2014

The role of neuropilin 2 in physiological and pathological angiogenesis and lymphangiogen

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

THE ROLE OF NEUROPILIN 2 IN PHYSIOLOGICAL AND PATHOLOGICAL ANGIOGENESIS AND LYMPHANGIOGENESIS

by

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B.S., University of Vermont, 2011

Submitted in partial fulfillment of the

requirements for the degree of

Master of Arts

2014

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ACKNOWLEDGMENTS

I would first like to thank Dr. Diane Bielenberg for giving me the opportunity to fulfill a lifelong dream of working on a cancer biology project. Her mentorship has helped me grow a great deal as a researcher and her continual kindness throughout the project has made it both fun and enjoyable.

I would also like to thank the previous members of the lab for laying the foundations for the thesis presented here, especially Matt Migliozzi and Nick Levonyak. Matt has been extremely helpful in bringing me up to speed in the lab and illustrating techniques that he used to great effect in his own excellent thesis. Nick's work has served as the leaping off point for my own research and without it, I would not have had such a clear direction to follow. I would like to thank my lab partner Erik Li for his continual patience and companionship during long hours in the lab. I would like to thank Dr. Hironao Nakayama from the Klagsbrun lab, whose guidance has proved indispensable.

I would like to thank the members of the Vascular Biology Program at Boston Children's Hospital for being so welcoming and friendly during my project. I have reaped tremendous benefit from all the presentations, lectures and conversations I have had with members from the many labs in the Vascular Biology Program.

I would like to acknowledge Ricardo Sanchez at Ricasan Histology Consultants for his histologic sections, which were an essential part of this investigation.

Finally, I would like to thank my parents. Without their continual support, encouragement and advice I would never have been able to reach for my dream of investigating cancer and pursuing a medical career. I owe them everything.

THE ROLE OF NEUROPILIN 2 IN PHYSIOLOGICAL AND PATHOLOGICAL ANGIOGENESIS AND LYMPHANGIOGENESIS

PATRICK MUCKA

ABSTRACT

The generation of new lymphatic vessels through lymphangiogenesis has been implicated in many disease states. This process has some overlap with the better studied angiogenesis pathway, but is under distinct molecular control. Specifically, it has been shown that VEGFR-3 and neuropilin-2 are important mediators of lymphangiogenesis. A greater understanding of this process could lead to new therapies for cancer and lymphedemas. We investigated lymphatic vessel growth in a mouse model with a focus on the effects of neuropilin-2 knockout. First, we induced an immunogenic response via delayed-type hypersensitivity to examine lymphangiogenesis in the physiologic state. Our neuropilin-2 knockout mouse model displayed a decreased ability to resolve inflammation on exposure to an allergen. Next, we subcutaneously injected a highly invasive melanoma to examine lymphangiogenesis in the pathologic state. We noted significantly reduced tumor growth in our neuropilin-2 knockout. In addition, the neuropilin-2 knockout mice displayed reduced vessel area in comparison to their wild-type littermates, suggesting that inhibition of neuropilin-2 may prove a potent antitumor therapeutic strategy. These results highlight

neuropilin-2’s important role as a mediator of physiological and pathological angiogenesis and lymphangiogenesis.

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LIST OF ABBREVIATIONS

ABC ..... Avidin/Biotinylated Enzyme Complex

aFGF ..... Acidic fibroblast growth factor

APS ..... Ammonium persulfate

bFGF ..... Basic fibroblast growth factor

BM ..... Basement membrane

DC ..... Dendritic cell

DMSO ..... Dimethyl sulfoxide

DNA ..... Deoxyribose nucleic acid

DTH ..... Delayed-type hypersensitivity

EC ..... Endothelial Cell

ECM ..... Extracellular matrix

EDTA ..... Ethylenediaminetetraacetic acid

FBS ..... Fetal bovine serum

GAPDH ..... Glyceraldehyde phosphate dehydrogenase

GFP ..... Green fluorescing protein

GPS ..... Glutamine-Penicillin-Streptomycin

HBSS ..... Hank's buffered saline solution

HIF-1α ..... Hypoxic inducible factor 1α

HIF-1β ..... Hypoxic inducible factor 1β

HUVEC ..... Human umbilical vein endothelial cells

IFN-γ ..... Interferon-γ

IL-1β ..... Interleukin 1β

KO ..... Knockout

LEC ..... Lymphatic endothelial cell

LM ..... Lymphatic malformation

MAM ..... Meprin/A5/PTPmu domain

MAPK ..... Mitogen activated protein kinase

MEK ..... Mitogen activate protein kinase kinase

MHC ..... Major histocompatibility complex

MMP ..... Matrix metalloproteinase

NRP1 ..... Neuropilin-1

NRP2 ..... Neuropilin-2

OCT ..... Optimal cutting temperature

PAE ..... Porcine aortic epithelial

PBS ..... Phosphate buffered saline

PDGF ..... Platelet derived growth factor

PI3K ..... Phosphatidylinositol 3 kinase

PIGF ..... Phosphatidylinositol-glycan F protein

PKC ..... Protein kinase C

PLCγ ..... Phospholipase C γ

RIPA ..... Radio immune precipitation assay

SEMA ..... Semaphorin

shRNA ..... Short hairpin ribose nucleic acid

sNRP ..... Soluble neuropilin

TAF ..... Tumor Angiogenic Factor

TAF ..... Tumor angiogenic factor

TBS ..... Tris buffered saline

TEMED ..... Tetramethylethylethylenediamine

TNB ..... Tris-NaCl-blocking

TNF-α ..... Tumor necrosis factor α

TNS ..... Trypsin neutralizing solution

TSP-1 ..... Thrombospondin-1

VEGF ..... Vascular endothelial growth factor

VEGFR ..... Vascular Endothelial Growth Factor

WT ..... Wild-type

INTRODUCTION

Angiogenesis and Lymphangiogenesis

Blood supply is essential for development and growth. During embryonic development, vasculogenesis occurs, producing a vascular network from precursor angioblasts (Noden, 1989). New vessels are derived from the existing vasculature through the endothelial cell (EC) dependent process of angiogenesis. During angiogenesis, ECs lining the vessel proliferate and sprout toward the region requiring nutrition. This process plays an important role in wound healing and immune response and is implicated in a wide variety of diseases including cancer, retinopathy and arthritis. Control over this phenomenon could lead to potent therapeutic strategies for millions suffering worldwide.

In response to angiogenic signaling, ECs first produce proteases called matrix metalloproteinases (MMPs), allowing their dissociation from the basement membrane (BM) and passage through it into the stroma. As the ECs move away from the existing vessel, one cell is selected as the tip cell. The tip cell continues to migrate into the perivascular stroma followed by a stalk of ECs and pericytes until another vessel is contacted, when further remodeling produces a nascent vessel (Figure 1) (Auerbach & Auerbach, 1994). As blood flow begins through the new vessel, nutrient exchange and oxygen perfusion occur, changing the microenvironment and removing the stimulus for angiogenic signaling.

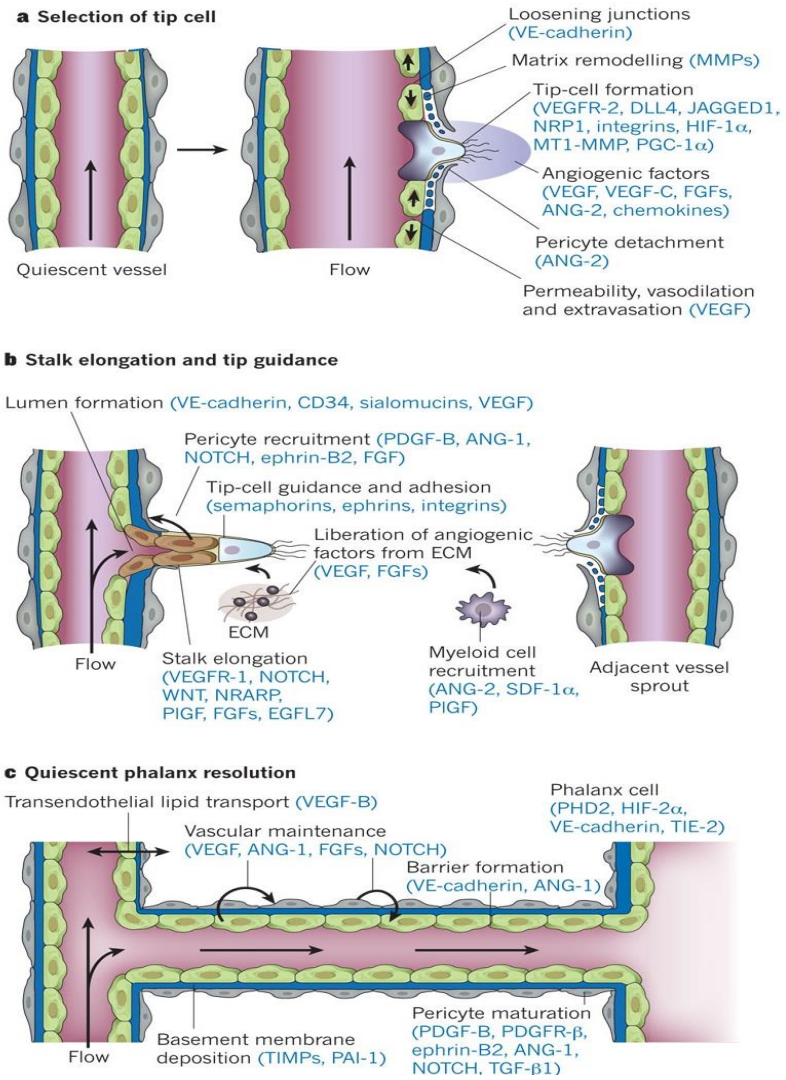

Figure 1 | **Angiogenesis:** a) Angiogenic signals induce ECs to remodel the BM using MMPs, a tip cell is selected. b) Tip cell is guided by more angiogenic signals within the stroma, stalk cells follow. ECM is remodeled by supporting stalk cells. c) The tip cell meets another vessel, pericytes surround nascent vessel and the BM is laid down by ECs lining the new vessel. From Carmeliet 2011.

Lymphangiogenesis is a parallel process to angiogenesis, referring to the generation of new lymphatic vessels from existing ones. While there is still

debate, evidence shows lymphatic vessels first develop during embryogenesis, arising from “lymphatic sacs” themselves derived from embryonic veins (Witte et al., 1997). As the blind-ended network of vessels draining the interstitium, lymphatic vessels serve to regulate interstitial pressure and provide immune surveillance by directing leukocytes to lymph nodes. The involvement of lymphatic vessels in many diseases has sparked intense study into the field, where research has largely focused on the control of lymphangiogenesis. As research has progressed, lymphangiogenesis has become a field distinct from angiogenesis under distinct molecular control and with some unique clinical implications.

Modern laboratory techniques have allowed the development of numerous models of physiologic and pathologic angiogenesis. However, the most dynamic of all models may also be the earliest. Tumors, as rapidly dividing cells, have high nutritional demands and their requirement of angiogenesis to supply nourishment would serve as the founding observation of this ever-expanding field.

Cancer

In 1971, Dr. Judah Folkman showed that angiogenesis was required for tumor growth and metastasis. He theorized that using an inhibitor of angiogenesis, or anti-angiogenic compound, could prove a potent therapeutic strategy for cancer patients (Folkman, 1971). By illustrating its necessity for tumor growth, Folkman had revealed angiogenesis as one of the critical processes in cancer cell biology.

Cancer biology is heavily focused on the key differences that separate our normal cells from malignant tumor cells. Research has elucidated several basic characteristics defining a malignant tumor cell, which Hanahan and Weinberg termed “Hallmarks of Cancer” (Figure 2). These characteristics are acquired by non-tumor cells along the path to malignancy. Development of these traits is not sequential; cells may acquire different traits at different times.

