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Lymphangiogenesis and Metastasis—A Closer Look at the Neuropilin/Semaphorin3 Axis

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Abstract

Metastasis is the leading cause of cancer-related deaths. Understanding how the lymphatic system responds to its environment and local stimuli may lead to therapies to combat metastasis and other lymphatic-associated diseases. This review compares lymphatic vessels and blood vessels, discusses markers of lymphatic vasculature, and elucidates some of the signaling motifs involved in lymphangiogenesis. Recent progress implicating the neuropilin and semaphorin axes in this process is discussed.

Introduction

The framework of the blood vascular system is that of a closed circulatory system that pumps blood from the heart to all organs and tissues in the body, delivering oxygen from red blood cells and nutrients from the serum. The lymphatic system is like a recycling system as it returns extra fluids that leak into the interstitial space back to the venous system. In peripheral tissues, the two systems never intermix, and homeostasis is only ensured when both systems are working properly.

Although both the blood and lymphatic systems are lined with endothelial cells (EC), the organization and function of each is different (Kaipainen & Bielenberg, 2010). Fluid and oxygen exchange takes place at the capillary bed, not in larger vessels like arteries or veins. Mature blood capillaries are surrounded by a basement membrane and supported by pericytes—components that ensure the stability of the blood capillary. Lymphatic capillaries, on the other hand, lack a continuous basal lamina and are not associated with pericytes, as either of these components would interfere with their function (Sauter et al., 1998). In contrast, terminal lymphatic endothelial cells (LEC) are attached to the extracellular matrix via anchoring filaments (elastic fibers) (Leak & Burke, 1968). Initial lymphatic capillaries have overlapping LEC with “button-like” junctions between cells that

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act as a primary valve or gate regulating the uptake of fluid (Baluk et al., 2007; Yao et al., 2012). When interstitial pressure increases, the anchoring filaments pull on the LEC to cause a small gap between cells. Oncotic and hydrostatic pressures drive fluid entry. Immune cells and tumor cells can also enter lymphatic capillaries with fluid uptake.

The lymphatic capillaries then drain the fluid through larger channels called collecting ducts. Collecting lymphatics are lined with LEC containing “zipper-like” adherens junctions that stain positively for VE cadherin (Baluk et al., 2007). Collecting vessels are surrounded by basement membrane and smooth muscle cells and do not have anchoring filaments or openings. An important feature of collecting lymphatics is the presence of valves that prevent fluid backflow (Figure 1F). These ducts are arranged into units called lymphangions—defined as the distance between two consecutive valves. Interestingly, the valve region of the lymphangion lacks smooth muscle coverage, presumably to ensure proper function of the valve. Ducts channel the fluid into the lymph node (LN) through afferent vessels into the sinus region. Immune and tumor cell entry into the LN may be an active and rate-limiting step depending on the cell type (von Andrian et al., 2003; Das et al., 2013). Fluid exits the LN through a single efferent vessel. Lymph may be filtered through several LNs before returning to the venous system through large lymphatic ducts which empty into subclavian veins. The thoracic duct, also called the left lymphatic duct, is the largest lymphatic vessel and drains into the left subclavian vein, while the right lymphatic duct drains into the right subclavian vein.

The structure and morphology of the lymphatic system is crucial to its function. Lymphatics regulate interstitial fluid volumes to prevent edema, absorb dietary fats in the intestines, transport antigens to the LN, and facilitate immune cell traffic to and from LNs. However, the structure of the lymphatic vasculature, especially that of lymphatic capillaries, makes them susceptible to invasion by migrating cancer cells and thus key players in the process of metastasis.

Lymphatic Vessels

Lymphatic Vessel Detection

In the last two decades, the understanding of the biology of the lymphatic system has advanced considerably due to the identification of relatively specific protein markers for lymphatic endothelial cells. The first marker thought to be restricted to lymphatic vessels was vascular endothelial growth factor receptor three (VEGFR3) (Kaipainen et al., 1995). Today, many lymphatic markers are used for detection including lymphatic vessel endothelial hyaluronan receptor-1 (LYVE1), podoplanin (PDPN), prospero homeobox protein 1 (prox1), CD31, and neuropilin 2 (NRP2) (see staining in Figures 1, 3). Antibodies to these proteins have enabled the detection of lymphatic vessels in tissues using immunohistostaining techniques. It is now appreciated that none of these markers is unique to lymphatic vessels, and their presence on other cell types can confound lymphatic detection.

