related to forms of congenital skeletal abnormalities like achondroplasia, Muenke coronal craniosynostosis and Crouzon syndrome with acanthosis nigricans(Vajo, Francomano et al. 2000; Hung, Lee et al. 2008).

FGFs induce angiogenesis through the activation of VEGF signaling(Javerzat, Auguste et al. 2002); Kubo *et al.* reported that *FGF2* activated*VEGF-C/VEGFR-3* signaling in cornea which resulted in lymphangiogenesis(Kubo, Cao et al. 2002). However, Shin *et al.*reportedthat *FGFR-3* is a direct target of *Prox1* in LECs(Shin, Min et al. 2006) and provided evidence that FGF signaling may have a direct role in lymphangiogenesis. They showed that forced expression of *Prox1* in BECs upregulates *FGFR-3* in these cells; PROX1 binds directly to *FGFR-3* promoter via its*homeoprospero* DNA binding domain and activates *FGFR-3* transcription. Using electrophoretic mobility shift assayassays (EMSA), they identified a new PROX1 binding sequence in the*FGFR-3* proximal promoter: CACGCCTCT. Further investigation disclosed that *FGFR-3* is expressed in human and mouse LECs and suppression of *FGFR-3* in these cells decreases their proliferation by 30 – 40 %. They also

showed that FGF2 binds directly to LECs and promotes their migration and proliferation independently from the *VEGF-C/VEGFR-3* signaling pathway(Shin, Min et al. 2006).

As mentioned earlier, FGFR-3 has several important developmental roles such as bone morphogenesis, development of the inner ear, and development of alveolar tissue in the lungs(Weinstein, Xu et al. 1998; Ornitz and Marie 2002). *FGFR-3* null mice show skeletal abnormalities including kyphosis, scoliosis and overgrowth of long bones and vertebrae(Colvin, Bohne et al. 1996).However Shin *et al.* reported that they could not find any lymphatic phenotype in the *FGFR-3* null mouse. They proposed that this is due to compensation by other FGF receptors(Shin, Min et al. 2006).

Disorders involving the Lymphatic Vasculature

Due to its crucial roles in body fluid homeostasis, fat absorption, and immune surveillance, the lymphatic vasculature is involved in many aspects of development, growth and other physiological functions. Malfunctions of the lymphatic vessels affectthe normal physiology of many other organs and systems(Alitalo, Tammela et al. 2005). Lymphatic vessels are also involved in cancer metastasis. It is shown that increased tumour lymphangiogenesis is correlated with poor prognosis in cancer patients (Miyahara, Tanuma et al. 2007).

Lymphedema

Simply stated, lymphedema is the failure of lymph transport. It is a devastating disorder, which profoundly affects the quality of life of millions of patients and their families. Nonetheless, existing medical literature regarding

diagnosis and treatment of this disorder is both inadequate and controversial(Board and Harlow 2002; Horning and Guhde 2007).

Lympedema occurs when there is an imbalance between the production of lymph within the tissue and its uptake by the lymphatic vessels. Different conditions are responsible for development of lymphedema including infection, trauma (e.g. burns and radiation) and transplantation. Congenital/developmental disorders of lymphatic vessels also lead to lymphedema(Board and Harlow 2002). Since formation of lymph fluid is a passive process and depends on many physiochemical aspects of tissue environment, disruption of lymph flow can occur by: blockage of lymphatic vessels and nodes, increased osmotic pressure within the interstitial space and any changes within extracellular matrix property(Swartz 2001). As lymphedema develops within a tissue the interstitial pressure increases. Consequently, inflammatory cytokines and enzymes are released which lead toward increased inflammation in tissue. Inflammation in turn aggravates the situation and over a long time period results in remodeling of extracellular matrix and fibrosis, infection and disability of the affected organ(Swartz 2001). When fibrosis happens in the interstitial space, the permeability of lymphatic capillaries is lost which leads to more edema(Oliver 2004).

Several pathologies involving lymph nodes (e.g. infection, tumour metastasis) or removal of lymph nodes by surgery can block the flow of lymph resulting in edema of upstream organs. A prominent example of the latter is post-mastectomy arm edema in women affected by breast cancer. Theoverall

incidence of arm edema in these patients is reported at 26% (Warren, Brorson et al. 2007).

Lymphedema is a complex state of regional edema resulting from various pathologies. It is estimated that the prevalence of lymphedema is relatively high. However, the majority of cases remain unrecognized and do not receive appropriate care(Rockson and Rivera 2008). Traditionally, lymphedema is divided to two categories; primary (genetic/congenital) and secondary (acquired)(Rockson 2006).

Primary Lymphedema

Primary lymphedema results fromdefects in lymphatic vasculature development. However the exact pathogenesis of most primary lymphedema is not completely understood. Primary lymphedema is classified according to the age of onset and other phenotypic characterizations(Connell, Brice et al. 2008).Lymphedemamay be present at birth or within two years after birth (congenital lymphedema), or emerge during puberty (Lymphedema praecox), or later in the life, after age 35 (Lymphedema tarda)(Rockson 2006).

Primary lymphedema is not a common form of edema. It affects 1.2/100,000 of the young population(Rockson and Rivera 2008). Today the basis of some forms of primary lymphedema has been determined.Mutations (heritable or sporadic cases) in some of the genes involved in development of lymphatic vessels (e.g. *VEGFR-3*and *FOXC2*) are found to be responsible for several categories of primary lymphedema(Brice, Child et al. 2005). Lymphedema praecox is the most common type of primary lymphedema, which affects women

more than men. In addition, primary lymphedema is commonly observed in the context of several other congenital syndromes (e.g. Turner's syndrome, Noonan's Syndrome and trisomy 18)(Connell, Brice et al. 2008). So far, two types of primary lymphedema have been described and investigated extensively: Milroy's disease and lymphedema-distichiasis.

Milroy's Disease

Milroy's disease is an autosomal dominant inherited disorder. It was first described by William Milroy in 1892 (Brice, Child et al. 2005). It was shown that mutations in *VEGFR-3* are responsible for most cases of Milroy's disease(Irrthum, Karkkainen et al. 2000). So far, almost 20 mutations in this gene have been reported and almost all of these mutations are in the kinase domains of *VEGFR-3* (exon 17- 26)(Irrthum, Karkkainen et al. 2000). However, not all cases with a Milroy's phenotype of lymphedema have a mutation in the*VEGFR-3* gene. It is estimated that only 70 percent of patients witha atypical Milroy's phenotype (bilateral or unilateral lower limb edema at birth or during 2 years after birth) have mutations in their *VEGFR-3* gene. The penetrance of the disease is reportedto be between 70 to 80 percent(Connell, Brice et al. 2008).

It was originally thought that due to *VEGFR-3* mutations, lymphatic aplasia is responsible for the clinical lymphedema. But recent studies using fluorescent microlymphangiography showed that there are remarkable amounts of initial lymphatic vessels in the affected limbs. However these vessels are not functional. Though it is believed that valve failure is involved in the pathology of Milroy's Disease, the exact mechanism of the disease is still unknown(Connell, Brice et

al. 2008).

Lymphedema–Distichiasis

Lymphedema–Distichiasis is a subgroup of primary lymphedema caused by mutations in the*FOXC2* (*forkhead transcription factor*) gene. Distichiasis is derived from the Greek word distikhos (two rows). Indeed, patients have two rows of eyelashes in the eyelids. The disease is inherited in an autosomal dominant form with a high degree of penetrance(Connell, Brice et al. 2008).

Lymphedema in Lymphedema–Distichiasis appears during puberty inthe lower limbs and is usually bilateral. Distichiasis can be seen from birth and is bilateral. Functional lymphangiography studies have suggested that reflux in the main collector lymphatic vessels is responsible for thelymphedema. It is also reported that in *FOXC2* knockout mice the development of lymphatic valves is abnormal(Connell, Brice et al. 2008).

Secondary Lymphedema

Secondary lymphedema occurs when lymphatic vessels are blocked or damaged. It happens frequently during medical procedures and, in fact iatrogenic lymphedema (e.g. due to surgery and radiation) is the most common form of lymphedema in developed countries(Rockson and Rivera 2008). Other pathological conditions including infections, burns and tumours are other causes of secondary lymphedema(Warren, Brorson et al. 2007).

Some infective and parasitic diseases may also involve lymphatic vessels andtherefore result in edema in upstream tissues. Lymphatic filariasis is a very well known example in developing countries. It is the most common cause of

secondary lymphedema in the world and is caused by nematode larva transmitted by mosquitos(Rockson 2006).

Lymphatic vessels and Cancer

Lymphatic vessels are amajor route for cancer metastasis(Das and Skobe 2008). Most tumours tend to metastasize to sentinel (regional) lymph nodes early in their development(Tuttle 2004). This event is highly important and determinant in the progress of malignancies(Jackson 2007). Patients with lymph node metastasis have a poorer prognosis and need more aggressive treatments(Miyahara, Tanuma et al. 2007). In addition, inhibition of lymph node metastasis is associated with better outcomes and decreaseddecreasedprogress of the disease throughout the body(Shimizu, Kubo et al. 2004). Until very recently, it was believed that in order to metastasize, cancer cells enter pre-existing lymphatic vessels and circulate until they are trapped in regional lymph nodes. However it is now very well documented that metastasis is not just a simple event and many signaling pathways and micro-environmental changes occurs to facilitate and promote lymph node metastasis(Nathanson 2007).

Cancer cells stimulate lymphangiogenesis by releasing lymphangiogenic factors to their microenvironment (Figure 4A).Many studies have confirmed the development of new lymphatic vessels close to and sometimes inside the tumour mass(Miyahara, Tanuma et al. 2007). These studies presented strong evidence that these events increase the rate of lymph node metastasis in patients(Miyahara, Tanuma et al. 2007). Though often abundant in number, intra-tumoural lymphatic vessels are not functional due to high interstitial

pressureinsidewithin the tumour. On the other hand, peri-tumoural lymphatic vessels are functionally active and play an important role in tumour cell circulation(Das and Skobe 2008). However, the importance of lymphatic metastasis varies among different malignancies(Karpanen and Alitalo 2001).

Tumours also induce lymphangiogenesis within regional lymph nodes (Figure 4B)(Ji 2009). This event can happen prior to any traceable lymph node metastasis. It seems that the primary tumour mass plays a role by sending lymphangiogenic signals to the sentinel lymph nodes through the draining lymphatic vessels(Harrell, Iritani et al. 2007; Hirakawa, Brown et al. 2007).

Recently many studies (both in human and animals) have focused on molecular pathways inducing lymphangiogenesis in a tumour context(Sleeman and Thiele 2009). It appears that VEGF-C/VEGFR-3 signaling is the most important player in tumour lymphangiogenesis. Inhibition of this pathway, i.e. using soluble VEGFR-3 or antibodies against VEGFR-3, is shown to reducereducetumour mediated lymphangiogenesis and metastasis(Jain and Padera 2002; Arigami, Natsugoe et al. 2009). Currently, many studies are in progress to evaluate clinical benefits of such approaches in cancer patients(Sleeman and Thiele 2009).

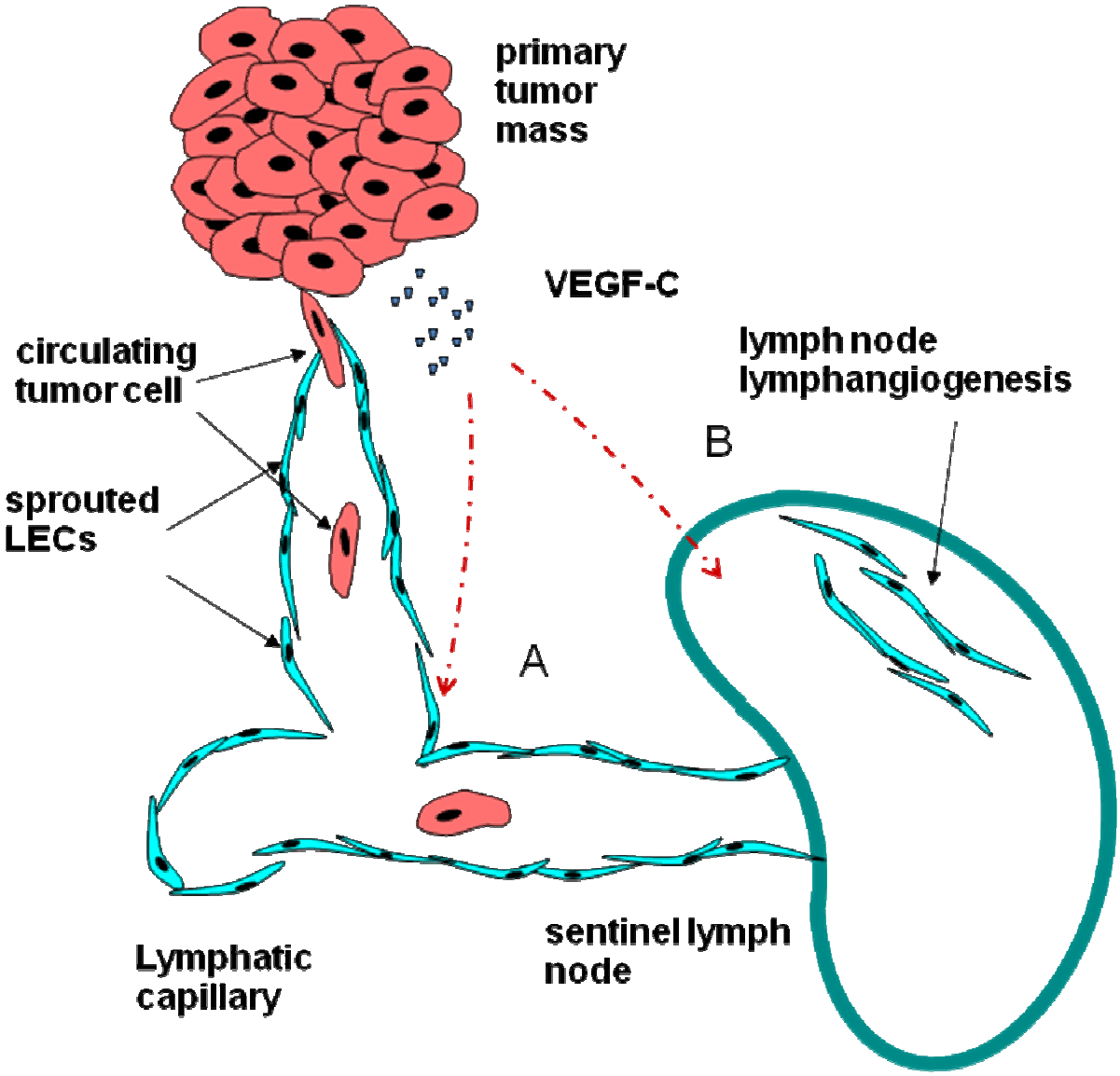

Figure 4 – Process of tumour lymphangiogenesis.

A) by releasing VEGF-C, tumor cells promote the formation of new lymphatic capillaries inside (intra-tumour lymphangiogenesis) and around the tumour mass (peri-tumoural lymphangiogenesis). This will facilitate and increase the mobilization of tumour cells and their metastasis. B) Tumours also induce lymphangiogenesis in sentinel lymph nodes even before arrival of cancer cells to the lymph node vessels (Harrell, Iritani et al. 2007; Hirakawa, Brown et al. 2007).

Rationale

The lymphatic vasculature has crucial roles in development, fluid homeostasis, immunosurveillance and fat absorption(Alitalo, Tammela et al. 2005). In addition, the role of lymphatic vessels in pathologic conditions such as tumour metastasis and lymphedema is well documented (Karkkainen and Alitalo 2002; Baluk, Tammela et al. 2005; Das and Skobe 2008). Although the lymphatic circulation was discovered at nearlythe same time as the systemic blood circulation, studies of the lymphatic vessels have been hampered by the fact that lymph is a transparent fluid and discrimination between these two types of vessels was only possible based on histological examination (Oliver and Harvey 2002; Oliver and Alitalo 2005). It was not until very recently that reliable markers have been discovered that specifically label lymphatic vessels(Wigle and Oliver 1999; Wigle, Harvey et al. 2002).

Prox1 was first cloned in the mouse based on its homology to *Prospero*, a divergent homeobox in *Drosophila*(Oliver, Sosa-Pineda et al. 1993). It is expressed in several organs during development such as liver, pancreas, lens and lymphatic vessels (Oliver, Sosa-Pineda et al. 1993). *Prox1*$^{-/-}$ mouse embryos die at E14.5-E15.0 due to multipledevelopmental developmentaldefects (Wigle, Chowdhury et al. 1999; Wigle and Oliver 1999; Sosa-Pineda, Wigle et al. 2000; Burke and Oliver 2002; Dyer, Livesey et al. 2003; Wang, Kilic et al. 2005; Risebro, Searles et al. 2009). The most striking finding in gross examination of the mutant embryos is a severe edema which correlates with the fact that *Prox1* null mousemice are completely devoid of lymphatic vessels(Wigle and Oliver

1999). In the wild type mouse, *Prox1* expressing endothelial cells bud off from central embryonic veins and migrate toward a specific signal (VEGF-C) to form lymphatic sacs. Lymphatic vesselsthen thensprout from these lymphatic sacs and spread all over the body and to form the lymphatic vasculature system(Wigle and Oliver 1999; Wigle, Harvey et al. 2002). Interestingly,adult lymphatic endothelial cells express *Prox1* and lymphatic vessels induced by pathological conditions express*Prox1*(Wigle, Harvey et al. 2002). Further investigation of the *Prox1*$^{-/-}$ mouse embryo revealed that endothelial cells which migrate from the venous wall not only fail to form lymphatic sacs andto express markers of the lymphatic phenotype, but instead adopt a blood endothelial phenotype as their default fate. These findings were strongly suggested that *Prox1* acts as a master regulator of lymphatic development(Wigle, Harvey et al. 2002).

This theory was further confirmed by subsequentexperiments in cultured venous endothelial cells. Forced over-expression of *Prox1* in these cells was sufficient to result in a switch towards a lymphatic phenotype; expression of lymphatic vessels markers was upregulated whereasexpression of blood vessels markers was suppressed(Hong, Harvey et al. 2002; Petrova, Makinen et al. 2002). In addition, siRNA mediated suppression of *Prox1* in lymphatic endothelial cells resulted in a reversal of the lymphatic phenotype to that of a blood endothelial cell (Johnson, Dillard et al. 2008). Using a conditional *Prox1* knockout mouse model, Johnson *et al.*also reported the reversal of the phenotype of the lymphatic vasculature toward the phenotype of the blood vasculature *in vivo* due to deletion of *Prox1*at different developmental stages. They reported that

lymphatic vessels were filled with blood and express blood vessel markers after deletion of *Prox1* in mouse embryos or even in mouse neonates until postnatal day 5(Johnson, Dillard et al. 2008). Thus, expression of *Prox1* is required both to induce lymphatic differentiation in endothelial cells and is required to maintain the lymphatic phenotype in the adult lymphatic endothelial cells.

Given the fact that *Prox1* is a homeobox gene, it is a reasonable hypothesis that its homeodomain product, PROX1, would be able to interact with chromatin extensively. Crystallographic studies have revealed that in Prospero, that the homeo and Prospero domains form a continuous single DNA binding domain consisting of six helices(Yousef and Matthews 2005). It is via the third helix that the homeoprospero domain largely binds to DNA(Yousef and Matthews 2005). Although PROX1 has also beenbeenshown to act as a co-repressor to suppress the expression ofsome genes such as *cholesterol 7-alpha hydroxylase* and *small heterodimer partner*via its interaction with transcription factor LRH-1(Qin, Gao et al. 2004). However, there are only a few published reports of a direct interaction between PROX1 and its target DNA sequences in mammalian cells. In addition, the exact mechanisms by which PROX1 regulates its target genes are still unknown and very little is known about the role of the different putative functional domains of PROX1 in regulation of its target genes. So far, only two direct target genes have been identified for PROX1 in mammals: *Fibroblast Growth Factor Receptor-3* (*FGFR-3*) in lymphatic endothelial cells(Shin, Min et al. 2006) and *βB1-Crystallin* in lens fiber cells(Chen, Taube et al. 2008).

The role of vascular endothelial growth factors and their receptors in the development of blood vessels is well documented.These molecules are the focus of studies of either excessive or deficient angiogenesis (e.g. cancer and ischemia)(Szuba, Skobe et al. 2002; Karkkainen, Haiko et al. 2004). It is shown that *VEGFR-3* and its ligands *VEGF-C* and *VEGF-D* are the dominant VEGF members involved in lymphatic vessel development (Veikkola, Jussila et al. 2001; Karkkainen, Haiko et al. 2004; Tammela, Petrova et al. 2005). During embryonic development, *VEGFR-3* is important for the development of the systemic blood vasculature (Dumont, Jussila et al. 1998). Later, its expression is largely restricted to the lymphatic vasculature and plays an important role in both physiological and pathological lymphangiogenesis (Veikkola, Jussila et al. 2001; Karkkainen, Haiko et al. 2004; Karpanen and Alitalo 2008). Mutations of *VEGFR-3* are responsible for a large proportion of primary (congenital) lymphedema (e.g. Milroy's disease) (Irrthum, Karkkainen et al. 2000; Iljin, Karkkainen et al. 2001; Szuba, Skobe et al. 2002; Brice, Child et al. 2005; Connell, Brice et al. 2008).The *VEGFR-3* gene is upregulated following*Prox1* overexpression inbloodfollowing *Prox1* over-expression in blood endothelial cells(Hong, Harvey et al. 2002; Petrova, Makinen et al. 2002). Its GC-rich promoter lacks both TATA and CAAT boxes (Iljin, Karkkainen et al. 2001). Bioinformatic analysis of its promoter reveals several PROX1 binding sites in its proximal region strongly suggesting that in order to upregulate *VEGFR-3*, PROX1 binds directly to its promoter.