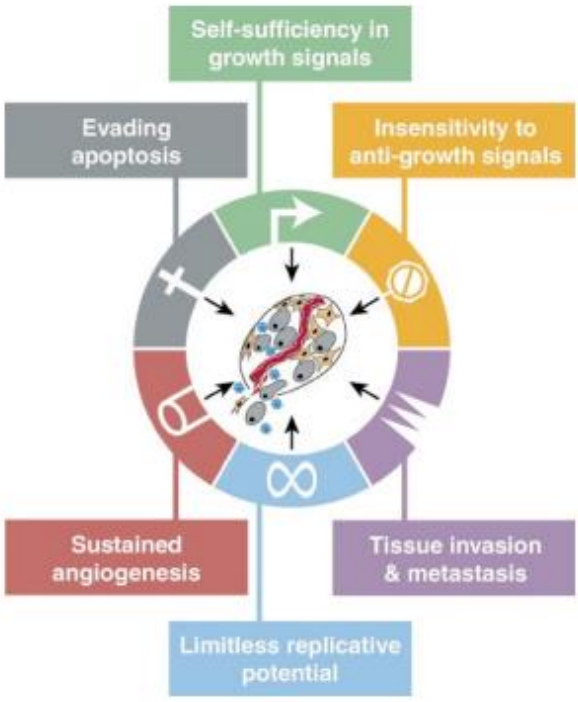

Figure 2 | Tumor Characteristics: Characteristics that tumor cells acquire on the way to malignancy. The tumor shown in the center reflects the theory of heterozygous tumor composition. From Hanahan and Weinberg 2001.

Sufficiency in growth signals normally reserved for cells completing programmed replication is characteristic of tumor cells. Tumor cells frequently produce their own growth signals, creating a microenvironment conducive to growth (Payne & Jones, 2011). Cancer cells also display insensitivity to anti-growth signals that would typically shut down growth due to contact inhibition or activation of tumor suppressor genes. In conjunction with these two signaling modifications, cancer cells frequently achieve unlimited replicative potential through up-regulation of telomerase activity (Blasco, 2002). Although cells have natural apoptotic mechanisms to defeat such replication, tumor cells circumvent these pathways. Pro-apoptotic signaling pathway molecules p53, cytochrome C

and caspase-8 have all been shown as deregulated in many cancers (Derksen et al., 2006; Hanahan and Weinberg, 2000). The ability to initiate angiogenesis has been confirmed as a defining feature of a malignant tumor cell (Hanahan and Weinberg, 2011). Angiogenesis was also shown to be required for metastasis. During growth, nearly all tumor cells metastasize from their primary site to distant locations throughout the body, the resultant growth of which cause 90% of all cancer deaths (Sporn, 1996). In theory, by creating an anti-angiogenic therapy, not only could growth be stopped but lethal metastasis prevented, giving antiangiogenic therapies great clinical relevance.

Current evidence supports a tumor composition that is heterogeneous, containing a variety tumor cells and additional stromal cells contributing to the tumor microenvironment (Hanahan and Weinberg, 2011; Naumov et al. 2006). Such coordination within the tumor produces an environment where single cells need not acquire all characteristics necessary for invasion and malignancy, they can benefit from the progression of other cells by being exposed to the enriched tumor microenvironment (Fidler, 2003).

Metastasis

Metastasis is the spread of tumor cells from the primary growth to a distant, or secondary, growth site (Naumov et al., 2006). Much like angiogenesis, metastasis is a complex sequential process reliant largely on the ability of migrating cells to traverse the various cellular barriers (Figure 3). Metastasis begins when tumor cells have acquired several of the previously discussed characteristics and have created a supportive tumor microenvironment (Egeblad et al., 2010). These cells recruit new vessels through angiogenesis, creating a route away from the tumor in addition to bringing nutrition in. The tumor cells express adhesion molecules such as modified laminin receptors to attach to the BM of the new vessels (Barsky et al, 1984a). Elements of the ECM and BM such as collagen pose a significant obstacle to migrating invasive cells. Tumor cells are able to pass through the basement membrane using MMPs and other proteases, which are frequently overexpressed in tumors (Mueller & Fusenig, 2004). Once in the vessel, the tumor cell is able to metastasize to a distant site under the direction of additional chemokines (Fidler 2002). Upon arrival, tumor cells adhere to the vessel wall and begin the process of extravasation in a similar fashion to the prior vessel intravasation. Tumor establishment and growth at the new site is dependent on the presence of a supportive microenvironment. If the tumor cells survive and are able to recruit new vessels, growth occurs, beginning the metastatic cycle again. The selective pressure exerted by the multiple barriers in metastasis assure that only the most invasive cells are able to

successfully become established tumor growths, resulting in a secondary tumor that is far more malignant than its primary.

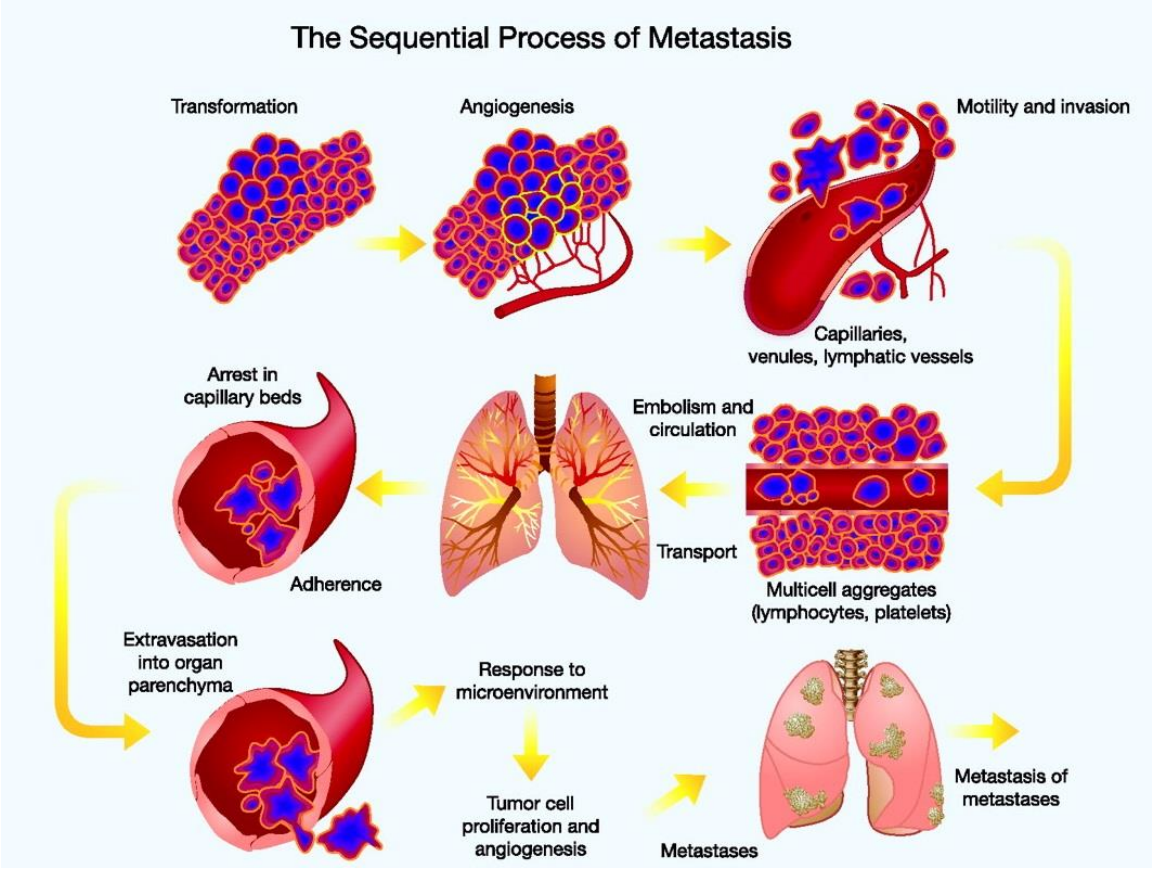

Figure 3 | Metastatic Sequence: The primary tumor (top left) recruits vessels through angiogenesis. Tumor cells (in blue) enter the circulation through capillaries, venules and lymphatic vessels (top right), travel through the circulation and adhere to the vessel wall at a distant site (center left). Tumor cells extravasate into the stroma, where their success in forming a new tumor depends on the microenvironment (bottom center). If the microenvironment is supportive of growth, the metastatic cycle begins again (bottom right). From Fidler 2010.

Examination of tumor models of angiogenesis has yielded important insight in the variable nature of vessel formation. Tumor vessels are disorganized

and have discontinuous basement membranes, causing them to be leaky (Nagy and Dvorak, 2012; Hashizume et al., 2000). Lacking the integrity of a normal BM and pericyte sheath, these vessels are easy targets for invasive cells (Liotta et al., 1991). Normalization of tumor vasculature occurs with the application of some antiangiogenic compounds, indicating that it may be possible to confound the invasive abilities of tumor cells through such therapy (Jain, 2005).

Lymphatics normally form discontinuous BMs with large interendothelial gaps without a pericyte sheath, making the vessels easier to penetrate than normal blood vessels (Sauter et al., 1998; Zwaans & Bielenberg, 2007). Thus, from a structural point of view, lymphatics represent less of a challenge to metastasis as tumor blood vessels. Once in the lymph, the tumor cell faces a far less harsh environment than found within the blood circulation enabling greater survivability and proliferation for the cell. In the clinic, carcinomas have been shown to preferentially metastasize through lymphatic vessels (Pepper, 2001). As such, finding a way to inhibit lymphangiogenesis may represent a viable therapeutic strategy alongside antiangiogenic therapies.

The cost of pathological angiogenesis to the host and the conceptual understanding of possible therapeutic intervention now apparent, the question of control over angiogenesis remains. The true players in angiogenesis stayed unknown for many years, but this did not prevent key investigators from formulating a theoretical framework for the elements to function within.

Control of Angiogenesis

In addition to his other findings in 1971, Judah Folkman was able to isolate a molecule he termed tumor angiogenic factor (TAF) (Folkman, 1971). This molecule was the first isolated molecule that could be termed pro-angiogenic; it had the ability to repeatedly induce physiologic angiogenesis and to activate tumors from dormancy, leading to pathologic angiogenesis. The hypothesis of an “angiogenic switch” or scale with angiogenic molecules on one side, antiangiogenic molecules on the other, began gaining traction within the field (Figure 4). By adding molecules to the angiogenic side, the scale can be tipped to favor angiogenesis and vice versa. The hypothesis proposed that normal cells contain a balance of pro/antiangiogenic molecules. During normal physiologic function, the cell can modify its microenvironment and that of neighboring cells, signaling for angiogenesis in response to hypoxia or immune reaction. When the needs of the cell are met, the microenvironment shifts back towards a balanced composition. When applied to tumor microenvironments, the hypothesis predicted an abundance of angiogenic molecules would be found driving the pathologic angiogenesis (Liotta et al., 1991).

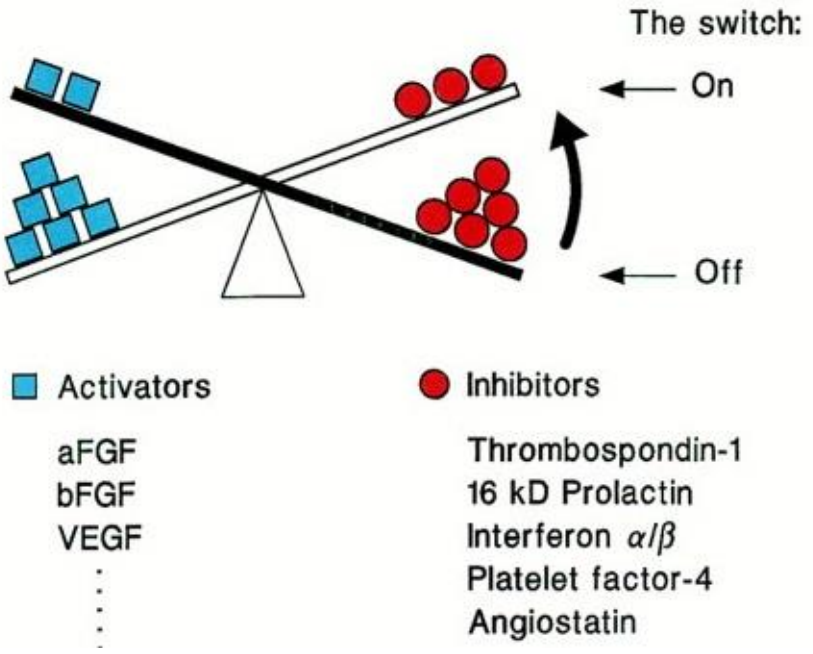

Figure 4 | Angiogenic Switch: The angiogenic switch, with angiogenic molecules in blue and antiangiogenic molecules in red. When angiogenic molecules "outweigh" antiangiogenic molecules, the switch is considered "on" and angiogenesis proceeds. From Hanahan and Folkman 1996.

