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The role of neuropilin 2 in physiological and pathological angiogenesis

Levonyak, Nicholas S.

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Thesis

THE ROLE OF NEUROPILIN 2 IN PHYSIOLOGICAL AND PATHOLOGICAL ANGIOGENESIS

by

NICHOLAS S. LEVONYAK

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Approved by

First Reader

Matthew Nugent, Ph.D.
Professor of Biochemistry

Second Reader

Diane Bielenberg, Ph.D.
Assistant Professor of Surgery
Harvard Medical School
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THE ROLE OF NEUROPILIN 2 IN PHYSIOLOGICAL AND PATHOLOGICAL ANGIOGENESIS

NICHOLAS S. LEVONYAK
Boston University School of Medicine, 2013
Major Professor: Matthew Nugent, Ph.D., Professor of Biochemistry

ABSTRACT

Neuropilin 2 (NRP2) is a transmembrane receptor protein that was first discovered on neurons and then endothelial cells. On endothelial cells, it serves as co-receptor with the vascular endothelial growth factor receptor (VEGFR) to bind VEGF and induce a pro-(lymph)angiogenic intracellular signal. In addition to VEGF, NRP2 is also a receptor for semaphorin 3F (SEMA3F), which upon binding to NRP2 and Plexin A1 induces a strong anti-angiogenic signal. It is our hypothesis that SEMA3F could be a potentially effective treatment for metastatic cancers. A greater understanding of the regulation and expression of its receptor, NRP2, is needed. While NRP2 has been most robustly studied for its role in the vasculature, recent studies have shown that it is expressed on other cell types as well such as dendritic cells, T-cells, and visceral smooth muscle cells. In this study, we used western blot and immunohistochemistry to explore various different organs and cell types in an attempt to locate other novel locations of NRP2 expression. In particular, we found several new tissues that express NRP2
including the uterus and adipose tissue. Interestingly, NRP2 is expressed much more strongly in brown adipose tissue than white adipose tissue. In addition, we found that expression of NRP2 in adult organs is weaker than during development but is apparent, particularly on lung vascular EC and the intestinal lymphatic lacteal. In addition, we used several in vivo angiogenesis assays in order to help understand how NRP2 is regulated in the mature vasculature. We found that in the cutaneous wound healing assay, Nrp2 knockout mice healed at the same rate as their wild-type and heterozygous littermates. However, when delayed type hypersensitivity reactions were induced in these mice, the Nrp2 knockouts demonstrated persistent swelling over a longer period of time in comparison to littermates. We also examined how the loss of NRP2 affected pathological angiogenesis by orthotopically injecting a murine syngeneic pancreatic adenocarcinoma cell line (Panc0H7) into the Nrp2 knockout mice. These mice displayed smaller tumors, less grossly apparent metastases, and less ascites. Taken together, these data suggest that NRP2 is important in physiological and pathological angiogenesis. Anti-NRP2 or SEMA3F strategies may represent promising anti-metastatic therapies.
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<tr>
<td>a-FGF</td>
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<td>DTH</td>
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<td>EC</td>
<td>endothelial cell</td>
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<td>horseradish peroxidase</td>
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<td>LEC</td>
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<td>MMP</td>
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<td>NIP</td>
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<td>Nrp</td>
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<tr>
<td>NRP</td>
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<tr>
<td>PDAC</td>
<td>pancreatic ductal adenocarcinoma</td>
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<td>PDGF</td>
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<td>SCG</td>
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<td>SMC</td>
<td>smooth muscle cell</td>
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<td>Abbreviation</td>
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<tr>
<td>TEMED</td>
<td>tetramethylethylenediamine</td>
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<td>TNB</td>
<td>Tris-NaCl blocking buffer</td>
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<td>TGF-α</td>
<td>transforming growth factor alpha</td>
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<td>VEGFR</td>
<td>vascular endothelial growth factor receptor</td>
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INTRODUCTION

The Process of Angiogenesis

Angiogenesis is the process by which new blood vessels are formed from existing blood vessels. This process is dependent upon endothelial cells (ECs) which line the lumen of all blood vessels and are the sole type of cell present in capillaries (W. Auerbach & R. Auerbach, 1994). This process is separate from vasculogenesis which involves the formation of blood vessels in embryogenesis via precursor angioblasts (Noden, 1989).

The angiogenic cascade can be stimulated both physiologically and pathologically. In both cases, this occurs in response to hypoxic cellular conditions which leads to the secretion of growth factors that induce ECs to proliferate and sprout (Chung, Lee, & Ferrara, 2010). ECs then secrete matrix metalloproteinases (MMPs) which are proteolytic enzymes that degrade the basement membrane and allow for newly formed ECs to migrate towards the growth factors into the perivascular stroma (Efferet & Strohmeyer, 1995). ECs proliferate from the edge of the migrating vessel and then organize themselves to form new three-dimensional capillary structures (W. Auerbach & R. Auerbach, 1994). The distinction between physiological and pathological angiogenesis involves the source of the growth factors. In physiological angiogenesis, the source is a hypoxic environment (stromal cells including fibroblasts, epithelial cells or immune cells) whereas in pathological angiogenesis, the source is a
tumor cell aberrantly releasing growth factors which may or may not be linked to hypoxia.

**Angiogenesis and Metastasis**

Angiogenesis is required for tumors to grow beyond one millimeter in size, and it is also an essential aspect of cancer metastasis (Folkman, 1971). Cancer metastasis is the process by which tumor cells travel within blood vessels and lymphatic vessels into distant sites in the body (Sturk, 2004). The metastatic process is sequential and requires the following in order to occur: primary tumor growth, tumor angiogenesis, tumor cell detachment and invasion into capillaries, survival within the circulation, eventual embolism and arrest within the capillaries of distant organs, adhesion to the vessel wall and extravastation, and finally the establishment of a new microenvironment at the secondary site which requires both proliferation and angiogenesis (Fidler, 2003; Figure 1).

This process is similar for metastasis via lymphangiogenesis with some key differences. Many lymphatic capillaries that drain tumors have either missing basement membranes or ones that lack laminin and collagen XVIII (Skobe et al., 2001) which may allow tumor cells to possess less invasive characteristics in order to invade the lymphatic system. In addition, extravasation may not be required in lymphatic metastasis (Sleeman, 2000). Ultimately, the tumor cells must reach the bloodstream. This can occur through several different mechanisms including drainage from the thoracic duct into the venous blood or
invasion into blood vessels within a lymph node itself (Haagensen et al., 1972 and Tobler & Detmar, 2006).

Figure 1 | Tumor Metastasis | The process of tumor metastasis involves a very precise step-wise process that begins when the primary tumor grows large enough to require a blood supply. Adapted from Fidler, 2003.

The process of cancer metastasis is incredibly detailed and requires both a highly invasive tumor cell and a new microenvironment that will sustain secondary tumor growth. This is the “seed”, invasive tumor cell, and “soil”, secondary site, hypothesis and both factors are absolute requirements in cancer metastasis (Fidler, 2002; Meric-Bernstam & Pollock, 2010).

Dr. Judah Folkman was the first to hypothesize that tumors required angiogenesis in 1971 when he discovered that tumors remain in a state of
dormancy unless they are able to establish a new blood supply (Folkman, 1971).

His discovery sparked the investigation of anti-angiogenic therapies for metastatic cancers which has been one of the most highly researched topics in cancer over the past 40 years. In addition, angiogenesis has been investigated in response to physiological conditions requiring new vessel growth that eventually resolves such as during endometrial thickening in the female menstrual cycle, cutaneous thickening following cell mediated Type IV hypersensitivity reactions (delayed type hypersensitivity) and those that do not resolve including wound healing or tissue regeneration (liver regeneration). Pathologic angiogenesis occurs during macular degeneration, psoriasis, arthritis, and cancer.

**Vascular Endothelial Growth Factor and its Receptors**

One of the key components of angiogenesis is the idea of the angiogenic switch. Dr. Folkman described how the body tightly regulates angiogenesis by balancing proangiogenic and antiangiogenic molecules (Hanahan & Folkman, 1996). Under normal physiological conditions or homeostasis, inhibitors of angiogenesis outweigh or equal the promoters and new blood vessels are not formed. However, when a pathological phenotype or hypoxic cell begins to secrete proangiogenic growth factors, the balance can be shifted towards angiogenesis (Figure 2).
The scale can be tipped towards angiogenesis by a host of endogenous proangiogenic growth factors including acidic fibroblast growth factor (a-FGF, FGF-1), basic fibroblast growth factor (b-FGF, FGF-2), vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), transforming growth factor alpha (TGF-α), platelet derived growth factor (PDGF), placental growth factor (PIGF), interleukin-8 (IL-8), and angiopeotin-1 and 2 (Ang-1 and Ang-2) (Hannahan & Folkman, 1996; Longo, 2012). VEGF, first discovered as vascular permeability factor, is perhaps the most crucial and most studied of the growth factors which regulate tumor angiogenesis (Dvorak et al., 1995). While it was first discovered in regards to vascular permeability, its role has also been confirmed in stimulation of EC proliferation, tube formation, and induction of
endothelial cells to synthesize proteolytic enzymes (Longo, 2012). Since its discovery in 1983, five VEGF-related genes have been identified including VEGF-A, VEGF-B, VEGF-C, VEGF-D and VEGF-E, all of which are approximately 40 kDa as dimers (Ferrara, 1996). Posttranslational modification of the VEGF gene makes several different isoforms, but VEGF_{121} and VEGF_{165} (denoted by the number of amino acids in the protein; 121 in human and 120 in mouse; 165 in human and 164 in mouse) are the two most common forms found overexpressed in human tumors (Takahashi, 2011). VEGF_{165} has a heparin-binding domain (exon 7) that aids in binding to the extracellular matrix and keeps the protein local to its site of secretion, while VEGF_{121} lacks this domain and is freely soluble and acts at a distance (Klagsbrun, D’Amore, 1996).