As mentioned above, *FGFR-3* is another important growth factor receptor which is upregulated by the over-expression of *Prox1* in endothelial cells(Shin,

Min et al. 2006). *FGFR-3* is a potent receptor for FGFs and has important roles in angiogenesis and wound healing(Hung, Lee et al. 2008). PROX1 regulates the expression of *FGFR-3* by directly binding to its promoter(Shin, Min et al. 2006). But the roles of other known PROX1 domains in regulating FGFR-3 expression are unknown.

Hypothesis

AsSince *Prox1* is a homeobox transcription factor, we hypothesized that PROX1 activates lymphatic growth factor receptors (e.g. FGFR-3/VEGFR-3) by directly binding to specific DNA sequences in their promoter regions through its homeoprospero domain.

Objectives

1) To determine the functional domains of PROX1 that are required for its activation of *VEGFR-3/FGFR-3* expression, looking at both protein and reporter gene expression.

2) To determine whether PROX1 directly binds to *VEGFR-3/FGFR-3* promotersusing Electrophoretic Mobility Shift Assays (EMSA) and Chromatin Immunoprecipitation (ChIP).

Methods and materials

Antibodies

Antibodies were purchased as follows: mouse monoclonal anti-FLAG antibody (cat# F3165) fromSigma-Aldrich (Saint Louis, MO, USA), mouse monoclonal anti-VEGFR-3 antibody(cat # MAB3757) from Chemicon (Temecula, CA, USA), mouse monoclonal anti-FGFR-3 antibody (cat#B9) from Santa Cruz Biotechnologies(CA, USA), mouse monoclonal anti-alpha tubulin antibody(cat # ab7291) from Abcam (Cambridge, MA, USA), and mouse monoclonal anti-PROX1 antibody (cat # MAB5654) from Chemicon (Millipore).

Cell culture

DMEM Cell culture medium was purchased from HyClone (Logan, Utah, USA). Fetal Bovine Serum (FBS) was purchased from Invitrogen (Carlsbad, CA, USA).Human embryonic kidney cells (HEK 293A) were used to perform luciferase gene reporter assays. HEK 293A cells were obtained from the American Type Culture Collection (ATCC) and cultured in a 7575 cm^2 cell culture flask in Dulbecco's Modified Eagles's Medium (DMEM) supplemented with 5% FBS, 100100 U/ml penicillin, and 100100 µg/ml streptomycin. The medium was replaced every 48 hours andand cells were grown until 80% confluency. Then cells were trypsinized and one fifth of the harvested cells were sub-cultured into a new 75cm^2 cell culture flask. Human umbilical vein endothelial cells (HUVECs) were used for immunoblotting studies of FGFR-3 and VEGFR-3 expression after infection with differentdifferentadenoviral constructs (Ad *EGFP,* Ad *wtProx1*

andAd *HDPDΔProx1*). HUVECs were obtained from Clonetics(Lonza, USA)and cultured in a 7575 cm^2 cell culture flask in HyQ's HAM's/F-12 medium with 2% FBS, 1 mM L-glutamine and 1% Gentamycin (HyClone, Logan, Utah, USA).The medium was replaced every 48 hours. HUVECs were grown until 80% confluency, then trypsinized and sub-cultured into new 75cm^2 cell culture flasks. Only HUVECs from passages 4 to 10 were used for infection with adenoviral infection and immunoblotting studies.Human neonatal dermal lymphatic microvascular endothelial cells (HMVEC-dLyNeo) were used for EMSAand ChIP. HMVECs were purchased from Clonetics (Lonza, USA)and cultured in a 7575 cm^2 cell culture flask in EGM2-MV medium with 5% FBS, 1 mM L-glutamine and 1% Gentamycin (Lonza, USA). The medium was replaced every 48 hours. HMVECs were grown until 80% confluency, then trypsinized and sub-cultured into new 7575 cm^2 cell culture flasks. Only HMVECs betweenpassages 4 to 10 were used for EMSA and ChIP assays.

Transfection and luciferase gene reporter assays:

HEK 293A cells were co-transfected with 1 µg of expression vector (different versions of *Prox1* in pCMV-Tag4A), 1 µg of reporter vector and 1 µg ofpcDNA3.1-lacZ (gift from Dr Mesaeli, University of Manitoba) as a control for transfection efficiency as follows:HEK 293A were grown in a 75cm^2 cell culture flaskuntil 80% confluency then trypsinized and for performing luciferase gene reporter assays,harvested cells were counted under the microscope, stained with Trypan Blue and using aahemocytometer. $1.2*10^5$ HEK 293 Cells were plated in each well of two 6-well culture plates with 2 ml of DMEM+5%FBS medium. Cells

were cultured overnight; in this situation the confluence of the cultured HEK 293A is about 70% after 24 hours.Cells were washed with 37°C PBS then 2 ml of 37°C OPTIMEM medium was added to each well. The plates were then returned to the cell culture incubator. Vectors were pipetted into a microcentrifuge tube with 0.5 ml of OPTIMEM medium. 7.5 µl of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) was incubated with 0.5 ml OPTIMUM for 5 minutes in room temperature in another microtube. Vectors were incubated with Lipofectamine for 20 minutes in room temperature and then the solution was added to each well of cell culture plates. After 48 hrs of transfection, cells were harvested and lyzed by adding 100 µl of NP-40 lysis buffer (1 M Tris, 10% NP-40, 50 mM DTT, pH 7.8). Luciferase activity measurements were performed using a Lumat LB 9507 Luminometer and with addition of luciferin buffer (20 mM Tricine, 1.07 mM $MgCO_3$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 µM coenzyme A, 470 µM beetle luciferin , 530 µM ATP). β-galactosidase activity was measured at 410 nm using the Dynex MRXTC Revelation instrument and after addition of ONPG buffer. β-galactosidase activity was used to for differences in transfection efficiency.

Infection and immunoblotting assays

To study the expression of FGFR-3 and VEGFR-3 following over-expression of *Prox1*, HUVECs were infected with different adenoviral constructs (i.e. Ad *EGFP* as the negative control, Ad*wtProx1* andAd *HDPDΔProx1*) as follows:HUVECs reached 80% confluence in a 75cm^2 cell culture flask (passages 5-9) were trypsinized, centrifuged down and resuspended in 5 ml of

media.HUVECs were counted, stained withwith Trypan Blue and using a hemacytometer, andthe required quantity of viral constructsfor each sample was calculated with different MOI for $3*10^5$ cells, and the viral construct for each sample was placed in a microcentrifuge tube.$3*10^5$ HUVECs were added to each sample, mixed well and then plated in a 3.5 cm culture dish. After 48 hr, cells were washed 2 times with cold PBS and harvested using New RIPA lysis buffer (50mM Tris, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 1% Sodium Deoxycholate, 0.1% SDS , pH=7.4) with protease inhibitors (Complete Mini, Roche). The DC standard protein assay kit was used to measure protein concentration of centrifuged cell lysates and 40 µgof µgtotal protein of each sample was incubated with sample buffer in 90ºC for 5 minutes.Each sample was loaded onto an 8% SDS-poly acrylamide gel. After electrophoresis at 150V for 30 minutes, the proteins were transferred to a nitrocellulose membrane.Membranes were blocked in 5% skim milk for 3 hours in TBS buffer. Membranes were then incubated overnight at 4ºC with a primary monoclonal antibody against either VEGFR-3 (1:500) or FGFR-3 (1:200) in 5% skim milk protein.Then membranes were incubated with a secondary anti-mouse antibody (1:10000) in 5% skim milk protein at room temperature for one hour.Membranes werethen probed by ECL buffer and visualized using a Bio-Rad Fluor-S^{TM} MultiImager machine. Then membranes were incubated with anti-FLAG Ab (1:10000) and anti-Tubulin Ab (1:10000) seperately and visualized as before.Using Quantity One software (BioRad), images were analyzed and band intensity was quantified. The amount of VEGFR-3 or FGFR-3 expression was normalized to the expression of Tubulin

on the same blot and normalized expression amounts wereused for statistical analyses.

Choromatin Immunoprecipitation Assays

ChIP experiments were performed as described (Le, Du et al. 2007). 15 *10^6LECcells were trypsinized, harvested and washed with 37ºC PBS. Then cells were fixed with 1% paraformaldehyde for 10 min at room temperature. Cells were washed three times with cold PBS. Fixed cells were sonicated using a Sonifier cell disruptor 350 in SDS lysis buffer (1% SDS, 50mM50mM Tris-HCl,pH8.1, 10m10m MEDTA) on ice to generate soluble chromatin complexes with DNA fragment lengths ranging between 300 and 600 bp. Samples were then centrifuged at the maximum speed of the microcentrifuge and the supernatant was transferred to a new tube. Samples were precleared with 60µl of 50% A/G sepharose beads for 1 hour.Mousemonoclonal antibody to PROX1 (Chemicon) was used to immunoprecipitate cross-linked DNA-PROX1 complexes with A/G beads (Pierce, USA). Beads were washed and then cross-linkslinks were reversed overnight in a65ºC water bath. Isolated genomic DNA was purified and used as a template for PCR amplification of target DNA sequences in the *VEGFR-3*and*FGFR-3* promoters (Table 1).

Table 1- Primers used for ChIP assays

Primer Name	5'/3'	Sequence
Human VEGFR-3-F	5'	CGGTGCACCCGAGCAGTG
Human VEGFR-3-R	3'	CCATCACCTCCGGAGCGCATC
Human FGFR-3-F	5'	TGTCGCGGAACCACAGAG
Human FGFR-3-R	3'	ACGTCAGAGGGCTCGCGC
Mouse VEGFR-3-F	5'	CAAGCTGGAAGCGACAGAGA
Mouse VEGFR-3-R	3'	GCTCTAGGCTGGGAGTGAGA
Mouse FGFR-3-F	5'	GGAGAAGCGTGCTGGTAGAC
Mouse FGFR-3-R	3'	AGATCCGAAGAGGCGTGGT

Electrophoretic Mobility Shift Assays (EMSA)

EMSA experiments were performed as described (Le, Du *et al.* 2007). Using*XhoI* and *Acc65I* restriction enzymes, the 266 bp VEGFR-3, 220 bp FGFR-3 and 90 bp VEGFR-3 probes were excised from their respective reporter plasmids and purified using a DNA gel purification kit (Qiagen). The ends of these probes were filled in with the large fragment of the DNA polymerase enzyme I (Klenow, Invitrogen, Carlsbad, CA) and labeled in the presence of radiolabeled ($\alpha^{32}P$) dGTP. Oligonucleotides were ordered as single stranded complementary DNA sequences (Invitrogen, Carlsbad, CA) and annealed in a PCR thermocycler. Then using T4 polynucleotide kinase (T4 PNK, Invitrogen, Carlsbad, CA) these oligonucleotide probes were end labeled with $\gamma^{32}P$-dATP. Nuclear extracts were obtained from bothHepG2 orHuman neonatal dermal lymphatic microvascular endothelial cells (HMVECs). Briefly, 10 million cells were harvested from cell culture flasks and washed with 1X PBS two times at 4ºC. Following centrifugation at 2000 r.p.m., 1 ml of Buffer A (20 mM HEPES/KOH pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 250 mM sucrose) was added to the pellet, placed on ice (15 min) and the suspension was then passed 40 times

through a 26G 0.5″ needle and centrifugedat 4500 r.p.m. (10 min.). The pellet was then washed with Buffer A and 10 µl of 10% NP-40 was added, rotated at 4°C (10 min.), centrifugedat 3000 r.p.m. (15 min.) and then 200 µl of Nuclear Extraction Buffer (20 mM HEPES/KOH pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol) was added to the pellet, rotated at 4°C (30 min.), centrifugedat 13 000 r.p.m. (15 min.) and the resultant supernatant was used as the nuclear extract. For each reaction, recombinant proteins (100 ng) or nuclear extracts (1 µg) were incubated with 1X binding buffer, poly(dI-dC), 1 mM PMSF and hot DNA probe. For 'cold competition' assays, unlabeled double-stranded oligonucleotides were added at 100-fold excess. Reactions were loaded ona 4% non-denaturing polyacrylamide (37.5: 1 acrylamide/bisacrylamide) gel in 0.5X Tris–Borate–EDTA solution and run at 350 V for 40 minutes. Gels were then dried and exposed to a film (XOMAT) overnight at −70°C. Autoradiography was developed and scanned and analyzed using Adobe Photoshop software.

Statistical analysis

The results were expressed as means ± SE, and statisticaldifferences between each test group and control group (from independent repeated experiments) were evaluated by the studentt-test and a *P* value less than 0.05 was considered as statistically significant.

Results

A) Prox1 activates the *FGFR-3* and *VEGFR-3* promoters by different mechanisms

Prox1,the mammalian orthologue of*Prospero*, is a homeobox gene that has profound transcriptional effects when it is expressed in endothelial cells. It encodes PROX1, a transcription factor of 737 amino acids with a predicted weight of 84 kDa. As mentioned earlier, several functional domains and motifs have been already identified in PROX1 (Figure 2A). Like its orthologue in *Drosophila,* PROX1contains two DNA binding domains at its carboxyl terminus: the *homeo* domain (*HD*) and *prospero domain* (*PD*). In addition it contains two nuclear receptor boxes (*NR1*&*NR2*) in its amino terminus. There is also a glutamine (Q) rich domain between amino acids 211 to 260 in PROX1.

To identify the role of different domains of PROX1 in activation of its targets, different versions of *Prox1* have been generated and cloned into the expression vector pCMV-4A in our lab (Figure 5). This vector encodes a FLAG epitope at the carboxyl end of the expressed peptide, which makes it possible to detect the exogenous *Prox1* using an anti-FLAG antibody. The different *Prox1* versions used in this project are listedin listedTable 2.

Three versions of *Prox1*: *PDΔ Prox1, HDΔ Prox1* and *HDPDΔ Prox1*,which lack either or both DNA binding domains were used in this study (Figure 5).

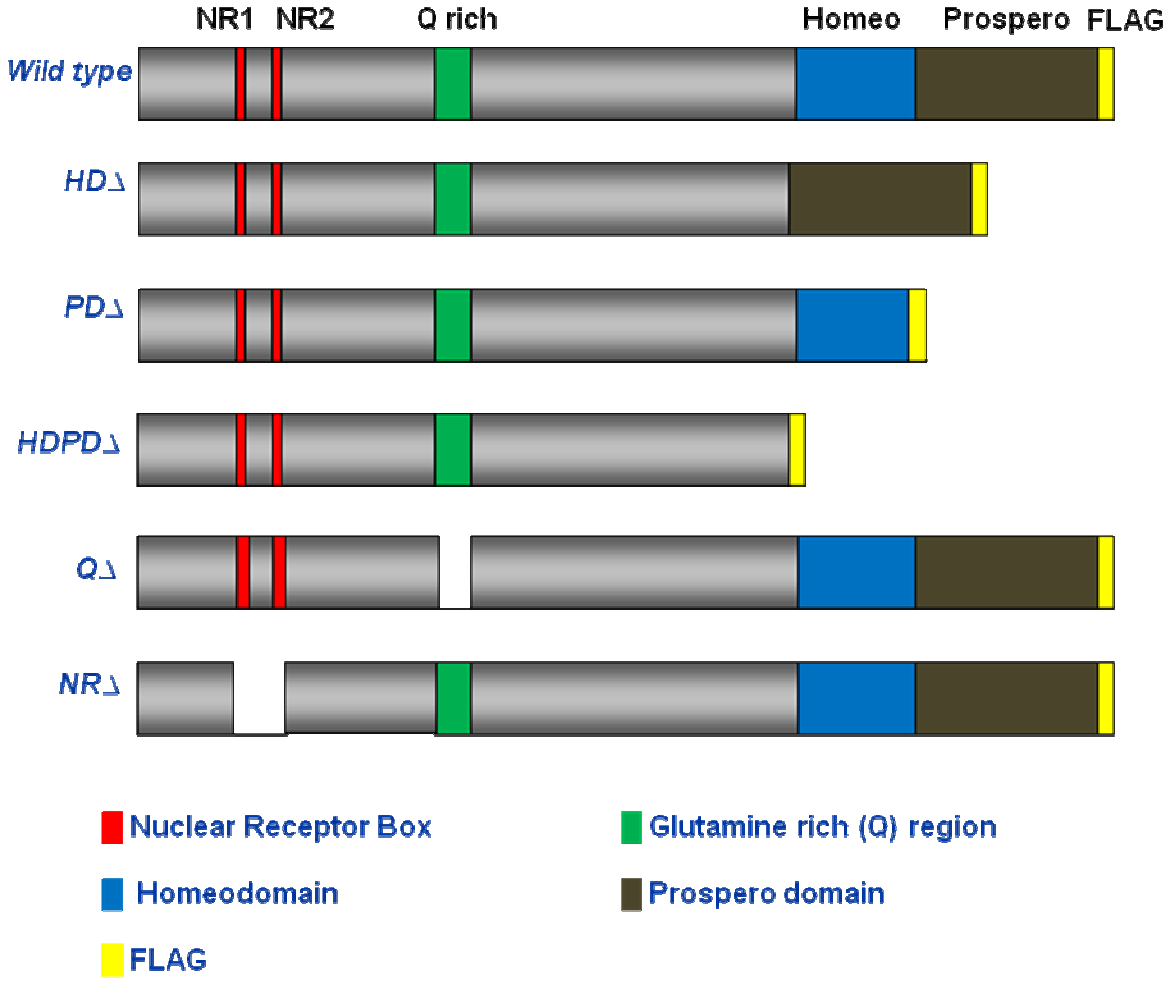

Figure 5 –Different versions of PROX1. To determine the role of different *Prox1* domains/motifs in regulation of its target genes, different deletion constructs of *Prox1* were generated and cloned into the pCMV-4A expression vector in our lab. This expression vector encodes a FLAG epitope at the carboxyl end of the expressed peptide. Note that three versions of *Prox1*: *PDΔ Prox1*, *HDΔ Prox1* and *HDPDΔ Prox1*, which lack either one or both of the DNA binding domains are termed as being "DNA binding deficient PROX1" in this study.

Table 2 - The different *Prox1* versions

Name of the construct	*Abbreviation*	*Deleted domain/s*	*Deleted amino acids*
wild type Prox1	*wt Prox1*	-	-
prospero deleted *Prox1*	*PDΔ Prox1*	*prospero*	638 - 737
homeo deleted *Prox1*	*HDΔ Prox1*	*homeo*	583 – 638
homeoprospero deleted *Prox1*	*HDPDΔ Prox1*	*homeo & prospero*	583 – 737
nuclear receptor boxes deleted *Prox1*	*NRΔ Prox1*	*nuclear receptorreceptor 1* and *nuclear receptorreceptor 2*	70 -97
glutamine deleted *Prox1*	*QΔProx1*	glutamineGlutamine-rich domain	211-260

Using luciferase reporter gene assays, it was shown in our laboratory that *wt Prox1* is able to activate a region 1.8 Kb upstream of the transcription start site of the *VEGFR-3*gene.Different versions of this promoter region were generated and cloned into the pGL3 vector. It was shown that a 90bp region (-90 to 0) proximal to the transcription initiation sitewas sufficient for PROX1 mediated activation of the *VEGFR-3* gene. However, the highest activity was observed with the proximal 266bp *VEGFR-3* promoter (-266 to 0). Therefore, we used this construct (266 bp *VEGFR-3*) for our subsequent luciferase reporter gene assays unless otherwise mentioned. PROX1 was also shown to activate the proximal 220220 bp mouse *FGFR-3* promoter inserted into the pGL2 vector (McEwen *et al.*, 1999). We used this vector in our luciferase reporter gene assays to study the

activation of *FGFR-3* gene by different versions of *Prox1*.

A.1) DNA-binding deficient versions of *Prox1* are not able to activate the 220bp *FGFR-3* promoter *in vitro*

Using the luciferase reporter gene assay, we first studied whether the DNA binding domains of PROX1 were required for activation of the 220 bp *FGFR-3* promoter. In this regard we studied the activity of the 220bp *FGFR-3*promoter mediated by different DNA binding deficient versions of*Prox1*. As seen in Figure 6A and 6B, only *wt Prox1* is able to activate the 220 bp *FGFR-3* promoter. Although DNA-binding deficient versions of *Prox1* activated the 220 bp *FGFR-3* promoter more than empty pCMV-4A vector, this activation was much less than the activation of the *FGFR-3* promoter wasmediated by *wt Prox1*. In our experiments, *wt Prox1* was able to activate the 220 bp *FGFR-3* promoter about 7 times more than empty vector, confirming the finding that PROX1 activates the *FGFR-3* promoter *in vitro*. On the other hand, *wt Prox1* activated the 220 bp *FGFR-3* promoter 3-fold higher than allof the DNA-binding deficient *Prox1* constructs. These findings show that for full activation of the *FGFR-3* gene, PROX1 requires its DNA binding domain (i.e. the *homeoprospero*- domain), supporting our hypothesis that PROX1 binds directly to the *FGFR-3* promoter region.