With the advent of molecular techniques in the 1980's, many angiogenic molecules were finally characterized. Basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF), platelet derived growth factor (PDGF), interleukin-1β (IL-1β), hypoxic inducible factor-1α/β (HIF-1α, HIF-1β) and vascular endothelial growth factor (VEGF) are a few of the key angiogenic molecules. These molecules function largely by acting as ligands, signaling through an equally diverse array of receptor tyrosine kinases (Carmeliet, 2000). To balance out such factors, there are also numerous endogenous antiangiogenic molecules such as thrombospondin-1 (TSP-1), α and β interferon,

semaphorin 3 and platelet factor-4. Many of these molecules have several isoforms allowing additional modulation to the angiogenic cascade, indicating that the “switch” may really be more like “scale”, with a wide range of responses depending not only on the “weight” but also the composition of each “load”.

The wide range of signaling options available allow the body to respond to many different physiologic and pathologic instigators of angiogenesis and lymphangiogenesis. Physiologic angiogenesis is most frequently driven by hypoxia. The 200μm diffusion limit of oxygen represents the maximum distance a cell can be placed from a capillary without hypoxic effects (Filho et al., 1994). Physiologic angiogenesis also occurs during some immune reactions to aid in leukocyte delivery to and interstitial drainage from the affected site. Pathologic angiogenesis can also be driven directly by hypoxia, but aberrant signaling is usually the main motivating force. Tumors are the most obvious example of signaling gone awry. When initiating growth, angiogenesis is likely triggered by hypoxia within the tumor (Filho et al., 1994). However, as the tumor grows and the cells acquire more invasive characteristics, some tumors may begin to overexpress various angiogenic molecules enriching the microenvironment. This leads to greater tumor vascularization, more growth and a further enriched tumor microenvironment. Thus, to bring angiogenesis back into balance within the tumor, angiogenic molecules must be removed or antiangiogenic molecules added. Because of its wide diversity of roles in angiogenic signaling, the VEGF family has been the focus of most therapeutic strategies to date.

VEGF and Receptors

Vascular endothelial growth factor (VEGF) was first described as a modulator of vascular permeability but has since been described as a powerful inducer of EC proliferation, migration and production of proteases. The VEGF family is comprised of five genes, VEGFA, VEGFB, VEGFC, VEGFD and VEGFE all coding for proteins that function as ~40kDa dimers (Ferrara, 1996). In addition, VEGFA undergoes mRNA processing to produce four separate isoforms $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$, of which $VEGF_{165}$ is the most abundant in both physiologic and pathologic condition. $VEGF_{165}$ contains a heparin-binding domain that aids in extracellular matrix binding, keeping this isoform local when secreted, as opposed to $VEGFA_{121}$ which lacks this domain and is freely soluble to disperse (Klagsbrun & D'Amore, 1996). The VEGFs belong to the cysteine knot growth factor family and all contain a ~100 amino acid homology domain containing 8 cysteine residues used for binding to receptors.

The VEGFs bind two separate families of receptors the VEGFR family of tyrosine kinase receptors and the neuropilin family (NRP1 and NRP2). The VEGFR family consists of three receptors VEGFR1, VEGFR2 and VEGFR3. The VEGFRs contain 7 extracellular immunoglobulin domains and have cytosolic tyrosine kinase domains (Ferrara et al., 2003). The receptors signal through normal tyrosine kinase behavior and have differential affinities for the VEGFs

(Figure 5). VEGFR1 binds VEGFA, VEGFB and PIGF and is expressed on macrophages, monocytes and ECs. VEGFR2 binds VEGFA, VEGFC, VEGFD and VEGFE and is expressed on ECs, hematopoietic cells, neuronal cells and some tumor cells (Roskoski, 2007). VEGFA binds VEGFR1 with 10 times the affinity it binds VEGFR2. However, this difference in affinity is compensated by the reduced amount of autophosphorylation by VEGFR1 when compared to VEGFR2 (Seetharam et al., 1995). The greater tyrosine kinase activity seen by VEGFR2 has led to its acceptance as the major regulator of VEGF-induced cell proliferation, migration and angiogenesis (Ferrara et al., 2003). The signal is primarily transduced by the PLCγ-PKC-Raf-MEK-MAPK pathway which initiates DNA synthesis in ECs, preparing the cells for mitosis (Shibuya & Claesson-Welsh, 2006). Of course as an instigator of angiogenesis, VEGFR2 also signals for cell migration, achieved using the Shb-PI3K pathway initiating actin organization and cell migration (Roskoski, 2007). Due to its potent signaling capabilities, VEGFR2 has been the primary target of antiangiogenic therapies. Finally, VEGFR3 binds only VEGFC and VEGFD and is found on vascular ECs during development. Studies in transgenic VEGFR3 knockout mice are embryonic lethal around E11, indicating a critical role for VEGFR3 in the developing vasculature (Dumont et al., 1998). In the adult organism VEGFR3 is expressed only in the ECs lining the lymphatic vasculature, making this receptor the focus of much study within the lymphangiogenesis field.

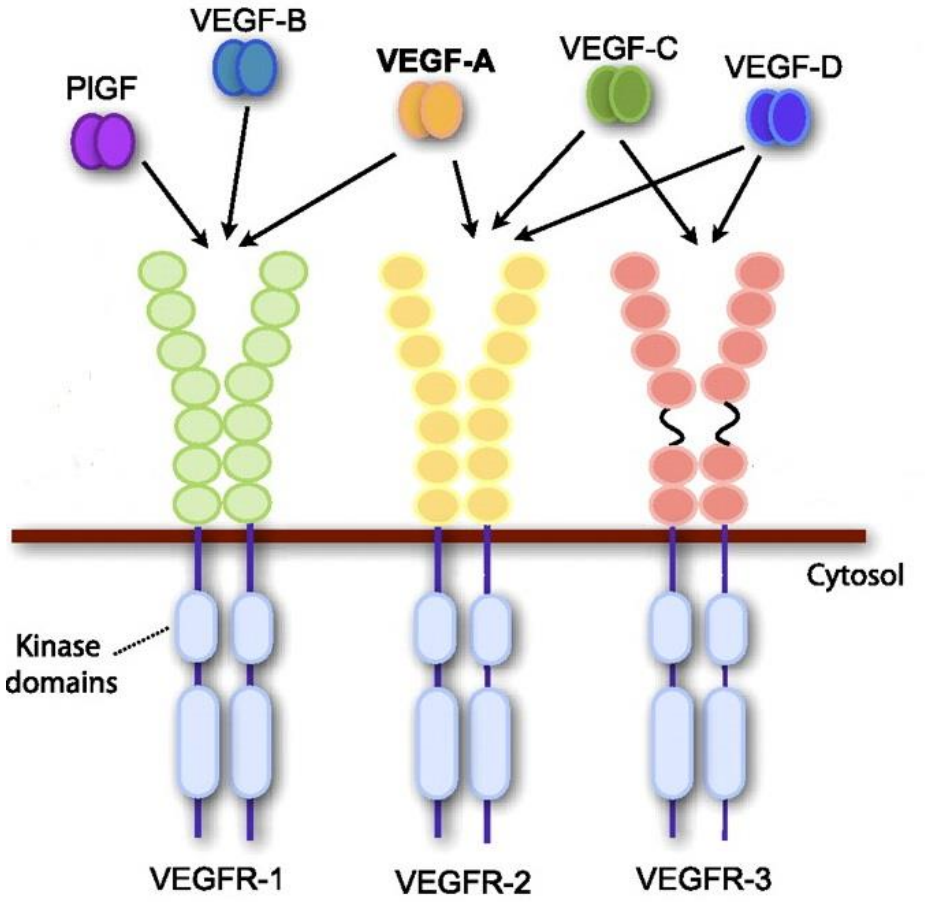

Figure 5 | VEGF and VEGF Receptors: Ligands of the VEGFRs are shown with indications of their binding preferences (Top). The VEGFRs (middle) contain six or seven immunoglobulin binding domains used to bind the ligands shown above. A short transmembrane area links to the intracellular kinase domains used to signal downstream pathways. Adapted from Ruiz de Almodovar et al. 2009

Neuropilin Receptors

The second family of receptors bound by the VEGFs is the neuropilins (NRPs), NRP1, NRP2 and soluble NRP. NRPs were originally discovered as receptors for semaphorins, playing a role in neuronal growth and guidance (Takagi et al., 1991). In 1998, NRP was shown to bind VEGF also, implicating the receptor as a regulator of angiogenesis (Soker et al., 1998). The NRP family receptors are both ~135kDa with ~44% amino acid homology (Giger et al., 1998). The structure contains a large extracellular domain divided into several subdomains, a transmembrane domain, and an intracellular domain. The extracellular domain contains two complement binding domains termed a1 and a2, two coagulation factor V/VII termed b1 and b2, and a meprin domain (MAM) designated c (Chen et al., 1997).

The c and transmembrane domains play a significant role in dimerization, which is an essential aspect of neuropilin signaling (Geretti et al, 2008). The b1/b2 domains contain heparin binding domains (HBD) and are necessary for receptor-ligand interaction for both VEGF and semaphorin-3s (Mamluk et al., 2002). The b1/b2 domains contain binding sites for VEGFs, which is mediated through interactions with amino acids corresponding to exon 7, as opposed to VEGFR-VEGF binding interactions with the conserved cysteine knot motif (Soker et al, 1996; Weismann et al., 1997). NRP1 and NRP2 have differing specificities for the VEGFs, with NRP1 binds VEGFA, VEGFB, VEGFE and PIGF2 while NRP2 binds VEGFA, VEGFC, VEGFD and PIGF2 (Klagsbrun et al., 2002; Gaur

et al., 2009). Signaling is achieved through association with VEGFRs also present in the membrane, with VEGF acting as a “bridge” between the two receptors. Both NRPs associate with VEGFR2, mediated by VEGFA, to enhance angiogenic signaling. NRP2 also associates with VEGFR3, mediated by VEGFC, to enhance lymphangiogenic signaling (Favier et al., 2006).

The a1/a2 domains on NRP contain binding sites for class 3 semaphorins (SEMA3s), which also require the b1/2 domain to bind NRP. The SEMA3s were the first NRP ligand discovered (Kolodkin et al., 1997). These molecules produce a collapsing cell phenotype produced by actin cytoskeleton depolymerization, leading to inhibition of migration (Sakurai et al., 2012; Bielenberg et al. 2008). SEMA3 signaling through NRP is achieved through dimerization with an additional receptor from the plexin family (Puschel, 2002). Plexins are capable of activating many intracellular signaling pathways including those that control cytoskeleton dynamics and proliferation such as Rho and Ras, as well as kinases like SRC, PI3K and MAPK (Gaur et al., 2009).

NRP’s modulation of VEGFR signaling presents it as an ideal target for antiangiogenic therapy and the activity of SEMA3s to inhibit VEGF-NRP binding reinforces this, suggesting that SEMA3s may make an excellent therapeutic agent. Investigation of NRP expression and function in murine models has proved essential for the study of this family of receptors.

Neuropilin Transgenic Mice

In 1997, NRP1 overexpression was shown to produce an embryonic lethal phenotype by Kitsukawa. Abnormalities were found in NRP-expressing organs within the developing nervous system, cardiovascular system and limbs. Embryos overexpressing NRP displayed excess vessels, dilation of vessels and an abnormal heart. The study determined that the cardiovascular abnormalities were the likely cause of lethality in the embryos indicating NRP1 is essential in the development of the nervous system (Kitsukawa et al., 1997). Further studies in a NRP1 knockout mouse produced similar embryonic lethality at around day E13. These also embryos show greatly disorganized vasculature which suggested that the phenotype was due to disruption of NRP1-VEGFR2 association (Kawasaki et al., 1999). Mice null for both NRPs are embryonic lethal at ~E8.5 with even more vascular disorganization and additional hemorrhaging, similar to a VEGFR2 knockout (Takashima et al. 2002).