The VEGFs bind to two distinct families of receptor proteins: three tyrosine kinase VEGF receptors and two other co-receptors known as neuropilin-1 and neuropilin-2 (NRP1, NRP2). All three VEGFRs have six-seven extracellular immunoglobulin domains and tyrosine kinase machinery in the cytosolic region (Ferrara, Gerber, & LeCouter, 2003). Upon binding to VEGF, the receptor undergoes typical tyrosine kinase behavior: dimerization and autophosphorylation which initiates an intracellular signaling cascade. VEGFR1 binds VEGF-A, VEGF-B, and PIGF and is found on macrophages, monocytes, and ECs (Roskoski, 2008). In general, VEGF-A binds VEGFR1 with the highest affinity but the receptor’s tyrosine kinase activity is relatively low in comparison to its other family members (Seetharam et al., 1995). VEGFR2 binds VEGF-A,
VEGF-C (mature form), VEGF-D (mature form), and VEGF-E and is found on ECs, hematopoietic cells, neuronal cells, and some tumor cells (Roskoski, 2007). Although, it has a slightly lower affinity for VEGF-A than VEGFR1, it induces a much greater tyrosine kinase activity and is thought to be the major regulator of VEGF-induced cell survival, mitogenesis, and angiogenesis (Ferrara, Gerber, & LeCouter, 2003). It primarily signals through the PLCγ-PKC-Raf-MEK-MAPK pathway which promotes DNA synthesis in ECs (Shibuya & Claesson-Welsh, 2003). In addition, it can activate the Shb-PI3K pathway which leads to actin stress fiber organization and cell migration (Roskoski, 2007). Due to its major role in angiogenesis, VEGFR2 has been highly investigated for potential antiangiogenic therapies. VEGFR3 is the smallest of the VEGF receptors at 110 kDa, binds VEGF-C and VEGF-D (both pro- and processed forms), and is only found on vascular ECs during development (Kukk et al., 1996). After development, it is expressed solely in the lymphatic vasculature by lymphatic ECs (Kaipainen et al., 1995). Transgenic VEGFR3 knockout mice are lethal at mid-gestation which implies that the receptor is essential for normal vasculature development (Dumont et al., 1998).
Figure 3 | The VEGF Receptors | VEGFR1, VEGF2, and VEGFR3 are the three tyrosine kinase receptors for the VEGF family of growth factors. Each ligand has differing affinity for each receptor. NRPs are also receptors for VEGF but are not shown. Adapted from Ruiz de Almodovar et al, 2009.

The Neuropilin Receptors

VEGF is also a ligand for a second family of protein receptors, the neuropilins (human: NRP; mouse: Nrp). Two NRPs have been identified – NRP1 and NRP2. They are each 130 kDa transmembrane glycoproteins which share 45% amino acid homology. Despite such similar primary structure, the NRP1 and
NRP2 genes map to entirely different chromosomes. In humans, they map to 10p12 and 2q34, respectively (Klagsbrun, Takashima & Mamluk, 2002). Each NRP protein has five extracellular domains which have been coined a1, a2, b1, b2, and c (Klagsbrun & Eichmann, 2005). In addition, each protein has an intracellular domain of 40 amino acids that has been shown to interact with a protein known as neuropilin interacting protein (NIP) or RGS–GAIP-interacting protein (GIPC) but no known signaling motif has yet been elucidated (Cai & Reed, 1999). Interestingly, both NRP proteins share the identical final three amino acids at their C-terminal end, SEA (Bielenberg et al., 2006). Recently, deletion of the SEA domain of NRP1 has been shown to inhibit PIGF signaling in medulloblastoma indicating that it may have a key role yet to be discovered (Snuderl et al., 2013).

The first identification of NRP came in 1987 when Fujisawa described an antigen that was specific for the monoclonal antibody A5 (Takagi et al., 1987). This mAb targeted the neuropile in the optic tectum of *Xenopus laevis* which ultimately led to NRP’s neurological nomenclature. It was not until 1995 that this antigen was given the name neuropilin when it was revealed by immunohistochemistry to be present on *Xenopus* principal olfactory axons (Satoda et al., 1995). In that same year, NRP was shown to be essential in development when Fujisawa described that chimeric mice overexpressing Nrp were embryonic lethal. The abnormalities found in the embryos included both nervous system and vascular defects. In particular, they found increased
numbers and dilation of blood vessels, malformed hearts, and defasciculation of nerve fibers (Kitsukawa et al., 1995). In 1997, a second NRP protein (NRP2) was identified in the developing mouse spinal cord which was expressed differentially from NRP1 (Chen et al., 1997).

The first known ligand for NRP, the semaphorin class III (SEMA3) family of axonal guidance molecules, was also identified in 1997 (Kolodkin et al., 1997). The semaphorins are divided into eight classes and the SEMA3s are a group of seven proteins which are all approximately 100 kDa and the only class that binds to NRPs (Bielenberg & Klagsbrun, 2007). The SEMA3 family has seven members, SEMA3A, B, C, D, E, F and G. In particular, SEMA3F specifically binds NRP2 while SEMA3A specifically binds NRP1, and SEMA3B binds both NRPs. The first SEMA3 reported to interact with NRP was SEMA3A which was shown to repel axons and cause a functional collapse of growth cones in the dorsal root ganglia (Fujisawa, 2002). In particular, the SEMA3s bind to the a1a2 and b1b2 domains of NRP (Mamluk et al., 2002) SEMA3s dimerize via a cysteine residue and this dimerization is essential for NRP binding (Adams et al., 1997). In the neuronal guidance pathway, NRP/SEMA3 requires another family of receptors, the plexins, in order to provide signaling capabilities (Puschel, 2002). In particular, SEMA3F signals via NRP2/Plexin A1 in tumor cells and EC (Shimizu et al., 2008). SEMA3E is an exception within the class 3 SEMAs as it does not bind directly to either NRP but binds directly to Plexin D1 (Klagsbrun and Shimizu, 2010).
VEGF was found to be a ligand for NRP on ECs in 1998 (Soker et al., 1998) and was later shown to bind to the b1b2 domain in NRP1 (Mamluk et al., 2002). Both NRPs bind VEGF$_{165}$ but vary in their specificity for the other VEGF family members. In particular, NRP1 binds VEGF-A,B, E, and PIGF2 while NRP2 binds VEGF-A,C,D and PIGF2 (Klagsbrun, Takashima, & Mamluk 2002; Gaur et al., 2009). Mechanistically, VEGF-A binds to both NRP1 and VEGFR2 forming a bridge between each receptor and enhancing the angiogenic signal (Soker et al., 2002). NRP2 is a co-receptor for VEGF-A with VEGFR2 and for VEGF-C/D with VEGFR3 (Favier et al., 2006). Because both the SEMA3s and VEGFs require binding to the b1b2 domain, they are competitive inhibitors of one another; and VEGF and SEMA3 have similar binding affinities for NRP (Mamluk et al., 2002).
Figure 4 | Neuropilin and its Ligands | A) The SEMA3s and their specificity for both NRP1 and NRP2. NRP requires the plexin family of receptors in order to bind the SEMA3s. B) The VEGF family of growth factors and its specificity for the NRP receptors. These require the VEGF family of receptors as co-receptors and are expressed differentially on cells. From Gaur et al., 2009.

Similar to the Nrp1 overexpressing chimeric mice, Nrp1 knockout mice are embryonic lethal at E12.5-E13.5 (Kawasaki et al., 1999). These embryos
displayed a greatly disorganized vasculature in the yolk sac suggesting that NRP1 is essential in embryonic vessel formation. Embryos that are null for both Nrp1 and Nrp2 show an even more drastic phenotype than the Nrp1 knockouts. These embryos survived only until E8.5 and presented with numerous hemorrhages and a vascular phenotype similar to VEGF or VEGFR2 knockout mice (Takashima et al., 2002).

**Nrp2 Transgenic Mice**

Nrp2 knockout mice show a loss in small lymphatic vessel and capillary formation during development but are viable (Yuan et al., 2002; Figure 5). This severe reduction in small lymphatic vessel number was found in many different tissues and correlated with a decrease in DNA synthesis in LECs. Other vessel types including arteries, veins and large lymphatic vessels all developed normally (Yuan et al., 2002). More recently, it was shown that double heterozygote Nrp2/VEGFR3 mice demonstrated an inability to sprout lymphatic vessels in response to VEGF-C only cementing the observation that proper Nrp2 function is important in lymphangiogenesis (Xu et al., 2010).

The abnormal lymphatic vessel structure in Nrp2 knockout mice has led many to believe that NRP2 may play a key role in lymphangiogenesis and cancer metastasis. An antibody that targeted the b1b2 binding domain of Nrp2 was shown to decrease VEGF-C mediated tumor lymphangiogenesis using a murine corneal micropocket assay (Caunt et al., 2008). In addition, it was found that the
anti-Nrp2 antibody caused a reduction in metastasis and concurrent disruption of tumor lymphatic vessel formation.

Despite its largely normal cardiovascular phenotype, the Nrp2 deficient mice show significant loss of neuronal function. In particular, they demonstrate defects in cranial nerve development, spinal sensory axons, and in hippocampal mossy fiber axons (Chen et al., 2000). This phenotype is likely observed as a result of loss of SEMA3F mediated repulsion which normally guides nerves into their proper orientation via Nrp2.

Most recently, Nrp2 knockout mice were shown to have an increase in smooth muscle (SM) dependent bladder contractility. The mice demonstrated increased bladder filling pressures and enhanced tension generation when bladder tissue when investigated ex vivo (Bielenberg et al., 2012). From this data, it is likely that Nrp2 plays a key role in relaxing the bladder and is now being investigated as a potential target to regulate visceral SM contractility.