A.2) DNA-binding deficient versions of *Prox1* are able to activate the 266bp *VEGFR-3* promoter *in vitro*

We used the 266 bp *VEGFR-3* promoter to investigate whether the PROX1 DNA-binding domains are required for PROX1-mediated activation in HEK 293 cells. Figures 7A and 7B show the reporter gene activity of

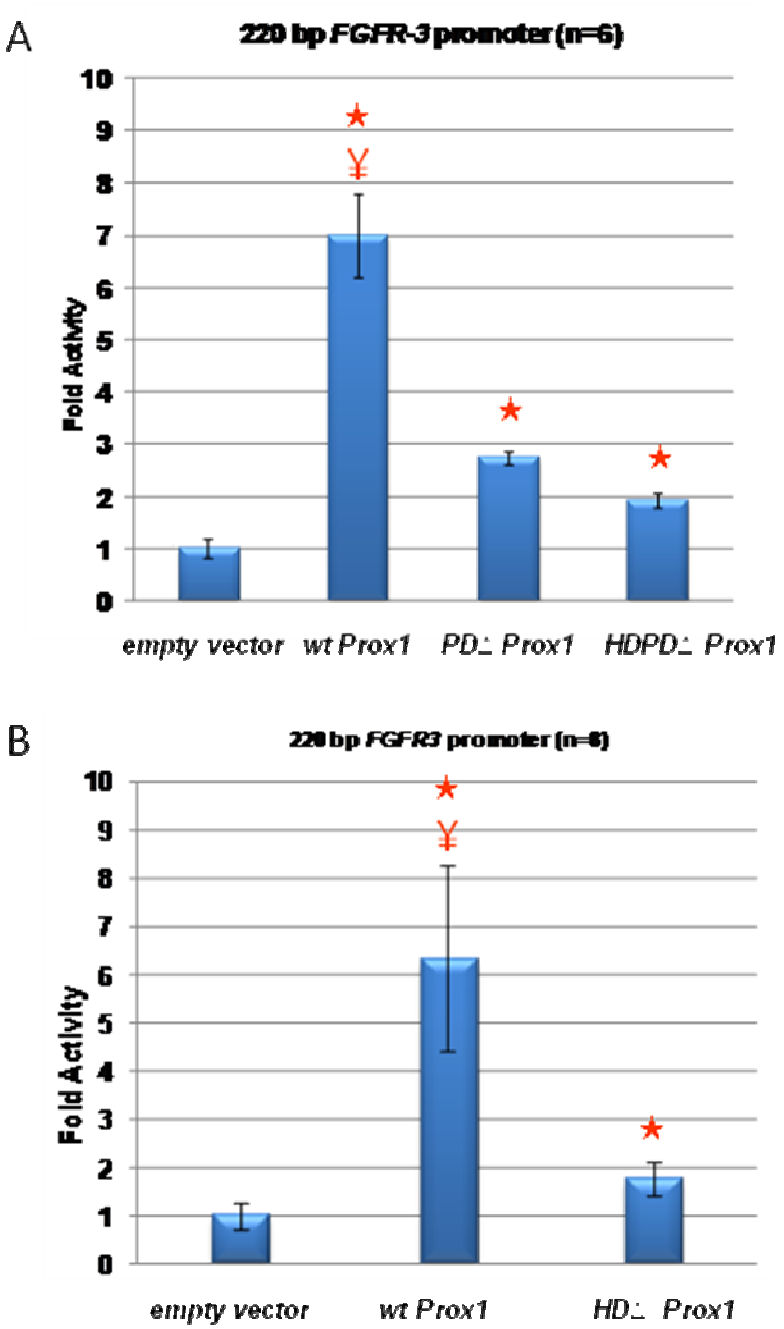

Figure 6 – DNA binding deficient versions of *Prox1* are not able to activate the 220 bp *FGFR-3* reporter construct. Using the luciferase gene reporter assay we showed that *wt Prox1* activates the 220 bp *FGFR-3* promoter significantly more than *PDΔ Prox1* and *HDPDΔ Prox1* (A) and *HDΔ Prox1* (B). Note the difference of reporter activity between *wt Prox1* and other DNA binding deficient versions of *Prox1* , which supports the hypothesis that in order to activate the *FGFR-3* promoter, *Prox1* requires both of its DNA binding domains.
★ indicates significantly different from control ($p<0.001$)
¥ indicates significantly different from other treatment ($p<0.01$)

the 266 bp *VEGFR-3* promoter after co-transfection with different *Prox1 constructs*. Both wild-type and DNA-binding deficient versions of *Prox1* were able to activate the 266 bp *VEGFR-3*promotersignificantly higher than the empty vector. In our experiments, *wt Prox1* was able to activate the 266 bp *VEGFR-3* promoter approximately6approximately 6-fold more than the empty vector. *PDΔ Prox1 and HDPDΔ Prox1* were able to activate the 266 bp *VEGFR-3* promoter about 4 to 5-fold more than the empty vector. The reduced activity by these versions of *Prox1* may be due to conformational changes in the resulting protein that decreases either their half-lives or makes protein-protien interactions less efficient. The level of activation of the 266 bp *VEGFR-3* promoter mediated by *HDΔ Prox1* is even higher than that for *wt Prox1* (about 10-fold more than the empty vector). This may be due to conformational changes in the protein, which increases its stability or the efficiency of its protein-protein interactions. Alternatively, it may reflect the importance of the *prospero* domain alone in the activation of the *VEGFR-3*promoter. These findings demonstrate that in order to activate the *VEGFR-3*gene*3* gene, PROX1 does not require its DNA-binding domains, supporting a co-activator role for PROX1 in the activation of the *VEGFR-3*gene

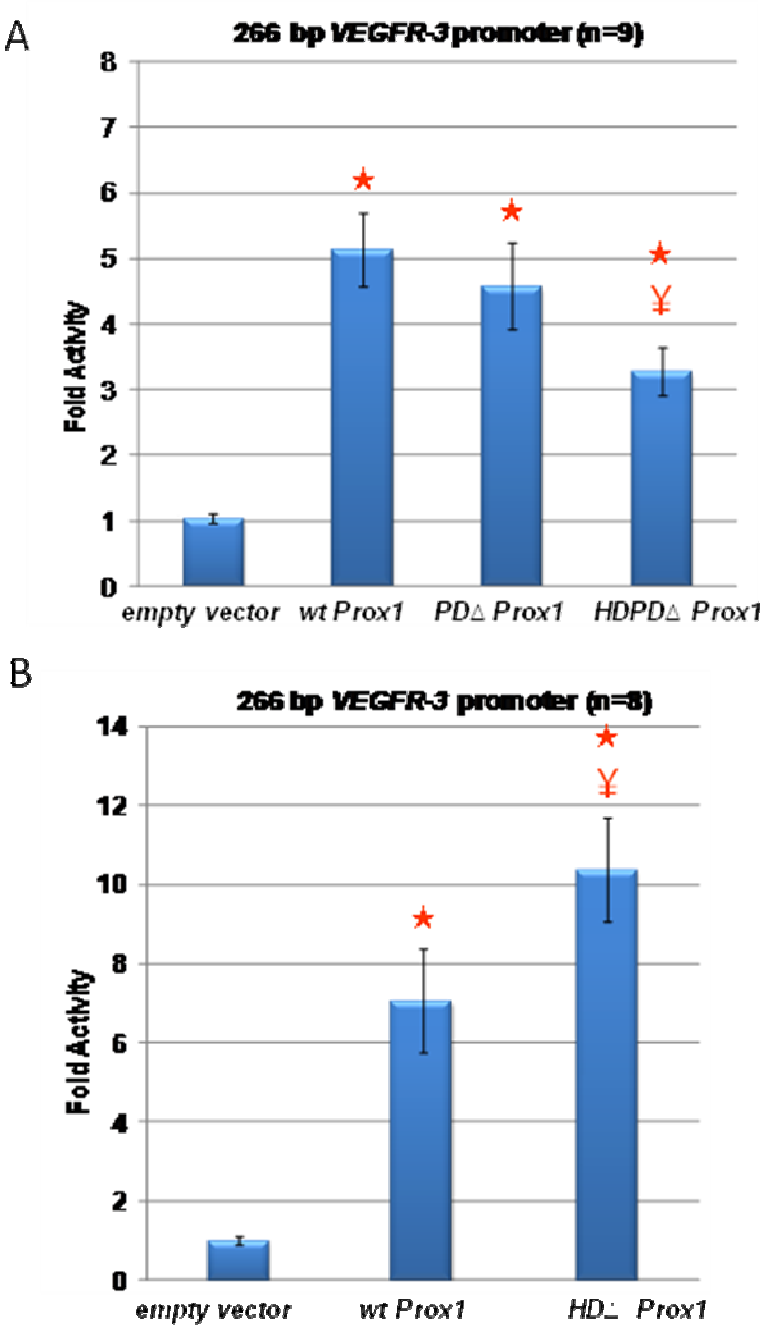

Figure 7 – DNA binding deficient versions of *Prox1* are able to activate the 266 bp *VEGFR-3* reporter construct. Both wild type and DNA binding defficient versions of *Prox1* were able to activate the 266 bp *VEGFR-3* promoter. A) The activation of the 266 bp *VEGFR-3* promoter by *PDΔ Prox1* is not significantly less than *wt Prox1*. Although *HDPDΔ Prox1* activates this promoter significantly less than *wt Prox1*, the amount of activation of the 266 bp *VEGFR-3* promoter is much higher than *empty vector* (more than 3 fold). B) In our experiments, *HDΔ Prox1* activated the 266 bp *VEGFR-3* promoter *significantly* more than *wt Prox1*.
★ indicates significantly different from control ($p < 0.001$)
¥ indicates significantly different from other treatment ($p < 0.01$)

B) *Prox1* requires different domains to induce the *FGFR-3* and *VEGFR-3* proteins in venous endothelial cells

To further investigate our findings usingluciferase reporter gene assays, we over-expressed *Prox1* in primary human umbilical venous endothelial cells (HUVECs) and measured protein levels of FGFR-3 and VEGFR-3. For this purpose, two adenoviral constructs were previously generated in our laboratory. These two versions of *Prox1* were Ad-*wt Prox1*and Ad-*HDPDΔ Prox1* (which lacks the entire *homeoprospero* domain from *Prox1* cDNA). We used Ad-*EGFP* as control for effects of viral infection. We infected HUVECs with different multiplicities of infection (MOIs) forforeach adenoviral construct (Ad-EGFP, Ad-*wt Prox1*and Ad-*HDPDΔ Prox1*). In each experiment, one sample was not infected to compare the expression of the FGFR-3 and the VEGFR-3proteins before and after infection by Western Blotting. Since our constructs were tagged with a FLAG epitope at their carboxyl terminaltermini, we detected expression of WT or HDPDΔPROX1 by using an anti-FLAG monoclonal antibody. WT PROX1 has a molecular weight of approximately 85 kDa, while HDPDΔ PROX1 has a predicted molecular weight of 75 kDa due to the absence of the *homeoprospero* domain.

B.1) DNA-deficient *Prox1* is not able to induce expression of *FGFR-3* in venous endothelial cells

The FGFR-3 protein is a membrane-bound protein and undergoes many glycosylation steps during its processing inside the cell. The mature form of the protein has a molecular weight of 130 kDa. The anti-FGFR-3 antibody detected

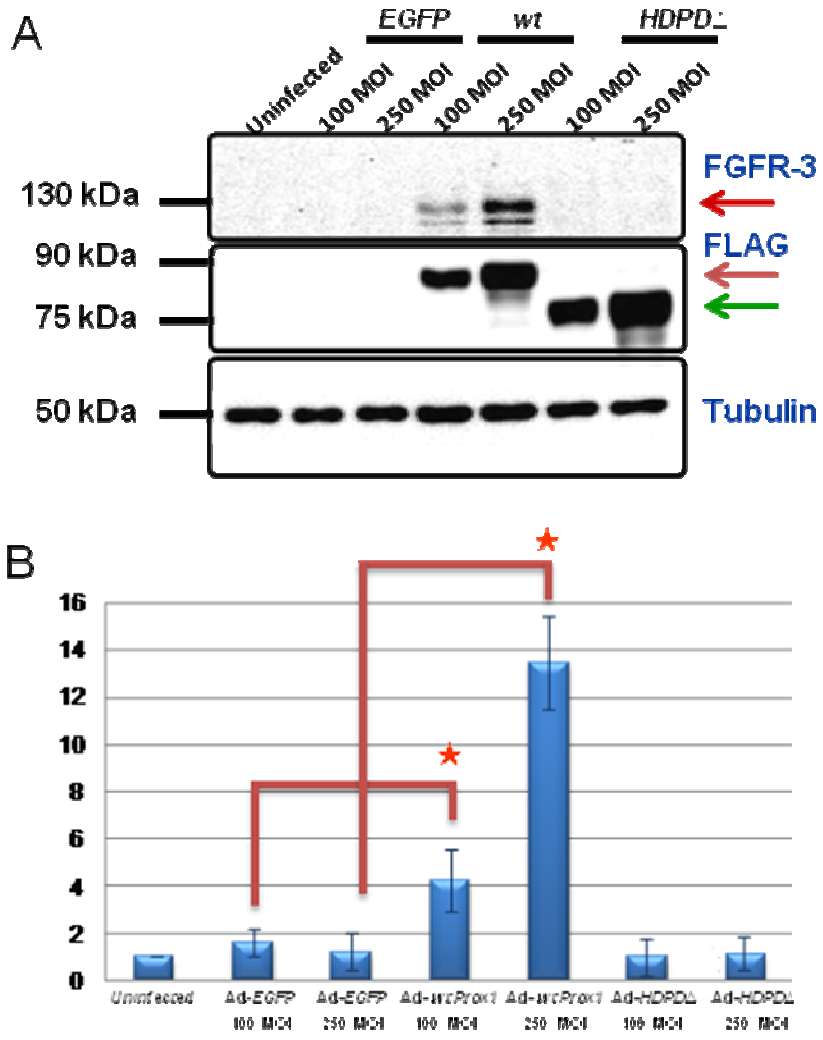

Figure 8 – DNA binding deficient *Prox1* is not able to induce expression of *FGFR-3* in venous endothelial cells. A) Using an anti-FLAG antibody we showed that both Ad-*wt Prox1* (blue arrow) and Ad-*HDPDΔ Prox1 (green arrow)* are expressed efficiently in HUVECs. The anti-FGFR-3 antibody recognizes two forms of FGFR-3, evident at 120 and 130 kDa. The latter isoform is the mature form of the protein (red arrow). Note that FGFR-3 protein only is detected in Ad-*wt Prox1* infected HUVECs showing that *Prox1* requires its DNA binding domains to induce *FGFR-3* expression in HUVECs. B) using the 130 kDa band (blue arrow) we quantified and analyzed FGFR-3 expression between different samples. The expression of FGFR-3 is significantly higher in HUVECs infected by Ad-*wt Prox1* than Ad-*EGFP*. In contrast, Ad-*HDPDΔ Prox1* did not induce the expression of *FGFR-3* in HUVECs.
★ indicates significantly different from control ($p < 0.01$).

two variants of FGFR-3: 120 kDa and 130 kDa. We infected HUVECs at two different MOIs: 100 and 250. As shown in Figure 8A, expression of *wt* and *HDPDΔProx1*is clearly detected at both 100 and 250 MOIs. In our experiments, the expression level of HDPDΔPROX1 was consistently higher than that of WT PROX1. This may be due to increased stability of the HDPDΔPROX1 protein due to conformational changes in the protein structure. We could only detect FGFR-3 expression in HUVECs which were infected by Ad-*wt Prox1.* Neither Ad-*EGFP* nor Ad-*HDPDΔ Prox1* were able to induceexpression ofof FGFR-3 in HUVECs at either MOI (Figure 8). This is in accordance with our luciferase reporter gene assays, which showed that the DNA-binding deficient versions of *Prox1* are not able to activate *FGFR-3* gene expression (Figure 6).

Quantification of FGFR-3 expression by *Prox1* is shown in Figure 8B. FGFR-3 expression in Ad-*wt Prox1* infectedHUVECsis significantly higher than uninfected, Ad-*EGFP* infected andAd-*HDPDΔ Prox1* infected HUVECs. Increasing the amount of Ad-*wt Prox1* dosedependently increased the amount of FGFR-3 expression, but increasing the amount of Ad-*HDPDΔ Prox1* from 100 MOI to 250 MOI had no effect on FGFR-3 levels. These results confirmed that in endothelial cells, *Prox1* requiresits intact DNA binding domain to activate the expression of the endogenous *FGFR-3*gene. In other words, the DNA-binding domain of PROX1 is necessary for *FGFR-3* gene activation.

B.2) DNA-binding deficient *Prox1* is able to induce expression of *VEGFR-3* in venous endothelial cells

We repeated our *Prox1* over-expression studies in HUVECs and we detected the expression of VEGFR-3 in these cells. We infected HUVECs at 50 and 100 MOIs and harvested 48 hours later. Similar to FGFR-3, VEGFR-3 is a membrane-bound receptor and undergoes several post-translational modifications. The mature form of VEGFR-3 has a disulfide bond between the 5th and the 6th extracellular Ig like domains. Therefore, when detected by denaturing-reducing immunoblotting techniques, these bonds are cleaved. Thus,the mature VEGFR-3 form is detected at a molecular weight of approximately 125 kDa. In addition, other partially processed forms of VEGFR-3 protein are detected at 175 and 190 kDa.We measured the level of the 125 kDa band of VEGFR-3 in our blotsin orderto in orderquantify the expression of mature VEGFR-3. As demonstrated in Figure 9B, uninfected HUVECs expressed low levels of VEGFR-3 protein. During embryonic life, *VEGFR-3* is expressed in the blood endothelial cells and it is only after the maturation of blood and lymphatic vasculature that the expression of *VEGFR-3* is limited to lymphatic endothelial cells. There was increased *VEGFR-3* expression in Ad-*EGFP* infected HUVECs when compared to uninfected HUVECs, indicating perhaps a non-specific activation of *VEGFR-3* gene by adenoviral infection. However the amount of VEGFR-3 protein (125 kDa) was significantly increasedin increasedboth Ad-*wt Prox1* and Ad-*HDPDΔ*

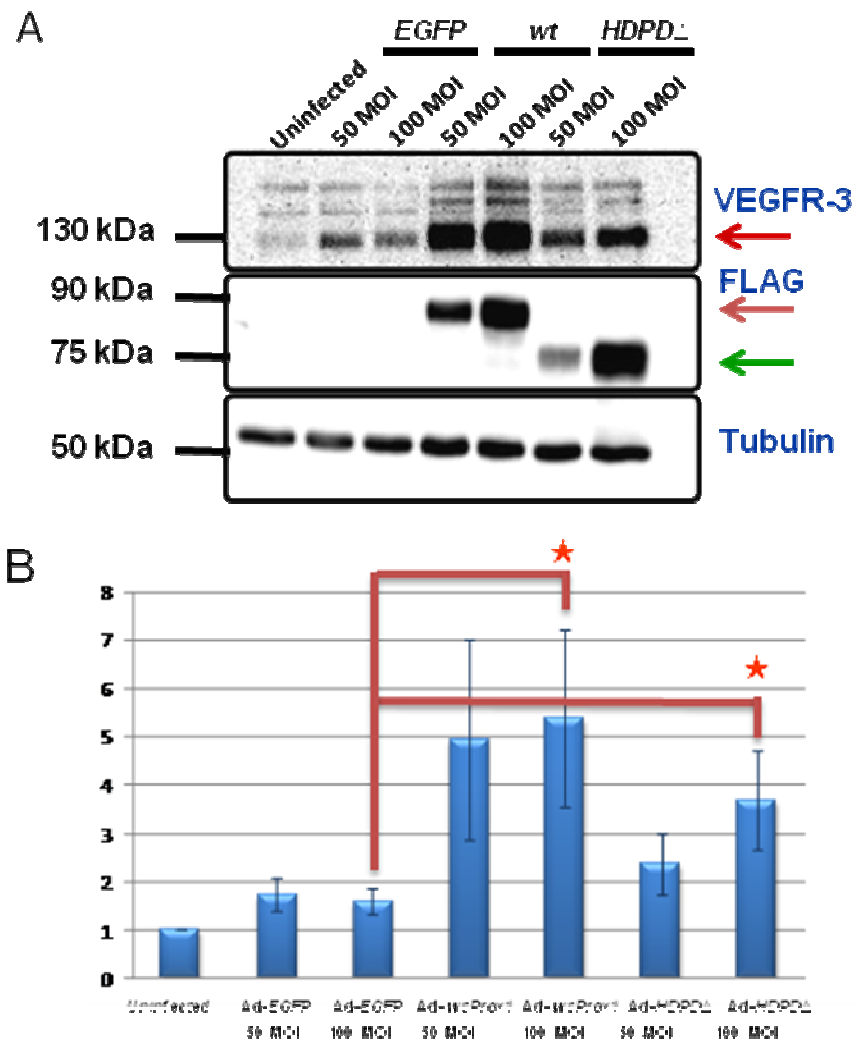

Figure 9 – A DNA binding deficient version of *Prox1* is able to induce expression of VEGFR-3 in venous endothelial cells. A) Using an anti-FLAG antibody we showed that both Ad-*wt Prox1* (blue arrow) and Ad-*HDPDΔ Prox1 (green arrow)* are expressed efficiently in HUVECs. The anti-VEGFR-3 antibody recognizes VEGFR-3 at different sizes and the 125 kDa (red arrow) represents the mature form of the protein. Note that a low level of VEGFR-3 expression is detected in uninfected HUVECs. However Ad-*wt Prox1* was able to induce VEGFR-3 significantly more than control Ad-*EGFP Prox1* at 100 MOI. Interestingly, Ad-*HDPDΔ Prox1* induces VEGFR-3 strongly in HUVECs at 100 MOI. B) Using the 125 kDa band (red arrow), we quantified and analyzed *VEGFR-3* expression among different samples. The expression of VEGFR-3 is significantly higher in Ad-*wt Prox1* and Ad-*HDPDΔ Prox1* than Ad-*EGFP Prox1* at 100 MOI, supporting that *Prox1* does not require its DNA binding domain to induce *VEGFR-3* expression in HUVECs. ★ indicates significantly different from control ($p < 0.05$).