LYVE1 is a cell surface receptor for hyaluronan found on LEC (Banerji et al., 1999). Macrophages also express LYVE1. PDPN is a mucin-type transmembrane glycoprotein with

extensive O-glycosylation expressed by LEC (Breiteneder-Geleff et al., 1999). Podocytes and some epithelial cells, especially those in the epidermis and mammary fat pad, also express PDPN. Platelet endothelial cell adhesion molecule-1 (PECAM1), also called CD31, is a pan-endothelial cell stain. CD31 stains blood EC and, to a lesser extent, LEC and macrophages. NRP2 is not exclusive to LEC and is expressed by EC, neurons, melanocytes and visceral smooth muscle (Bielenberg & Klagsbrun, 2007; Bielenberg et al., 2012). Prox1 is a transcription factor found in many cell types. Prox1 is a master regulator of many lymphatic vessel-associated genes (Wigle & Oliver, 1999). Nuclear staining for prox1 is useful for distinguishing lymphatic vessels from blood vessels, which lack prox1 (Figure 1D).

Another method to detect lymphatic vessels and to measure their functionality is lymphangiography. This technique involves injecting a dye (either colored or fluorescent) or a radioactive tracer into the tissue of interest and visualizing the drainage of the dye/tracer through lymphatic channels (Jain et al., 2012). This method is commonly used in murine models, especially in the ear or tail (Figure 2). The blood vascular plexus (seen visibly through the skin due to RBCs in the blood) resembles the branches of a tree—going from thick to ever-thinner branches. In contrast, the cutaneous lymphatic plexus, visualized with lymphangiography, can be seen as a network of circular, interconnecting channels. The looping pattern ensures that lymph will flow even when one pathway becomes blocked. During inflammation, lymphangiography can reveal aberrant lymphatic flow as the pooling of dye or the dilation of vessels (Figure 2B). Alternately, the dye may not flow at all in cases of severe lymphedema. Lymphangiographic techniques are important for identifying sentinel LNs from primary tumors for biopsy or surgery.

Tissues (or tumors) vary in their microvessel density depending on their requirements for oxygen and nutrients. Similarly, each organ or tissue differs in its lymphatic vessel density (LVD). For instance, the footpad has a very high LVD as does the tongue and the pubic areas (Zwaans & Bielenberg, 2007). More than half of the total lymph volume in the body comes from lymph formed in the liver and drains into capillaries in the triad region (Alexander et al., 2010). The lymphatics in the diaphragm are important for draining excess peritoneal fluid. Interestingly, mice are born without lymphatics in the diaphragm (Poggi et al., 1991; Yuan et al., 2002). Lymphatic vessels sprout perinatally in the diaphragm, making this organ a unique model for studying lymphangiogenesis. Even within a certain tissue type such as the skin, location can matter—for instance, leg skin (dermis) has a higher LVD than does forearm skin (possibly from the increased load due to gravity) (Stanton et al., 1999).

Some tissues are devoid of lymphatics altogether, such as the central nervous system including the spinal cord, brain and retina. This absence of LEC is presumably to avoid immune cell infiltration into these locations and maintain them as relatively “immune privileged.” However, the lack of lymphatic vessels can also have deleterious consequences in times of trauma or disease. For example, in diabetic retinopathy, leaky vessels result in fluid accumulation and swelling; since the retina lacks lymphatics, there is no system in place to drain the fluid and vision becomes impaired. Similarly, the edema that is associated with brain trauma and stroke are a major cause of the neural damage associated with these events. The study of the lymphatic system is growing rapidly. Just eight years ago, zebrafish

were discovered to have a lymphatic system (Yaniv et al., 2006). Tissues once thought to be lacking lymphatics such as the placenta, the choroid, and the retro-orbital adipose may actually have lymphatic vessels (Bellini et al., 2012; Dickinson et al., 2006).

Lymphatic Vessel Development

Although Hippocrates described lymph in the 5th century B.C as “white blood,” it was not until the 1620's that the anatomist Aselli made a more detailed description of the lymphatic system (Kaipainen & Bielenberg, 2011). At the turn of the 20th century, several scientists proposed theories as to the origin or development of the lymphatic system and Sabin's hypothesis has been most supported by modern day mechanisms (Sabin, 1902; Oliver & Detmar, 2002). Briefly, lymphatic vessels branch from veins during embryonic development and further develop through the process of lymphangiogenesis (Yaniv et al., 2006; Srinivasan et al., 2007). The step-by-step maturation of a complex lymphatic vascular network from its venous origins has been delineated by Oliver and includes: (1) lymphatic competence, (2) lymphatic bias, (3) lymphatic specification and (4) LEC differentiation (Yang & Oliver, 2014). Each step coincides with the up- and down-regulation of various LEC markers.