In our laboratory, we have both Nrp2\textsuperscript{+/LacZ} and Nrp2\textsuperscript{+/gfp} transgenic mice. In both cases, the mice have an insert after exon 1 where either LacZ or GFP is knocked-in while Nrp2 is knocked out. The mice maintain the endogenous Nrp2 promoter and therefore the expressed LacZ or GFP represents endogenous Nrp2 in vivo. Interestingly, despite their similarities in genetic creation, the Nrp2\textsuperscript{LacZ/LacZ} mice die at birth whereas the Nrp2\textsuperscript{gfp/gfp} mice are viable.
Neuropilin Expression

Despite being first encountered in neuropiles, a constantly growing knowledge of NRP has shown the receptor to be found in a variety of different organs and cell types (Gagnon et al., 2000). In the nervous system, NRP1 is expressed on both sensory and sympathetic neurons. NRP2, however, is only found on sympathetic neurons and, thus, only sympathetic neurons such as the superior cervical ganglia (SCG) are repelled by SEMA3F (Bagri and Tessier-Lavigne, 2002).

In the embryonic endothelium, there is also a stark contrast in NRP expression. In the early vascular structures of the chick known as blood islands, both NRP1 and NRP2 are expressed. Further along in development, however, NRP1 and NRP2 are found preferentially on arterial and venous EC, respectively.
(Herzog, Kalcheim, Kahane, Neufield, 2001). In addition, lymphatic EC have been shown to preferentially express NRP2 which gives further precedent to the lymphatic phenotype found in the Nrp2 knockout mice (Yuan et al., 2002). After birth, NRPs are dramatically down-regulated on neurons and EC. In the mature (adult) endothelium, the pattern of NRP2 expression is more heterogeneous but can be found on blood capillaries, veins, and lymphatics (Bielenberg et al., 2006).

As the wealth of NRP knowledge grows, new cell types are being discovered that express NRP with unknown functions. In particular, NRP1 has been shown to be widely expressed in various types of epithelial cells (Gagnon et al., 2000; Kurschat et al., 2006). NRP2 has recently been shown to be expressed in visceral (but not vascular) smooth muscle cells (SMCs) and most strongly in the bladder (Bielenberg et al., 2012). Due to the increasing number of cell types which express NRP, there is a great incentive to understand more fully the underlying mechanism of NRP expression beyond solely neuronal and vascular activity.

**Neuropilins in Cancer**

The NRPs are expressed in a wide range of tumor types. In general, carcinomas more commonly express NRP1 while other non-carcinoma tumor types commonly express NRP2 but there is some overlap (Bielenberg et al., 2006, Figure 6). The first two cancer cell lines reported to express NRP1 were the prostate cancer cell line PC3 and the breast cancer cell line MDA-MB-231 (Soker et al., 1998). Since then, NRP1 has been found in tumor biopsies from
bladder, kidney, colon, pancreas, skin, ovarian, and lung carcinomas as well
(Wey, Stoeltzing, & Ellis, 2004; Neufeld et al., 2005)

Figure 6 | NRP1/2 Expression in Human Cancer Cell Lines | RT-PCR of various carcinoma and non-carcinoma human cell lines shows that NRP1 is more commonly expressed in carcinomas while NRP2 is more strongly expressed in cells of other origins. However, there is some overlap in expression. Cell lines from left-right: PC3, MDAMB231, 253JB5, SN12PM6, A375SM, U87MG, Sy5Y, HUVEC. From Bielenberg et al., 2006.

Malignant prostate carcinoma tissue samples were shown to have a ten-fold increase in NRP1 expression compared to normal tissue (Vanveldhuizen, 2003). In addition, when NRP1 was overexpressed in AT2.1 rat prostate cancer cells, the resulting tumors increased in size up to 7-fold over control tumors and were less apoptotic (Miao et al., 2000). Histology indicated
that the tumors had increased vessel density, increased levels of VEGF protein, and hyperproliferative vessels within the tumor (Miao et al., 2000). How tumor cells that express only NRP1 and no tyrosine kinase VEGFRs (and therefore cannot have VEGF autocrine signaling) could increase in size compared to tumor cells not expressing NRP1 was perplexing. One hypothesis derived from this finding is that NRP1 could be acting as a reservoir and holding VEGF on the tumor surface or within the tumor microenvironment thus increasing the gradient of VEGF toward the tumor which would chemo-attract new vessel sprouts. This hypothesis could be expanded to include NRP2 which may sequester VEGF-A, C, and D and therefore induce both angiogenesis and lymphangiogenesis.
Figure 7 | VEGF Sequestration Hypothesis | The hypothesis that cells which express NRP act as a reservoir for VEGF by binding VEGF and holding it near the cell surface at a high concentration/gradient. This high concentration of VEGF then causes an accelerated and stronger angiogenic response.

Several breast cancer cell lines have been shown to express NRP. Rat carcinogen-induced carcinomas express both NRP1 and NRP2 (Heffelfinger et al., 2004). There are also numerous examples of invasive or metastatic mammary carcinomas cell lines that express NRP1 while non-metastatic cell lines do not (Soker et al., 1998). In addition, a peptide inhibitor of NRP1 resulted in an increase in apoptosis in breast cancer cells (Barr et al., 2005).
NRP2 is most commonly expressed in non-carcinomas and has been found in melanoma cells, glioblastoma cells, and neuroblastoma cells. Its strong expression in these cell lines indicate that it likely has a neural crest origin (Bielenberg et al., 2006). However, its involvement in carcinomas has been shown as well. In particular, several studies have shown increased levels of NRP2 in the most common type of pancreatic cancer, pancreatic ductal adenocarcinoma (PDAC).

In three different pancreatic cancer cell lines (ASPC-1, CAPAN-1, PANC-1), there were higher mRNA levels of both NRP1 and NRP2 than any of the three VEGF receptors. This was a significant increase from normal pancreatic tissue which is devoid of NRP1 expression and lowly expresses NRP2 only in islet and acinar cells (Fukahi et al., 2004). Another study used shRNA to knockdown NRP2 expression in PDAC cells leading to decreased Akt activation and reduced survival signaling when phospho-Akt was measured. In addition, shRNA-NRP2 caused up to a 70% decrease in migration, and a 50% -70% decrease in invasion ability. There was no affect on PDAC proliferation in vitro. When these cells were grown in vivo, tumors were 63% - 95% smaller than controls and had reduced blood vessel lumen size (Dallas et al., 2008). Taken together, these data suggest that systemic inhibition of NRP2 inhibits tumorigenicity and progression (direct effects on NRP2-expressing tumor cells) as well as tumor size and angiogenesis (direct effects on NRP2-expressing vessels).
PDAC is the fourth most deadly cancer in the United States with a median survival of only 6 months (Jemal et al., 2008). PDAC has a five-year survival of less than 5% which is the lowest of any solid tumor (Jemal et al., 2008; Jemal et al., 2009). Because only 20% of pancreatic tissue is necessary for normal physiological function, it is rarely the primary tumor that causes mortality (Douglass and Penetrante, 1990). Instead, the most common cause of death is due to liver failure caused by metastasis (Douglass and Penetrante, 1990). The discouraging survival data for PDAC show that a potential therapeutic target is urgently needed.

**Class 3 Semaphorins in Angiogenesis**

Although the SEMA3s were first discovered as axonal guidance molecules, it has been shown that they have a key role in angiogenesis and vasculogenesis as well. Of the SEMA3s, while SEMA3A and SEMA3F are considered antiangiogenic, SEMA3C has proangiogenic properties. SEMA3A and SEMA3F are capable of inhibiting EC proliferation, survival, and cord formation with differing opinions on the mechanism (Shimizu et al., 2008; Acevado et al., 2008; Banu et al., 2006). Some have suggested that SEMA3A and SEMA3F inactivate integrins on ECs and cause disruption of angiogenesis and vascular remodeling (Serini et al., 2003). Others argue that they act by inhibiting either VEGF or b-FGF induced angiogenesis and cause a pro-apoptotic and cell repellant phenotype (Guttmann-Raviv et al., 2007). While others suggest that SEMA3F de-polymerizes F-actin stress fibers (called “collapse”) in both
tumor cells and EC and thereby inhibits migration, angiogenesis, and metastasis (Shimizu et al., 2008). In addition, numerous studies have shown that cancer cells transfected with SEMA3F cause ensuing tumors to become less vascularized, have decreased metastatic potential, and show an encapsulated phenotype (Bielenberg et al., 2004; Kessler et al., 2004; Kigel et al., 2008; Wu et al., 2011).

Due to their antitumor and antimetastatic properties, the SEMA3s have been shown to be tumor suppressor genes in some instances. For example, loss of heterozygosity of the 3p21.3 region has been positively correlated with lung cancer (Roche et al., 1996; Sekido et al., 1996). This region was later shown to be connected to a deletion of SEMA3B and SEMA3F (Futamura et al., 2007). In addition, deletion of SEMA3F has been correlated with advanced stage lung cancer and melanoma (Bielenberg et al., 2004; Lantuejoul et al., 2003).

**In vivo Angiogenesis Assays**

A number of assays have been developed that highlight angiogenesis in its vast number of physiological roles. In this study, we utilized two of these assays while investigating our Nrp2 transgenic mice: cutaneous wound healing and induced delayed-type hypersensitivity.

**A) Cutaneous Wound Healing**

Acute wound healing occurs in a sequential manner that involves four overlapping yet definitive phases: hemostasis, inflammation, proliferation, and remodeling (see Figure 8). Hemostasis begins immediately following infliction of
the wound with constriction of blood vessels and fibrin clot formation in order to control the bleeding (Gosain and DiPietro, 2004). The early inflammatory phase (about 24 hours after wounding), is characterized by a large number of polymorphonuclear leukocytes (PMNs) or neutrophils that arrive at the wound site and engulf bacteria to prevent infection (Broughton et al., 2006). The accumulation of macrophages around the wound site identifies the late inflammatory phase (about 48 hours after wounding). Similar to neutrophils, macrophages endocytose bacteria and damaged tissue. Near the end of the inflammatory phase, however, they begin to recruit cells for the proliferative phase and are stimulated by the low oxygen content to recruit EC’s and other cells necessary for angiogenesis (Mosser and Edwards, 2008).