Prox1 infected HUVECs at 100 MOI. T,he amount of VEGFR-3 was increased with a low level of infection (50 MOI), but it was significantly higher than Ad-*EGFP* infected HUVECs at a higher level of infection (100 MOI) in Ad-*wt Prox1* and Ad-*HDPDΔ Prox1* infected HUVECs (Figure 9B). This indicates that both wild type and DNA-binding deficient versions of *Prox1*were able to induce the expression of the *VEGFR-3* gene in primary venous endothelial cells. However the level of VEGFR-3 induced by Ad-*wt Prox1* was more than the level induced by Ad-*HDPDΔ Prox1*. This is in accordance with our luciferase reporter gene assays; *HDPDΔ Prox1* activated the 266 bp *VEGFR-3* promoter less than *wt Prox1*. It appears that the *homeoprospero* domain of *Prox1* is essential for its full expression of *VEGFR-3* gene.

C.1) Deletion of nuclear receptor boxes (NRs) or the glutamine-rich domain (Q) in *Prox1* decreases *Prox1* mediated activation of the 220 bp *FGFR-3* reporter gene.

We furtherinvestigated the role of other known functional domains of *Prox1* in the activation of the *FGFR-3*/*VEGFR-3* genes. *Prox1*has two nuclear receptor boxes (*NR1*& and *NR2*) at its amino terminal. It also contains a glutamine (Q) rich domain (amino acids 211 to 260)(Yousef and Matthews 2005). These domains are important for PROX1 trans-activation activity and its interaction with other transcription factors(Yamazaki, Yoshimatsu et al. 2009). Using luciferase reporter gene assays we examined the role of these domains in activation of the 220 bp *FGFR-3*/266bp*VEGFR-3* promoters.

First we looked at the roleof ofthe nuclear receptor boxes or the glutamine-rich domain of PROX1 in the activation of the 220 bp *FGFR-3*promoter. As shown in Figure 10A, versions of *Prox1* which lack either nuclear receptor boxes (i.e. *NRΔ Prox1*) or the glutamine-rich domain (i.e. QΔ Prox1)were able to activate the 220bp *FGFR-3* promoter significantly higher than empty vector (more than 4 and 3-fold, respectively). However, the amount of activation by *NRΔ Prox1* and *QΔ Prox1* was significantly less than *wt Prox1*(almost 6-fold, compared to the empty vector). These results show that for full activation of the *FGFR-3* promoter by PROX1 these domains are required.

C.2) Deletion of nuclear receptor box (NRs) or the glutamine-rich (Q) domains in *Prox1* did not significantly alter the*Prox1* mediated activation of the 266 bp VEGFR-3 promoter.

Using luciferase reporter gene assays, we also studied the activation of the 266 bp VE*GFR-3* promoter by *wt Prox1*, *NRΔ Prox1* or *QΔ* Prox1 in HEK 293 cells. As shown in Figure 10B, both *NRΔ Prox1* and *QΔ Prox1* activated the 266 bp *VEGFR-3* promoter comparable to the activation induced by *wt Prox1*. In other words, deletion of the nuclear receptor boxes or the glutamine-rich domain of PROX1 did not have a significant effect on *Prox1*mediated activation of the 266 bp *VEGFR-3*promoter.

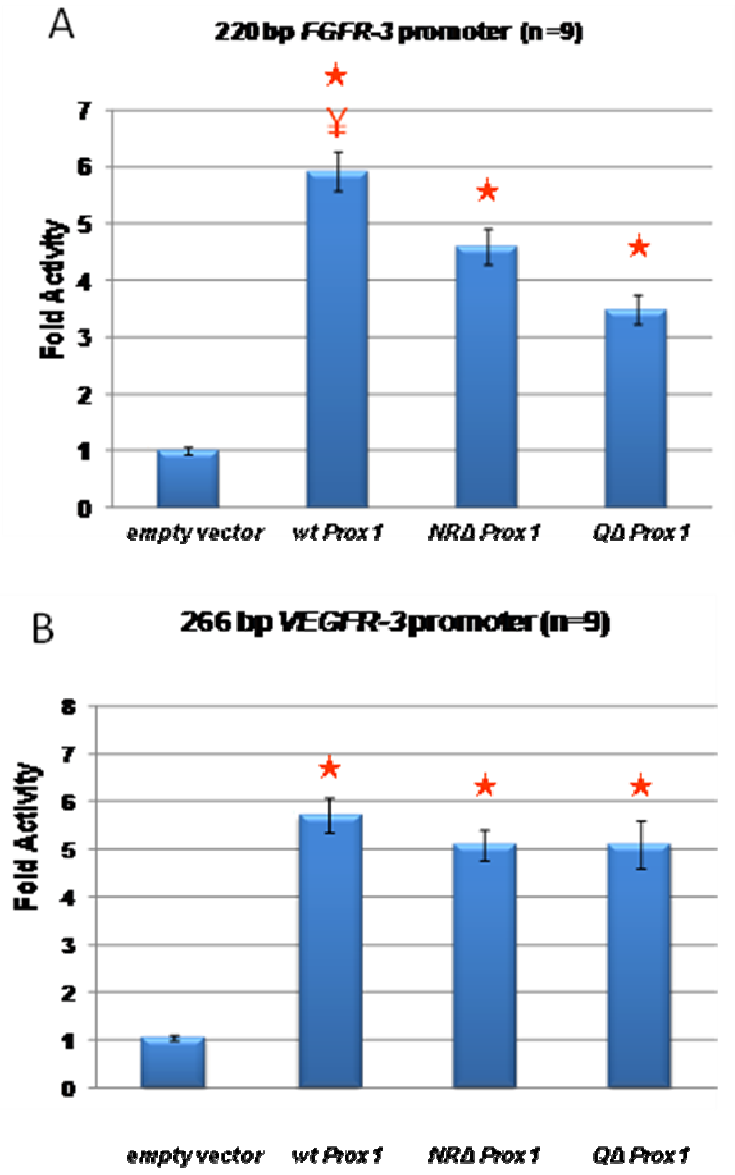

Figure 10 – Nuclear Receptor Boxes 1 & 2 and the Glutamine rich Domain are not essential for *Prox1* mediated activation of *FGFR-3* and *VEGFR-3* genes. To show the role of nuclear receptor boxes (NR1 & NR2) and the glutamine rich (Q) domain, we tested whether *NRΔ Prox1* or *QΔ Prox1* can activate (A) the 220 bp *FGFR-3* or (B) 266 bp *VEGFR-3* promoters. A) *NRΔ Prox1* and *QΔ Prox1* were able to activate the 220 bp *FGFR-3* promoter, although to a significant lower extent than *wt Prox1*. B) *NRΔ Prox1* and *QΔ Prox1* activated the 266 bp *VEGFR-3* promoter comparable to *wt Prox1*.
★ indicates significantly different from control (p<05)
¥ indicates significantly different from other treatment (p<001)

D) *Prox1* does not interact with *CBP/p300* to activate the 266 bp *VEGFR-3* promoter

Previous reports showed that PROX1 interacts with CBP/p300to activate the *βB2-CrytallinCrystallin* promoter (Chen, Dowhan et al. 2002). We repeated our luciferase reporter gene assays to examine whether there is any potential interaction between PROX1and CBP in the activation of the 266 bp *VEGFR-3* promoter. As seen in Figure 11, we did not observe any synergy between *Prox1* and CBP in the activation of the 266 bp *VEGFR-3* promoter. CBP alone did not activate the 266 bp *VEGFR-3* promoter significantly higher than the empty vector. In addition co-transfection of *Prox1* and *CBP* did not increase the activity of the 266 bp *VEGFR-3* promoter over transfection with *wtProx1* alone. These results don support a role for CBP in the activation of the 266 bp *VEGFR-3* promoter.

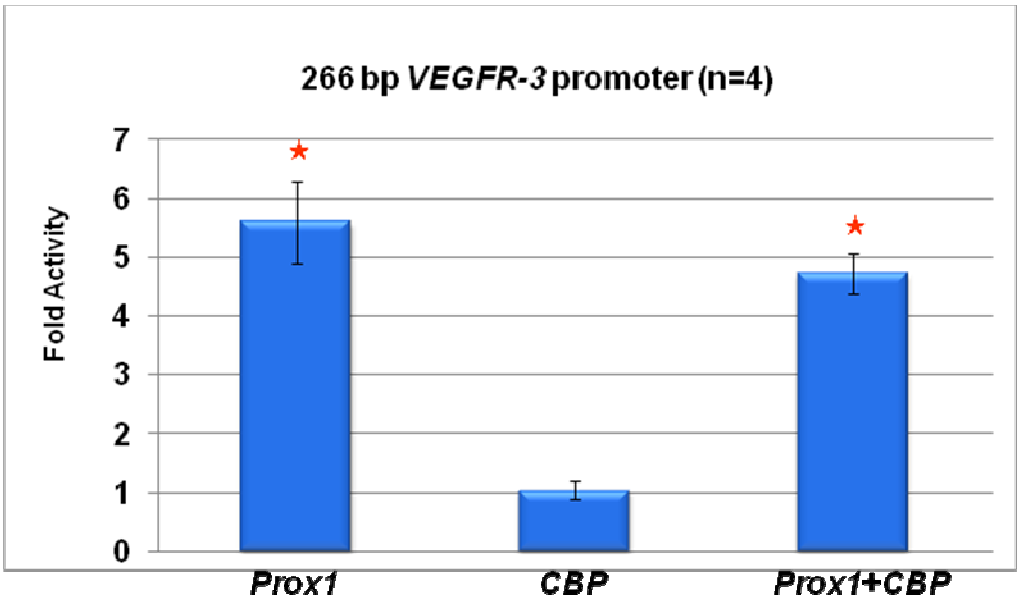

Figure 11 – *Prox1* does not show any synergy with CBP in its activation of the 266 bp *VEGFR-3* promoter. To investigate whether CBP has a role in *Prox1* mediated activation of the 266 bp *VEGFR-3* promoter, we carried out luciferase reporter gene assays with *wt Prox1* and *CBP*. However neither *CBP* alone or in combination with *wt Prox1* activated the 266 bp *VEGFR-3* reporter construct more than that seen for *wt Prox1* alone.
★ indicates significantly different from control ($p < 0.01$)

E) *Prox1* does not interact with *SP1* to activate the 266 bp *VEGFR-3* promoter

The results from our luciferase reporter gene assays and immunoblotting showed that *Prox1* does not require its DNA-binding domain to activate the 266 bp *VEGFR-3*promoter. To *3* promoter, sofurther investigatewhether other putative partners of PROX1 could contribute towards the activation of the *VEGFR-3* gene,we first analyzed the *VEGFR-3* promoter using bioinformatics tools. Previously, in our lab it was shown that the proximal 90 bp *VEGFR-3* promoter is sufficient and necessary for activation by *wt Prox1*. We analyzed the sequence of this proximal promoter for potential transcription factor binding sites using the Transcription Regulatory Element Search (TRES) (http://bioportal.bic.nus.edu.sg/tres).

As shown in Figure 12, the 90 bp *VEGFR-3* promoter has a very high GC rich content (~ 90%) and our analysis of this promoter sequence identified *SP1* (*SPECIFICITY PROTEIN 1*) as being a potential transcription transcriptional theactivator of the *VEGFR-3* gene. Thus weexamined whether there was any synergy between *Prox1* and *SP1* in the activation of the 266 bp *VEGFR-3* promoter.

E.1)*SP1* does not show synergy with *Prox1* in the activation of the 266 bp *VEGFR-3* promoter

We performedluciferase reporter gene assays using the 266 bp *VEGFR-3* promoterand andco-transfecting *wtProx1* and *SP1* to determine whether there is any potential interaction between these two transcription factors in the activation of the *VEGFR-3*gene (Figure 13). In contrast to *wt Prox1,* which fully activated the 266 bp *VEGFR-3* promoter, exogenous *SP1* did not alter the activity

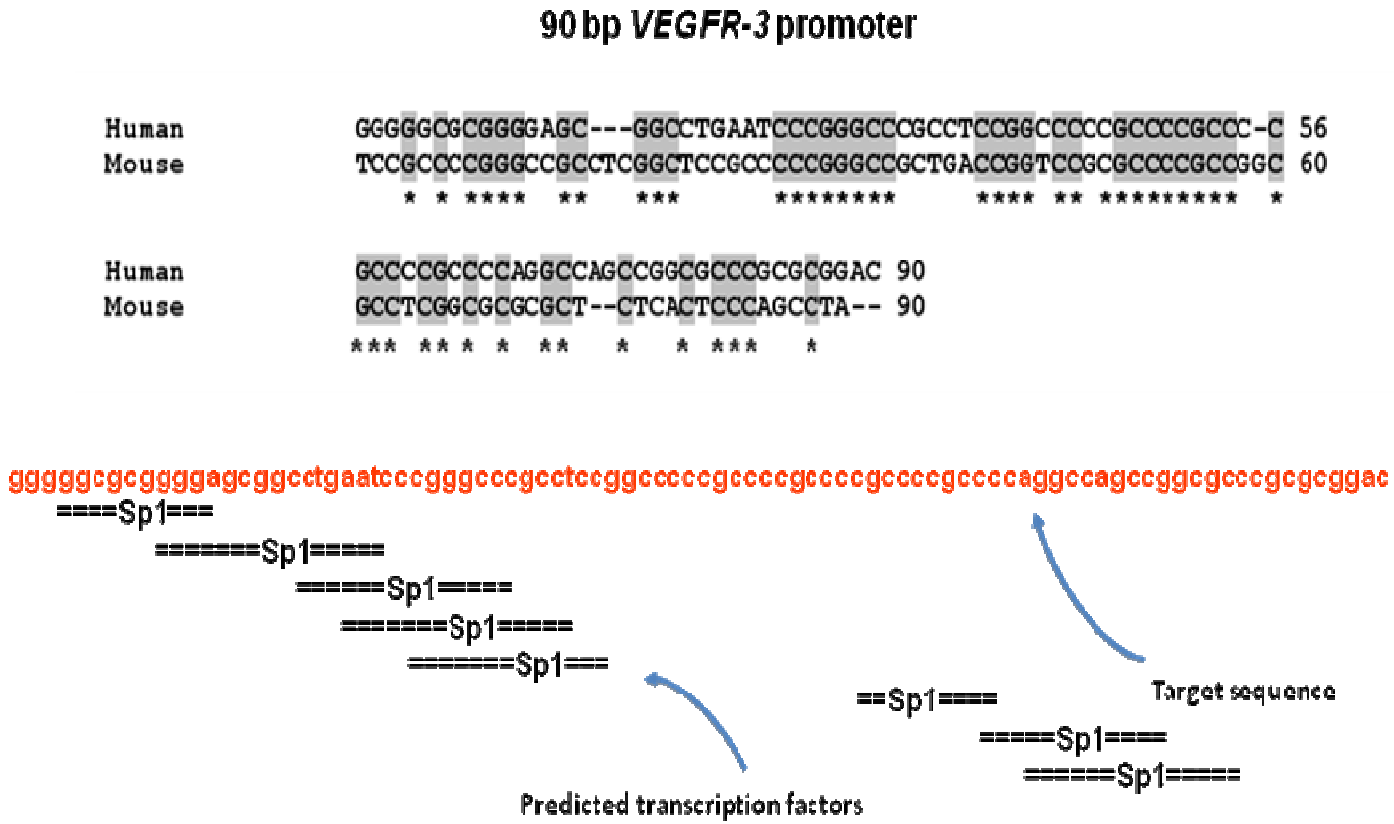

Figure 12 – SP1 is a predicted transcription factor partner of PROX1 on the 90 bp *VEGFR-3* promoter. Comparative analysis of mouse and human 90bp *VEGFR-3* promoter shows that there are many conserved SP1 predicted binding sites in this DNA sequence.

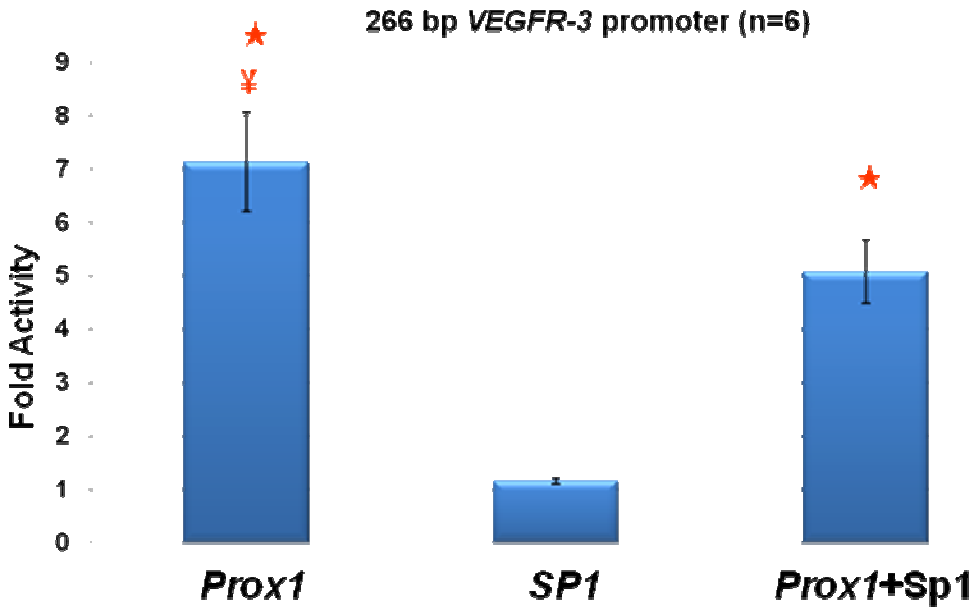

Figure 13 – Exogenous *SP1* shows no synergy with *Prox1* in the activation of the 266 bp *VEGFR-3* promoter. To investigate whether SP1 has a role in *Prox1* mediated activation of the 266 bp *VEGFR-3* promoter we performed luciferase reporter gene assays with *wt Prox1* and *SP1*. However neither *SP1* alone or in combination with *wt Prox1* were able to activate the 266 bp *VEGFR-3* promoter more than *wt Prox1 alone*.
★ indicates significantly different from control ($p < 0.01$)
¥ indicates significantly different from other treatment ($p < 0.05$)

of this promoter. Suprisingly, the combination of *wt Prox1* and *SP1*significantly decreased*Prox1* mediated *VEGFR-3* activation.

E.2) Mithramycin did not alter the activation of the 266 bp *VEGFR-3* promoter by *wt Prox1*

SP1 is ubiquitously expressed in all mammalian cells. So there is a possibility ectopic*SP1* does not activate the 266 bp *VEGFR-3* promoter because promoter may be saturated by the endogenous SP1. In this regard, we decided to block the effect of endogenous SP1 and assess the effect on the 266 bp *VEGFR-3* promoter activity. Mithramycin is a well known inhibitor of SP1-like transcription factors(Blume, Snyder et al. 1991). We repeated our luciferasereporter gene assays with wt *Prox1* in the presence of Mithramycin. Using the MTS cell viability assay we first determined non-toxic doses of Mithramycin for HEK 293 cells. As shown in Figure 14A, Mithramycin doses over 250 nM are significantly toxic to HEK 293 cells and decrease cell viability by more than 20% by 24 hours andby70% byand by 70% at 48 hours. However doses under 200 nM of Mithramycin did not decrease HEK 293 cells viability more than 20%within the first 24 hours (Figure 14B). So, we used doses less than 200 nM and treated our cells for 24 hours with Mithramycin and repeatedour luciferase reporter gene assays with the 266 bp *VEGFR-3*promoter. First we determined the effect of Mithramycin on the basal luciferase reporter activity of the 266 bp *VEGFR-3* promoter in HEK 293 cells. Mithramycin was able to efficientlysuppress the 266 bp *VEGR-3* promoter activity in HEK 293 cells (Figure 15A). This could indicate

that endogenous SP1-like transcription factors have important roles in controllingfor the basal activity of the 266 bp *VEGFR-3* promoter. To test whether Mithramycin is able to counteract *Prox1*mediated activation of the 266bp *VEGFR-3* promoter, we repeated the *wt Prox1*/266 bp *VEGFR-3*reporter assay in the presence of different non-toxic doses of Mithramycin. Mithramycin was not able to significantly decrease the activation of the 266 bp *VEGFR-3* promoter mediated by *Prox1*. This indicates that the *Prox1* mediated activation of 266bp *VEGFR-3*is likely independent of SP1 activity.