Vascular endothelial growth factor-C (VEGF-C) and its tyrosine kinase receptor VEGFR3 are crucial mediators of lymphangiogenesis. VEGF-C/VEGFR3 signaling has been indicated as the pivotal signaling pathway during the initial induction of lymphatic sprouts (Karkkainen et al., 2004). Heterozygous *Vegfc*^{+/-} mice, although viable, develop cutaneous hypoplasia of lymphatic vessels and lymphedema whereas homozygous knockouts (*Vegfc*^{-/-}) are embryonic lethal--lacking any functional lymphatic vasculature (Karkkainen et al., 2004; Hägerling et al., 2013). Heterozygous mutations of the VEGFR3 gene are also associated with the swelling of extremities in mouse models due to lymphatic anomalies (Karkkainen et al., 2001). Mice expressing soluble VEGFR3 (sVEGFR3) show lymphatic vessel regression and inhibition of lymphangiogenesis (Mäkinen, et al., 2001; Singh et al., 2013).

Neuropilin (NRP) Receptors

NRPs comprise another family of transmembrane VEGF receptors. Originally discovered for their pivotal role in embryonic neuronal development, Klagsbrun and colleagues later discovered the role of NRPs as VEGF co-receptors and thus angiogenic regulators (Kolodkin et al., 1997; Soker et al., 1998). The NRP family consists of two distinct 130 kDa transmembrane glycoproteins—NRP1 and NRP2. The two proteins are structurally similar, sharing 44% amino acid homology, yet map to separate chromosomes (NRP1:10p12; NRP2:2q34) (Giger et al., 1998; Rossignol et al., 1999). The structure of both NRP receptors consists of five extracellular domains (a1, a2, b1, b2, and c), a transmembrane domain, and a short intracellular domain with a PDZ binding motif (Klagsbrun & Eichmann, 2005; Bielenberg et al., 2006). The NRPs are unique receptors in that they can bind to two dissimilar families of ligands—the VEGF family of angiogenic proteins and the class 3 semaphorin (SEMA3) family of guidance molecules. NRP1 and NRP2 bind multiple ligands (Gaur et al., 2009); this review will focus on those with vascular and lymphatic consequences.

NRP Ligands and Signaling

Each NRP has differential affinity for individual ligands and co-receptors. Within the VEGF family, NRP1 binds VEGF-A, -B, -E and placenta growth factor (PlGF2), whereas NRP2 binds VEGF-A, -C, -D, and PlGF2 (Klagsbrun et al., 2002; Gaur et al., 2009). Furthermore, NRP1 may complex with VEGFR1 or VEGFR2 while NRP2 interacts with VEGFR1, 2, and 3 (Soker et al., 1998; Favier et al., 2006; Koch et al., 2014). Through these complexes NRPs are able to enhance VEGFR-mediated pro-angiogenic and pro-lymphangiogenic signaling.

VEGF ligands bind the b1/b2 region of NRP receptors, forming a bridge between NRP and its respective VEGFR (Soker et al., 2002; Mamluk et al., 2002; Appleton et al., 2007; Pan et al., 2007). The binding of VEGF-A to NRP1 enhances its affinity for VEGFR2 (Soker et al., 2002). By coupling with VEGFR, NRP is able to further enhance VEGFR phosphorylation and induce a stronger pro-angiogenic response. Furthermore, NRP1 can complex with VEGFR2 in the same cell (*cis*) or on adjacent cells (*trans*). Within the same cell (likely an EC), VEGF binds NRP1 and VEGFR2 and a complex is rapidly formed; internalization of the complex ensues (Koch et al., 2014). In *cis*, signaling occurs through PLC γ , ERK1 and ERK2. When NRP1 is expressed on one cell (EC or tumor cell) and VEGFR2 on another cell (EC), complex formation via VEGF binding is delayed and subsequent signaling through ERK2 and PLC γ is sustained (Koch et al., 2014). Arguably, NRP1 and 2 are necessary for optimal signaling of the VEGFR2 and VEGFR3 receptors --as mutant mouse studies have confirmed (refer to section 3.4). The differential expression patterns of NRPs and their co-receptors in various cells and tissues ultimately regulate their function and signaling activities (refer to section 3.2).