The proliferative phase begins about 72 hours after wounding and key components of this phase include collagen and granulation tissue formation, fibroblast migration, and re-epithelialization at the wound site (Gosain and DiPietro, 2004). Concurrently, angiogenesis is essential in this phase of wound healing and is directly stimulated by hypoxia and lactic acid in the wound site. The final phase of healing is the remodeling phase which includes capillary regression to normal levels and is characterized by equal levels of collagen synthesis and breakdown (Greenhalgh, 1998).

In the cutaneous wound healing assay employed in this study, a punch of skin is removed from the back of the mouse and wound healing is monitored over a ten day period. Because angiogenesis is most crucial from day three
onward during the proliferative phase, we paid careful attention to that range in the healing process. It is of importance to note that the angiogenesis induced in this assay is largely hypoxia dependent.

Figure 8 | Wound Healing Steps | Wound healing begins with injury (a) where the clotting cascade is immediately induced identified by the fibrin clot (b). In the early inflammatory phase (c), neutrophils (PMNs) are the primary cell type present. In the late inflammatory phase (d), the PMNs are replaced by macrophages. In the proliferative phase (e), fibroblasts, keratinocytes, and ECs are recruited to the wound site to re-epithelialize the tissue and angiogenesis is paramount here. Remodeling (f) is a long term process after wounding. From Beanes et al., 2003.
B) Induced Delayed-Type Hypersensitivity Reaction

Delayed type hypersensitivity reactions of type IV hypersensitivity reactions are thus named because their response to an allergen is non-immediate and can take up to several days to reach its full impact. This is in stark contrast to immediate hypersensitivity reactions which usually occur within 12 minutes of exposure. Type IV hypersensitivity reactions are unique in that they are entirely cell mediated and do not involve an antibody response (Uzzaman & Cho, 2012).

When exposed to the allergen, CD4+ helper T cells known as Th-1 cells become activated and secrete IFN-γ which is a potent stimulator of macrophages. The macrophages secrete large amounts of TNF-α and IL-1, both of which cause even more macrophages to be recruited and result in a significant inflammatory response (Czarnobilska et al., 2007).

In order for macrophages and other factors to accumulate at the site of allergen affliction, some modification to the vasculature and/or lymphatic vasculature is required. For example, mice over expressing VEGF_{164} display prolonged swelling when exposed to the same DTH assay employed in this study (Detmar et al., 1998). The angiogenesis and lymphangiogenesis anticipated in this assay is mediated by growth factors and, unlike the wound healing assay, not by hypoxia. Therefore, these two assays allow us to explore angiogenesis from two different origins.
In conclusion, the wound healing assay allows us to explore hypoxia induced angiogenesis whereas the DTH assay allows us to explore growth factor/cytokine induced angiogenesis. Tumor angiogenesis and metastasis can be induced by both mechanisms so it is imperative to study angiogenesis from both perspectives.
SPECIFIC AIMS

It is our laboratory’s hypothesis that NRP2 is pivotal in the growth and metastasis of cancer. In particular, we believe that using SEMA3F to target NRP2 could be potentially efficacious in a number of different metastatic tumor types. Because NRP2 is the target of this treatment, it is essential to more fully understand where NRP2 is expressed physiologically. A more robust understanding of the organs and cell types which express NRP2 could give an idea of how specific SEMA3F therapy may be on tumor angiogenesis or lymphangiogenesis. Previous studies in our laboratory have shown that adult expression of NRP2 on ECs is not well understood and very heterogeneous. If NRP2 could eventually be used as a therapeutic target, its regulation and expression pattern should be uncovered. We will also use Nrp2/GFP and Nrp2/LacZ transgenic mice to more fully understand how the loss of NRP2 affects angiogenesis in both the physiological and pathological settings. Our hypothesis is that NRP2 is important for normal angiogenesis. Therefore, physiological processes involving angiogenesis such as wound healing and tissue expansion resulting from delayed-type hypersensitivity reactions would be impacted by the loss of NRP2. In addition, we predict that tumor angiogenesis and growth would also be impeded in the knockout mice.
In summary, the three main aims of this study were:

1. To identify the organs and cell types which most strongly express NRP2 *in vivo*.
2. To analyze the role of NRP2 is physiological angiogenesis including wound healing and delayed type hypersensitivity reaction.
3. To analyze the role of NRP2 in pathological angiogenesis; in particular tumor metastasis via angiogenesis and lymphangiogenesis.
METHODS

Protein Isolation

Tissue samples were flash frozen in liquid nitrogen and stored at -80°C. Tissue samples were pulverized using a 59014N Biopulverizer (BioSpec Products) and samples were re-suspended in Radio Immuno Precipitation Assay (RIPA) Buffer (50 mM Tris-Hcl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium deoxysulfate; Boston BioProducts) containing EDTA-free protease inhibitor cocktail (Roche Applied Science). Samples were then agitated using Vibra Cell sonicator (Sonics Materials) until tissue samples became homogenized in solution. Samples were placed on ice for 15 minutes and then centrifuged for 10 minutes at 14,000 RPM and 4°C. The supernatant was then collected and stored at -20°C.

Protein Assay

Total protein concentration of tissue sample lysates was quantified using Bio-Rad DC Protein Assay Kit utilizing the Lowry protein assay method. Protein concentrations were determined using a standardized serial dilution of Albumin from Bovine Serum (BSA, Fisher) in a 96-well flat bottom plate (Corning). Each well contained 5 µL of protein sample, 25 µL of 2% Protein Assay Solution S in Solution A, and 200 µL of Solution B (Bio-Rad). Samples were incubated in colorimetric solution for 15 minutes and then read in VersaMax Microplate Reader (Molecular Devices) at 750 nm using Softmax Pro 48.
Program. Data was then collected in Microsoft Excel and sample concentrations were determined using best-fit line obtained from standard BSA samples.

**SDS-PAGE Gel Preparation**

The separating layer of gel was prepared by combining 3.75 mL of 30% acrylamide/0.8% bisacrylamide (National Diagnostics), 3.75 mL of 4x Tris-HCl/SDS pH 8 (National Diagnostics), 7.5 mL H₂O, 50 µL of 10% ammonium persulfate, and 10 µL Tetramethylethylenediamine (TEMED; JT Baker Inc.). Separating solution was added between two glass plates separated by a 1.5mm-thick spacer in a gel-casting apparatus and allowed to harden for 30-60 minutes.

The stacking layer of gel was prepared by combining 0.65 mL of 30% acrylamide/0.8% bisacrylamide (National Diagnostics), 1.25 mL of 4x Tris-HCl/SDS pH 8 (National Diagnostics), 3.05 mL H₂O, 25 µL of 10% ammonium persulfate, and 5 µL TEMED. Stacking solution was then added above hardened separating layer in gel cast. A 10-well comb was added to the stacking layer and allowed to harden for 30-60 minutes. Prepared gels were either used immediately or stored at 4°C in a moist environment.

**Western Blot**

Protein samples were prepared in equal concentrations to ensure equal loading. 6X SDS Reducing Sample Buffer (Boston BioProducts) was added to each sample at a final concentration of 1X. Samples were boiled for 5-10 minutes and then loaded into 7.5% SDS-PAGE gel immersed in Running Buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3; Boston BioProducts). Proteins were
separated via electrophoresis for 2 hours at 100 V and then transferred to nitrocellulose membrane (Bio-Rad). Membranes were immersed in Transfer Buffer (25 mM Tris, 192 mM glycine, pH 8.4; Boston BioProducts) with 20% methanol and allowed to transfer overnight at 4°C and 75 mA or two hours at room temperature and 300 mA.

Membranes were then blocked with 5% blotting-grade blocker non-fat dry milk (Bio-Rad) in Tris Buffered Saline (10 mM Tris-HCl, 150 mM NaCl, pH 7.4) with 1% Tween-20 (TBS-T) for 1 hour at room temperature with mild agitation. Blocked membranes were then incubated with rabbit anti-mouse Nrp2 primary antibody (Cell Signaling) or mouse anti-GAPDH primary antibody (Millipore) for two hours at room temperature or overnight at 4°C. Membranes were subsequently washed three times in TBS-T, 15 minutes each, and then incubated with horseradish peroxidase (HRP) linked anti-rabbit or anti-mouse IgG secondary antibody (GE Healthcare) for one hour at room temperature. After three subsequent washes in TBS-T, Western Lightning Plus-ECL Oxidizing and Enhanced Luminol Reagent (Perkin Elmer) were mixed in equal proportions and added to membranes for 3 minutes, exposed to Hyblot Film (Denville) for up to 20 minutes, depending on band intensity.

For immunoprobing with a different primary antibody, membranes were incubated in Restore Western Blot Stripping Buffer (Thermo Scientific) for 15 minutes for removal of previous antibody. Membranes were then blocked, incubated, and exposed according to procedure above.
**Immunohistochemistry**

Paraffin embedded sections were warmed at 58°C on slide warmer for 15-20 minutes or until dry. Sections were then deparaffinized in xylene for 4 minutes and rehydrated in decreasing concentrations of ethanol for 2 minutes each (100% twice, 95% twice, 70%, 50%). Slides were placed in PBS for 5 minutes and then subjected to either sodium citrate or proteinase K antigen retrieval. For sodium citrate, sections were placed in coplin jar filled with 10mM sodium citrate buffer (pH 6) and then heated using microwave to 100°C for 10 minutes. For proteinase K, sections were incubated at 37°C in 20 µg/mL proteinase K (Roche) in PBS for 10 minutes. Following either method, slides were then washed in PBS 3 times for 3 minutes each. 3% hydrogen peroxide in methanol was then added to each slide for 12 minutes to block endogenous peroxidase enzymes and then washed again in PBS 3 times, 3 minutes each. Sections were then protein blocked with Tris-NaCl blocking buffer (TNB, Perkin Elmer) for 30 minutes followed by incubation overnight at 4°C with desired primary antibody (see Table 1 for specific protocol for each antibody).