NRP2 knockout mice are not embryonic lethal and display a loss of lymphatic capillary and small vessel formation during development while all other vessels including arteries, veins and large lymphatic vessels developed normally (Yuan et al., 2002)(Figure 6). This phenotype corresponded with a reduction in DNA synthesis in lymphatic endothelial cells (LECs), indicating that these cells were not proliferating in the mutants. A similar phenotype was more recently described in double heterozygote NRP2/VEGFR3 mice which were unable to sprout lymphatic vessels in response to VEGFC, further implicating NRP2 as an

important mediator of lymphangiogenesis (Xu et al, 2010). Reinforcing this idea, a reduction of VEGFC mediated tumor lymphangiogenesis and metastasis was shown after treatment of mice with an anti-NRP2 antibody (Caunt et al., 2008).

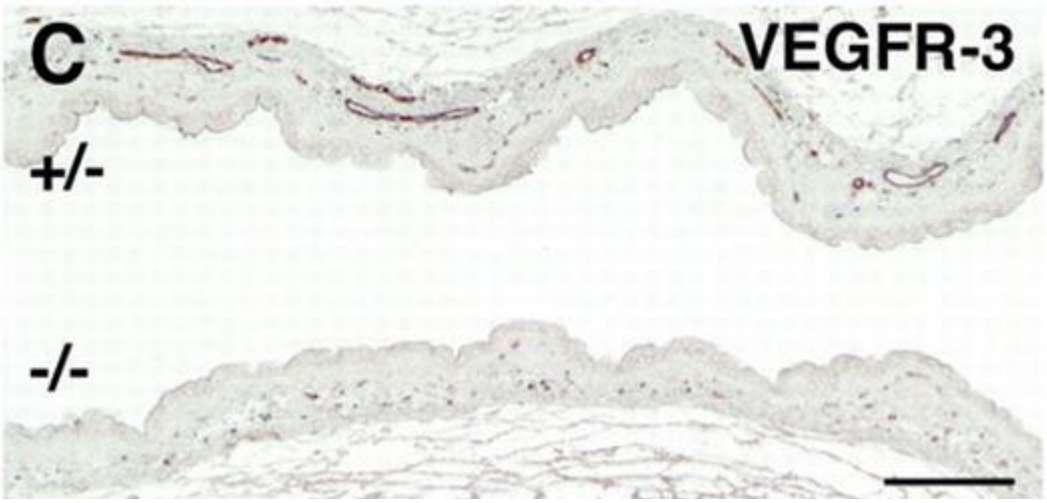

Figure 6 | Compromised Lymphatic Development in VEGFR3 Knockouts: Brown staining is for VEGFR3, a lymphatic marker. Along the top, a VEGFR3 mutant mouse with one allele knocked out is shown to have lymphatic vessels. Below, a mutant mouse with both alleles of VEGFR3 knocked out shows compromised development of small lymphatic vessels. From Yuan et al. 2002

In our laboratory, we have $Nrp2^{gfp/gfp}$ (NRP2 KO) and Lyve/Cre mice. The Nrp2 KO mice have a GFP gene knocked in at the location of the Nrp2 gene while the Nrp2 gene is knocked out. These mice do not have a different phenotype than normal Nrp2 knockout mice other than the presence of GFP in Nrp2 expressing tissues, allowing for quick genotyping of wild-type mice. The Lyve/Cre mice contain a Cre gene following a wild-type Lyve-1 promoter. Lyve-1 is a lymphatic marker first identified as a hyaluronan receptor (Jackson et al., 2001). Cre is a tyrosine recombinase derived from the P1 bacteriophage which is

able to carry out site-specific recombination. The Cre enzyme recognizes the presence and orientation of loxP sites found within the DNA. Genes bookended by loxP are termed “floxed” as are the mice hosting them e.g. floxed Nrp2 mice. By breeding a Lyve/Cre mouse with a floxed Nrp2 mouse, a tissue specific knockout is produced in the offspring, which do not express Nrp2 in lymphatic vessels. This knockout is essential to the deeper investigation of the role NRP2 plays in lymphangiogenesis because it allows us to explore the role of NRP2 expression on lymphatics alone. Such study is required due to the broad and varied expression of NRPs within the body.

Physiologic Neuropilin Expression

Neuropilins are expressed in a continually expanding variety of organs and cell types further implicating them in additional roles far beyond their initial neuronal guidance role and the angiogenesis mediation investigated in this thesis (Wild et al., 2012). NRP1 and NRP2 are expressed differentially in the epithelia of various organs, but expression is not mutually exclusive. A study of NRP1 indicated its expression in the epithelia of breast, lung and blood vessels (Gagnon et al., 2000). Previous studies by our group have shown NRP2 expression in uterus, skin, lung, intestine and brain whole organ lysates (Figure 7). During development, NRP1 and NRP2 are expressed differentially in arterial and venous ECs respectively (Herzog et al., 2001). In the mature organism expression is not as clearly defined with ECs in blood, vein and lymphatic

vessels all variably expressing NRP2 (Bielenberg et al., 2006). Previous work by Nick Levonyak in the lab has shown variable expression in the central lacteal of intestinal villi, as well as lung ECs, providing further evidence for this NRP2's heterogenous expression. Immunohistochemical staining also revealed high expression of NRP2 in cutaneous vessels during the same study. Due to this finding, it was hypothesized that both wound healing and resolution of allergen-induced inflammation (two important physiologic angiogenesis processes) would be compromised. Interestingly, Levonyak found that wound healing was not compromised in NRP2 knockout mice, suggesting that it may be possible to compensate through another pathway. In addition, NRP2 expression on LECs is completely exclusive of NRP1 expression giving further precedent for the lymphatic phenotype found in NRP2 KO mice (Yuan et al., 2002; Bielenberg et al., 2004).

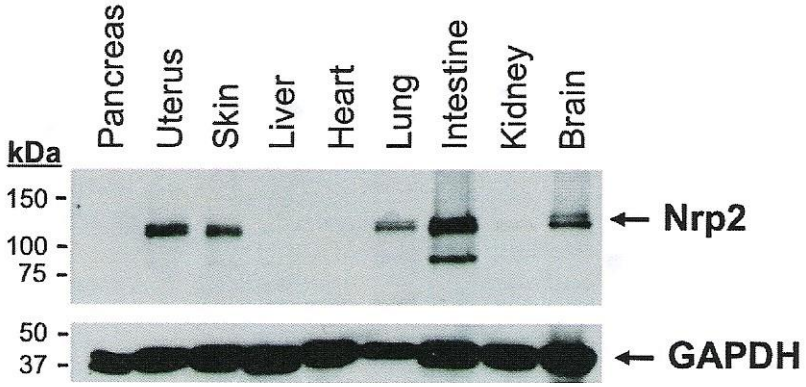

Figure 7 | NRP2 expression measured by western blot: NRP2 protein levels in various tissues were measured by western blot using whole organ lysates. Also present is a band around 85kDa, representing sNRP. GAPDH expression was measured to check loading equivalence of samples. From Nick Levonyak, 2013

NRPs are not limited to guidance and migration roles. Currently, the role of NRP in immune response is also being heavily investigated since NRPs are expressed on leukocytes as well as macrophages and dendritic cells (DCs) (Stepanova et al., 2007; de Paulis et al., 2006). NRP1 has been shown to mediate the interaction between DCs and T-cells and inhibition of this interaction was shown to prevent DC-mediated proliferation of T-cells (Tordjman et al., 2002). Roles like this one are still being discovered for NRPs, implicating them in inflammation, a field which is rapidly converging with the study of cancer. NRPs may also be excellent targets for transplant immunity therapies. Knockdown of NRP2 by shRNA has shown enhanced the survival of corneal allografts by inhibiting local lymphangiogenesis (Tang et al., 2010). The expanding role of

NRP in the immune response along with the growing number of normal cells shown to express NRPs increasingly implicates them in physiologic functioning.

Pathologic Neuropilin Expression

Neuropilins are found in a wide variety of tumor types. Their expression is not mutually exclusive, but one is typically predominant if both are present. NRP1 expression is found in greater levels in carcinomas, while NRP2 is more heavily expressed in melanomas, glioblastomas and neuroblastomas. Thus, NRP2 is found more often in tumors originating for neural crest cells, although there are notable exceptions such as pancreatic ductal adenocarcinoma, which highly expresses NRP2 (Bielenberg et al., 2006; Fukahi et al., 2004).

NRP expression was first reported in PC3 prostate and MDA-MD-231 breast cancer cell lines (Soker et al., 1998). Further studies have shown NRP expression in bladder, colon, pancreas, kidney, ovarian, skin and lung carcinomas (Wey et al., 2004; Neufeld et al., 2005). Interestingly, some of these tissues normally do not express NRPs or do not express NRP1 or NRP2 while expressing the other. The former was shown in three pancreatic cancer cell lines, which expressed high levels of NRP1 and NRP2. Normally, pancreatic cells are completely devoid NRP1 and lowly express NRP2 (Fukahi et al., 2004). Malignant prostate carcinoma samples showed a ten-fold increase in NRP1 over their normally very low levels, with higher levels of NRP1 correlating with more invasive phenotypes (Vanveldhuizen et al., 2003).

Histological analysis of prostate tumors overexpressing Nrp1 in rats showed increased vessel density and proliferation, along with high levels of VEGF, indicating that angiogenesis occurred (Miao et al., 2000). These tumors were shown to be VEGFR negative, removing the possibility of autocrine signaling as the cause of the increased tumor growth. Even without direct signaling, VEGF may still have initiated angiogenesis by creating a gradient in which the tumor microenvironment was more supportive of angiogenesis than the surrounding tissue, thus attracting sprouting vessels. In a theory termed the "VEGF sequestration" hypothesis, NRP1 was proposed as a reservoir for VEGF, keeping the concentration in the tumor higher than would be possible with free VEGF. It is possible that NRP2 also functions in this manner with VEGFA, VEGFC and VEGFD. More recently, NRP2 knockdown via shRNA produced tumor cells which displayed markedly impaired migration, invasion and growth capabilities (Dallas et al., 2008). In addition, tumor size was reduced by as much as 95% in the tumors. Considered together, these findings show that inhibition of NRP2 inhibits tumorigenicity and progression through direct action on NRP2-expressing tumor cell as well as tumor growth and angiogenesis by the direct effects on NRP2-expressing vessels.

Cancer is not the only pathology of consequence for our group; lymphatic malformation (LM), also known as lymphangioma, represents an area that has received relatively little study, yet has the prospect of profound clinical impact. LMs consist of masses of abnormal lymphatic vessels that continually proliferate,

ultimately forming an edematous mass. These vascular anomalies commonly occur in the head and neck reason, which can drastically affect function of the respiratory and digestive tract. In addition, they are commonly visible to naked eye and can be disfiguring, which has a negative impact on patient outlook (Figure 8). Currently, the only treatments available are surgical options to remove the LM, which do nothing to prevent the anomaly from coming back (Perkins & Manning, 2010). In addition, there is no animal model for this disorder, which has led to a clinical focus in the field. The development of a model would allow the underlying mechanisms driving LMs to be studied and is consequently the focus of many already working in the vascular biology field. Our current understanding of NRP as a mediator of lymphangiogenesis suggests that it may be possible to use an antilymphangiogenic compound, such as SEMA3F, to suppress the growth of LMs.