In contrast, soluble NRPs (sNRP), secreted receptors consisting of only the A and B ligand-binding domains, may act as a sink, sequestering VEGF or hepatocyte growth factor (HGF) ligands and thereby inhibiting their pro-angiogenic and pro-lymphangiogenic effects (Panigrahy et al., 2014). Transgenic mice overexpressing sNrpl in the skin experienced reduced vascular permeability in response to both VEGF₁₆₅ and VEGF₁₂₁ (Mamluk et al., 2005). In this case, the lack of available ligand decreases the cellular response to VEGF. Tumors overexpressing sNrpl seem to suffer from “VEGF-withdrawal” and are apoptotic and hemorrhagic (Gagnon et al., 2000). Alternately, NRPs may be shed from the cell surface by ADAMs (a disintegrin and metalloprotease) (Romi et al., 2014).

NRP receptors possess a short cytoplasmic domain. Both NRPs end in the same three terminal amino acids: S-E-A, which are responsible for binding to synectins, PDZ-domain containing proteins (Cai & Reed, 1999; Prahst et al., 2008). The cytoplasmic PDZ-binding domain of NRP is required for NRP/VEGFR2 complexing (Prahst et al., 2008). Synectin^{-/-} mice have arterial morphogenesis defects with a lack of mural cell coverage (Paye et al., 2009). NRP1's cytoplasmic domain plays an important role in the endocytic trafficking of VEGFR2 and the subsequent signaling through ERK to promote arteriogenesis (Lanahan et al., 2013). In medulloblastoma, the SEA motif of NRP1 was crucial for PlGF signaling (Snuderl et al., 2013). The once enigmatic intracellular domain of NRP is evolving into a potential therapeutic target.

The mechanism of NRP2 and VEGFR3 interaction with VEGF-C/D is important for functional lymphangiogenesis. VEGF-C and -D both bind NRP2 and VEGFR3 to enhance VEGFR3 phosphorylation and induce a stronger lymphangiogenic response (Favier et al., 2006). VEGFR3 signaling results in the subsequent activation of PI3K and phosphorylation of downstream serine kinases AKT and ERK, which regulate LEC functions (Coso et al., 2012). The ability to mediate LEC proliferation, migration, and survival makes many of the aforementioned pathways essential for normal lymphangiogenesis. However, in pathologic conditions, these pathways can be hijacked by tumors in order to facilitate metastasis. For example, VEGF-C overexpressing tumors have large lymphatic vessels and metastasize readily to regional LNs (Skobe et al., 2001). On the flip side, SEMA3F can bind the $\alpha 1/\alpha 2/\beta 1$ region of NRP2, inhibiting the association between NRP2 and several pro-angiogenic ligands (Bielenberg et al., 2004; Geretti et al., 2007; Vander Kooi et al., 2007). SEMA3F may inhibit lymphangiogenesis via several mechanisms (discussed in greater detail below).

NRPs also interact with other proteins as well including HGF, transforming growth factor- β , platelet-derived growth factor, and integrins. HGF regulates angiogenesis and peritumoral lymphangiogenesis, increases the invasiveness of several types of tumors, and facilitates metastasis (Cao et al., 2006). HGF promotes LEC proliferation and migration *in vitro* and lymphatic vessel formation *in vivo* (Kajiya et al., 2005). Biacore techniques were first used to detect HGF binding to NRP1 (West et al., 2005). Subsequently, NRP1 and NRP2 were demonstrated to act as co-receptors with c-met similar to their function with VEGF/VEGFR (Sulpice et al., 2008). sNRP1 binds HGF and may regulate liver development and regeneration (Panigrahy et al., 2014). A better understanding of the HGF/NRP pathway may present more options for therapeutic regulation of pathologic angiogenesis and lymphangiogenesis.

NRP/Semaphorin Interactions

NRPs also interact with another family of ligands known as class 3 semaphorins (SEMA3). In fact, SEMA3 were the first known ligands of NRP receptors (Kolodkin et al., 1997). The entire SEMA family consists of eight classes (SEMA1–7; SEMAV); of which, classes 3–7 are found in vertebrates (reviewed in Neufeld & Kessler, 2008). SEMA3 (A-G) are the only class of vertebrate SEMA that are secreted and not membrane bound. The SEMA3 protein structure consists of an N-terminal SEMA domain, a plexin-semaphorin-integrin (PSI) domain, an Ig loop, and a C-terminal basic domain. The N-terminal SEMA domain is approximately 500 amino acids in length and forms a seven-bladed β -propeller motif that binds NRP and may also facilitate SEMA3 homodimerization (Gherardi et al., 2004). Within the C-terminal domain are cysteine-rich regions that form disulfide bridges, also necessary for dimerization and overall bioactivity (Klostermann et al., 1998). NRPs are co-receptors for the SEMA3 family. SEMA3/NRP complexes interact with larger transmembrane proteins called plexins, which transduce the signal cascade (plexins are reviewed in Peralta et al., 2012). NRPs differ in their binding affinity and functionality via the SEMA3s (Gaur et al., 2009). The majority of class 3 SEMAs (SEMA3B, SEMA3C, SEMA3F, and SEMA3G) bind NRP2. SEMA3A binds NRP1, while SEMA3E binds neither NRP1 nor NRP2. Rather, SEMA3E binds and signals directly through Plexin D1 (Gu et al., 2005).