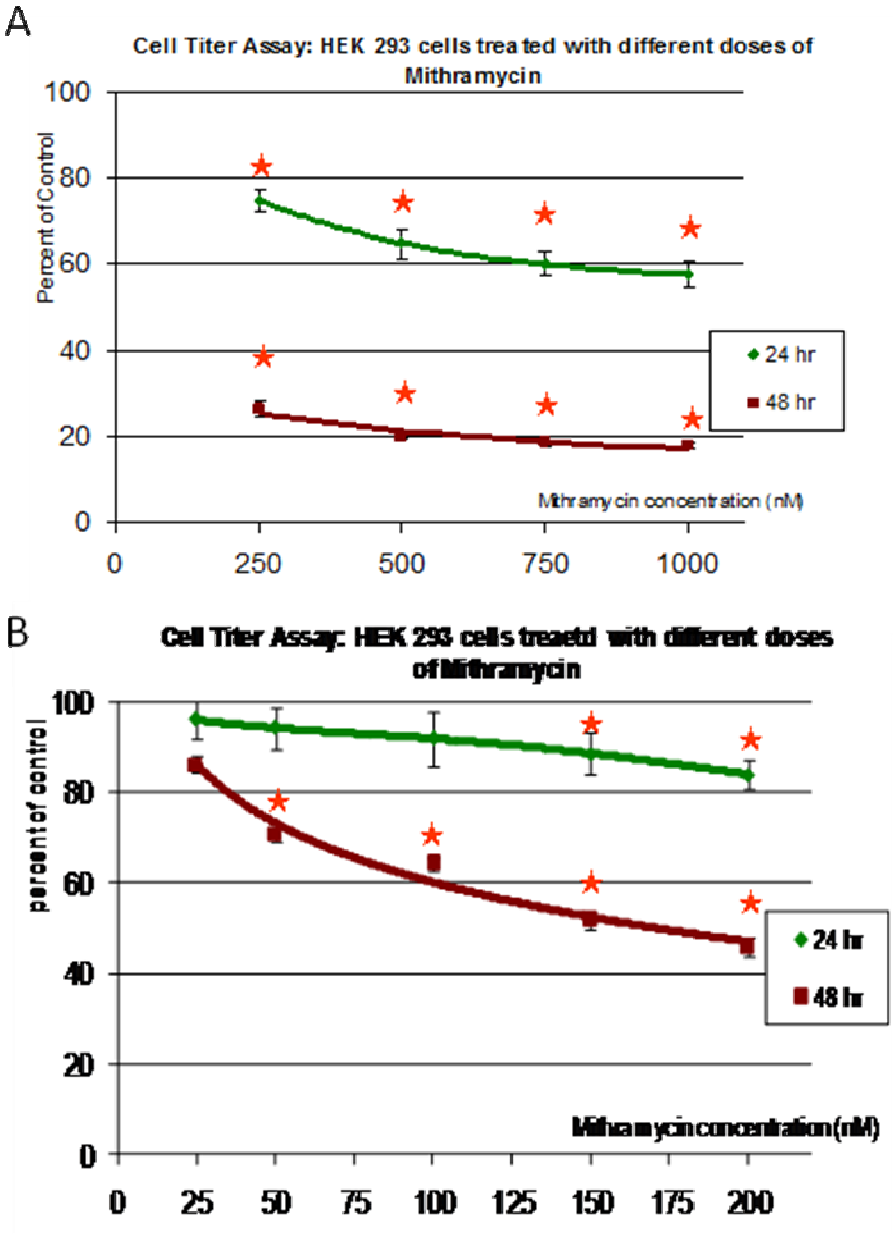

Figure 14 – Mithramycin doses under 200 nM does not decrease HEK 293 cell viability significantly during first 24 hours. To determine non-toxic doses of Mithramycin for HEK 293 cells we used the MTS cell viability assay and determined that Mithramycin doses less than 200nM did not decrease cell viability less than 80% during the first 24 hours.
★ indicates significantly different from control ($p < 0.01$)

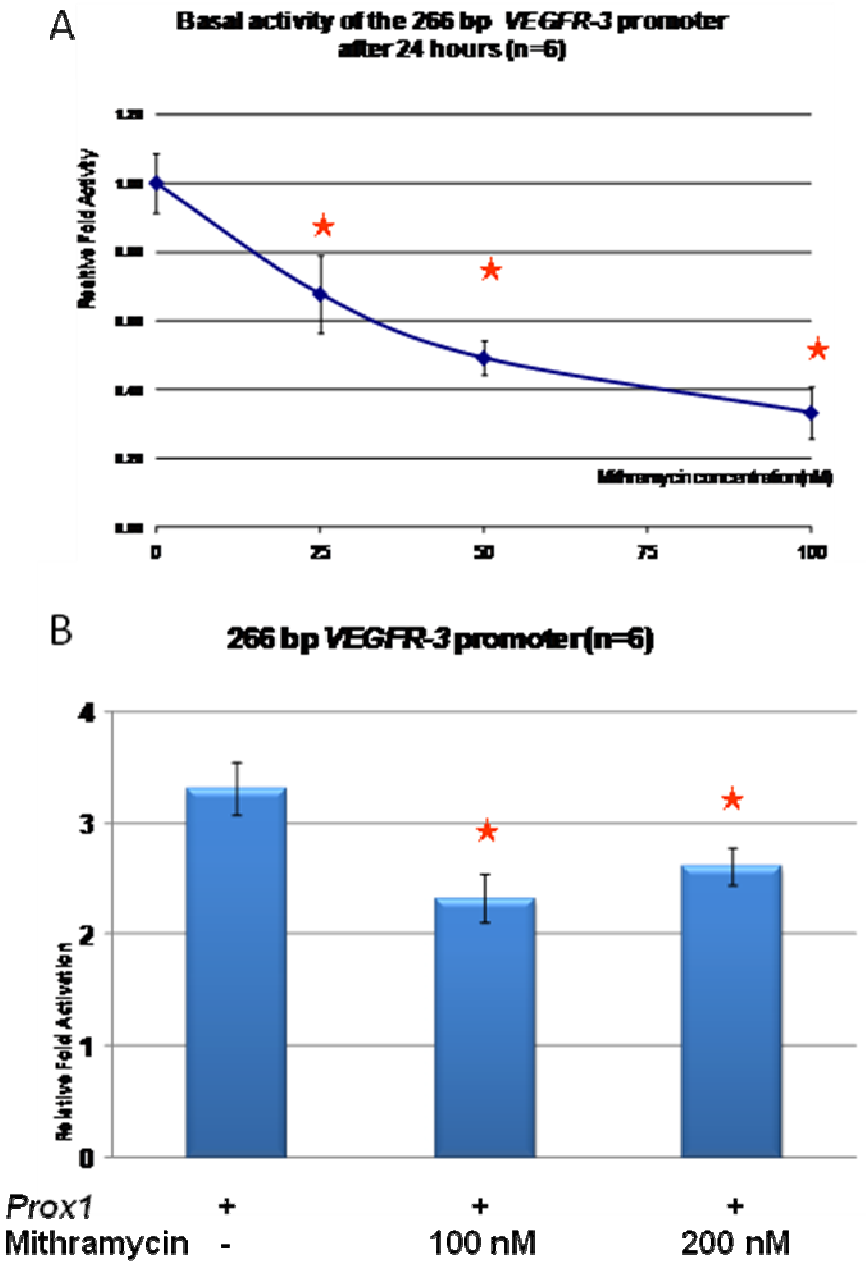

Figure 15 – Mithramycin does not affect *Prox1* mediated activation of the 266 bp *VEGFR-3* promoter. A) Mithramycin decreases the basal activity of the 266 bp *VEGFR-3* promoter to approximately 30% of control at 100 nM. B) However *Prox1* is able to activate the 266 bp *VEGFR-3* promoter in the presence of Mithramycin, supporting that PROX1 probably does not interact with SP1 transcription factor family to activate the *VEGFR-3* promoter.
★ indicates significantly different from control ($p < 0.01$)

F) PROX1 binds to proximal regions of the *VEGFR-3* and *FGFR-3* promoters

To confirm that activation of *VEGFR-3* and *FGFR-3* genes by *Prox1* in endothelial cells is due to PROX1 recruitment to the promoter regions of these two genes (either bound directly on DNA or indirectly via other bound transcription factors), we conducted a set of experiments to investigate whether PROX1 is localized to the *VEGFR-3* and *FGFR-3* promoters. First,weexamined PROX1 binding to the promoter regions of *VEGFR-3*and *FGFR-3* genes by using Electrophoretic Mobility Shift Assays (EMSA).

We used three versions of recombinant PROX1 that were previously produced in our lab: wild type PROX1 (WT PROX1), Homeodomain deleted PROX1 (HDΔ PROX1) and DNA-binding deficient PROX1 (DBD PROX1). Since these proteins were generated using a Maltose Binding Protein (MBP) fusion protein, we used recombinant MBP as a control in our assays. We excised the 266 bp *VEGFR-3*, 220 bp *FGFR-3* and 90 bp *VEGFR-3* promoters from their reporter vectors and labeled them with α-P^{32} as our radiolabeled DNA probes.

F.1) PROX1 binds to the 220 bp *FGFR-3* promoter in vitro

Using EMSA, we first investigated whether PROX1 binds directly to the 220 bp *FGFR-3* promoter. As shown in Figure 16A, MBP did not shift the 220 bp *FGFR-3* probe (lane 2). WT PROX1effectivelyshifted effectivelythis probe (lane 3). In contrast, neither HDΔ PROX1 nor DBD PROX1 shifted the 220 bp *FGFR-3* probe (lane 4 and 5), indicating that binding of PROX1 to the 220 bp *FGFR-3* promoter is via a PROX1 DNA-binding domain. When “cold” (i.e. non-radiolabeled) probe was added to the sample containing WT PROX1 (lane 6), the observed shift was

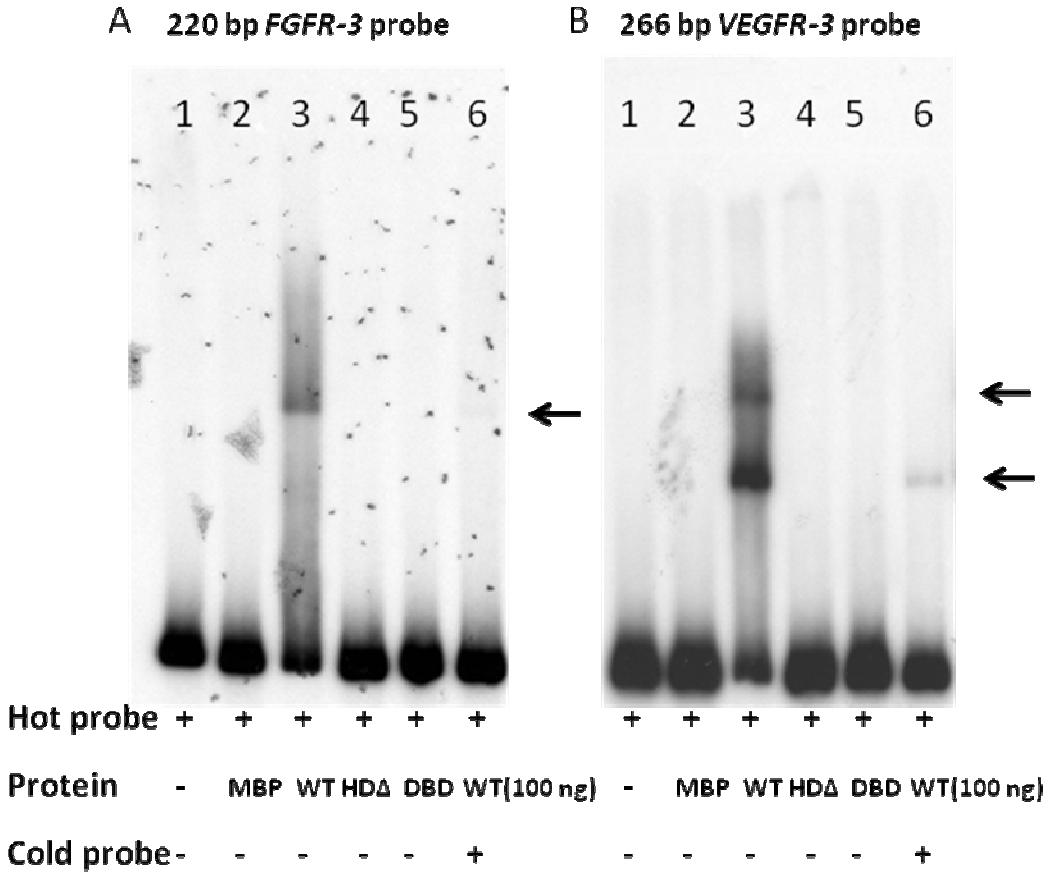

Figure 16 – PROX1 binds to the proximal regions of the *VEGFR-3* and *FGFR-3* promoters. A) WT PROX1 shifted the labeled 220 bp *FGFR-3* probe in EMSA (arrow in lane 3). But DNA binding deficient versions of PROX1 did not shift the probe (lane 4 and 5) and the shift was significantly reduced when cold probe was added to the sample (arrow in lane 6). B) WT PROX1 shifted the 266bp *VEGFR-3* hot probe (arrows in lane 3). DNA binding deficient versions of PROX1 did not shift this probe (lane 4 and 5) and the shift was significantly reduced when cold probe was added to the sample (arrow in lane 6).

competed out. These findings indicate that WT PROX1 binds directly and specifically to the 220 bp *FGFR-3*promoter via its DNA-binding domain in our *in vitro* EMSA assay.

F.2) PROX1 binds to the 266 bp *VEGFR-3* promoter *in vitro*

We further tested whether PROX1 binds to the 266 bp *VEGFR-3* radio-labeled probe (Figure 16B). MBP did not shift the 266 bp *VEGFR-3*probe (lane 2), whereas WT PROX1 shifted this probe (lane 3). Neither HDΔ PROX1 nor DBD PROX1 shifted the 266 bp *VEGFR-3* probe (lanes 4 and 5). When cold probe was added to the sample containing WT PROX1 (lane 6), the observed shift was reduced showing a competition between the cold and labeled 266 bp *VEGFR-3* probes in binding to WT PROX1. These findings indicate that WT PROX1 binds directly and specifically to the 266 bp *VEGFR-3* promoter via its DNA-binding domains in our *in vitro* EMSA.

F.3) PROX1 binds to the 90 bp *VEGFR-3* promoter *in vitro*

Since we previously observed that the proximal 90 bp *VEGFR-3* promoter is essential for activation by PROX1, we investigated whether WT PROX1 is able to bind to the 90 bp *VEGFR-3* promoter using EMSA (Figure 17).

First we showed that only WT PROX1 was able to bind to the labeled 90 bp *VEGFR-3* probe (Figure 17A). MBP, the control protein, did not shift the the 90 bp *VEGFR-3* probe (lane 2). Only WT PROX1, but not HDΔ PROX1 or DBD PROX1 shifted this probe (lanes 3, 4 and 5). This indicates that WT PROX1 also

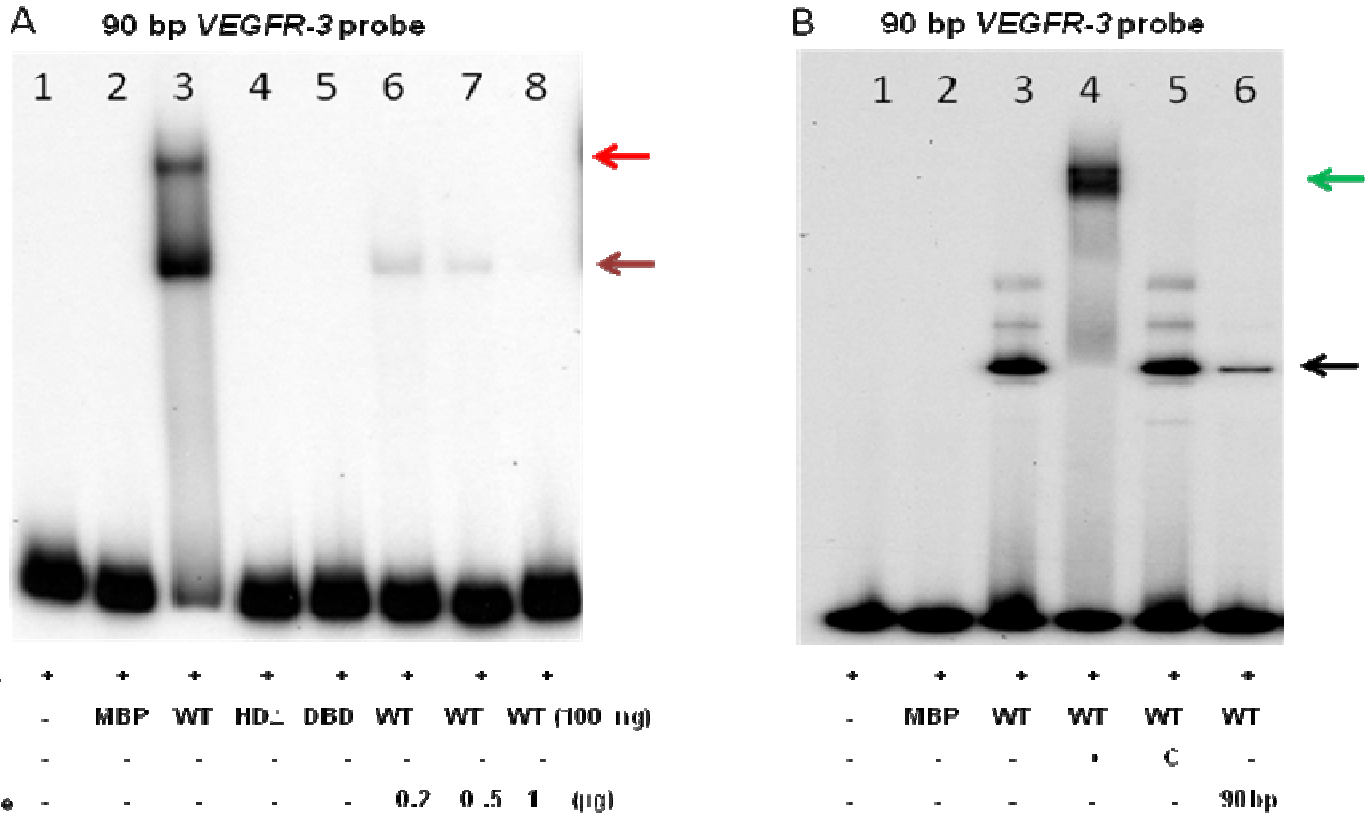

Figure 17– PROX1 binds to the proximal 90 bp *VEGFR-3* promoter. A) WT PROX1 shifted the labeled 90 bp *VEGFR-3* probe (arrows in lane 3). DNA binding deficient versions of PROX1 did not shift the probe (lane 4 and 5) and the shift was reduced when cold probe was added to the sample in a dose dependent manner (lane 6, 7 and 8). B) Using a monoclonal antibody against PROX1 (Chemicon) resulted in a supershift of WT PROX1 and the labeled 90 bp *VEGFR-3* probe (green arrow in lane 4). This supershift was not observed with the control antibody (mouse IgG in lane 5). The band shift was reduced when cold probe was added to the sample (black arrow in lane 6).

binds to the promoter via itshomeodomain. When the cold 90 bp *VEGFR-3*probe was added to the sample containing WT PROX1 (lane 6), the observed shift was reduced showing a competition between the cold and labeled 90 bp *VEGFR-3*probes in binding to WT PROX1.

We further confirmed that WT PROX1 binds to 90bp *VEGFR-3*promoter by using an antibody against PROX1 in our EMSA (Figure 17B). Addition of anti-PROX1 Ab to the sample (lane 4) resulted in a "supershift", whereas a control mouse IgG did not result in a supershift (lane 5). Finally, addition of the unlabeled (cold) 90bp *VEGFR-3*probe to the sample competed with the labeled probe for binding to WT PROX1 and reduced the shift (lane 6). So, in addition to our previous EMSA that showed WT PROX1 binds directly to the 90 bp *VEGFR-3*promoter via its DNA-binding domains, these results confirm that the binding of WT PROX1 to the 90 bp *VEGFR-3*promoter is specific.

G) PROX1 binding to the 90 bp *VEGFR-3* promoter is via a conserved binding site

To further investigate whether binding of WT PROX1 to 90 bp VEGFR-3 is via a specific conserved binding site, we divided this probe into four overlapping oligonucleotide probes which we designated asV1, V2, V3 and V4 (Fig 18). We used these probes to determine the exact PROX1 binding region within the 90 bp *VEGFR-3* promoter and whether this binding is via a conserved binding site.

Figure 18 – Generation of four overlapping oligonucleotides that span the 90 bp *VEGFR-3* promoter. In order to find the PROX1 binding site in the 90 bp *VEGFR-3* promoter, we generated four overlapping oligonucleotides (V1, V2, V3 and V4) from this promoter region. The forward oligonucleotides and related reverse complementary oligonucleotides (not shown) were annealed and used in EMSA.