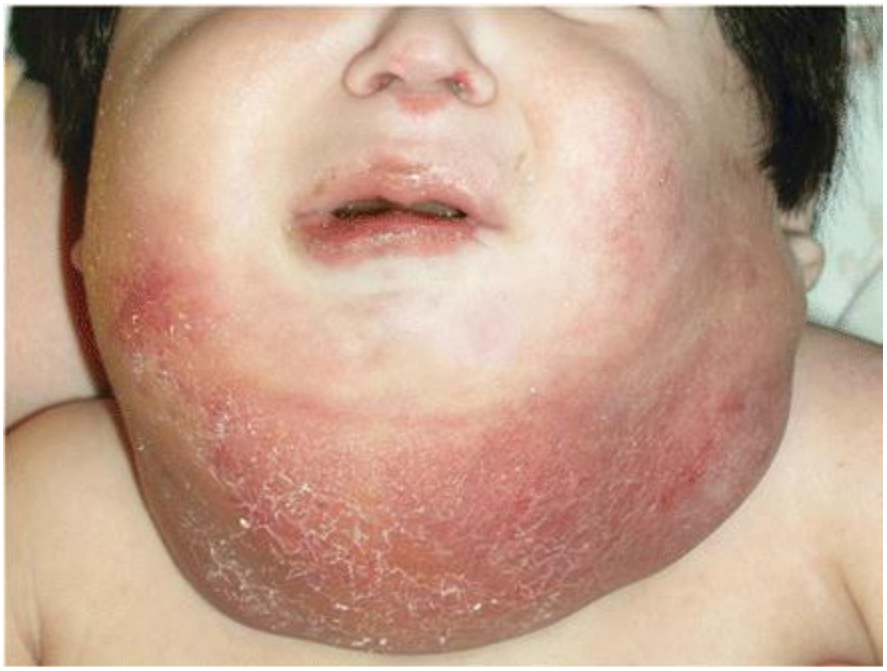

Figure 8 | Lymphatic malformation in a patient: As seen here, lymphatic malformations are characterized by red, edematous swellings that commonly occur in the head and neck. As the malformation grows, it can become “hot” due to extensive, but dysfunctional vascularization and painful, due to the increased interstitial pressure from dysfunctional drainage. From Perkins and Manning 2012.

Class 3 Semaphorins

Class 3 Semaphorins (SEMA3s) were first discovered as axonal guidance molecules, but their role has been expanded to include action in angiogenesis as well. There are 7 SEMA3s (SEMA3A-G), which are structurally composed of an N-terminal SEMA domain, a plexin-semaphorin-integrin (PSI) domain, an Ig loop and a C-terminal basic domain. They also contain several endoprotease cleavage sites that play roles in regulating the activity of SEMA3s. The SEMA

domain contains a 7-bladed β propeller motif that is used for NRP binding (Gherardi et al, 2004). The C-terminal domain has been proven to be essential for SEMA3 dimerization (Klostermann et al., 1998). Most SEMA3s bind NRP2, with the notable exception SEMA3A, which requires NRP1 for signaling (Figure 9) (Chen et al, 1997).

Several theories exist as to SEMA3's mechanism of action, but most recent evidence supports their depolymerization of F-actin filaments as the cause of their inhibitory action (Shimizu et al. 2008). This result was confirmed by Matt Migliozzi in the lab in porcine aortic epithelial cells overexpressing NRP2. This mechanism of action causes the cytoskeleton to lose shape, preventing ECs from extending processes needed for motility. Others have suggested that these effects are due to the inhibition of VEGF-NRP interaction or inactivation of integrins on ECs, disrupting angiogenesis (Guttmann-Raviv et al. 2007; Serini et al., 2003). The SEMA3s act as competitive inhibitors of the VEGFs, with higher concentrations of SEMA3A shown to be necessary to produce growth cone collapse in the presence of VEGFA (Miao et al., 1999).

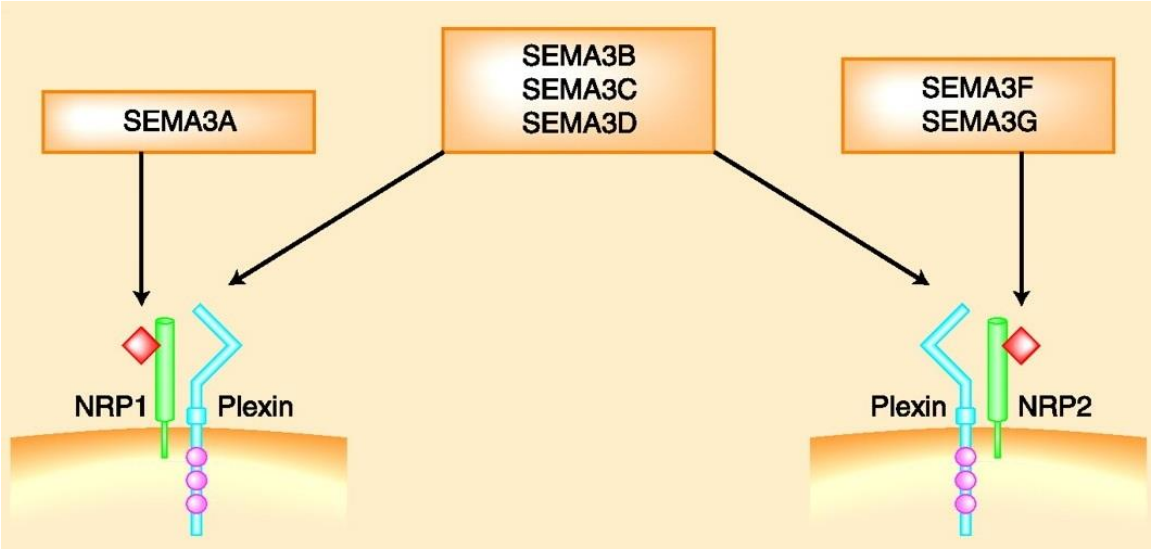

Figure 9 | SEMA3-NRP-Plexin Interaction: Class 3 semaphorins are shown (red diamond) with binding preferences for the NRPs (green). The SEMA-NRP complex is shown associated with the transmembrane plexin receptor (blue), which uses kinase (domains in purple) activity to activate downstream targets. Adapted from Gaur et al., 2009

SEMA3A and SEMA3F are capable of inhibiting EC proliferation and migration, making them promising as antiangiogenic therapeutics. Tumors overexpressing SEMA3A have decreased vascularization and metastatic potential (Bachelder et al., 2003; Moretti et al., 2008; Vacca et al, 2006). Previous work by our group and others has shown that many of these effects are recapitulated with SEMA3F (Bielenberg et al., 2004; Kessler et al., 2004). In addition, Matt Migliozzi from the lab has shown that SEMA3F is effective in reducing tumor vascularization *in vivo*. Critically, he found that there was a reduction in the lymphatics surrounding the tumor, indicating that SEMA3F may be able to confound tumor cell access to lymphatic vessels and thus their ability

to metastasize *in vivo*. Loss of expression of SEMA3s has been correlated with advanced stage lung cancers and melanomas, indicating that their therapeutic replacement may be a viable treatment option *in vivo* (Bielenberg et al, 2004; Lantuejoul et al., 2003). Taken together, these findings suggest that SEMA3F may be an effective antitumor and antimetastatic therapeutic.

In vivo Assays

Many assays have been designed to study angiogenesis in both physiologic and pathologic conditions. Physiologic assays, like physiologic angiogenesis, are more frequently driven by hypoxia or immune response as in wound healing and allergic reaction. On the other hand pathologic assays, such as tumor growth assays, reflect aberrant signaling (i.e. VEGFC overexpression in our B16F10 line) not seen in physiologic conditions. In this study, we made use of one physiologic assay, the delayed-type hypersensitivity assay and one pathologic assay, the tumor growth assay.

Delayed-type hypersensitivity (DTH) reactions are type IV hypersensitivity reactions and are so named because the response to allergen exposure is not immediate, requiring hours to days to reach full effect. This is in stark contrast to immediate hypersensitivity reactions in which the full response occurs in seconds to minutes. Type IV hypersensitivity reactions are unique among the hypersensitivities in that they are completely cell-mediated and do not involve an antibody response (Uzzaman & Cho, 2012).

As a cell-mediated response, the key players in DTH are macrophages and leukocytes, which are NRP2 expressing cells. Initial exposure to an allergen causes macrophages to phagocytize the molecule and present it to Th-1 cells on a class II major histocompatibility complex (MHC). Th-1 cells are activated by this interaction, causing proliferation, in essence “priming” the system for a second exposure. On second exposure to the allergen, Th-1 cells recognize the allergen and secrete interferon-γ (IFN-γ), which is a potent stimulator of macrophages. Once at the exposure site, macrophages secrete tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1) both of which are inflammatory and recruit additional macrophages leading to further inflammation. As the concentration of allergen decreases, less macrophages are recruited until finally the inflammatory response is resolved (Czarnobliska et al., 2007)

Of course, for this inflammation to be resolved the leukocytes and macrophages must first reach the site of exposure. Macrophages local to the exposure are the first to respond, yet others must extravasate to reach the allergen. During DTH reactions, vascular remodeling occurs, allowing responding immune cells to be deposited closer to the site of exposure. Studies in VEGFA overexpressing transgenic mice have shown prolonged inflammatory response and enlarged lymphatics using the same DTH assay used in this study (Kunstfeld et al, 2004). When VEGFA signaling was blocked through systemic application of VEGFR1 and VEGFR2 antibodies, inflammation did not occur, suggesting that being able to turn the angiogenic switch on and off is important in normal

physiologic functioning. This assay allowed us to examine the role NRP2 plays in physiologic angiogenesis, which has not been previously described.

The second *in vivo* assay used in this study is a tumor growth study using mouse B16 melanoma. We studied angiogenesis in cancer for the reasons discussed above, but there are two additional reasons our group used B16 melanoma for this study. B16 is one of the few murine cancer models that are NRP2 positive, as was previously confirmed by Meetu Seth in our lab. This finding is crucial for our study of NRP2's role in tumor angiogenesis. Melanomas preferentially use Nrp2-positive lymphatics to metastasize, thus an exploration of Nrp's role in this could reveal why degree of lymphangiogenesis correlates with poor outcome in the clinic. In addition, there is no shortage of evidence supporting melanoma as the most lethal skin cancer.. By further investigating this highly invasive melanoma, we hope to determine if SEMA3F could be an effective antitumor and antimetastatic agent for this deadly form of cancer.

METHODS

Cell Culture

General Cell Culture

B16F10 melanoma cells, B16F10 melanoma cells transfected with VEGFC, A375SM melanoma cells, porcine aortic endothelial (PAE) cells transfected with either neuropilin1 (PAEnrp1) or neuropilin2 (PAEnrp2) and human umbilical vein endothelial (HUVEC) cells were removed from storage in liquid nitrogen and rapidly thawed in 37°C water bath. The cells were cultured in 100mm plates with 10mL of complete media with appropriate media (Table 1). Media was made complete by supplementing the appropriate media with 10% Fetal Bovine Serum (FBS) and 1% glutamine-penicillin-streptomycin (GPS). Cells were cultured in humidified 37°C incubators at either 5% or 10% CO2 (Table 1). After 24 hours, the media was aspirated and replaced with 10mL of appropriate media. Media was aspirated and replaced with fresh every 4 days until plates were visually fully confluent.

Table 1 | Cell Culture Information: Cell lines were cultured in corresponding media. All cells were placed in humidified 37°C incubators with the listed 5% or 10% CO2.

Cell Line	Media	CO2 %
A375SM Clean	MEM	10
B16F10	DMEM	5
B16F10 VEGFC	DMEM	5
HUVEC	EGM2	5
LM	EGM2-MV	5 or 10
PAE Nrp1	Ham's F12 Modified	5
PAE Nrp2	Ham's F12 Modified	5

Once confluent, media was aspirated and plates were washed with 3mL 0.05% Trypsin ethylenediaminetetraacetic acid (EDTA) followed by an additional 1mL Trypsin-EDTA(Gibco). Plates were incubated at 37°C in humidified incubators for 5 to 10 minutes to allow cells to trypsinize and lift off plates. Trypsin-EDTA was then inactivated using either media or trypsin neutralizing solution (TNS)(Gibco). If TNS was used, cells were centrifuged at 1000rpm for 5 minutes and resuspended in appropriate media. Cells were added to new 100mm plates with 10mL fresh media.