Protein convertases regulate the cleavage of SEMA3 proteins. The enzymatic cleavage has differential effects on SEMA3 bioactivity. SEMA3A and SEMA3B are normally repulsive cues or inhibitory ligands of NRP; but once processed, their activity is ablated (Adams et al., 1997; Varshavsky et al., 2008). SEMA3G can bind NRP2 in its full-length form and bind both NRPs in its cleaved form (Kutschera et al., 2011).

SEMA3F preferentially binds NRP2, which, as mentioned above, is a mediator of lymphangiogenesis. SEMA3F-expressing cells can repel EC and LEC *in vitro* (Bielenberg et al., 2004). Tumors overexpressing SEMA3F have dramatically reduced microvessel density and do not metastasize to regional LNs (Bielenberg et al., 2004; Kessler et al., 2004; Kigel et al., 2008). The SEMA3F locus (3p21.3) is commonly deleted in small cell lung carcinoma, indicating SEMA3F may be a tumor suppressor gene (Sekido et al., 1996). The strong anti-tumor/anti-metastasis effect of SEMA3F is likely due to multiple mechanisms: i) SEMA3F can directly inhibit the migration of tumor cells expressing NRP2, ii) SEMA3F can inhibit tumor neovascularization or lymphangiogenesis by competing with VEGF-A for NRP1 or NRP2, or VEGF-C/D for NRP2, iii) SEMA3F can directly signal through NRP2/plexinA (regardless of VEGF expression) in EC or LEC to inhibit migration, collapse cell shape or repel. VEGF- and SEMA3-binding induce different endocytosis mechanisms for NRP and signal very differently inside the cell (Salikhova et al., 2008). The signal transduction pathway of SEMA3F/NRP2/plexin has been mapped through Abelson (Abl2) tyrosine kinase, RhoA, and cofilin—ultimately resulting in a depolymerized f-actin cytoskeleton and immobilized cells (Shimizu et al., 2008).

Others suggest that SEMA3 regulates integrin signaling in EC and thus mediates downstream phosphorylation of AKT and integrin-linked kinase (ILK) (Bielenberg et al., 2004; Kusy et al., 2005; Potiron et al., 2007). ILK is an upstream regulator of ERK1/2 (Potiron et al., 2007). Inhibition of ERK1/2 affects cell differentiation, survival, metabolism, proliferation, and VEGF synthesis possibly providing another means by which SEMA3F may obstruct tumor progression and growth (Kessler et al., 2004; Lloyd et al., 2006). SEMA3F inhibition of AKT, a well-documented kinase with an arsenal of downstream targets, resulted in a significant decrease in HIF-1 α expression, a major regulator of VEGF synthesis (Cassinelli et al., 2013; Potiron et al., 2007).

NRP Localization

The Nrp1 receptor is localized to arteries and the Nrp2 receptor to veins and lymphatic vessels during development (Herzog et al., 2001; Yuan et al., 2002). In the adult, both Nrps are downregulated on quiescent EC but become upregulated during times of inflammation, ischemia or injury (Klagsbrun et al., 2002; Bielenberg et al., 2006). Adult blood vessels express Nrp1 or Nrp2 (Berge et al., 2011; Bielenberg et al., 2012). Lymphatic vessels express NRP2 in capillary endothelial cells and along the length of collecting ducts (Figure 3), but valves do not express NRP2 (Bouvier et al., 2012). Rather, valves express Nrp1; and Sema3A has been shown to regulate valve formation and function (Jurisic et al., 2012; Bouvier et al., 2012). Nrps are not exclusive to vascular cells. Nrp1 is also expressed on epithelial cells, vascular SMC, T cells, dendritic cells, and neurons. Nrp2 is found on visceral SMC, T cells, melanocytes, and neurons.