G.1) Unlabeled V1 and V3 compete with the labeled 90 bp *VEGFR-3* probe in binding with WT PROX1

Using EMSA, we first investigated whether unlabeled V1, V2, V3 and V4 compete with the labeled 90 bp *VEGFR-3* probe in binding to WT PROX1 (Figure 19). Altogether, MBP did not shift the labeled 90 bp *VEGFR-3* probe (lane 2), WT PROX1 shifted this probe (lane 3); this shift was reduced when unlabeled 90 bp *VEGFR-3* probe was added to the sample (lane 4). The gel shift was also markedly reduced when unlabeled V1 and V3 probes were added to the sample (lane 5 and 7). In contrast, adding unlabeled V2 and V4 to the sample did not compete out the gel shift as effectively as the unlabeled V1 and V3 probes (lanes 6 and 8). These results indicated that both V1 and V3 specifically compete with the 90 bp *VEGFR-3* promoter in binding to WT PROX1.

G.2) PROX1 binds to V1 and V3 in EMSA

We further investigated the binding of V1 and V3 probes to WT PROX1. We used labeled oligonucleotides (V1, V2, V3 and V4) and performed EMSA with WT PROX1 (Figure 20). WT PROX1 shifted labeled V1 (lane 3) and V3 (lane 9) clearlyvery. There was a consistent faint shift with V2 probe (lane 5), but no shift was observed with V4 probe(lane 12). Addition of unlabeled V1 and V3 to the samples decreased the shifts by WT PROX1. These results show that WT PROX1 binds specifically to V1 and V3, two oligonucleotides derived from the proximal 90 bp *VEGFR-3* promoter.

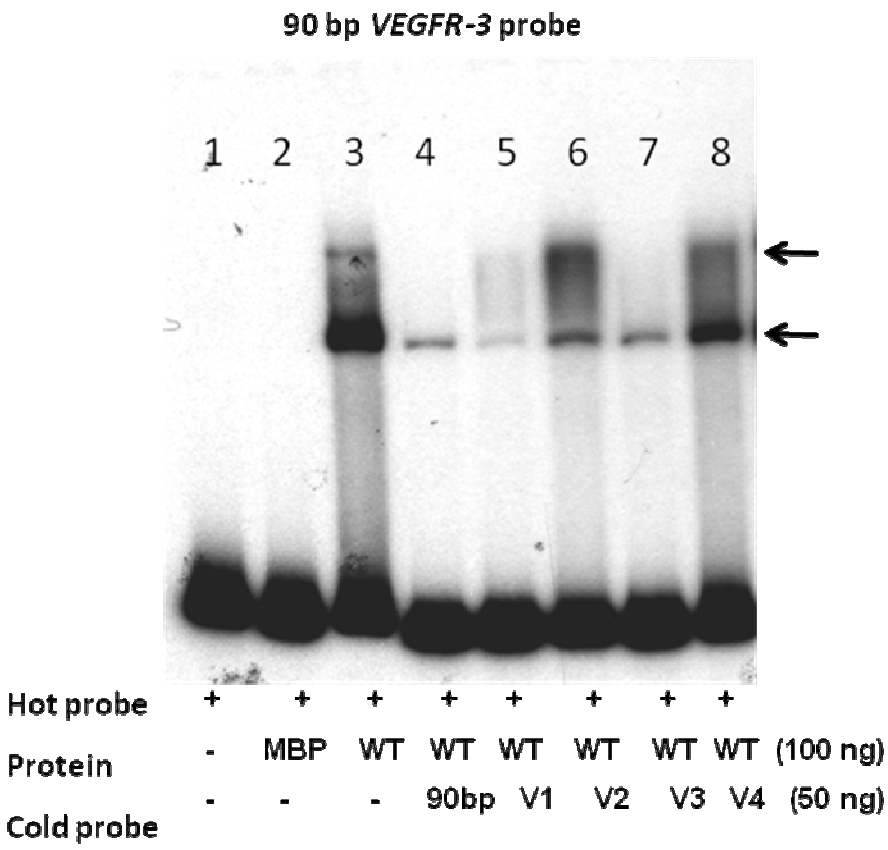

Figure 19 – V1 and V3 compete for binding by PROX1 with the 90 bp *VEGFR-3* probe. We used unlabeled oligonucleotides (V1, V2, V3 and V4) to determine whether they compete with labeled 90 bp *VEGFR-3* in binding by WT PROX1. The shift by WT PROX1 (lane 3) was reduced when unlabeled 90 bp *VEGFR-3* probe was added to the sample (lane 4). The shift was also reduced when unlabeled V1 (lane 5) and V3 (lane 7) were added to the sample. Addition of unlabeled V2 (lane 6) altered the pattern of shift. However addition of V4 (lane 8) to the sample did not reduce the band shift significantly.

16

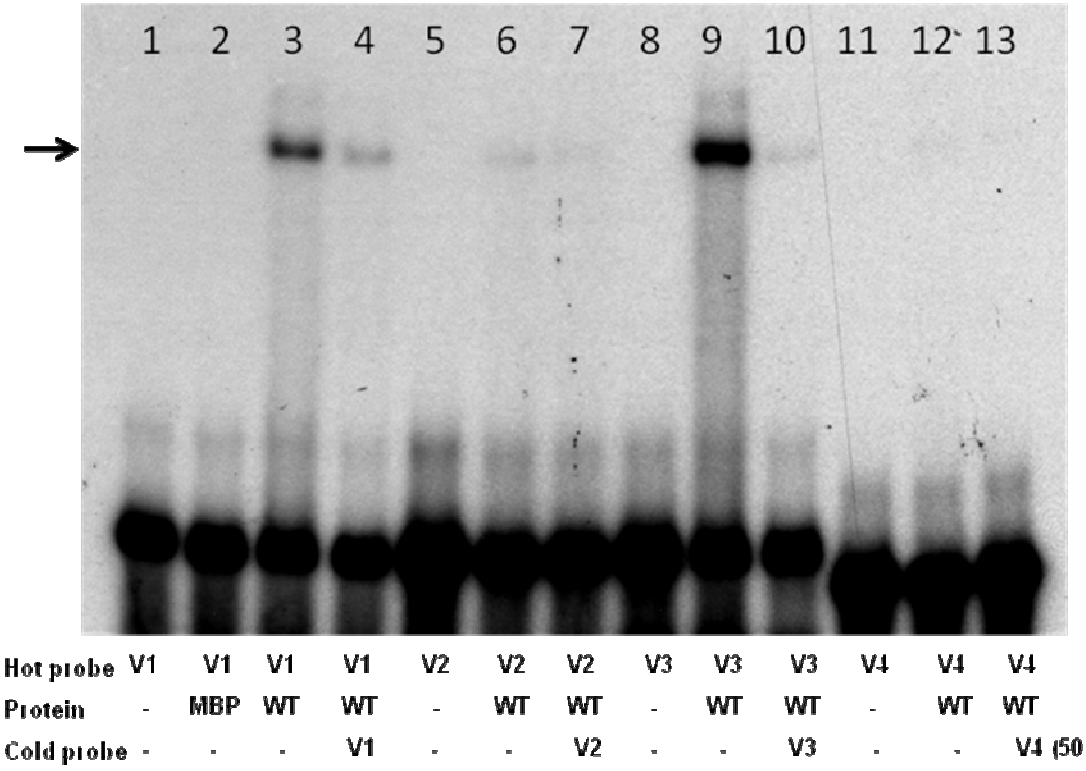

Figure 20 –WT PROX1 specifically binds to V1 and V3. We labeled V1, V2, V3 and V4 and ran an EMSA with WT PROX1. WT PROX1 clearly shifted labeled V1 (lane 3) and V3 (lane 9), minimally shifted labeled V2 (lane 6) but did not shift V4 at all (lane 12). The shifts with V1 and V3 were significantly reduced when unlabeled V1 (lane 4) and V3 (lane 10) were added to the related sample.

G.3) PROX1 binds to V1 and V3 via a "CGCCTCGGC" conserved *cis* element

To determine which *cis* elements on the *VEGFR-3* promoter are required for binding of PROX1, we analyzed the two oligonucleotides bound by WT PROX1, V1 and V3.We found a conserved DNA sequence between V1 and V3: "CGCCTCGGC"(Figure 21A). Of interest, this sequence was also evident in V4 although binding of this oligonucleotide by WT PROX1 was not detected *in vitro*. We mutated this site in both V1 and V3 to investigate the role of this sequence in binding of WT PROX1 to the *VEGFR-3* promoter (Figure 21B). We denoted the mutant version of V1 as M1 and the mutant version of V3 as M3. We used unlabeled M1 and M3 in our EMSA to investigate whether these mutantscompeted with labeled V1 and V3 in binding with WT PROX1. Since V4 did not show any shift in the previous EMSA we used this oligonucleotide as a negative control for binding (Figure 22). As expected, WT PROX1 shifted labeled V1 (lane 3) and V3 (lane 8) probes. These shifts were decreased when unlabeled probe (V1 in lane 4 or V3 in lane 9) was added to the sample. However adding unlabeled mutant oligonucleotide did not decrease the gel shift significantly (M1 in lane 5 and M3 in lane 10). Addition of unlabeled V4 also did not decrease the shift (lanes 6 and 11). These results support the hypothesis that PROX1 binding to the 90 bp *VEGFR-3* promoter via a "CGCCTCGGC" consensus sequence.

A **90 bp VEGFR-3 proximal promoter**

TCCGCCCCGGGCCGCCTCGGCTCCGCCCCCGGGCCGCTGACCGGTCCGCGCCCCGCCGGCGCCTCGGCGCGCGCTCTCACTCCCAGCCTA

V1: TCCGCCCCGGGCCGCCTCGGCTCCGCCCCCGGGCCGCTGA

V2: CTCCGCCCCCGGGCCGCTGACCGGTCCGCGCCCCGCCGGC

V3: CCGGTCCGCGCCCCGCCGGCGCCTCGGCGCGCGCTCTCAC

V4: GCCTCGGCGCGCGCTCTCACTCCCAGCCTA

B

M1:

TCCGCCCCGGGCCGCCCGGCGCCCGCCCCCGGGCCGCTGA

M3:

CCGGTCCGCGCCCCGCCCACTCCGCGGCGCGCGCTCTCAC

Figure 21 – Mutation of the "CGCCTCGGC" conserved *cis* element in V1 and V3. A) We found a conserved DNA sequence between V1 and V3 (blue sequences in V1 and V3). B) Using elements from V4 (as the negative control), we mutated both of these conserved sequences (red sequences in M1 and M3).

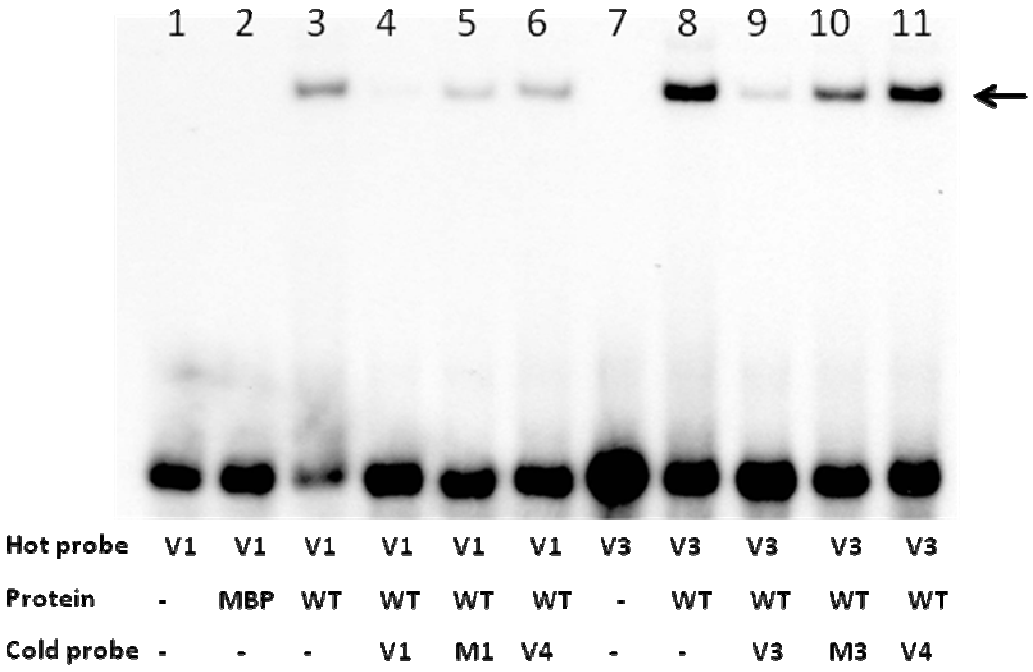

Figure 22 – mV1 and mV3 do not significantly compete with V1 and V3 in binding with WT PROX1. WT PROX1 shifted labeled V1 (lane 3) and V3 (lane 8). The shifts were reduced when unlabeled V1 (lane 4) and V3 (lane 9) were added to the related samples. However addition of M1 (lane 5) and M3 (lane 10) and V4 (lane 6 and 11) to the related samples did not significantly reduce the shift, supporting that the conserved sequence of CGCCTCGGC is essential for binding of WT PROX1 to the probes derived from the *VEGFR-3* promoter.

G.4) Mutated V1 and V3 do not compete with the 90 bp *VEGFR-3*in binding with WT PROX1

Next, we conducted an EMSA with the labeled 90 bp *VEGFR-3* probe and the unlabeled M1 and M3 probes to determine whether mutation ofthe the“CGCCTCGGC” sequence affects the ability of WT PROX1 to bind to the 90 bp *VEGFR-3* promoter (Figure 23). MBP did not shift the 90 bp *VEGFR-3* probe (lane 2). As demonstrated previously, WT PROX1 shifted this probe (lane 3) , which was decreased when unlabeled 90 bp *VEGFR-3* probe was added to the sample (lane 4). Unlabeled V1 and V3 also decreased the shift of labeled 90 bp *VEGFR-3* probe by WT PROX1 (lanes 5 and 7). However, when unlabeled M1 (lane 6), M3 (lane 8) or V4 (lane 9) were added to the samples, the amount of the shifted band was not reduced. These results confirm that WT *Prox1* binds to the 90 bp *VEGFR-3* specifically and this binding is via the two consensus sequences of “CGCCTCGGC” in the *VEGFR-3* promoter.

H) Nuclear extracts (NE) of lymphatic endothelial cells (LECs) interact with the the 90 bp *VEGFR-3* probe

PROX1 is a transcription factor localized to nuclei of LECs. We repeated our EMSA and used nuclear extracts (NE) obtained from cultured primary human LECs to investigate whether nuclear proteins within these extracts, similar to recombinant WT PROX1, are able to shift the labeled 90 bp *VEGFR-3*probe (Figure 24). MBP did not shift the probe (lane 2), while recombinant WT PROX1 shifted the 90 bp *VEGFR-3*probe (lane 3). NE also shifted this probe (lane 4). Addition of unlabeled 90 bp *VEGFR-3*probe (lane 5) or unlabeled V3 (lane 7) to

the samples decreased this shift, whereas addition of M3 (lane 6) did not the decrease the shift. As seenevident in Figure 24, the size of bands is not the same between sample with recombinant PROX1 and sample with LEC nuclear extract. This is due to the fact that our recombinant PROX1 is not the full length protein or . that a complex of proteins, including PROX1, is binding to this region. These results illustrate thatproteins from the nuclei of LECs bind specifically to the 90 bp *VEGFR-3*promoter via the conserved “CGCCTCGGC” *cis* element.

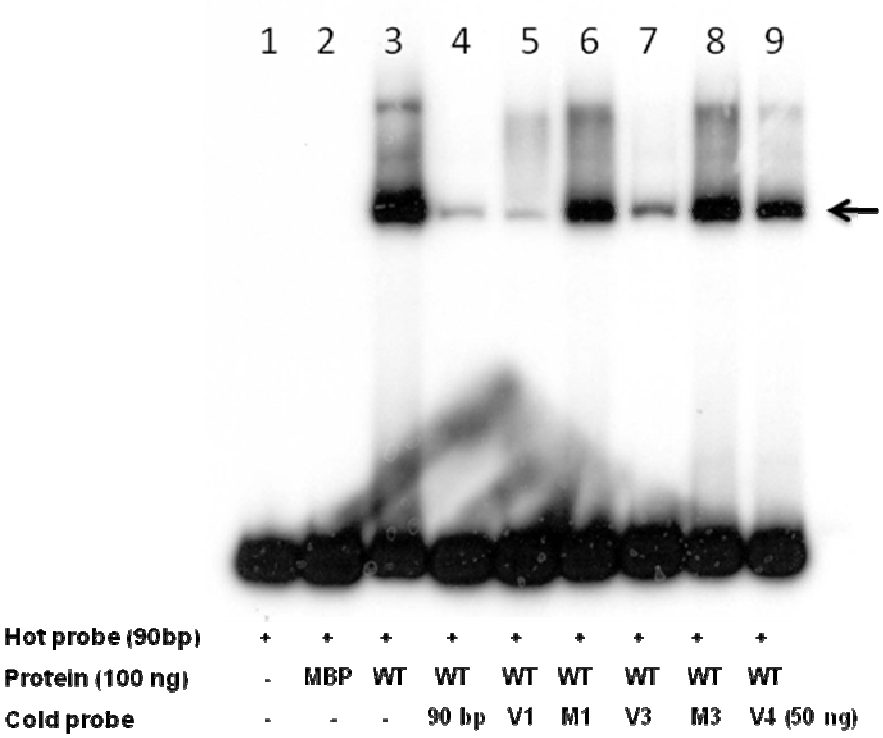

Figure 23 – M1 and M3 do not compete with the labeled 90 bp *VEGFR-3* probe in binding to WT PROX1. WT PROX1 shifts the labeled 90 bp *VEGFR-3* probe (lane 3). This shift is reduced when unlabeled 90 bp *VEGFR-3* (lane 4), V1 (lane 5) or V3 (lane 7) are added to the sample. However addition of M1 (lane 6), M3 (lane 8) or V4 (lane 9) do not decrease the shift, supporting the finding WT PROX1 does not bind to M1 and M3.

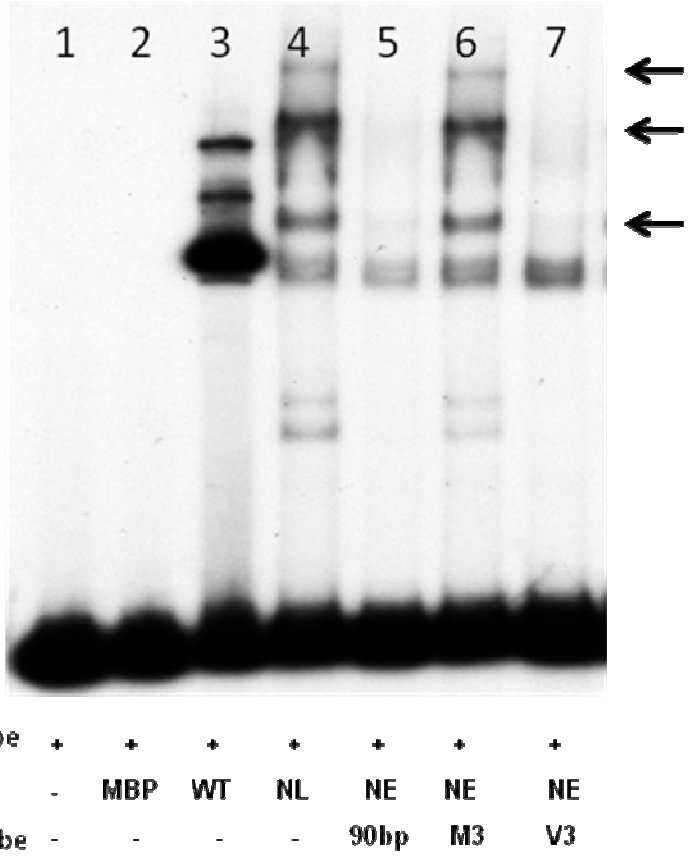

Figure 24 – Nuclear extracts (NE) for lymphatic endothelial cells (LEC) shifts the 90 bp *VEGFR-3* probe. Using labeled 90 bp *VEGFR-3* probe we performed an EMSA with recombinant WT PROX1 or nuclear extract (NE) from LECs. WT PROX1 shifted the labeled probe (lane 3). The labeled 90 bp *VEGFR-3* probe was also shifted with nuclear extract from LECs (lane 4). Most of the shifts were reduced (arrows) when unlabeled 90 bp *VEGFR-3* probe (lane 5) or V3 were added to the sample. In contrast these shifst were not reduced when unlabeled mV3 was added to the sample (lane 6). This data Supports that the conserved CGCCTCGGC sequence in the 90 bp *VEGFR-3* promoter is essential for binding of some nuclear proteins in LECs to this promoter.

I) Chromatin immunoprecipitation (ChIP) assay to assess whether PROX1 is localized to the promoter regions of the *FGFR-3* and *VEGFR-3* genes

We carried out a series of ChIP assays to test whether PROX1 is localized to either the *VEGFR-3* or *FGFR-3* promoters in endothelial cells *in situ*. For ChIP assays, we used three different sources of *Prox1* expressing endothelial cells as the input material: HUVECs over-expressing wt *Prox1*, cultured lymphatic endothelial cells (LECs) derived from human neonatal foreskin, and the skin of E16 mouse embryos which is is a rich sourcein lymphatic vessels . Figure 25 shows the results of our ChIP assay. Unfortunately, all of our antibody negative samples contained a positive band. This result indicates that our immunoprecipitations were not specific and require further optimization.