Cells were frozen down as stock for later use by first trypsinizing cells, then resuspending them in freezing media composed of 70% appropriate media (Table 1), 20% (FBS) and 10% Dimethylsulfoxide (DMSO). Cells were pipetted into cryogenic vials and placed in a Mr.Frosty insulated with 250mL isopropanol.

Tubes were frozen overnight to -80°C and then placed in liquid nitrogen for storage.

Protein Isolation

Once visually confluent, cells were placed on ice and washed with phosphate buffered saline twice (PBS). Protein Lysis Buffer was prepared by dissolving one ULTRA mini tablet protease inhibitor in 10ml radio immune precipitation assay buffer (RIPA)(Roche). PBS was removed and 500µL of Lysis Buffer was added. Plates were scraped, ultimately sweeping lysis product to one side of the plate. Liquid was transferred to small tube and allowed to sit on ice for 20 minutes. The tube was then centrifuged at 14,000rpm for 10 minutes to pellet cell bodies, after which the supernatant was removed and frozen as cell lysate and the pellet discarded.

Protein Analysis

Protein Assay

Cell lysates were assayed to determine total protein concentration using a Bio-Rad DC Protein Assay. A standard was prepared in a 96 well plate by serially diluting BSA and pipetting 5µL aliquots in triplicate. 5µL samples were added in duplicate to the plate. 20µL Protein Assay Solution S (Bio-Rad) was added to

1mL of Protein Assay Solution A (Bio-Rad) to yield a 2% A+S solution. 25µL of A+S solution was added to each well except those used to serially dilute the BSA standard. 200µL Protein Assay Solution S (Bio-Rad) the same wells as A+S solution. The plate was allowed to incubate at room temperature for 5 minutes before being read by a VERSAmax microplate reader (Molecular Devices) at 750nm. The standard readings were uses to produce a graph in Microsoft Excel. The best-fit line formula was used to calculate the protein concentrations of each sample.

Casting an SDS-Page Gel for Western Blot

The resolving layer solution was made with 3.75mL of 30% acrylamide/0.8% bis-acrylamide (National Diagnostics), 3.75mL of 4x 1.5M tris-HCl/0.4% SDS pH 8.8 (National Diagnostics), 7.5mL sterilized distilled water, 50µL of 10% ammonium persulfate (APS), and 10µL tetramethylethylethylenediamine (TEMED). This solution was pipetted into a gel mold (Bio-Rad) and topped with a thin layer of methanol to produce a flat surface for the stacking layer. The cast was allowed to incubate at room temperature for approximately 30 minutes to allow the resolving layer to polymerize. The methanol layer was discarded and a stacking layer solution of 1.25mL 0.5 M tris-HCl/0.4% SDS pH 6.8 (National Diagnostics), 0.65mL 30% acrylamide/0.8% bis-acrylamide, 3.05mL of sterilized distilled water, 25µL APS and 5µL TEMED was mixed and pipetted into the gel cast on top of the resolving layer. A 10 or 15 well

gel comb was then placed in the stacking layer with care to prevent any bubble formation in the wells. The gel was allowed to incubate at room temperature for 30 minutes to allow the stacking layer to polymerize. The gel was removed from casting apparatus and wrapped in a wet paper towel, then in plastic wrap and stored at 4°C.

Western Blot

Cultured cells were analyzed for NRP2 expression via western blot. Cell lysate samples were diluted with distilled water to allow loading of 40µg of protein per well. 5µL 6X-reducing SDS Sample Buffer was added to all samples, including Precision Plus Protein Standard #161-0374 (Bio-Rad). All samples and ladder were boiled for 5 minutes and spun down. The SDS-Page gels were immersed in 1X running buffer (Bio-Rad). All wells of the gel were flushed with 1X running buffer to remove salt deposits. 30µL of each prepared sample and ladder was added to each well. The lid was placed on the running apparatus and connected to the power supply. Gels were typically run for 2 hours at 90V. After completion of the run, the gel mold was removed from the apparatus and separated, freeing the gel. The protein samples were then transferred to nitrocellulose membrane (Bio-Rad) by running for 2 hours at 300mA in 1X transfer buffer (Boston Bioproducts). Upon completion, ladder positions were marked on the membrane. The membrane was then blocked by incubating the membrane in 3% milk (3g dry non-fat mile blotting grade blocker (Bio-Rad) in 1X

Tris Buffered Saline (TBS)) for 30 minutes. The membrane was then washed three times in TBS-T (1% Tween 20 (Sigma), 1X TBS) for 10 minutes per wash with agitation. The membrane was incubated with rabbit anti-nrp2 monoclonal antibody (Cell Signaling Technologies) at 1:1000 concentration in TBS-T for 2 hours at room temperature with agitation. The primary antibody solution was then poured off the membrane and saved for later use. The membrane was then washed in TBS-T as previously described, three times for 10 minutes each with agitation. Secondary antibody solution was the prepared with HRP-conjugated anti-rabbit antibody (GE Healthcare UK. Secondary antibody solution was pipetted onto the membrane and incubated for 1 hour with agitation. The membranes were washed in TBS-T a final three times for 10 minutes each with agitation. A 1:1 solution of Oxidizing reagent and Enhanced Luminol Reagent prepared from a Western Lighting Plus-ECL kit (Perkin Elmer). The membrane was incubated in this solution for 5 minutes before exposure.

In vivo

Mouse Genotyping

Three separate lines of mice were maintained, two C57BL/6 “black 6” lines and one NU/J “nude” line (Jackson Laboratories). The primary line maintained was a neuropilin2 knock-out green fluorescing protein (GFP) knock-in black 6

mouse line. To genotype, the mice were anesthetized and a portion of the ear removed. The ear was examined via fluorescence microscopy. Presence of the knockout allele was determined by noting GFP fluorescence in the hair follicle. If GFP fluorescence was present, the ear was analyzed using Transnetyx mail-order genotyping service. This service probed the sample for the presence of a wild-type neuropilin2 gene with presence indicating a heterozygote and absence indicating a knockout. The second black 6 line contained a Cre gene under control of a Lyve-1 promoter, producing a tissue specific knockout upon breeding with a loxP-NRP2 mouse (floxed NRP2) obtained from another lab. These mice were genotyped using Transnetyx with a two-probe system. Samples were probed for the presence of the Cre gene, and then probed for a wild-type Lyve gene. Outcomes of this analysis are seen in (Figure 10).

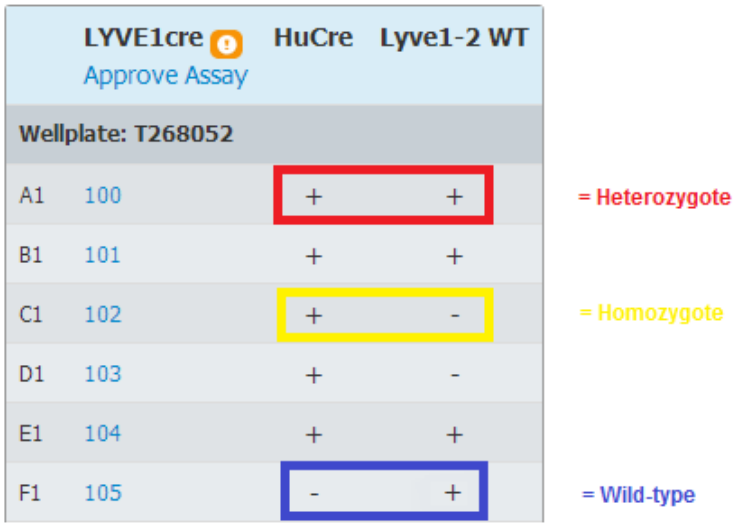

Figure 10 | Genotyping Report: a sample genotyping report from Transnetyx. Genotype is indicated by corresponding colored text. In red, the heterozygote possesses both the Cre gene and the wild-type Lyve-1 promoter. In yellow the homozygote posseses the Cre gene but lacks the wild-type Lyve-1 promoter. In blue, the wild-type mouse does not possess the Cre gene, but does have the wild-type Lyve-1 promoter.

Delayed-Type Hypersensitivity Assay

Ear swelling was examined in wild type and NRP2 knockout mice by inducing a delayed-type hypersensitivity reaction with 4-ethoxymethylene-2-phenyl-oxazolin-5-one (oxazolin)(Aldrich). A 2% oxazolin solution was made in 1:4 olive oil:acetone. To prime the immunologic response, mice were anesthetized and 50µL 2% oxazolin was pipetted onto the ventral abdomen along with 5µL per paw. Mice were left for 5 days to allow their allergic response to oxazolin to develop. After 5 days an initial baseline ear thickness measurement was taken with a No. 7326 caliper (Mitutoyo) and the allergic response was induced by pipetting 10µL 1% oxazolin to each side of the ear to be measured. Measurements were taken every 24 hours thereafter for up to 11 days. Ears were removed from mice on days 1, 4 and 7 for histological analysis. The thickness difference was calculated for each mouse and graphed in Excel.

Tumor Growth Assay

B16F10 melanoma cells overexpressing VEGFC were obtained originally from the Jain laboratory (Isaka et al., 2004) and cultured (Table 1) until plates were visually confluent. Plates were trypsinized and the cells counted using a hemocytometer to give an estimate of cell number needing approximately 1.3 x 10^6 cells per mouse. The cells were spun down and resuspended in enough Hank's Buffered Salt Solution (HBSS) to yield 1.1x10^6 cells per 100µL. Mice

were anesthetized and injected subcutaneously with 100µL of cell solution. Mice were monitored every 48 hours after injection and measurements of length and width of the tumor were taken with a mechanical caliper once the tumors were detectable. Mice were sacrificed when the average of the length and the width exceeded 10mm. The tumor volume was estimated using the formula length*width^2*0.52 and graphed in Excel. Tumors were removed from mice and preserved for histological analysis.

Immunohistochemistry

Fixation

Ears from the DTH assay and tumors from the tumor growth assay were fixed in paraffin or frozen section. Paraffin fixed tissue samples were left in formalin overnight, and then placed in Phosphate Buffered Solution (PBS) for histological preparation. Frozen section tissue samples were snap frozen in OCT using liquid nitrogen.

Immunostaining

Paraffin section slides were de-paraffinized with xylene and re-hydrated to PBS through an ethanol ladder. Antigen retrieval was performed with Proteinase K at 20µg/mL (Roche) at 37°C for 15 minutes. A hydrophobic ring was drawn

around the tissue sample using a marker pen. The slides were washed in PBS three times for 3 minutes per wash. Endogenous peroxidases were blocked with 3% hydrogen peroxide (Sigma) in methanol for 12 minutes at room temperature. The slides were washed in PBS three times for 3 minutes per wash. The slides were blocked with Tris-NaCl-blocking buffer (TNB) for 1 hour at room temperature. Primary antibody was prepared at appropriate concentration in TNB (Table 2). 250µL primary antibody solution was added per slide. Slides were incubated overnight at 4°C. The slides were washed three times for 3 minutes per wash. Secondary antibody solution was prepared at the appropriate concentration in TNB (Table 2). 250µL secondary antibody solution was added to each slide and allowed to incubate for 1 hour at room temperature. During this time the corresponding Vectastain Avidin/Biotinylated Enzyme Complex (ABC) kit (Vector Labs) was prepared. The slides were washed three times for 3 minutes per wash. The ABC kit was pipetted onto the slides and left to incubate for 30 minutes at room temperature. The slides were washed as before. The DAB Peroxidase Substrate or Red Alkaline Phosphatase Substrate kits (Vector Labs) were then added to each slide and observed for stain development (TABLE 2).