NRP Mutant Mice

The connection between NRPs and the vasculature has been most convincingly shown by transgenic and mutant mouse studies (Table 1). The first evidence came in 1995, several years before NRP was shown to bind VEGF—mice overexpressing Nrp1 showed a severe and excessive vascular phenotype with dilated vessels, ectopic sprouting, and malformed hearts (Kitsukawa et al., 1995). Subsequently, the majority of studies have focused on knockout or mutant models. Nrp1-null mice are embryonic lethal at E12.5 and have severe vascular defects including the transposition of great vessels (Kawasaki et al., 1999). Nrp2-null mice are viable but have reduced lymphatic capillary numbers (Yuan et al., 2002). Intriguingly, Nrp2^{lacz/lacz} mice succumb after birth but nrp2^{gfp/gfp} mice survive, although they are smaller than wild-type littermates (Takashima et al., 2002; Yuan et al., 2002). Nrp1 and Nrp2 double knockout mice have avascular yolk sacs and die at E8.5, an earlier developmental time point than Nrp1^{-/-} alone (Takashima et al., 2002). Mice with a knockout of one Nrp and heterozygous for the other Nrp (Nrp1^{-/-} Nrp2^{+/-} or Nrp1^{+/-} Nrp2^{-/-}) survive until E10.5 and die of vascular defects including sparse and malformed vessels, absence of large vessels and branching defects in the yolk sac (Takashima et al., 2002).

To distinguish the relative importance of SEMA3 and VEGF to the development of the neuronal system, vascular system and cardiovascular system, knockin mice called Nrp1^{Sema-} were created with a mutation in the CUB domain of Nrp1, such that they could not bind Sema3a (Gu et al., 2003). These mice develop a normal vascular system, but have heart defects—suggesting that Sema3a/Nrp1 is not required for vessel formation. Nrp1^{Sema-} mice were later found to have smaller lymphatic valves similar to Sema3a^{-/-} and Plexina1^{-/-} mice (Bouvree et al., 2012).

As mentioned above, Nrp1/VEGFR2 complex formation is dependent on the cytoplasmic domain of Nrp. Mice lacking this domain (Nrp1^{cyto} /) have malformed arteries and abnormal vascular patterning and resemble synectin^{-/-} mice in some aspects (Fantin et al., 2011; Lanahan et al., 2013). Mice lacking VEGF/Nrp1 interactions in an EC-specific Nrp1 knockout (Tie2cre-Nrp1^{f/f}) are embryonic lethal and suffer from widespread vascular deficiencies (Gu et al., 2003). Another EC-specific Nrp1 knockout (EC-SCLcre-Nrp1^{f/f}) lacks VEGF- and Sema3a-induced permeability (Acevedo et al., 2008). Mice engineered with a mutation (Y297A) in the b1 binding domain of Nrp1 lack VEGF binding and display reduced growth and diminished angiogenesis (Fantin et al., 2014). These mice do not die *in utero* like Nrp1^{-/-} mice and therefore can be used to evaluate physiological and pathological angiogenesis. In fact, tumor angiogenesis is reduced in Nrp1^{Y297A/Y297A} mice (Fantin et al., 2014).

Although, many genes have surfaced as important mediators of lymphatic vessel development, valve formation, proliferation, and migration, those affecting the VEGFR3/NRP2 signaling pathway appear to have especially deleterious effects (Yang & Oliver, 2014). Although mice lacking one allele of Nrp2 (Nrp2^{+/-}) are phenotypically normal, mice heterozygous for Nrp2 and VEGFR3 have major lymphatic defects (Xu et al., 2010).

Nrp2^{+/-}Vegfr3^{+/-} mice have branching and sprouting problems in lymphatic vessels but Nrp2^{+/-}Vegfr2^{+/-} mice do not (Xu et al., 2010).

Pathologic Lymphangiogenesis and Tumor Metastasis

Growth of solid tumors beyond 1–2 mm³ in volume is angiogenesis-dependent (Folkman, 2007). New capillary formation not only supplies the tumor with essential nutrients but also provides a means for tumor cell dissemination through the circulation. Tumor vessels are especially leaky, and lymphangiogenesis occurs in and around tumors to enlarge and expand existing lymphatic vessels to accommodate the increased need for drainage. Some angiogenic factors, such as VEGF-A, also induce lymphangiogenesis. Tumors secrete a myriad of lymphangiogenic molecules including VEGF-C/D, HGF, IL1 β , and angiopoietin-2.