The next day, slides were then washed 3 times, 3 minutes each with either PBS or TNT buffer (1x TBS, 0.5% Tween-20) depending on the method of signal amplification. Sections were then incubated in corresponding secondary antibody diluted in TNB for one hour at room temperature. Signal was then amplified using a variety of methods depending on the primary antibody including
tyramide amplification (Perkin Elmer) and Avidin-HRP Amplification Kit (ABC; Vector). Antibody binding was detecting using DAB Chromagen Kit (Vector) and incubated in DAB solution for up to 20 minutes using light microscopy. Brown precipitates denoted positive signal. Slides were then rinsed in water, counterstained using hematoxylin (Sigma-Aldrich) and Tacha’s Bluing Solution (BioCare Medical) to stain all nuclei blue and then dried overnight. The following day, slides were mounted using xylene and permount (Fisher).

For fluorescence imaging, antigen was visualized using FITC (green color) or Texas Red and then counterstained using Hoescht solution (blue color, Invitrogen). Slides were mounted using Clear-Mount with Tris-Buffer (Electron Microscopy Sciences), sealed with clear nail polish and then stored in dark at 4°C.

Table 1 | IHC Specifics | Details on methods used for each particular IHC antibody.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Antigen Retrieval</th>
<th>Secondary Antibody</th>
<th>Amplification Method</th>
<th>Wash Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-mouse Nrp2 (Cell Signaling) – Dilute 1:100</td>
<td>Sodium citrate pH 6</td>
<td>Biotinylated anti-rabbit (Vector) – Dilute 1:300</td>
<td>ABC amplification kit (Vector) or SA-FITC (1:100)</td>
<td>PBS</td>
</tr>
<tr>
<td>Rat anti-mouse CD31 (BD Pharmigen) – Dilute 1:100</td>
<td>Proteinase K</td>
<td>Biotinylated mouse-absorbed, anti-rat (Vector) – Dilute 1:300</td>
<td>Streptavidin (SA) HRP (Perkin Elmer; 1:100); Biotinylated tyramide (Perkin Elmer; 1:50); SA-HRP (1:100)</td>
<td>TNT after primary antibody; PBS before</td>
</tr>
<tr>
<td>Chicken anti-mouse GFP (Abcam) – Dilute 1:500</td>
<td>Sodium citrate pH 6</td>
<td>Anti-chicken IgY HRP (Abcam) – Dilute 1:500</td>
<td>None</td>
<td>PBS</td>
</tr>
<tr>
<td>Hamster anti-mouse podoplanin (ReliaTech) – Dilute 1:100</td>
<td>Proteinase K</td>
<td>Anti-Syrian hamster HRP (Vector) – Dilute 1:300</td>
<td>Biotinylated tyramide (1:50); SA-HRP or SA-FITC (1:100)</td>
<td>TNT after primary; PBS before</td>
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**Mice**

Mice were maintained under pathogen-free conditions in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All procedures and care for the animals was in accordance with current regulations set by the US Department of Agriculture, Department of Health and Human Services, NIH, and approved by the Institutional Animal Care and Use Committee (IACUC) at Boston Children’s Hospital.

Nrp2<sup>+/gfp</sup> mice (Nrp2<sup>tm1.2Mom/MomJ</sup>, Stock #006700) were purchased from The Jackson Laboratory and maintained in C57BI6 (black coat) background. Nrp2<sup>+/gfp</sup> mice were bred with other heterozygotes to yield Nrp2<sup>gfp/gfp</sup> which were viable and fertile. They were used in this study to decipher the role of NRP2 in both pathological and physiological angiogenesis. Experiments were performed on wild-type (Nrp2<sup>+/+</sup> or WT), heterozygote (Nrp2<sup>+/gfp</sup> or Hets), and knockout (Nrp2<sup>gfp/gfp</sup> or KOs) littermates obtained from breeding.

**Genotyping of Nrp2 Transgenic Mice**

Bred mice were weaned at 3 weeks and genotyped. A different piece of ear was taken from each mouse in a cage to use for genotyping and distinguishing the mice in the future. Because these are GFP transgenic mice
and NRP2 is expressed by melanocytes in the hair follicles, the ears were observed under the fluorescent microscope for evidence of GFP. Mice were then placed in two groups based on the results of microscopy – WT group (no GFP positive hair follicles) and Het/KO group (GFP positive hair follicles). See Figure 9 for an example of each result. The ears in the Het/KO group were then sent to Transnetyx where PCR was used to determine if they were NRP2+ (heterozygous) or Nrp2- (knockout).

Figure 9 | Genotyping of NRP2 Transgenic Mice | A. Fluorescence microscopy of GFP-positive hair follicles from mouse indicating GFP is present (4X). B. Fluorescence microscopy of GFP negative hair follicles indicating that no GFP is present. Note: hairs are auto-fluorescent and appear green in all mice. C. Report from Transnetyx indicating a Het mouse (+) or a KO mouse (-).
**Wound Healing Assay**

One day prior to assay, fur from back of mice was shaved to reveal bare skin on the entire dorsal midline. Five mice from each group (WT, Het, and KO) were used in wound healing assay. On the day of the wounding, mice were anesthetized in a chamber containing 4% isoflurane and the back of mouse was sterilized with ethanol and iodine swabs. The mice were then laid on their sides, skin pinched back, and a 6 mm biopsy punch was used to make two 6 mm full-thickness subcutaneous wounds on the back of each mouse. Mice were given analgesic (buprenorphine) once every twelve hours for the next 36 hours.

Immediately following wounding, transparency paper was used to outline the wound and measure its size. Wound size measurement was repeated every other day until all wounds were healed. IP Labs software was used to quantify the area of the wounds after they were traced onto transparency paper and digitally scanned. Percent of area healed was plotted versus the time after wounding for each group and compared using Excel. Unpaired *t*-test was used for statistical analysis.

**Delayed Type Hypersensitivity Reactions**

DTH reactions were induced in the ear skin of Nrp2 transgenic mice (*n*=6 mice per WT, Het, & KO group). Skin was sensitized by topical application of 2% oxazolone (4-ethoxymethylene-2-phenyl-2-oxazoline-5-one; Sigma) in acetone/olive oil solution in 4:1 vol/vol solution. This solution was added to each paw (5 µL) and abdomen (50 µL). On the fifth day after sensitization (day 0), 20
µL (10 µL each side) of a 1% oxazolone solution was applied to one ear of each mouse. The thickness of the ears was measured using a sensitive caliper daily for 11 days. The increase in ear thickness from baseline (day 0) for each mouse was used to determine the amount of inflammation. The change in ear thickness was plotted versus time using Excel. Unpaired t-test was used for statistical analysis.

**Pancreatic Tumor Inoculation**

Panc0H7 cells, a mouse C57BI6 pancreatic adenocarcinoma cell line (syngeneic to our Nrp2 transgenic mice), were provided by Dr. Keping Xie (MD Anderson Cancer Center, Houston, TX) and cultured in collaboration with Dr. Dipak Panigrahy (Boston Children’s Hospital). The cells were mixed in a 1:1 volume ratio with matrigel (BD Biosciences), a material composed of extracellular matrix proteins that aids in tumor-take and hardens to keep tumor cells from leaking from the injection site. The cells were prepared so that 40 µL of solution would contain 10^6 total Panc0H7 cells and that volume was injected into the pancreas of each mouse.

The cells were aspirated into a 1 mL syringe and a precision push button dispensing device (Hamilton) was added to the syringe along with a 30 gauge needle for injection. The syringe was kept on ice between each injection so that the matrigel would not harden prematurely. The Panc0H7(matrigel) mixture was injected orthotopically into female WT, Het, and KO Nrp2 transgenic mice (n=5 from each group).
Mice were anesthetized in a chamber containing 4% isoflurane and then transferred to the operating table where they were given isoflurane throughout the procedure via a nose cone. The skin around the incision site was sanitized with iodine and alcohol before a 1-1.5 cm incision was made on the left abdomen of the mouse. The mouse spleen was then externalized with a cotton swab in order to expose the attached pancreatic tissue. The tumor cell and matrigel mixture (40 µL) was then injected orthotopically into the mouse pancreas and held in place for ten seconds so that the matrigel would solidify. After solidification, the spleen and pancreas were returned to the abdomen and wound clips (Autoclip) were used to close the incision. Mice were allowed to recover in cages under a heating lamp and were given analgesic every 12 hours for 48 hours after surgery. The staples were removed one week after injection when the incision was healed and tumor size was monitored every 2 days by palpation.

Seventeen days after injection, all mice were sacrificed and tumors were resected. The pancreas, spleen, and liver were weighed and then fixed in 10% buffered formalin overnight, transferred to 70% ethanol and finally processed and embedded in paraffin. In addition, levels of ascites and other remarkable observations in the tumor bearing mice were noted. Tumor weights and organ weights were averaged among the groups and graphed. Unpaired t-test was used for statistical analysis.
RESULTS

Nrp2 is expressed in numerous different organs and cell types

To deduce Nrp2 expression in murine organs, WT mice were sacrificed and then protein was isolated from brain, heart, lung, skin, pancreas, liver, uterus, and intestine. These protein lysates were separated by 7.5% SDS-PAGE, transferred to nitrocellulose, then immunoblotted with anti-Nrp2 antibody to quantify relative Nrp2 protein expression amongst organs (see Figure 10). Of the organs studied, the intestine most strongly expressed Nrp2 which confirmed previously reported data (Bielenberg et al. 2012). The intestine also showed the highest levels of soluble Nrp2 (sNrp2). Previous studies in the lab have shown that the band observed at 80-85 kDa is likely sNrp2 but it is also possible that this band could be attributed to degraded protein. In addition, the brain, skin, lung, and uterus all showed relatively higher levels of Nrp2 than other organs. This is the first time uterus has ever been shown to express Nrp2. Because Nrp2 is expressed on a variety of different cell types, western blot analysis only describes the relative amount of protein in each organ, but tells us nothing about the nature of its expression.
Figure 10 | Nrp2 expression measured by western blot | Nrp2 protein levels were measured from WT organ lysates. The anti-NRP2 antibody also detects what is likely a soluble form of Nrp2 (sNrp2) which is around 80-85 kDa. The blot was stripped and reprobed for GAPDH, to evaluate loading.