Antigen	Marker	1° Ab	1° Conc.	2° Ab	2° Conc.	Vectastain ABC kit	Substrate Kit
CD-31	Pan-vessel marker	Anti-mouse CD-31 (Cell Signaling Technologies #3568)	1:200	Biotinylated anti-syrian hamster	1:200	Elite (Vector Labs pk-6200)	DAB kit (Vector Labs SK-4100)
Lyve-1	lymphatic marker	Anti-mouse LYVE-1 (ReliaTech 10 2-PA50)	1:80	Biotinylated anti-rabbit	1:200	Standard (Vector Labs ak-5000)	Red AP kit (SK-5100)
Podo	lymphatic marker	Anti-mouse Podo (Relia-Tech 103-M40)	1:100	Biotinylated anti-rat	1:200	Standard (Vector Labs ak-5000)	Red AP kit (SK-5100)

Table 2 | Immunohistochemical Staining: listed are the antigen, marker, 1° and 2° Ab concentrations, Vectastain ABC kit and Substrate kit for each

RESULTS

NRP2 deficient mice show maintained ear swelling

Due to NRP2's extensive expression in cutaneous lymphatic vessels, we hypothesized that NRP2 KO mice would maintain ear swelling in response to oxazalone-induced DTH reaction. 24 hours after induction, both wild-type (WT) and KO mice displayed swelling of more than 300μm, although the KOs did not swell to as great a degree as our wild-type mice (Figure 11a). After 72 hours, the KO mice showed a greater degree of swelling than their WT littermates, which persisted until the ears were taken on day 7 for histological analysis (Figure 11a).

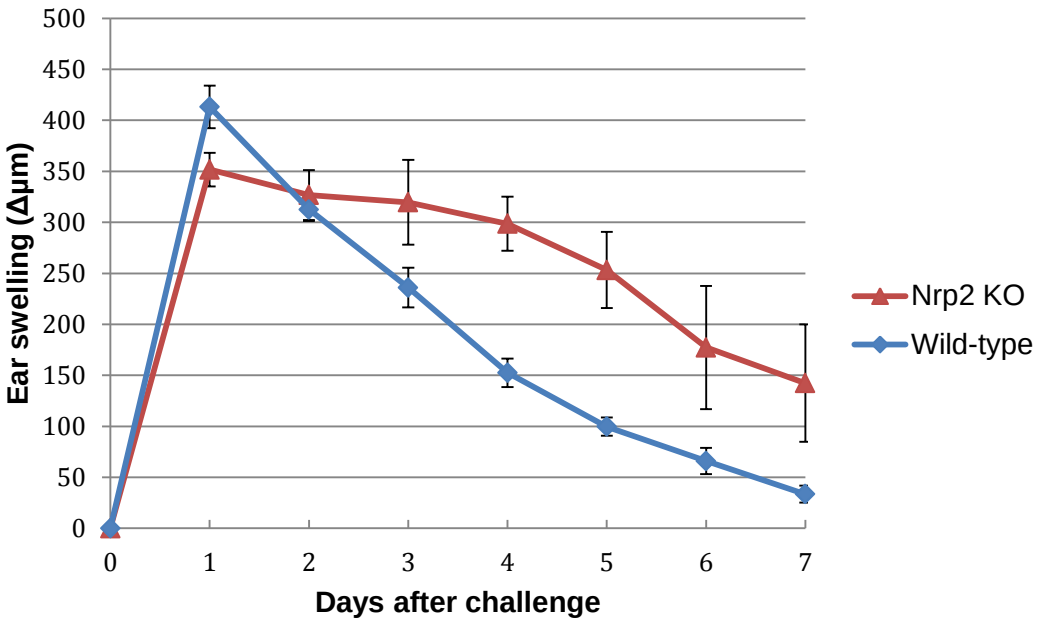

Figure 11a | DTH Assay: NRP2 KO mice (Red triangle, red line) show prolonged ear swelling in comparison to WT littermates (blue diamond, blue line). Ear swelling is expressed as difference in μm of the ear.

Previously, this experiment was conducted by Nick Levonyak from the lab (Figure 11b). During that experiment, KO and WT littermates showed the same degree of swelling. From day 2 onward, the KO mice showed significantly increased swelling over the WTs, which persisted to the end of the 11 day trial. The KO mice were monitored thereafter, finding that swelling was still persistent at 21 days.

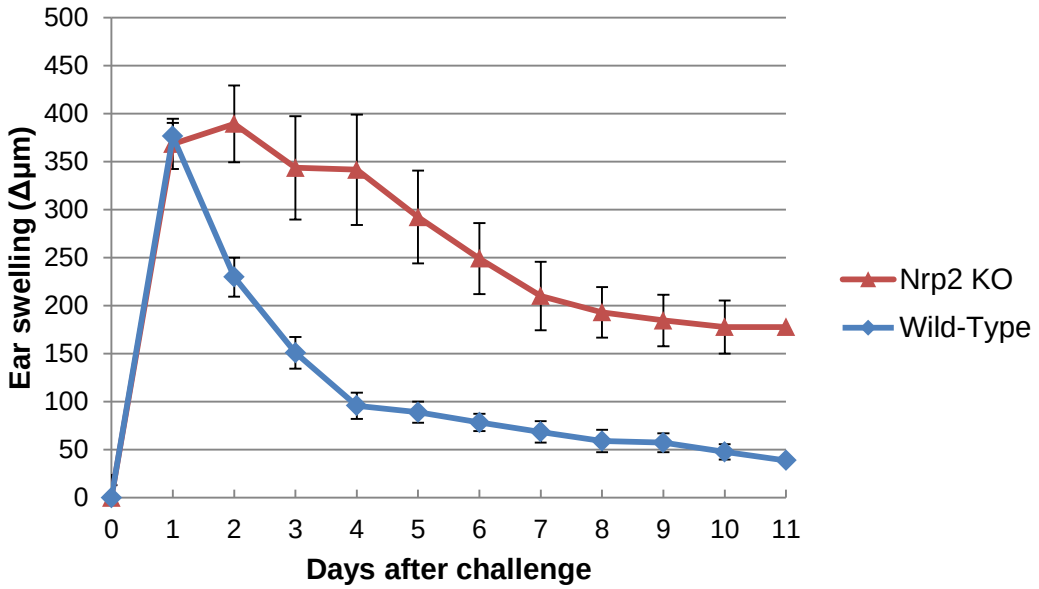

Figure 11b | DTH Assay: Nrp2 KO mice (Red triangle, red line) show prolonged ear swelling in comparsion to WT littermates (blue diamond, blue line). Ear swelling is expressed as difference in μm of the ear.

Histological analysis was performed on WT ears taken at days 1, 4 and 7, and on KO ears taken at day 1 and 7. Marked swelling can be seen in both WT and KO ear sections from day 1 (Figure 12). Previous experiments by Nick Levonyak have shown the characteristic prolonged swelling in histological sections of KO ears and a return to normal in WT ears. Ears were stained for the pan-vessel marker CD-31 and lymphatic marker Lyve-1. Preliminary examination of CD-31 stained ears did not reveal obvious differences between the WT and KO at any day 1 or day 7. The mean vessel diameter and vessel density should be analyzed via software to confirm this. Lyve-1 staining revealed drastic dilation of lymphatics on day 1 in both the WT and the KO (Figure 11). We hypothesize that the prolonged swelling found in NRP2 KOs is due to maintained dilation of large lymphatic vessels. Comparison of Lyve-1 staining on day 7 indicates that the lymphatic vessels of NRP2 KO mice may remain dilated beyond those of their WT counterparts. Again, quantitative analysis via software is necessary to make a firm conclusion in this regard.

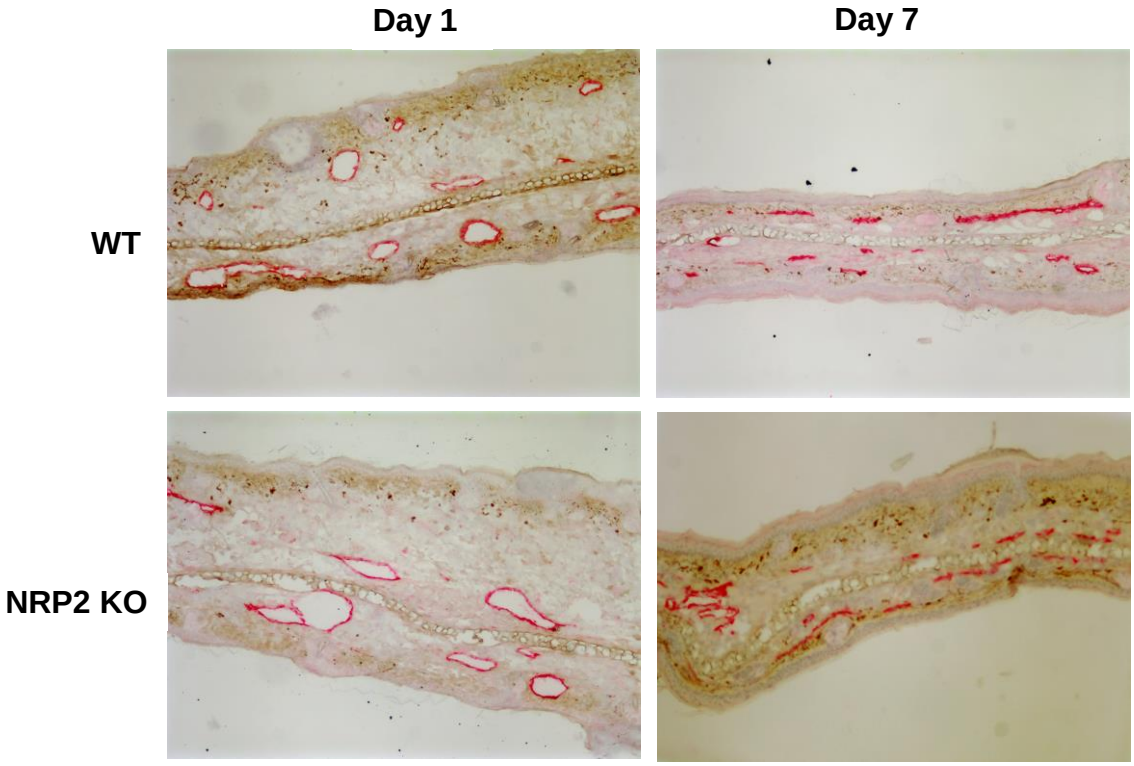

Figure 12 | Histology of DTH ear swelling in NRP2 KO and WT mice: Ear sections of WT (top) and KO (bottom) mice (10X). Sections were stained with Lyve-1, a lymphatic marker (red), then counter-stained hematoxylin (faint blue-grey).

B16F10 Melanoma expresses NRP2

Expression of NRP2 was verified via western blot in three candidate B16F10 melanoma cell lines for our tumor growth assay. Two of these cell lines were transfected to over-express VEGFC while the third was a normal B16F10 melanoma line. All three were confirmed to express NRP2 (Figure 13).

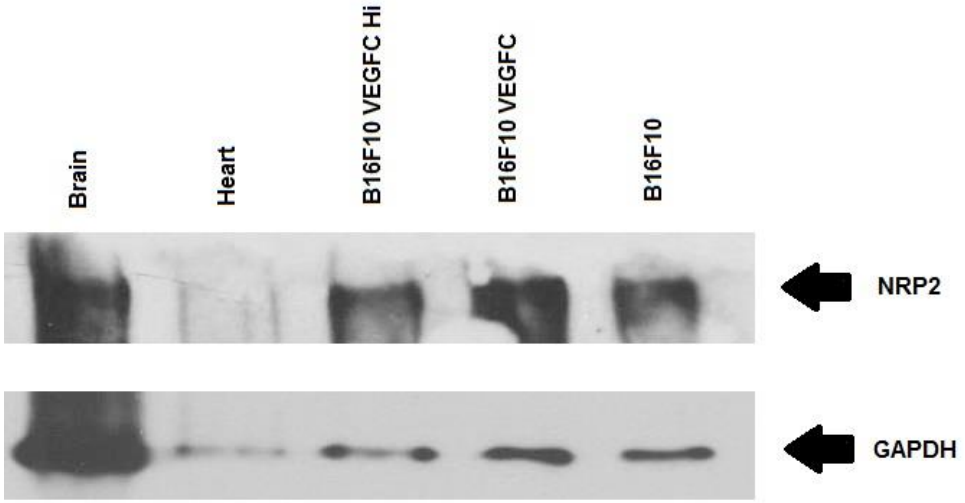

Figure 13 | B16F10 Melanoma expresses NRP2: NRP2 protein levels were verified by a band at ~130kDa. Loading equivalence was checked via GAPDH protein levels, seen in bands at ~37kDa. Brain lysate was used as a positive control, while heart lysate was used as a negative control for NRP2 protein presence.