Due to the unique structure of lymphatic capillaries and their permissive entry of immune cells, migratory tumor cells can enter the lymphatic system readily. In fact, the lymphatic system helps to “clear” tumor cells from the body by filtering them in the nodes and exposing the cells to phagocytic macrophages. Lymphatic invasion was found to correlate more strongly with LN metastasis in human melanoma specimens than vascular invasion (Doeden et al., 2009). Enlarged peritumoral lymphatic vessels are often seen to contain a “plug” of tumor cells inside the vessel (Figure 4A) (Banyard et al., 2014; Enquist et al., 2014). It is unclear whether tumor cells invade the lymphatic vessel as a single cell and proceed to grow inside the vessel or whether the cells invade as a “sheet” using collective migration (Friedl & Wolf, 2003). Tumor cell entry into the sinus of the sentinel LN may not be a passive process as previously thought. Gated access to the LN required CCR8 receptor expression by tumor cells and CCL1 expression by LEC in the sinus (Das et al., 2013). CCL1 is a cytokine used to attract and recruit monocytes, dendritic cells, NK cells and immature B cells to the LN. Other mechanisms may be uncovered which regulate tumor cell admission to selective LNs.

Nearly all malignant carcinomas have been found to metastasize to regional LNs (Zwaans & Bielenberg, 2007). In fact, the TNM staging system for tumor classification is based upon this fact. Yet, the importance of tumor-associated lymphangiogenesis and the relative contribution of lymphatic dissemination to the existence of metastasis in distant organs is still debated. Measurement of tumor-associated LVD and area has been associated with metastasis in many tumor types. In fact, lymphangiogenesis was more predictive of metastasis in human melanoma than tumor thickness (Dadras et al., 2005). However, metastasis to certain locations may not require initial metastasis to the LN. For example, colon carcinoma metastasizes to regional LNs and the liver. When implanted in the colonic mucosa in mice, tumor cells metastasized to liver; and systemic, weekly treatment with anti-lymphangiogenic anti-VEGF-C antibody inhibited LN metastasis but did not inhibit liver metastasis (Enquist et al., 2014).

Anti-lymphangiogenic therapy may be more effective in melanoma and carcinomas seen to metastasize early to LNs such as breast cancer and oral squamous cell carcinoma. Many new strategies to target lymphatic vessels are emerging (reviewed by Zwaans & Bielenberg,

2007; Alitalo & Detmar, 2012; Karaman & Detmar, 2014). Many of the molecules and receptors mentioned in this review are the target of potential drug therapies. These include antibodies to receptors such as VEGFR2, VEGFR3, and NRP2. For instance, anti-NRP2 antibodies inhibited lymphangiogenesis and metastasis in preclinical models (Caunt et al., 2008). Ligands such as VEGF-C and VEGF-A can also be targeted. SEMA3F protein therapy may also inhibit metastasis. Future studies using nanotechnology, siRNA strategies and microRNA may prove to be effective.

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Highlights

- Markers specific to lymphatic vessels
- Neuropilins in development
- Neuropilins in disease and cancer
- Transgenic and mutant neuropilin mice
- Targeting lymphangiogenesis to inhibit metastasis

Figure 1. Lymphatic-associated proteins

No proteins are exclusive to only LEC but several are relatively restricted in their staining profile. FFPE human skin sections were stained for LYVE1 (A), PDPN (D2-40) (B) and CD31 (C). LEC (arrows) stain weaker for CD31 than do EC. Prox1 (red) stains the nuclei of LEC (D); CV, cardinal vein. A lymphatic vessel (l) stained for VEGFR3 (green) in a mouse skin cryosection (E); red blood cells in an artery (a) and vein (v) were autofluorescent. Pdpn staining outlines the LEC and the valves in a large lymphatic vessel surrounding a skin tumor in a mouse FFPE section (F). Brown color denotes positive staining in A–C, F. Sections were counterstained with hematoxylin (blue) in A, C, F. All images in panels A–C, E–F were taken in the Bielenberg laboratory. Panel D is taken with permission from Copyright Clearance Center from Qu et al, 2010, originally published in *Development* 137:1285–1295.

Figure 2. Lymphangiography

Balb/c mouse ears were challenged with vehicle (A) or antigen (B) during delayed-type hypersensitivity reactions. Injection of blue dye into the ears allowed visualization of the lymphatic channels (lymphangiography). The lymphatic plexus in the ear is intricate with many branches and circular connections within the network (blue) (A). In an inflammatory state (B), lymphatic vessels are dilated and thicker in appearance and areas of pooling (arrows) can be observed. Images are from the Bielenberg laboratory.

Figure 3. NRP2 mediates lymphatic sprouting

Illustration of intestinal villi in the intestine (A). Each villi contains a blood capillary loop (red/blue) and a central lymphatic capillary called a lacteal (green). Lacteals express Nrp2 (blue) in $Nrp2^{LacZ/+}$ mice cryosections stained with x-gal and counterstained with eosin (pink) (B). Anti-Nrp2 antibodies (brown) also detect the lacteal in immunostaining (C); counterstained with hematoxylin (blue). Mouse intestinal lacteals at P8 were stained with LYVE1 (green) in control-treated (D) and anti-Nrp2^B-treated mice (E). Scale bar = 125 microns. Nrp2 antibodies inhibited lymphatic sprouting. Panels A–C are from the Bielenberg laboratory. Panels D–E are from ©Xu et al., 2010, originally published in JCB, doi:10.1083/jcb.200903137.