Nrp2 is differentially expressed on EC and LEC in organs

Based on our growing knowledge of NRP expression on various different cell types, organs were then explored for their differential expression of Nrp2. The lung and intestine were chosen for investigation based on their high expression of Nrp2 in the western blot analysis.

Serial sections of Nrp2<sup>+/gfp</sup> (Het) mouse colons were stained with podoplanin, a lymphatic EC marker, as well as Nrp2 (see Figure 11A&B). Previous studies have shown that smooth muscle in the bladder highly expresses Nrp2 and Figure 11A demonstrates it does in the colon as well. Figure 11C provides a schematic of the intestinal villus which features the lacteal, a lymphatic vessel the travels up each villus as well as blood vessel capillaries.
wrapping around the lacteal. Podoplanin staining (Figure 11B) shows that each villus, cut longitudinal, has this podoplanin positive lacteal. Interestingly, many of these lacteals are also positive for Nrp2 (Figure 11A) but the staining appears to show that not every lacteal is positive. This may be the result of tissue sectioning or there could be something regulating differential expression of Nrp2 on the lacteal.

![Figure 11 | Nrp2 expression on colon lacteal](image)

Figure 11 | Nrp2 expression on colon lacteal | A) Section of mouse colon stained with Nrp2 antibody (brown). Arrows point to lacteals positive for Nrp2 and star signifies the Nrp2 positive smooth muscle (10X). B) Serial section of mouse colon stained with podoplanin (brown) which is expressed on lymphatic EC in the lacteal while SM layer is negative (10X). C) Diagram of intestinal villus adapted from Hole’s Essentials of Human Anatomy & Physiology, 9/e, 2006, McGraw-Hill.

Based on the western blot (Figure 10), we then explored the expression of Nrp2 on lung ECs. Serial sections of mouse lung tissue were stained with CD31, an EC marker, and Nrp2 antibodies (Figure 12). As expected, CD31 is
strongly expressed on pulmonary veins and arteries in the lung (Figure 12A/C on “v” and “a”) but not bronchioles. Nrp2 was expressed on EC’s of those same veins but its expression was very heterogeneous and it was only expressed on a few ECs per vessel (Figure 12B). In addition, Nrp2 expression was confined to pulmonary veins and not present on pulmonary arteries (Figure 12D, arrows). This specific expression pattern has been previously reported in other tissues during development (Herzog, Kalcheim, Kahane, Neufield, 2001).

Figure 12 | Nrp2 expression on lung EC | Sections of lung tissue were stained with CD31 (A,C) and Nrp2 (B, D) antibodies (10X). CD31 is expressed on all endothelial cells in the lung while Nrp2 is present only on endothelial cells of the pulmonary veins (D). In D, arrows point to Nrp2 positive areas of the vessels. (a= artery/arteriole, v=vein/venule, br=bronchiole).
**Nrp2 is more highly expressed on brown adipose tissue than white**

Previously in the lab, Bernadette Zwaans stained Nrp2/LacZ E18 embryos with X-gal, which produces a blue precipitate in locations where Nrp2 is endogenously expressed. Interestingly, the scapular area of the embryo was very positive (blue; Figure 13). The scapular region in young mammals is highly composed of brown adipose tissue (BAT).

![WT and Het embryos](image)

**Figure 13 | Nrp2/LacZ E18 Embryo strongly positive in scapular region** | E18 Nrp2+/LacZ embryo was stained with X-gal and blue regions signify positive Nrp2 expression. The circle surrounds the scapular region of the embryo which is largely composed of BAT. WT (Nrp2+/+) embryo shows no blue because it has no LacZ gene and does not form any blue precipitate when exposed to X-gal.
Nrp2 protein expression was then explored in white adipose tissue (WAT) and BAT from WT and Het mice via western blot. As shown in Figure 14, BAT much more strongly expresses Nrp2 than WAT in adult mice (compare lanes 1 and 3). This is a novel finding and we are the first group to explore expression of Nrp2 in WAT and BAT. P3 brown fat strongly expresses Nrp2 (Figure 14, lane 5). As expected, heterozygous Nrp2 mice express approximately half as much Nrp2 protein in BAT as WT mice in both adult (compare lanes 3-4) and newborn mice (compare lanes 5-6). Nrp2+/- WAT (lane 2) was not equally loaded so it could not be compared to Nrp2+/- BAT. P3 WAT was not isolated. This finding was confirmed by the gene portal system, BioGPS, which shows higher levels of Nrp2 RNA in BAT than WAT (Lattin et al., 2008).

Figure 14 | Nrp2 expression is higher in brown adipose than white adipose tissue |
Protein isolated from WT and Het Nrp2 transgenic mice (both adult and 3-day old) was immunoprobed with anti-Nrp2 antibody. Brown fat lysates had higher levels of Nrp2.
**Nrp2 expression in mouse skin**

Previous studies by Bernadette Zwaans in the lab have shown that Nrp2 is expressed on a number of different cell types in the skin (see Figure 15). Based on the X-gal staining, Nrp2 appears to be very highly expressed on vessel ECs during development but there are less Nrp2 positive vessels in adulthood (Figure 15B). It is, however, expressed on melanocytes and SM pili that are found in the skin of adult mice. Our western blot analysis (Figure 10) showed the skin to be a high expresser of Nrp2, even in the adult. The expression of Nrp2 in the skin leads us to hypothesize that both cutaneous wound healing and delayed type hypersensitivity reactions may both be affected by loss of Nrp2.

**Figure 15 | Nrp2 expression in E18 and adult mouse skin** | A) Section of dorsal skin taken from Nrp2+/-LacZ E18 embryo showing Nrp2 expression (blue) on many vessels (20X). B) Section of dorsal skin taken from Nrp2+/-LacZ adult mouse showing reduced Nrp2 expression on vessels but strong expression in melanocytes (red arrow) and arrector pili SMC (yellow arrow) (20X).
Wound healing not affected by loss of Nrp2

Based on Nrp2 expression in the skin, we anticipated that cutaneous wound healing would be slowed in Nrp2 knockout mice compared to their Het and WT littermates. Wound healing assays were performed on Nrp2 transgenic mice with differing results between two trials. Figure 16A illustrates the first wound healing assay where 6 mm sub-cutaneous wounds were made on the back of five WT, Het, and KO mice. In this trial, the WT mice healed at a slower, yet not statistically significant, rate than the Het and KO groups. Due to the fact that the KO mice are deficient for a key angiogenic regulator, these results were puzzling. In the wound healing process, angiogenesis does not begin until the proliferative phase, which begins about 72 hours after wound infliction (Falanga, 2005). The KO mice have the shallowest rate of healing from day 3 onward, however this finding was also not statistically significant.

Because the WT wound healing data was puzzling in the first trial, the assay was repeated with only WT and Het mice (Figure 16B). In this trial, there was virtually no difference between the two groups indicating that the first WT trial may have been due to trial error. The assay was not repeated with KO mice but if the KO data from the first trial were superimposed into the second trial, there would be very little difference between the three groups. The only slight difference would be a sharp decrease on day 1 whereas the WT/Het groups both showed very little contraction over the first 24 hours.
Figure 16 | Wound Healing Assay | A) First trial of assay showing reduced rate of wound healing in WT mice (dotted line, circles) and KO (solid line, triangles)/Het (dotted line, squares) groups healing at approximately the same rate. B) Second trial of assay done with only Het and WT groups showing a quicker rate of healing for the WT group than in trial number one. C) Sections of skin surrounding wound from Nrp2/LacZ transgenic mice at Day 0, Day 3, and Day 5 with arrow pointing towards the wound site (10X). D) Day 5 section at higher magnification (20X).
Figure 16C/D shows histological data at various time points after dorsal incisional wounds (not punch biopsy) were performed in Nrp2/LacZ transgenic mice. Bernandette Zwaans provided this data. During both time points after the wound was made, there are very few Nrp2 positive (blue) vessels. There are, however, a great number of new blood vessels infiltrating the wound as indicated by the corresponding CD31 (brown) staining. These results may indicate that wound healing is not significantly dependent upon Nrp2 positive vessels.

**Nrp2 deficient mice show stronger, delayed ear swelling**

Because Nrp2 has been shown to be important in proper lymphatic vessel formation, we hypothesized that Nrp2 KO mice may respond differently to inflammation than their littermates. An oxazalone induced delayed-type hypersensitivity assay using the Nrp2 transgenic mice was performed (Figure 17). All mice in the three groups swelled to about the same size one day after being challenged with oxazalone (350-400 µm). After day one, Nrp2 deficient KO mice displayed persistent swelling of at least 300 µm until day five that was of statistical significance. In fact, as WT and Het mice began to recover on day two, the KO mice actually showed increased swelling on the second day. Nearly all WT and Het mice recovered to normal ear size by the end of the 11 day trial. KO mice, however, took considerably longer to fully recover to normal ear size with some showing persistent swelling at 21 days.
Interestingly, the Het group behaved very similarly to the WT s, reaching the same level of swelling and then recovering at nearly an identical pace. The Het mice produce half as much Nrp2 as their WT littermates but that appears to be enough for physiological recovery from swelling. The KO’s significant inability to recover at the same rate as their littermates indicates that Nrp2 may play a key role in draining lymph after swelling.