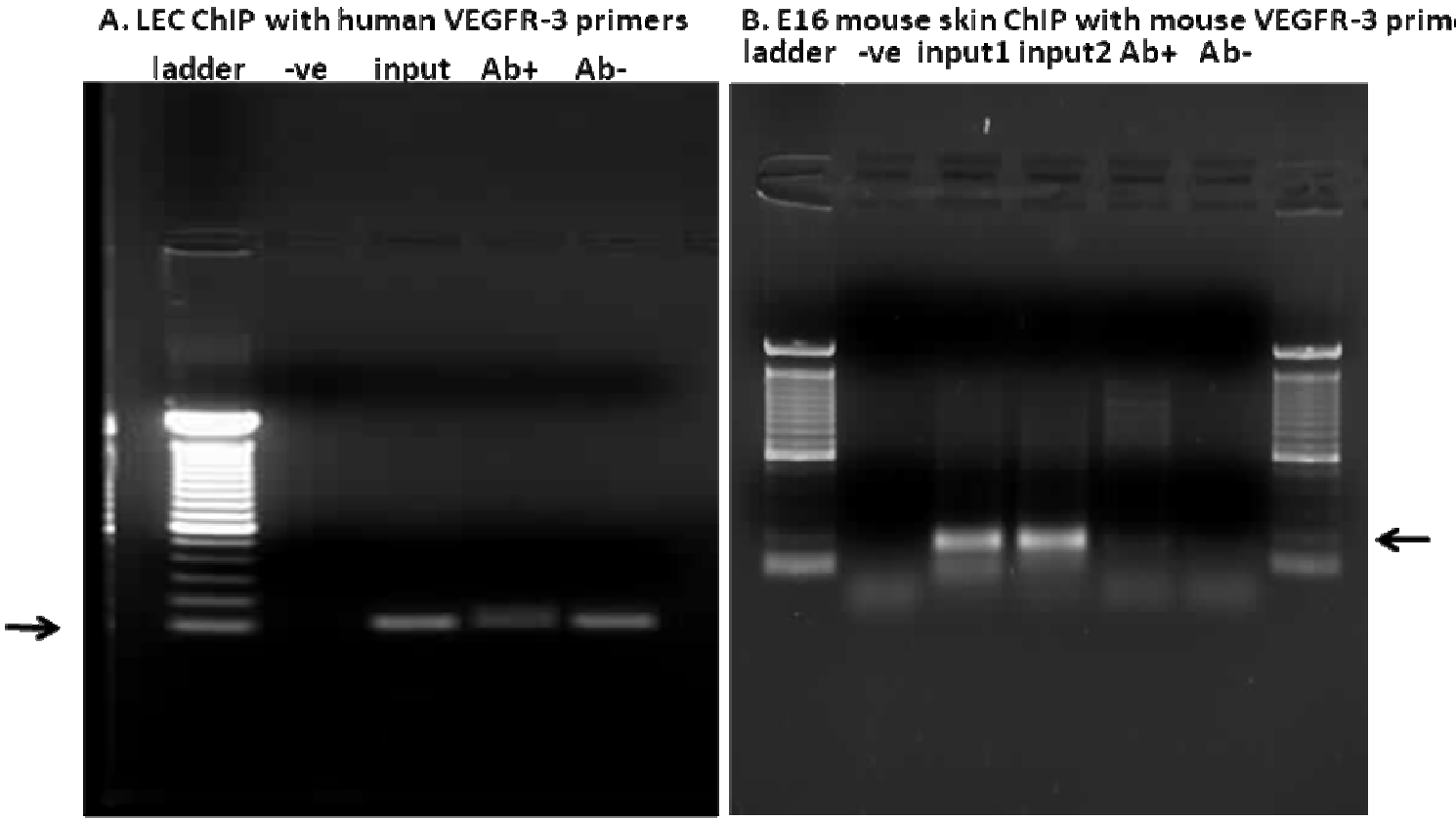

Figure 25 – Chromatin immunoprecipitation (ChIP) assays in A) LECs and B) E16 mouse skin. We ran a set of ChIP assays with both cultured cells and mouse embryonic tissue to determine whether PROX1 is localized to the *VEGFR-3* promoter. Though a positive band in anti-PROX1 Ab positive samples supports the localization of PROX1 in these cells, positive bands in antibody negative samples made these results inconclusive.

Discussion

The lymphatic vasculature acts a complementary system for the systemic blood circulation and plays important roles in normal physiology (Wigle and Oliver 1999). Recently, the significance of lymphatic vessels has been highlighted by its emerging role in the development of different human diseases like cancer (Das and Skobe 2008). However, little is known about the mechanisms that regulate normal and pathologic lymphangiogenesis.

Prox1, a homeobox gene, not only acts as a specific marker for lymphatic vessels, but is also required for their developmentand maintenance of the lymphatic phenotype(Wigle and Oliver 1999; Wigle, Harvey et al. 2002). *Prox1* induces, maintains and regulates the lymphatic phenotype (Wigle, Harvey et al. 2002). Lack of *Prox1* function at any stage of liferesults in the reversal ofchanges the lymphatic vessel phenotype towards a blood vessel phenotype (Johnson, Dillard et al. 2008). On the other hand, *Prox1* expression is sufficient to switch the blood vessel phenotype towards a lymphatic phenotype (Hong, Harvey et al. 2002; Petrova, Makinen et al. 2002). These exciting properties of a single molecule make it a fascinating subject for studying mechanisms involved in cellfate decisions.

Here we showed that Prox1 uses distinct mechanisms to regulate two growth factor receptors (i.e. *VEGFR-3* and *FGFR-3*) in lymphatic endothelial cells. Based on our results, PROX1 activates transcription of the *FGFR-3* gene through direct directly to the binding to its promoter region . On the other hand,

PROX1 regulates the expression of *VEGFR-3* as a transcriptional co-activator, since PROX1 does not require its DNA binding domains to activate its transcription.

Unlike other homeobox genes, *Prox1* contains a unique DNA binding domain. This domain consists of two distinct DNA binding domains: the *homeo* and *prospero* domains. Together they form a single DNA binding domain (the *homeoprospero* domain), which binds DNA at its major groove (Yousef and Matthews 2005). So far, some target DNA sequences of this domain have been identified in *Drosophila*. However, it appears that the targets of PROX1 in mammals are more diverse than what was initially assumed and includes both DNA *cis* elements and chromatin-associated proteins (Chen, Taube et al. 2008). PROX1 contains many functional domains and motifs and can interact with a variety of DNA *cis* elements and other transcription factors (Yamazaki, Yoshimatsu et al. 2009). These multiple pathways explain how PROX1 is a potent transcription factor and a key regulator of cell phenotype. *Prox1* null mice have several developmental abnormalities in organs such as liver, lens, heart, pancreas and lymphatic vessels (Wigle, Chowdhury et al. 1999; Wigle and Oliver 1999; Sosa-Pineda, Wigle et al. 2000; Wigle, Harvey et al. 2002). In addition, it is shown that *Prox1*over*Prox1* over-expressionin blood endothelial cells changes the expression of approximately 300 genes profoundly, profoundly affecting the endothelial cell transcriptome (Petrova, Makinen et al. 2002). Silencing of *Prox1* in mature differentiated lymphatic endothelial cells suppressed lymphatic markers(Johnson, Dillard et al. 2008).

Both VEGFR-3 and FGFR-3 are receptor tyrosine kinases (RTKs) andare areupregulated by *Prox1* over-expression in blood endothelial cells (Hong, Harvey et al. 2002; Petrova, Makinen et al. 2002). *VEGFR-3* null mice die before the development of lymphatic vessels due to VEGFR-3's pivotal role in vasculogenesis in the early embryonic period (Dumont, Jussila et al. 1998). However, *VEGF-C* null mice (the main ligand for VEGFR-3) survive longer and have arrested lymphatic development (Karkkainen, Haiko et al. 2004). FGF signaling is also shown to have important functions in angiogenesis and lymphangiogenesis (Shin, Min et al. 2006).

First, we investigated the role of the two known *Prox1* DNA binding domains in the activation of the *FGFR-3* and *VEGFR-3* promoters. Previously, Shin *et al.*showed that PROX1 activates the transcription of *FGFR-3* via binding to its proximal 220 bp promoter (Shin, Min et al. 2006). Previous studies in our lab also demonstrated that the proximal 90 pairsbp of the *VEGFR-3* promoter are essential for activation of this gene by PROX1; however maximal promoter activity is seen with a proximal 266 bp promoter.

While DNA-binding deficient versions of *Prox1* were not able to activate the 220 bp *FGFR-3* promoter (Figure 6), these constructs were able to efficiently activate the 266 bp *VEGFR-3* promoter (Figure 7). In our luciferase reporter gene experiments, *wtProx1* activated the 220 bp *FGFR-3* promoter significantly. Constructs lacking either the *homeo*or the *prospero* domain or both domains activated this promoter to a much lesser extent than *wt Prox1*. These findings show that in order to activate the *FGFR-3* gene *Prox1* requires an intact

*homeoprospero*domain. Thisresult resultsupports the hypothesis that PROX1 binds directly to the *FGFR-3* promoter. This is in accordance with the findings of Shin *et al.* that PROX1 binds directly to the *FGFR-3* promoter and activates its transcription. They showed that this binding is via a newly identifiedconsensus PROX1 binding site in the*FGFR-3* proximal promoter: CACGCCTCT(Shin, Min et al. 2006).

On the other hand, both wild type and DNA- binding deficient versions of *Prox1* were able to activate the 266 bp *VEGFR-3* promoter in our experiments. Thus, for activation of the *VEGFR-3* promoter, *Prox1*does not require itsDNA binding domains and potentially acts as a co-activator. However, the level of activation of the 266 bp *VEGFR-3* promoterby *HDPDΔ Prox1* (though convincingly more than the empty vector) was significantly lower thanforfor *wt Prox1*. In contrast, *HDΔ Prox1* activated the 266 bp *VEGFR-3* promoter to a greater extent than did *wt Prox1*. These findings suggest that conformational changes in HDPDΔ PROX1 may affect the function of the protein in a way that decreases its activity as a co-activator of the *VEGFR-3*gene. However, the deletion of the HD leads to a more efficient interaction of HDΔ PROX1 with its co-factors, thereby increasing the activation of the 266 bp *VEGFR-3* promoter. In addition to its role in binding to DNA, the homeodomain is required for protein-protein interactions and post-translational modification may affect its functions. What conformational alterations occur in these mutant proteins and how these changes affect binding to co-factors are questions that will be answered in future studies.

To establish how our luciferase reporter assays in HEK 293 cells correlate with the effects of *Prox1* on expression theof the respective endogenous genes in endothelial cells, we over-expressed *Prox1* in these cells. We used an *EGFP* adenoviral construct as the negative control. We observed that only the wild type version of *Prox1* was able to induce the expression of FGFR-3 in HUVECs. This confirmed our earlier reporter gene assays results that in order to activate the *FGFR-3*gene, an intact DNA-binding domain is necessary for *Prox1* function. We also observed that both wild type and DNA-binding deficient versions of *Prox1* were able to significantly induce *VEGFR-3* expression as compared to the *EGFP* construct. These results agree with our *VEGFR-3* luciferase reporter assays, confirming that *Prox1* does not require its DNA-binding domains to activate *VEGFR-3* expression. This supports the modala model thatwhere *Prox1* is a co-activator of *VEGFR-3* gene. expression. However, the quantity of VEGFR-3 protein induced by over-expression of *HDPDΔ Prox1* in HUVECs was less than what was induced by the over-expression of *wt Prox1*, supporting that the *homeoprospero* domain is important for the **full** activation of the *VEGFR-3* gene. This was in agreement with our luciferase reporter gene assays which showed that *HDPDΔ Prox1* did not activate the 266 bp *VEGFR-3* promoter as efficiently as did *wt Prox1*. Whether it is a conformational change or loss of protein-protein interactions that affects the ability of PROX1 to interact with its targets, or that PROX1 is required to bind to the promoter region of the *VEGFR-3* gene to maximize its effect on *VEGFR-3* expression, remains to be answered.

We further studied the role of other known domains of *Prox1* in the

activation of the 220 bp *FGFR-3* and the 266 bp *VEGFR-3* promoters. *Prox1* contains two nuclear receptor boxes (NR1 and NR2) and one glutamine-rich domain (Q) in its amino terminus (Yousef and Matthews 2005). PROX1 interacts with several nuclear receptors; in fact it acts as a co-repressor for all of its known interacting nuclear receptor partners(Yamazaki, Yoshimatsu et al. 2009). These nuclear receptors interact with PROX1 through anLRKLL motif withinNR1 in PROX1 (Kamiya, Kakinuma et al. 2008). We repeated the 220 bp *FGFR-3*andthe 266 bp *VEGFR-3* luciferase reporter gene assays with the constructs of *Prox1*, which either lacked both nuclear receptor boxes (*NRΔ Prox1*) or the glutamine-rich domain (*QΔ Prox1*). We observed that deletion of nuclear receptor boxes or the glutamine-rich domain reduced but did not eliminate *Prox1*mediated activation of *FGFR-3* promoter. We also found that the *NRΔ Prox1* or the *QΔ Prox1* constructs induced activity of the 266 bp *VEGFR-3* promoter was comparable to that of the *wt Prox1*. These findings show that PROX1 uses other domains/motifs to interact with its co-factors and transactivate *VEGFR-3* transcription. The fact that the deletion of the nuclear receptor boxes did not have any effect in the activation of *VEGFR-3* promoter suggests that PROX1 may not interact with a nuclear receptor in order to activate *VEGFR-3*.

Indeed, the only reported example of *Prox1* acting as a co-activator is its activation of the *βB2-Crystallin* gene. In this case,*Prox1* interacts with other transcription factors (CBP/p300 and c-Maf) and enhances *βB2-Crystallin* promoter activity (Chen, Dowhan et al. 2002). We investigated whether *Prox1* synergistically interacts with CBP/p300 in activation of the *VEGFR-3* promoter.

The *CBP* construct did not enhance *Prox1* mediated activation of the *VEGFR-3* promoter.

To determine what transcription factor P*rox1* interacts with to activate the *VEGFR-3* gene, we used a bioinformatics analysis to study the 90 bp DNA sequence in the 5' flanking region of the gene. The proximal 90 bp promoter region of the *VEGFR-3* gene is GC rich (about 90%) and contains several SP1 binding sites. SP1 was also proposed by Ilgin *et al.* as a potential transcription factor required for *VEGFR-3* gene expression(Iljin, Karkkainen et al. 2001). However, we failed to find any synergy between *Prox1* and SP1 in the activation of the *VEGFR-3* promoter. First, luciferase reporter assays showed that exogenous SP1 decreases *Prox1* mediated activationof 266 bp *VEGFR-3* promoter. Second, we inhibited endogenous SP1 by Mithramycin and determined the effect on *Prox1* mediated activation of the *VEGFR-3* promoter. Again, we were not able to show that treatment of cells with Mithramycin greatly decreases the activation of the 266 bp *VEGFR-3* reporter gene by *Prox1.* So it remains to be shown with which transcription factors *Prox1* interacts to activate the *VEGFR-3* gene.

In order to investigate the direct interaction between PROX1 protein and promoter regions of *FGFR-3*and *VEGFR-3* gene, we undertook a series of electrophoretic mobility shift assays (EMSA). In this regard we used several versions of recombinant PROX1 proteins produced in our lab: WT PROX1, HDΔ PROX1 and DBD PROX1; the latter two lack the ability to bind to DNA. As our reporter and immunoblotting assays had indicated earlier, we observed that only

WT PROX1 is able to bind to the 220 bp *FGFR-3* promoter *in vitro*. This supported our postulate that PROX1 binds directly to the promoter region of the *FGFR-3* gene through its DNA binding domain and thereby activates its transcription. However, to our surprise we found that WT PROX1 also binds to the 266 bp *VEGFR-3* promoter. We repeated our EMSA with a shorter version of the *VEGFR-3* promoter, i.e. the 90 bp *VEGFR-3* promoter. We observed that WT PROX1 bound to this promoter. Further experiments using mutated oligonucleotides revealed a conserved *cis* DNA element; "CGCCTCGGC" to which PROX1 specifically binds. Whether this DNA-protein interaction has any physiological significance should be answered by specific targeted mutagenesis in an *in vivo* model. What we know now is that the *homeoprospero* domain is important for the full activation of *VEGFR-3* gene expression. We also were able to show that proteins in nuclear extracts obtainedfrom lymphatic endothelial cells bind to and shift the 90bp *VEGFR-3* promoter. Our hypothesis is that the binding of PROX1 to the *VEGFR-3* promoter stabilizes its localization to the promoter and enhances its interaction with other transcription factors, thereby resulting in full activation of the gene. Finally, we attempted to show that PROX1 is localized to the promoter region of the *FGFR-3* and *VEGFR-3* genes by Chromatin Immunoprecipitation (ChIP) assays. Although our antibody against PROX1 was able to pull down a DNA-protein complex containing elements from the *VEGFR-3* promoter, the positive band in the negative control samples precluded a conclusive result.

Prox1 is a unique homeobox gene. It contains a *homeoprospero* domain, a

conserved DNA binding domain among different species. Here we showed that *Prox1* uses distinct mechanisms to activate its target genes. It binds directly to the *FGFR-3* promoter and activates its transcription. This binding is through the *homeoprospero* DNA binding domain. On the other hand, PROX1 does not require its DNA binding domain to activate *VEGFR-3* transcription. This indicates that PROX1 acts as aco-activator in the case of the *VEGFR-3* gene and interacts with other transcription factors to activate its transcription. However, we also showed that PROX1 has the ability to bind to the *VEGFR-3* promoter via a specific *cis* DNA element. This may be an additional mechanism that *Prox1* uses to enhance its transcriptional activity (Figure 26).

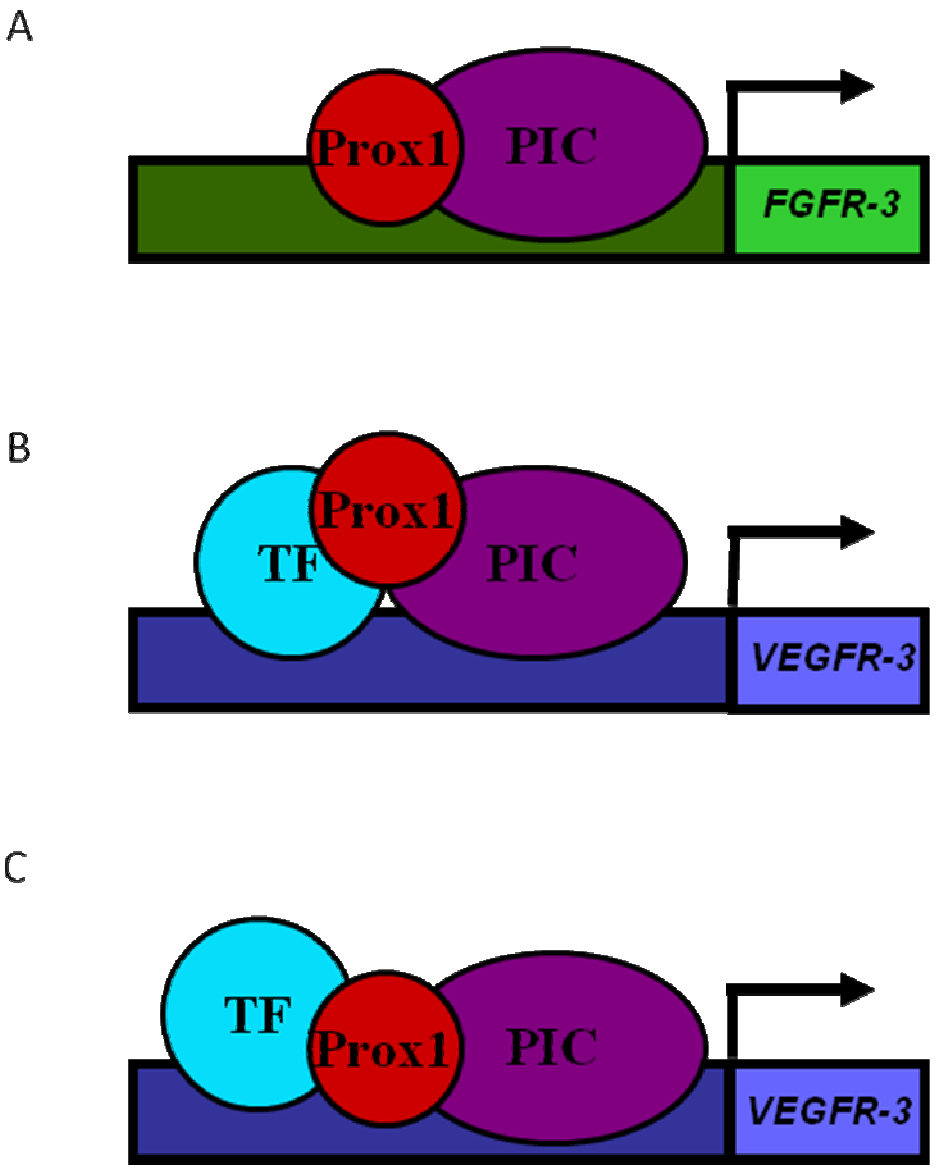

Figure 26 – PROX1 utilizes distinct mechanisms to activate *FGFR-3* and *VEGFR-3* genes. A) PROX1 requires an intact DNA binding domain supporting that PROX1 binds directly to the promoter region of *FGFR-3* gene and activates its transcription in endothelial cells. B) In contrast, DNA binding deficient versions of PROX1 activate *VEGFR-3* gene, supporting that PROX1 activates this gene as a co-activator. C) However, the results from our EMSA studies suggest that PROX1 may bind directly to the promoter region of *VEGFR-3* to enhance its interaction with its cofactors. (PIC: pre-initiation complex)

Conclusions

In this study we were able to:

1- Determine thethedifferent functional domains of *Prox1*that are *Prox1* required for regulation of *VEGFR-3* and *FGFR-3* gene expression.