Figure 4. Tumor-associated lymphatic vessels

Pdpn staining (brown) of mouse lymphatic vessels (lv) at the periphery (A) and inside (B) a human prostate tumor growing in the prostate of a nude mouse; blood vessel (bv) and prostate gland (g). Metastatic tumor cells (arrow) are seen inside the lymphatic vessel (A). Intratumoral lymphatic vessels are not collapsed (B). Images are from the Bielenberg laboratory.

Table 1

Mutant and transgenic mouse studies involving the Neuropilin/SEMA3 axis.

Mutant Mouse	Phenotype*	Reference
Overexpression		
Nrp1	embryonic lethal; excess/dilated vessels, malformed hearts, ectopic sprouting	Kitsukawa et al., 1995
K14-sNrp1	reduced skin vessel area; reduced permeability	Mamluk et al., 2005
Knockout/null		
Nrp1^{-/-}	embryonic lethal E12.5; vascular defects: transposition of great vessels, disorganized/insufficient vascular networks	Kitsukawa et al., 1997; Kawasaki et al., 1999; Takashima et al., 2002
Nrp2^{-/-} or gtp/gfp	viable; reduced lymphatic capillary number; reduced ischemia-induced retinal NV	Yuan et al., 2002; Shen et al., 2004
Nrp2^{-/-} or lacz/lacz	perinatal lethal; no apparent vascular phenotype	Takashima et al., 2002
Nrp1^{-/-}Nrp2^{+/-}	embryonic lethal E8.5; avascular yolk sacs	Takashima et al., 2002
Nrp1^{-/-}Nrp2^{+/-} Nrp1^{+/-}Nrp2^{-/-}	embryonic lethal E10–10.5; sparse capillary networks; lack large vessels; branching defects in yolk sacs	Takashima et al., 2002
Nrp1^{sema-}	small lymphatic valves; instability of the alveolar-capillary interface; cardiovascular defects	Bouvrée et al., 2012; Joza et al., 2013; Gu et al., 2003
Nrp1^{cyto} /	abnormal vascular patterning; impaired arteriogenesis	Fantin et al., 2011; Lanahan et al., 2013
Nrp1^{Y297A/Y297A}	viable; reduced size, growth and angiogenesis; reduced tumor growth and angiogenesis	Fantin et al., 2014
Nrp1^{sema-}Nrp2^{-/-}	cardiovascular defects; truncus arteriosus	Gu et al., 2003
Nrp2^{+/-}Vegfr3^{+/-}	lymphatic sprouting and branching defects	Xu et al., 2010
Nrp2^{+/-}Vegfr2^{+/-}	normal lymphatic sprouting	Xu et al., 2010
Sema3a^{-/-}	small lymphatic valves covered in SMC; normal lymphatic sprouting; cardiovascular defects; abnormal trunk vessels	Bouvrée et al., 2012; Behar et al., 1996
Plexina1^{-/-}	small lymphatic valves	Bouvrée et al., 2012
Tissue-specific		
Tie2cre-Nrp1^{fl/fl}	embryonic lethal; systemic vascular deficiencies associated with loss of VEGF/Nrp1	Gu et al., 2003
EC-SCLcre-Nrp1^{fl/fl}	lack VEGF- and Sema3a-induced permeability; vessel quiescence; reduced tumor initiation and angiogenesis in xenograft model	Acevedo et al., 2008; Koch et al., 2014
Esr1cre-Nrp1^{fl/fl}	lack Sema3a-induced permeability in DR models; airspace enlargement and apoptosis following chronic CS exposure in lung model	Cerani et al., 2013; Le et al., 2009; Joza et al., 2013
K14cre-Nrp1^{fl/fl}	lack of tumor initiation after carcinogen exposure; increased apoptosis following UVB	Beck et al., 2011; Riese et al., 2012
MMTVcre-Nrp2^{fl/fl}	defects in mammary gland branching and morphogenesis	Goel et al., 2011
SM22αcre-Nrp2^{fl/fl}	visceral smooth muscle increased contractility; bladder incontinence	Bielenberg et al., 2012

* Description of vascular-associated phenotypes; neuronal phenotypes are omitted. CS, cigarette smoke; DR, diabetic retinopathy; NV, neovascularization; SMC, smooth muscle cells.