**Figure 17 | DTH Assay** | NRP2 KO mice show prolonged ear swelling in comparison to WT and Het groups. DTH reactions were induced by oxazolone application to the ear and then monitored over the next 11 days. Ear swelling is expressed by the increase in micrometers of the ear. The ear swelling of the KO group (solid triangle, solid line) was prolonged in comparison to the Het (solid square, dashed line) and WT (solid circle, dashed line) groups. * indicates P<0.01
The DTH was performed a second time and mice were sacrificed at day 4 and ears were taken for histology. Figure 18 shows both H&E (A-C) and CD31 (D-F) fluorescent staining of ears from all three groups (WT, Het, and KO). KO mice showed dramatically higher swelling, as expected, on day 4 than both Hets and WTs. Interestingly, the outer region of the ear displayed the most dramatic swelling in all mice. CD31 staining showed a greater number of large vessels in the Het and WT ears in comparison to knockout ears. This is only a preliminary observation and vessel density should be analyzed more thoroughly in these mice.

![Figure 18](image)

**Figure 18 | Histology of DTH ear swelling in Nrp2 transgenic mice** | Panels A-C illustrate H&E staining of WT, Het, and KO ears displaying largest swelling in the KO and larger swelling in the outer ear in all three groups (10X). Panels D-F show fluorescent CD31 staining of just one field of each ear displaying smaller vessels in the KO ear (20X).
Nrp2 deficient mice have smaller tumors and less metastasis

Because Nrp2 has been demonstrated to play a key role in angiogenesis and lymphangiogenesis, we anticipated that Nrp2 KO mice would have fewer metastases than their WT and Het littermates. A fast growing syngeneic murine pancreatic adenocarcinoma cell line, Panc0H7, was injected orthotopically into the pancreas of WT, Het, and KO Nrp2 transgenic mice. This is an extremely fast growing cell line and the mice were sacrificed after just 17 days of growth. Despite the short time of growth, mice displayed significant tumor growth, metastasis, and development of ascites.

Figure 19 | Panc0H7 tumors grown in Nrp2 transgenic mice | A) Mass comparison of the pancreas, liver, and spleen between the WT, Het, and KO groups. KO pancreas mass was significantly lower than WT pancreas mass and KO liver mass was significantly lower than both Het and WT liver masses. B) Gross depiction of WT, Het, and KO pancreatic tumors depicting a stepwise decrease in size from WT to Het to KO groups. * indicates P<0.01.
Figure 19A displays the average mass of the pancreas and two of the most common metastatic sites, liver and spleen. Average pancreatic mass decreased in a stepwise fashion from WT (1.11 g) to Het (0.91 g) to KO (0.69 g), but only the difference between KO and WT was of statistical significance. Similarly, there was a stepwise decrease in mass seen in both the liver and spleen. There was no statistical significance between the spleen masses but the lower liver mass of the knockout was significant. The average liver weight in the KO group was 0.70 g whereas the Het and WT groups had average liver weights of 0.98 and 0.99 g, respectively. Figure 19B shows a gross depiction of all the resected pancreatic tumors. There was considerably more healthy pancreatic tissue in the KO group than both the Het and WT groups.

In humans, PDAC mortality is most often caused by liver failure due to metastasis to the liver. In this model, gross liver metastases were not observed in any group. This is likely due to the short overall time of growth. However, WT and Het livers were larger than KO livers suggesting that histology may show microscopic liver metastases in the WT/Het groups. In addition, there was a reduction in overall metastases in the KO group (Table 2). All of the WT ($n=4$) and Het ($n=4$) showed many intestinal, diaphragm, and spleen metastases whereas the KO group showed either an absence of metastases or only a few per site. In addition, the KO mice displayed considerably less ascites and appeared to have more healthy liver tissue.
Table 2 | PancOH7 metastasis and ascites in Nrp2 transgenic mice | Qualitative analysis of intestinal, diaphragm, and spleen metastases in WT, Het, and KO mice showing reduced metastases in KO group. In addition, KO mice had reduced amounts of ascites. *Each of the 5 KO mice showed just one small metastasis to the diaphragm while each in WT and Het groups displayed many metastases **The one KO mouse with spleen metastasis had one small metastasis while the Het and WT groups showed many metastases.

<table>
<thead>
<tr>
<th>Mice Groups</th>
<th>WT (n=4)</th>
<th>Het (n=4)</th>
<th>KO (n=5)</th>
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<tbody>
<tr>
<td><strong>Intestinal Mets</strong></td>
<td>4/4</td>
<td>4/4</td>
<td>2/5</td>
</tr>
<tr>
<td><strong>Diaphragm Mets</strong></td>
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<td>4/4</td>
<td>5/5*</td>
</tr>
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<td>4/4</td>
<td>1/5**</td>
</tr>
<tr>
<td><strong>Ascites (&gt;1mL)</strong></td>
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DISCUSSION

As our understanding of NRP2 increases so does the complexity of its function and expression. Because its expression is becoming more and more widespread, an immunoblot for NRP2 protein expression in various organs paints a rather limited picture of its involvement in angiogenesis and other physiological phenomena. For example, the intestine displayed the highest level of protein in Figure 10, but a large amount of that expression is limited to the visceral smooth muscle. To the same token, the majority of the protein expression seen in the brain in Figure 1 is due to NRP2’s role on neurons. Our novel finding that NRP2 is more highly expressed in brown adipose tissue than white is the most recent discovery involving NRP2’s interestingly complicated expression pattern.

Adipose tissue in mammals can be divided into two distinct types, white and brown, which have almost entirely opposite functions. The main function of WAT is that of a storage depot; it stores excess calories as triacylglycerides which can be later metabolized in the starved state. BAT, on the other hand, is filled with mitochondria which burn calories at a high rate in order to produce heat when the organism is in cold conditions (Saely, Geiger, Drexel, 2012). For many years, BAT was thought to be solely found in newborn mammals, but recent studies have shown that adults too have BAT in multiple locations throughout the body (Wehrli et al., 2007). BAT has also been identified as a more vascularized tissue than WAT (Cinti, 2009). This finding gives some precedence to our discovery, but it is unknown whether the large difference in NRP2 expression is
solely due to a higher vessel density. It is possible that NRP2 could also be expressed on the adipocytes, with a function that is currently unknown. Double staining of BAT sections using CD31 and NRP2 or NRP2 and perilipin (an adipocyte marker) could elicit which cell types express NRP2 in BAT. Because the amount of WAT is directly correlated with incidence of type II diabetes mellitus, the calorie-burning BAT has been identified as a potential therapeutic tool (Bartelt and Heeren, 2012). NRP2 could potentially play a key role in this process.

Because we are still learning which cell types express NRP2, a western blot cannot shed much light onto which organs would be most sensitive to anti-angiogenic therapies targeting NRP2. In order to help understand this, IHC is essential. However, IHC is further complicated by the fact that all of our Nrp2 antibodies detect both the soluble and receptor version of Nrp2. Positive indication of Nrp2 could be either full length or sNrp2 and developing methods to distinguish between the expression of the two is needed. Based on IHC, it does not appear as if there is a set of rules for where and how NRP2 is expressed on ECs. From the IHC data, it is clear that NRP2 is most frequently found on LECs in adult mice, but is not expressed on every LEC. This is particularly true on the intestinal lacteal; many do express NRP2 but it does not appear that they all do. Further, NRP2 expression is also seen on some vascular ECs but its expression again shows very little consistency. This is particularly true in lung EC. Figure 12 shows that many pulmonary vein EC express NRP2 but certainly not every EC in
the vessel. For the most part, there was no NRP2 expressed on LEC in the lung which was also surprising. This complicated expression pattern begs the question: what is regulating NRP2 expression?

Different physiological angiogenic assays could elicit information into how NRP2 is regulated in adult physiology. It did not appear as if cutaneous wound healing was dependent upon NRP2 as the Nrp2 KO mice healed at approximately the same rate as their Het and WT littermates. Angiogenesis is involved in wound healing, but it appears as if this method is largely NRP2 independent. It is potentially possible, however, that the Nrp2 KO mice over express other angiogenic factors in order to compensate for the loss of NRP2. The shallower slope of healing in KOs after angiogenesis traditionally begins indicates that perhaps the loss of NRP2 has some role, albeit very minimal. Because the histology of the wound healing site showed very few positive vessels (Figure 16C/D), however, the data suggests that it is more likely that adequate wound healing is not heavily dependent upon NRP2. For potential anti-NRP2 cancer therapy, this is ultimately a positive finding. It is unlikely that patients being treated with SEMA3F or another molecule that targets NRP2 would have any problems healing from wounds that they may encounter during treatment.

Interestingly, the site of wound healing may also be crucial in determining whether NRP2 plays a role. In a previous study performed in our lab by Bernadette Zwaans and Dr. Diane Bielenberg that has not yet been published, a
corneal suture assay in the eye of the mouse shows drastic NRP2 positive vessel growth in response to the suture assay (Figure 20). The shape of these vessels appear thick and more lymphatic looking while the CD31 vessels are thin and wispy. This may indicate that the corneal suture assay induces NRP2 dependent lymphangiogenesis while cutaneous wound healing may not require a large amount of lymphangiogenesis causing it to be NRP2 independent.

Figure 20 | Corneal Suture Assay | Corneal suture assay induces large increase in NRP2 positive lymphatic vessels. A) X-gal staining of cornea after suture assays shows many newly sprouted vessels (blue) that are positive for NRP2. B) The same eye stained with anti-CD31 antibody shows many blood vessels growing in the cornea after the assay which do not overlap with the x-gal staining suggesting that blood capillaries do not express Nrp2 in the suture model.