NRP2 deficient mice have smaller tumors with smaller blood vessels

In our tumor growth assay, we injected one million B16F10-VEGFC cells, which over-express VEGFC and were shown to express NRP2 in the western blot shown above. We hypothesized, based on previous studies by Nick Levonyak, that the tumors would be smaller in the NRP2 KO mice. This was confirmed by the tumor growth assay, which showed that NRP2 KO mice have significantly smaller tumors that their WT littermates from day 9 after injection onwards (Figure 14). We believe that the tumors in the KO mice remain dormant and are not capable of triggering the angiogenic switch.

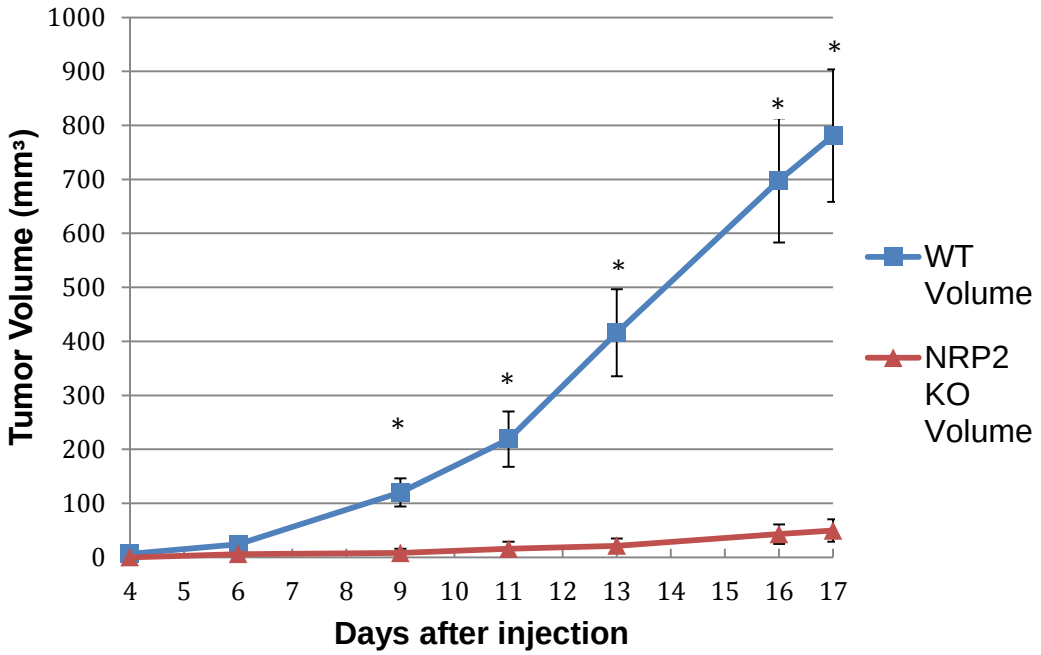

Figure 14 | NRP2 KO mice have smaller tumors: NRP2 KO mice (red triangle, red line) display significantly reduced tumor growth from their WT littermates (blue square, blue line). * indicates P<.01.

WT mice were sacrificed at day 17 after injection due to tumor burden and tumors were resected for histological preparation. Tumors from the KO mice were surgically removed and prepared for histology in the same manner. Tumor sections were stained for the pan-vessel marker CD-31 and the lymphatic marker Lyve-1. We hypothesized that tumors from NRP2 KO mice would display a reduced mean vessel diameter and vessel density. In addition, we expected to see increased intratumoral lymphatics. Tumors grown in NRP2 KO mice showed smaller vessels than their WT counterparts (Figure 15). However, vessel density did not appear to be affected. Both diameter and density should be quantified via software to confirm this. Intratumoral lymphatics did not appear to increase,

although they should be counted via software to confirm this finding. In addition, peritumoral lymphatics should also be quantified in the same manner.

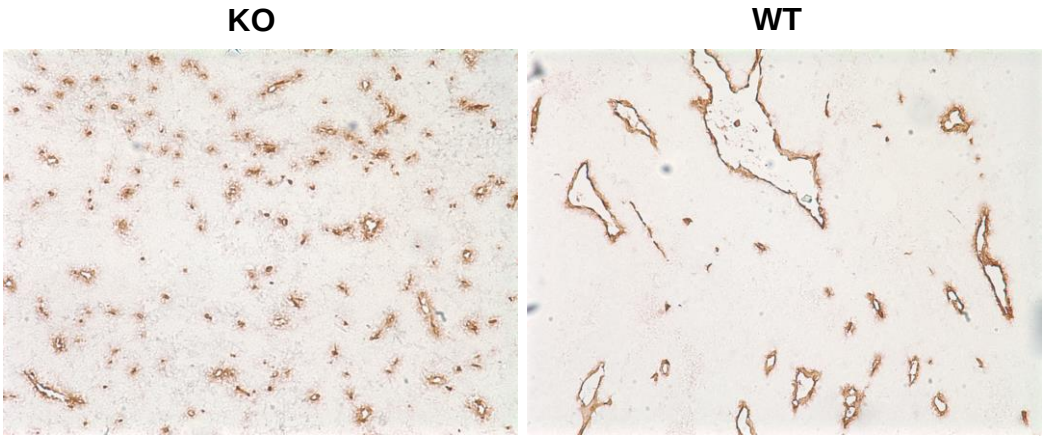

Figure 15 | NRP2 KO mice have smaller vessel lumens: Tumors were removed from KO (left) and WT (right) mice and placed in frozen section. Sections were stained for CD-31 (brown). Images were taken within the tumor.

DISCUSSION

Our increasing knowledge of the diversity of NRP2 expression and function demands deeper exploration. In the DTH assay, we propose that the prolonged swelling in NRP2 KO ears is due to maintained dilation of large lymphatic vessels. These vessels may remain dilated due to the mutant mouse's inability to initiate lymphangiogenesis through NRP2 signaling. In the future, we will confirm that angiogenesis/ lymphangiogenesis are occurring by staining for the proliferation marker Ki-67. Without smaller lymphatic vessels which arise through lymphangiogenesis, the remaining larger lymphatics are the only route for fluid to drain from the interstitium. We propose that these large lymphatics remain so dilated that they are not functional, possibly because the lymphatic valves are so far apart that they cannot prevent backflow. Investigating the functionality of lymphatic vessels has proven difficult for the lab, due to the difficulty in injecting dye into a mouse ear that is typically ~500μm thick. In addition, our black mice make it difficult to see dark colored dyes in the ear, further confounding our efforts, although in the future we intend to attempt this technique with radio-labelled dyes. Recently, it was shown that the SEMA3A-NRP1-Plexin signaling axis is essential in lymphatic valve formation (Bouvree et al., 2012). It is plausible that NRP2 may also play a role in this process due to its high expression on lymphatic vessels. Differences in the KO and Wild-type vasculature must also be considered. It is possible that the blood vessels in the

KO are leakier, thus resulting in a net leakage of fluid into the interstitium even if the KO maintains normal lymphatic functionality

Angiogenesis/ lymphangiogenesis do not represent the only possible explanations for the prolonged swelling seen in NRP2 KO ears. Because NRP2 is also expressed on cells of the immune system, there may be an as yet undefined role for NRP2 in the migration of these cells to the site of inflammation. This is another area that has been explored in NRP1 studies, where it was shown that NRP1 mediates T-cell migration to tumor sites in a VEGF-dependent manner (Hansen, 2013). It is plausible that NRP2 deficient mice are not capable of bringing in leukocytes to resolve the cause of their inflammation, which could lead to the prolonged inflammation we observed. To address this, we propose the use of tissue specific knockouts to observe the specific roles NRP2 plays in cells that express it. Our lab plans to repeat the DTH experiment using a lymphatic-specific NRP2 KO to examine the role NRP2 plays in physiologic inflammation without also implicating NRP2 functions elsewhere in the organism. The data from such a study will be especially insightful when compared with a leukocyte-specific NRP2 KO currently being developed by a collaborating lab. Finally, a tamoxifen-induced NRP2 KO mouse would allow us to address any difference that occurs during the development of the mouse vasculature due to NRP2 being knocked out because Nrp2 would only be knocked out during the experiment in this model, not during development of the vasculature. Taken together, the data we present here regarding physiological angiogenesis and

lymphangiogenesis open up more questions than they answer, but further study of this phenomenon using the DTH assay promises to deepen our understanding of NRP2's function.

The drastically reduced tumor growth seen in the NRP2 KO mice in our B16F10-VEGFC tumor growth assay gives great precedent to the possibility of an antiangiogenic therapy being highly effective. This result is given even greater weight when considered alongside the previous work of Nick Levonyak in our lab, which showed smaller, less metastatic pancreatic cancer tumors in NRP2 KO mice. Examination of the CD-31 staining reveals that the vessels in the KO tumors are much smaller in diameter, although they appear to be equally numerous as in their WT counterparts. We believe the reduction in tumor growth seen in the NRP2 KO is due to this inhibition of effective angiogenesis, depriving the hungry tumor cells of nutrients and oxygen. Work by Matt Migliozzi in our lab has shown that SEMA3F treatment can be effective at preventing tumor growth and metastasis in nude mice with A375SM human tumors, which are also NRP2 positive. In the future, we would like to repeat this tumor growth assay with B16F10 melanoma in WT mice and apply a SEMA3F treatment, which we believe will be highly effective at reducing both tumor growth and metastasis.

Thus, in our KO model, anti-NRP2 therapy is only simulated in the host, since the tumor can still respond to any NRP2 signaling produced within the tumor microenvironment or produced by the host. In a clinical setting however,

this would not be the case, as both patient and tumor (depending on which tumor type) would be NRP2 positive and able to respond to anti-NRP2/ antiangiogenic signaling. We expect this would greatly increase the antitumor effects seen here not only because of SEMA3F's antiangiogenic signaling mediated through the NRP2-Plexin pathway, but also due to its inhibition of VEGF-VEGFR binding. In the future, we would like to repeat this experiment with a NRP2-negative tumor in NRP2 KO mice, where we would expect no difference from the WT, due to lack of NRP2-axis signaling from the tumor. In addition, B16F10 melanoma is highly aggressive, so much so that the mice are typically sacrificed in ~2.5 weeks due to tumor burden. By repeating this experiment with a slower growing tumor, we would be able to determine if the reduction in growth is maintained or if the tumor is capable of surmounting the lack of host NRP2 by signaling through other pathways. Repeating this experiment with a tumor which expresses Nrp2 on the tumor cells but not the vasculature, such as T241, we would expect to see no difference between WT and our KO.

In one of his final reviews, Dr. Judah Folkman stated his belief that within the next decades, antiangiogenic and angiogenic therapies would revolutionize medicine and bring relief to millions suffering worldwide from a myriad of diseases. The data presented here show that we are approaching this time, especially in regard to antitumor agents such as SEMA3F, but the data also show that for every door we close, two more open. As always, further study is required to deepen our understanding of angiogenesis, which appears to become

more complicated to more it is investigated. One aspect that has not changed however, is the promise held within control over this powerful biological phenomenon

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CURRICULUM VITAE

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YEAR OF BIRTH: 1989

Education

Boston University School of Medicine, Boston, MA
Master of Arts, Medical Science
Expected graduation: May 2013

The University of Vermont, Burlington, VT
Bachelor of Science, Biochemistry
Graduated: May 2011

Relevant Experience

- **Harvard Medical School/ Children's Hospital, Boston, MA**
  Vascular Biology Program – Graduate Research Student (October 2013 – present)

- **The University of Vermont, Burlington, VT**
  Pharmacology Department – Undergraduate Researcher (2010)

- **National Institute of Health, Bethesda, MD**
  National Heart, Lung and Blood Institute – Intern (2006/2007)

Volunteer Experience

- **bWell Center, Boston University Medical Center, Boston, MA** – Volunteer (05/2013-09/2013)
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- **APEX Weekend Tutor, Prospect Hill Academy, Cambridge, MA –** Volunteer Tutor (2013-present)