2- Show that in contrast to *Prox1* mediated activation of the *FGFR-3 gene*, *Prox1* does not require its DNA-binding domain to activate the *VEGFR-3* gene.

3- Reveal that *Prox1* does not requireits nuclear receptor boxes to activate the *VEGFR-3* gene.

4- Find that *Prox1* does not requireits glutamine-rich domain to activate the *VEGFR-3* gene.

5- Show that CBP does not synergistically interact with *Prox1* to activate the *VEGFR-3* promoter.

6- Show that lossmodulation of SP1 function does not affect *Prox1* activation of the *VEGFR-3*promoter.

7- Demonstratethat recombinant PROX1 is able to bind to the*VEGFR-3* promoter *in vitro.*

8- Determine a consensus sequence in the *VEGFR-3* promoter to which PROX1 specifically binds.

Future Directions

1- In the future we will show that PROX1 is localized to the promoter region of *VEGFR-3*gene in lymphatic endothelial cells by the chromatin immunoprecipitation (ChIP) assay. This will strongly support our hypothesis that *Prox1* is a co-activator of *VEGFR-3* expression.

2- Determine the role of other domains/motifs of PROX1 protein in the activation of the *VEGFR-3* gene. Recently a new conserved *prospero* domain 1 (PD1) has been described, localized to the amino terminal of PROX1. It is critical to determine whether this domain has any role in the activation of the *VEGFR-3* gene.

3- We will also identify with which proteins PROX1 interacts on the promoter region of *VEGFR-3* to activate its transcription. This can be done by conducting immunoprecipitation and mass spectrometry assays to find PROX1 interacting partners in lymphatic endothelial cells.

4- We will find more direct target genes of *Prox1* in lymphatic endothelial cells using the ChIP-on-chip assay. PROX1 is a homeodomain protein and likely interacts with many DNA *cis* elements in endothelial cells. Yet, currently there are very few known direct targets of PROX1.

References

Alitalo, K., T. Tammela, et al. (2005). "Lymphangiogenesis in development and human disease." Nature**438**(7070): 946-53.

Andrade, M. and A. Jacomo (2007). "Anatomy of the human lymphatic system." Cancer Treat Res**135**: 55-77.

Arigami, T., S. Natsugoe, et al. (2009). "Vascular endothelial growth factor-C and -D expression correlates with lymph node micrometastasis in pN0 early gastric cancer." J Surg Oncol**99**(3): 148-53.

Baluk, P., J. Fuxe, et al. (2007). "Functionally specialized junctions between endothelial cells of lymphatic vessels." J Exp Med**204**(10): 2349-62.

Baluk, P., T. Tammela, et al. (2005). "Pathogenesis of persistent lymphatic vessel hyperplasia in chronic airway inflammation." J Clin Invest**115**(2): 247-57.

Belecky-Adams, T., S. Tomarev, et al. (1997). "Pax-6, Prox 1, and Chx10 homeobox gene expression correlates with phenotypic fate of retinal precursor cells." Invest Ophthalmol Vis Sci**38**(7): 1293-303.

Blume, S. W., R. C. Snyder, et al. (1991). "Mithramycin inhibits SP1 binding and selectively inhibits transcriptional activity of the dihydrofolate reductase gene in vitro and in vivo." J Clin Invest**88**(5): 1613-21.

Board, J. and W. Harlow (2002). "Lymphoedema 1: components and function of the lymphatic system." Br J Nurs**11**(5): 304-9.

Brice, G., A. H. Child, et al. (2005). "Milroy disease and the VEGFR-3 mutation phenotype." J Med Genet**42**(2): 98-102.

Burke, Z. and G. Oliver (2002). "Prox1 is an early specific marker for the developing liver and pancreas in the mammalian foregut endoderm." Mech Dev**118**(1-2): 147-55.

Chang, L. K., G. Garcia-Cardena, et al. (2004). "Dose-dependent response of FGF-2 for lymphangiogenesis." Proc Natl Acad Sci U S A**101**(32): 11658-63.

Chen, Q., D. H. Dowhan, et al. (2002). "CREB-binding protein/p300 co-activation of crystallin gene expression." J Biol Chem**277**(27): 24081-9.

Chen, X., J. R. Taube, et al. (2008). "Dual roles for Prox1 in the regulation of the chicken betaB1-crystallin promoter." Invest Ophthalmol Vis Sci**49**(4): 1542-52.

Chu-Lagraff, Q., D. M. Wright, et al. (1991). "The prospero gene encodes a divergent homeodomain protein that controls neuronal identity in Drosophila." Development**Suppl 2**: 79-85.

Colvin, J. S., B. A. Bohne, et al. (1996). "Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3." Nat Genet**12**(4): 390-7.

Connell, F., G. Brice, et al. (2008). "Phenotypic characterization of primary lymphedema." Ann N Y Acad Sci**1131**: 140-6.

Cui, W., S. I. Tomarev, et al. (2004). "Mafs, Prox1, and Pax6 can regulate chicken betaB1-crystallin gene expression." J Biol Chem**279**(12): 11088-95.

Das, S. and M. Skobe (2008). "Lymphatic vessel activation in cancer." Ann N Y Acad Sci**1131**: 235-41.

Demidenko, Z., P. Badenhorst, et al. (2001). "Regulated nuclear export of the homeodomain transcription factor Prospero." Development**128**(8): 1359-67.

Doe, C. Q., Q. Chu-LaGraff, et al. (1991). "The prospero gene specifies cell fates in the Drosophila central nervous system." Cell**65**(3): 451-64.

Douville, J. M. and J. T. Wigle (2007). "Regulation and function of homeodomain proteins in the embryonic and adult vascular systems." Can J Physiol Pharmacol**85**(1): 55-65.
Dumont, D. J., L. Jussila, et al. (1998). "Cardiovascular failure in mouse embryos deficient in VEGF receptor-3." Science**282**(5390): 946-9.
Dyer, M. A., F. J. Livesey, et al. (2003). "Prox1 function controls progenitor cell proliferation and horizontal cell genesis in the mammalian retina." Nat Genet**34**(1): 53-8.
Gehring, W. J., Y. Q. Qian, et al. (1994). "Homeodomain-DNA recognition." Cell**78**(2): 211-23.
Glasgow, E. and S. I. Tomarev (1998). "Restricted expression of the homeobox gene prox 1 in developing zebrafish." Mech Dev**76**(1-2): 175-8.
Harrell, M. I., B. M. Iritani, et al. (2007). "Tumor-induced sentinel lymph node lymphangiogenesis and increased lymph flow precede melanoma metastasis." Am J Pathol**170**(2): 774-86.
Hassan, B., L. Li, et al. (1997). "Prospero is a panneural transcription factor that modulates homeodomain protein activity." Proc Natl Acad Sci U S A**94**(20): 10991-6.
Hirakawa, S., L. F. Brown, et al. (2007). "VEGF-C-induced lymphangiogenesis in sentinel lymph nodes promotes tumor metastasis to distant sites." Blood**109**(3): 1010-7.
Hong, Y. K., N. Harvey, et al. (2002). "Prox1 is a master control gene in the program specifying lymphatic endothelial cell fate." Dev Dyn**225**(3): 351-7.
Horning, K. M. and J. Guhde (2007). "Lymphedema: an under-treated problem." Medsurg Nurs**16**(4): 221-7; quiz 228.
Hung, C. C., C. N. Lee, et al. (2008). "Genotyping of the G1138A mutation of the FGFR3 gene in patients with achondroplasia using high-resolution melting analysis." Clin Biochem**41**(3): 162-6.
Iljin, K., M. J. Karkkainen, et al. (2001). "VEGFR3 gene structure, regulatory region, and sequence polymorphisms." Faseb J**15**(6): 1028-36.
Irrthum, A., M. J. Karkkainen, et al. (2000). "Congenital hereditary lymphedema caused by a mutation that inactivates VEGFR3 tyrosine kinase." Am J Hum Genet**67**(2): 295-301.
Jackson, D. G. (2007). "Lymphatic markers, tumour lymphangiogenesis and lymph node metastasis." Cancer Treat Res**135**: 39-53.
Jain, R. K. and T. P. Padera (2002). "Prevention and treatment of lymphatic metastasis by antilymphangiogenic therapy." J Natl Cancer Inst**94**(11): 785-7.
Javerzat, S., P. Auguste, et al. (2002). "The role of fibroblast growth factors in vascular development." Trends Mol Med**8**(10): 483-9.
Ji, R. C. (2009). "Lymph node lymphangiogenesis: a new concept for modulating tumor metastasis and inflammatory process." Histol Histopathol**24**(3): 377-84.
Johnson, N. C., M. E. Dillard, et al. (2008). "Lymphatic endothelial cell identity is reversible and its maintenance requires Prox1 activity." Genes Dev**22**(23): 3282-91.

Kaipainen, A., J. Korhonen, et al. (1995). "Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development." Proc Natl Acad Sci U S A**92**(8): 3566-70.
Kamiya, A., S. Kakinuma, et al. (2008). "Prospero-related homeobox 1 and liver receptor homolog 1 coordinately regulate long-term proliferation of murine fetal hepatoblasts." Hepatology**48**(1): 252-64.
Karkkainen, M. J. and K. Alitalo (2002). "Lymphatic endothelial regulation, lymphoedema, and lymph node metastasis." Semin Cell Dev Biol**13**(1): 9-18.
Karkkainen, M. J., P. Haiko, et al. (2004). "Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins." Nat Immunol**5**(1): 74-80.
Karkkainen, M. J. and T. V. Petrova (2000). "Vascular endothelial growth factor receptors in the regulation of angiogenesis and lymphangiogenesis." Oncogene**19**(49): 5598-605.
Karpanen, T. and K. Alitalo (2001). "Lymphatic vessels as targets of tumor therapy?" J Exp Med**194**(6): F37-42.
Karpanen, T. and K. Alitalo (2008). "Molecular biology and pathology of lymphangiogenesis." Annu Rev Pathol**3**: 367-97.
Kirjavainen, A., M. Sulg, et al. (2008). "Prox1 interacts with Atoh1 and Gfi1, and regulates cellular differentiation in the inner ear sensory epithelia." Dev Biol**322**(1): 33-45.
Kubo, H., R. Cao, et al. (2002). "Blockade of vascular endothelial growth factor receptor-3 signaling inhibits fibroblast growth factor-2-induced lymphangiogenesis in mouse cornea." Proc Natl Acad Sci U S A**99**(13): 8868-73.
Le, T. N., G. Du, et al. (2007). "Dlx homeobox genes promote cortical interneuron migration from the basal forebrain by direct repression of the semaphorin receptor neuropilin-2." J Biol Chem**282**(26): 19071-81.
Lengler, J., E. Krausz, et al. (2001). "Antagonistic action of Six3 and Prox1 at the gamma-crystallin promoter." Nucleic Acids Res**29**(2): 515-26.
Makinen, T., T. Veikkola, et al. (2001). "Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3." Embo J**20**(17): 4762-73.
McEwen, D. G. and D. M. Ornitz (1998). "Regulation of the fibroblast growth factor receptor 3 promoter and intron I enhancer by Sp1 family transcription factors." J Biol Chem**273**(9): 5349-57.
Miyahara, M., J. Tanuma, et al. (2007). "Tumor lymphangiogenesis correlates with lymph node metastasis and clinicopathologic parameters in oral squamous cell carcinoma." Cancer**110**(6): 1287-94.
Nathanson, S. D. (2007). "Preclinical models of regional lymph node tumor metastasis." Cancer Treat Res**135**: 129-56.
Nishijima, I. and A. Ohtoshi (2006). "Characterization of a novel prospero-related homeobox gene, Prox2." Mol Genet Genomics**275**(5): 471-8.
Ogino, H. and K. Yasuda (2000). "Sequential activation of transcription factors in lens induction." Dev Growth Differ**42**(5): 437-48.
Ohtani, O. and Y. Ohtani (2008). "Organization and developmental aspects of lymphatic vessels." Arch Histol Cytol**71**(1): 1-22.

Oliver, G. (2004). "Lymphatic vasculature development." Nat Rev Immunol**4**(1): 35-45.
Oliver, G. and K. Alitalo (2005). "The lymphatic vasculature: recent progress and paradigms." Annu Rev Cell Dev Biol**21**: 457-83.
Oliver, G. and N. Harvey (2002). "A stepwise model of the development of lymphatic vasculature." Ann N Y Acad Sci**979**: 159-65; discussion 188-96.
Oliver, G., B. Sosa-Pineda, et al. (1993). "Prox 1, a prospero-related homeobox gene expressed during mouse development." Mech Dev**44**(1): 3-16.
Oliver, G. and R. S. Srinivasan (2008). "Lymphatic vasculature development: current concepts." Ann N Y Acad Sci**1131**: 75-81.
Ornitz, D. M. and P. J. Marie (2002). "FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease." Genes Dev**16**(12): 1446-65.
Otrock, Z. K., J. A. Makarem, et al. (2007). "Vascular endothelial growth factor family of ligands and receptors: review." Blood Cells Mol Dis**38**(3): 258-68.
Pan, M. R., T. M. Chang, et al. (2009). "Sumoylation of Prox1 controls its ability to induce VEGFR3 expression and lymphatic phenotypes in endothelial cells." J Cell Sci**122**(Pt 18): 3358-64.
Papoutsi, M., J. Dudas, et al. (2007). "Gene regulation by homeobox transcription factor Prox1 in murine hepatoblasts." Cell Tissue Res**330**(2): 209-20.
Perez-Castro, A. V., J. Wilson, et al. (1995). "Genomic organization of the mouse fibroblast growth factor receptor 3 (Fgfr3) gene." Genomics**30**(2): 157-62.
Perez-Castro, A. V., J. Wilson, et al. (1997). "Genomic organization of the human fibroblast growth factor receptor 3 (FGFR3) gene and comparative sequence analysis with the mouse Fgfr3 gene." Genomics**41**(1): 10-6.
Petrova, T. V., T. Makinen, et al. (2002). "Lymphatic endothelial reprogramming of vascular endothelial cells by the Prox-1 homeobox transcription factor." Embo J**21**(17): 4593-9.
Petrova, T. V., A. Nykanen, et al. (2008). "Transcription factor PROX1 induces colon cancer progression by promoting the transition from benign to highly dysplastic phenotype." Cancer Cell**13**(5): 407-19.
Qin, J., D. M. Gao, et al. (2004). "Prospero-related homeobox (Prox1) is a corepressor of human liver receptor homolog-1 and suppresses the transcription of the cholesterol 7-alpha-hydroxylase gene." Mol Endocrinol**18**(10): 2424-39.
Risebro, C. A., R. G. Searles, et al. (2009). "Prox1 maintains muscle structure and growth in the developing heart." Development**136**(3): 495-505.
Rockson, S. G. (2006). "Lymphedema." Curr Treat Options Cardiovasc Med**8**(2): 129-36.
Rockson, S. G. and K. K. Rivera (2008). "Estimating the population burden of lymphedema." Ann N Y Acad Sci**1131**: 147-54.
Roskoski, R., Jr. (2008). "VEGF receptor protein-tyrosine kinases: structure and regulation." Biochem Biophys Res Commun**375**(3): 287-91.
Sabin, F. R. (1916). "The Method of Growth of the Lymphatic System." Science**44**(1127): 145-158.
Shan, S. F., L. F. Wang, et al. (2008). "Modulation of transcriptional corepressor activity of prospero-related homeobox protein (Prox1) by SUMO modification." FEBS Lett**582**(27): 3723-8.

Shimizu, K., H. Kubo, et al. (2004). "Suppression of VEGFR-3 signaling inhibits lymph node metastasis in gastric cancer." Cancer Sci**95**(4): 328-33.
Shin, J. W., M. Min, et al. (2006). "Prox1 promotes lineage-specific expression of fibroblast growth factor (FGF) receptor-3 in lymphatic endothelium: a role for FGF signaling in lymphangiogenesis." Mol Biol Cell**17**(2): 576-84.
Shin, S. Y., H. J. Lee, et al. (2005). "The regulators of VEGF expression in mouse ovaries." Yonsei Med J**46**(5): 679-86.
Sleeman, J. P. and W. Thiele (2009). "Tumor metastasis and the lymphatic vasculature." Int J Cancer.
Song, K. H., T. Li, et al. (2006). "A Prospero-related homeodomain protein is a novel co-regulator of hepatocyte nuclear factor 4alpha that regulates the cholesterol 7alpha-hydroxylase gene." J Biol Chem**281**(15): 10081-8.
Sosa-Pineda, B., J. T. Wigle, et al. (2000). "Hepatocyte migration during liver development requires Prox1." Nat Genet**25**(3): 254-5.
Spana, E. P. and C. Q. Doe (1995). "The prospero transcription factor is asymmetrically localized to the cell cortex during neuroblast mitosis in Drosophila." Development**121**(10): 3187-95.
Steffensen, K. R., E. Holter, et al. (2004). "Functional conservation of interactions between a homeodomain cofactor and a mammalian FTZ-F1 homologue." EMBO Rep**5**(6): 613-9.
Swartz, M. A. (2001). "The physiology of the lymphatic system." Adv Drug Deliv Rev**50**(1-2): 3-20.
Szuba, A., M. Skobe, et al. (2002). "Therapeutic lymphangiogenesis with human recombinant VEGF-C." Faseb J**16**(14): 1985-7.
Tammela, T., T. V. Petrova, et al. (2005). "Molecular lymphangiogenesis: new players." Trends Cell Biol**15**(8): 434-41.
Tammela, T., G. Zarkada, et al. (2008). "Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation." Nature**454**(7204): 656-60.
Torii, M., F. Matsuzaki, et al. (1999). "Transcription factors Mash-1 and Prox-1 delineate early steps in differentiation of neural stem cells in the developing central nervous system." Development**126**(3): 443-56.
Tuttle, T. M. (2004). "Technical advances in sentinel lymph node biopsy for breast cancer." Am Surg**70**(5): 407-13.
Vajo, Z., C. A. Francomano, et al. (2000). "The molecular and genetic basis of fibroblast growth factor receptor 3 disorders: the achondroplasia family of skeletal dysplasias, Muenke craniosynostosis, and Crouzon syndrome with acanthosis nigricans." Endocr Rev**21**(1): 23-39.
Veikkola, T., L. Jussila, et al. (2001). "Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice." Embo J**20**(6): 1223-31.
Wang, J., G. Kilic, et al. (2005). "Prox1 activity controls pancreas morphogenesis and participates in the production of "secondary transition" pancreatic endocrine cells." Dev Biol**286**(1): 182-94.
Wang, L., J. Zhu, et al. (2008). "Repression of interferon-gamma expression in T cells by Prospero-related homeobox protein." Cell Res**18**(9): 911-20.

Warren, A. G., H. Brorson, et al. (2007). "Lymphedema: a comprehensive review." Ann Plast Surg**59**(4): 464-72.
Weinstein, M., X. Xu, et al. (1998). "FGFR-3 and FGFR-4 function cooperatively to direct alveogenesis in the murine lung." Development**125**(18): 3615-23.
Wigle, J. T., K. Chowdhury, et al. (1999). "Prox1 function is crucial for mouse lens-fibre elongation." Nat Genet**21**(3): 318-22.
Wigle, J. T., N. Harvey, et al. (2002). "An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype." Embo J**21**(7): 1505-13.
Wigle, J. T. and G. Oliver (1999). "Prox1 function is required for the development of the murine lymphatic system." Cell**98**(6): 769-78.
Wilting, J., M. Papoutsi, et al. (2002). "The transcription factor Prox1 is a marker for lymphatic endothelial cells in normal and diseased human tissues." Faseb J**16**(10): 1271-3.
Witte, M. H., K. Jones, et al. (2007). "Landmarks and advances in translational lymphology." Cancer Treat Res**135**: 1-16.
Yamazaki, T., Y. Yoshimatsu, et al. (2009). "COUP-TFII regulates the functions of Prox1 in lymphatic endothelial cells through direct interaction." Genes Cells**14**(3): 425-34.
Yousef, M. S. and B. W. Matthews (2005). "Structural basis of Prospero-DNA interaction: implications for transcription regulation in developing cells." Structure**13**(4): 601-7.
Zinovieva, R. D., M. K. Duncan, et al. (1996). "Structure and chromosomal localization of the human homeobox gene Prox 1." Genomics**35**(3): 517-22.