Our data indicates that NRP2 does play a role in recovery from swelling as indicated by the persistent swelling seen in KO mice ears after being challenged with allergen. It has been shown previously that a VEGF-C adenovirus in the ear can drastically increase lymphatic vessel sprouting in mice. These new lymphatic
vessels also persisted after the stimulation was removed (Dvorak & Mihm, 1972; Wirzenius et al., 2007). It is likely that the swelling due to DTH also induces lymphangiogenesis and lymphatic vessel sprouting in order to help drain the accumulated lymph. The persistent swelling seen in KO mice likely indicates that their lymphatic vessels are not capable of draining lymph at the same rate as their WT and Het littermates. Perhaps the mice have reduced numbers of lymphatic vessels or flawed lymphatic vessels that impede the draining of lymph. Preliminary CD31 staining does show a reduced number of large vessels in the DTH ears of KO mice. Previous DTH studies on other transgenic mice also compliment these findings. Mice over expressing VEGF_{164} show even more prolonged and drastic swelling than what is seen in our NRP2 KO mice (Detmar et al.,1998). When this mouse line is crossed with mice over expressing sNRP1 in the skin, that swelling is almost entirely reduced as sNRP1 acts as an inhibitor of VEGF_{164} induced vascular permeability (Mamluk et al., 2005). Our data suggests that NRP2 plays a pivotal role in lymphatic vessel formation and lymphangiogenesis and would be an excellent target for anti-lymphangiogenic therapies. Previous studies have shown that targeting NRP2 with an antibody does inhibit tumor lymphangeogenesis and our results here correlate with those findings (Caunt et al., 2008).

A stark difference between wound healing and DTH is in how angiogenesis (or lymphangiogenesis) is induced. In wound healing, blood vessels are physically removed in the formation of the wound and, therefore, the
ensuing angiogenesis is induced by hypoxia and the necessity for new blood vessels to supply the wounded area. DTH, however, does not involve hypoxia and is largely driven by growth factor and cytokine release. Growth factors also mediate angiogenesis and lymphangiogenesis in the corneal suture assay shown in Figure 20. It is possible that NRP2 does not respond to hypoxia induced angiogenesis but is instead more sensitive to certain growth factors released during DTH and corneal suture assays. It has been shown previously that NRP2 in SMC is upregulated by TGF-β and IGF while it is downregulated by PDGF and HBEGF (Bielenberg et al., 2012). It is unknown whether NRP2 in EC behaves similarly but it is possible that platelets in wound healing release PDGF that actually decreases NRP2 expression while other growth factors released during DTH induce NRP2 expression. We know that DTH involves the T-cell mediated release of many different growth factors at very high levels. It is possible that one or many of these growth factors somehow induce NRP2 expression.

Further NRP2 studies should be aimed towards understanding NRP2’s intricate regulation. Our data shows that many factors could impact NRP2 regulation and another possible factor could be ischemia. A hind limb ischemia assay performed in Michael Klagsbrun’s laboratory caused a great induction of NRP2, even in the femoral artery, which does not express NRP2 when unchallenged (Figure 21; Klagsbrun, Takashima, Mamluk, 2002). It would be interesting if NRP2 were induced by ischemia but not hypoxia. At first thought, this seems perplexing, but it could be possible that NRP2 induction is a stop gap
mechanism only utilized in severe ischemic events. Perhaps less severe hypoxic events do not necessitate induction of NRP2 expression.

![Pre-ischemia](image1.png) ![Post-ischemia](image2.png)

**Figure 21 | Hind limb ischemia in NRP2 mice** | Hind limb ischemia assay showing an increase in NRP2 expression after ischemia. Arrow points to femoral artery. From Klagsbrun, Takashima and Mamluk, 2002.

The significant decrease in tumor size and metastasis seen in our PDAC model demonstrates the potential efficacy of anti-NRP2 therapy. It is likely that using SEMA3F or another inhibitor of NRP2 would have an even more drastic effect on tumor growth and metastasis. In the KO model, the mouse does not make NRP2 but the tumor cells themselves still have it. In a therapeutic setting using SEMA3F, the NRP2 made by the tumor cells would also be inhibited. Our model here gives precedence to using SEMA3F as a potential antitumor agent. It is important to note that SEMA3F is not only an inhibitor of VEGF, but uses Plexin A1 to relay its own anti-angiogenic signal thereby tackling angiogenesis in two different ways. Further studies utilizing these mice in combination with a
classical VEGF inhibitor such as anti-VEGF antibodies or VEGFR2 kinase inhibitors would likely show even more inhibition of tumor growth and angiogenesis.

Despite not having any grossly visible liver metastasis in this model, it is still possible that some micrometastases are present and sections should be stained with cytokeratin to investigate their presence. In addition, tumors should be stained for EC and LEC markers in order to see if the KO mice have lower vessel density. A decrease in vessel density in the KO mice would be expected given the phenotype of the mouse and would be a large contributing factor in their reduced tumor size and metastases.

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<th>Method</th>
<th>Outcome</th>
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<td>Anti-NRP2 antibody</td>
<td>Inhibition of lymphangiogenesis</td>
<td>Caunt et al., 2008</td>
</tr>
<tr>
<td>SEMA3F overexpression</td>
<td>Inhibition of angiogenesis</td>
<td>Bielenberg et al., 2004; Kessler et al., 2004; Kigel et al., 2010, Wu et al., 2011</td>
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<td>NRP2 shRNA</td>
<td>Inhibition of tumor growth</td>
<td>Dallas et al., 2008</td>
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<tr>
<td>NRP2 binding peptide</td>
<td>Tumor EC Homing</td>
<td>Roth et al., 2012</td>
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More extensive NRP2 research has led to exciting discoveries indicating its presence and function in cell types beyond just neurons and ECs. In addition, many groups have attempted to use NRP2 as a target for cancer therapy utilizing a variety of different methods (see Table 3). Most all of the attempted methods have been successful to a certain degree and that list could be expanded to
include the Nrp2 knockout model included in this study. Targeting NRP2 can be approached from a wide range of perspectives and its success warrants a greater look into the mechanisms that underlie its expression in the adult vasculature. The data presented in this report indicate that NRP2’s physiological role is widespread and complexly regulated. Anti-NRP2 therapies do demonstrate potentially significant efficacy, but more research into NRP2 is necessary in order to more fully understand exactly where and how that target is expressed.
REFERENCES


Heffelfinger, S. C., Yan, M., Gear, R. B., Schneider, J., LaDow, K., & Warshawsky, D. (2004). Inhibition of VEGFR2 prevents DMBA-induced mammary tumor formation. *Laboratory investigation; a journal of technical methods and pathology, 84*(8), 989–998. doi:10.1038/labinvest.3700128


Klagsbrun, Michael, & Shimizu, A. (2010). Semaphorin 3E, an exception to the rule. *Journal of Clinical Investigation, 120*(8), 2658–2660. doi:10.1172/JCI44110


Sekido, Y., Bader, S., Latif, F., Chen, J. Y., Duh, F. M., Wei, M. H., ... Minna, J. D. (1996). Human semaphorins A(V) and IV reside in the 3p21.3 small cell lung cancer deletion region and demonstrate distinct expression patterns.
Proceedings of the National Academy of Sciences of the United States of America, 93(9), 4120–4125.


VITA

Nicholas S. Levonyak
6405 Bandera Ave • Dallas, TX 75225 • (214) 519-1511
nlevonyak@gmail.com
Born: 1989

EDUCATION

Boston University School of Medicine
Boston, MA
Candidate for Masters of Arts, Medical Sciences, May 2013

Gonzaga University
Spokane, WA
Bachelor of Science, Biochemistry, May 2011

RESEARCH EXPERIENCE

2012-present
Boston Children’s Hospital, Boston, MA
Vascular Biology Program
Graduate Thesis Research
“The Role of Neuropilin 2 in Physiological and Pathological Angiogenesis”
Advisor: Diane Bielenberg, PhD

2010-2011
Gonzaga University, Spokane, WA
Department of Chemistry and Biochemistry
Undergraduate Thesis Research
“Spectroscopic Characterization of α and γ-1 Melanocyte Stimulating Hormones”
Advisor: Matthew E. Cremeens, PhD

PUBLICATIONS


POSTER PRESENTATIONS

Levonyak N, Zwaans B, Seth M, Bielenberg DR. (2013) The Role of Neuropilin 2 in Physiological and Pathological Angiogenesis. Dr. M. Judah Folkman Research Day: Boston Children’s Hospital, Boston, MA.


WORK EXPERIENCE

2008-2010  DAVA Oncology, Dallas, TX
            - Supported the DAVA medical oncology team in their efforts to accelerate clinical trial enrollment
            - Contacted research sites personnel as a clinical trial specialist to uncover and solve accrual challenges

TEACHING EXPERIENCE

2009-2011  Teaching Assistant, Cellular Biology Lab and Genetics & Evolution Lab.
           Gonzaga University Biology Department
           Course Organizer: Ann-Scott H. Ettinger

2009-2010  Teaching Assistant, Organic Chemistry I and Organic Chemistry II Labs
           Gonzaga University Department of Chemistry and Biochemistry
           Course Organizer: Dr. Gemma D. D'Ambruoso, PhD

COMMUNITY SERVICE

2010-2011  St. Margaret’s Women’s Shelter, Spokane, WA
            Tutor, Childcare Provider, Work on Organic Farm
2008-2009  Hutton Settlement, Spokane, WA
          Tutor

AWARDS & HONORS

2008-2010  Gonzaga University President’s List (>3.70 GPA)
          Four times

2008-2011  Gonzaga University Dean’s List (3.50-3.70 GPA)
          Three times

INSTITUTIONAL & CLINICAL EXPERIENCE

2012-present  Brigham and Women’s Hospital, Boston, MA
              Volunteer Ambassador in Central Transport & Patient
              Access Services

2008– 2009  Mary Crowley Cancer Research Center, Dallas, TX
            Shadowing, Writing of review articles