

INVOLVEMENT OF NEUROPILIN-1 IN THE AUTOCRINE MECHANISM OF  
VASCULAR ENDOTHELIAL GROWTH FACTOR –165 IN BREAST CANCER  
PROGRESSION

by

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ABSTRACT

Vascular Endothelial Growth Factor (VEGF) is a potent inducer of angiogenesis through chemotatic, mitogenic and anti-apoptotic effects on endothelial cells in cancer progression. The autocrine effect of VEGF on epithelial breast cancer cells is addressed in the following studies, which focus on the VEGF receptor, Neuropilin-1 (NRP-1).

Although Neuropilin-1 is a specific receptor for the VEGF-165 isoform, NRP-1 requires interaction with other cell surface proteins in order to transduce signals intracellularly. Due to the lack of typical receptors responsible for NRP-1 mediated VEGF signaling on the surface of breast epithelial cells, expression of NRP-1 co-receptors was assessed *in vitro* in breast carcinoma cell lines and *in vivo* using human breast tissue samples. NRP-1 co-receptors (Plexin-A1 and L1 Cellular Adhesion Molecule- L1CAM) were previously believed to be functionally isolated to neuronal development. Using RNA and protein analysis, the expression of the

NRP-1 co-receptors in breast carcinoma cell lines was shown. Co-expression and co-localization of Plexin-A1 and NRP-1, and L1-CAM and NRP-1, was shown in human breast tissues. We also determined that Plexin-A1 expressing breast cancer cells respond to VEGF stimulation in culture by actin rearrangement. Mechanistic studies revealed the involvement of LIM Kinase in this process. In addition, the role of L1-CAM in anti-apoptotic effect of VEGF on MCF-7 cells in hypoxic conditions was determined. Mechanistic studies revealed an increased phosphorylation of Akt is involved in this process. A novel isoform of NRP-1 was isolated in our laboratory, and the effect of this isoform in vivo was characterized. We created a transgenic mouse designed to overexpress the novel NRP-1 isoform under the direction of the muscle creatine kinase promoter. In this study the generation of the transgenic animals to assess the autocrine effect of VEGF in mammary tumor formation and progression is described.

These studies suggest new mechanisms of action for the autocrine effect of VEGF-165 on breast cancer cells and a novel method for assessing these findings in vivo. Through the discovery of Plexin-A1 as a factor affecting VEGF-mediated migration of tumor cells, and L1-CAM as a receptor involved in VEGF mediated suppression of apoptosis, our understanding of the potency of VEGF as a cancer promoting molecule is greatly increased. By generating a transgenic mouse model that expresses high levels of a soluble VEGF-binding NRP-1 receptor, a powerful model for the study the autocrine effect of VEGF in tumor progression has been developed.

INDEX WORDS: Neuropilin-1, VEGF, Plexin-A1, L1-CAM, migration, breast carcinoma, Neuropilin-1 isoform

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## DEDICATION

To my Mom and Dad, I love you and thank God for you everyday.

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## CHAPTER 1

### INTRODUCTION

The growth and progression of malignant tumors is a complex process relying on various distinct signal transducing growth-promoting cues, including neovascularization. Vascular endothelial growth factor (VEGF), along with basic fibroblast growth factor, is the main stimulator of angiogenesis in the tumor microenvironment (Folkman, 1997). Until recently, VEGF was thought to act exclusively on endothelial cells via known receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR), which provide mitogenic, anti-apoptotic, and chemotactic stimuli (Matsumoto and Claesson-Welsh, 2001). However, the discovery of the VEGF<sub>165</sub> isoform specific receptor, Neuropilin-1 (NPR-1), which is located on both endothelial and tumor cells, supports the evidence that that VEGF is able to directly promote tumor growth and survival in an autocrine fashion (Soker et al., 1998). (Soker et al., 1998). The autocrine effect of VEGF on breast cancer cells has been previously demonstrated in our laboratory (Yoshiji, 1996). More recently, others have shown that the VEGF autocrine response requires NRP-1 expression to prevent apoptosis in breast cancer cells (Bachelder et al., 2001). Other studies (Pidgeon et al., 2001) suggest that VEGF directly regulates the expression of the anti-apoptotic protein bcl-2 on mouse primary mammary tumor cultures. However, the mechanism of VEGF-autocrine action remains largely unknown.

Neuropilin-1 was first discovered (Kolodkin et al., 1997 ; He and Tessier-Lavigne, 1997) as a semaphorin receptor responsible for axonal guidance in the developing brain. The semaphorin family consists of over 20 different proteins, which can act as repulsive or attractive cues to neuronal outgrowth. Studies on dorsal root ganglions have shown the importance of Neuropilin-1 in the repulsive action of the prototypical semaphorin, Sema3A (Kolodkin et al., 1997; He and Tessier-Lavigne, 1997). NRP-1 binds selectively to the different semaphorins and is believed to be responsible for providing binding specificity to semaphorin complexes (Kolodkin and Ginty, 1997). Presenting with only a short cytoplasmic domain, NRP-1 is believed to require other signal-transducing proteins in order to create a functional receptor complex (Rohm et al., 2000). NRP-1 has been shown to complex with KDR resulting in increased VEGF<sub>165</sub> binding affinity as well as enhanced mitogenicity and chemotaxis of endothelial cells (Soker et al., 1998). Breast cancer cell lines and tumor samples from patients are traditionally believed to lack both KDR and FLT-1. Therefore, we set out to establish the presence and possible function of other NRP-1 co-receptors expressed in breast cancer cell lines. In this study, we describe for the first time the presence of NRP-1 neuronal co-receptors Plexin-A1 and L1-Cellular Adhesion Molecule (L1-CAM) in breast cancer cell lines and in human breast tumor samples. We also show that NRP-1 is able to interact with both PlexinA1 and L1-CAM in breast cancer cells. These studies provide evidence for both Plexin-A1 and L1-CAM as novel signal-transducing proteins for migratory and anti-apoptotic actions, respectively, of VEGF-165. To date, two soluble splice variants of NRP-1 have been discovered and examined in VEGF signaling *in vitro* (Gagnon et al., 2000). Rat prostate tumor cells engineered to overexpress a soluble NRP-1 isoform are able to inhibit angiogenesis

(Gagnon et al., 2000; Rossignol et al., 2000). In our laboratory, we have isolated a novel NRP-1 splice variant that retains VEGF binding sites, but lacks the transmembrane and cytoplasmic NRP-1 domains. In order to determine the effect of inhibition of VEGF signaling through NRP-1 in mammary tumorigenesis, we generated transgenic mice to overexpress our novel NRP-1 isoform under the direction of the muscle creatine kinase (MCK) promoter (Lee et al., 1993) in order to achieve high circulating levels of this soluble isoform. Here we describe the generation and confirmation of this transgenic line. Ongoing studies involve crossing the transgenic mice, which overexpress this soluble NRP-1 with transgenic mice containing the mouse mammary tumor virus (MMTV) polyoma middle T-antigen (MT). MMTV-MT mice spontaneously form mammary tumors (Maglione et al., 2001). By crossing MMTV-MT mice with our transgenic line with the soluble NRP-1, which will act as a soluble VEGF receptor, we will be able to establish *in vivo* the role of the soluble NRP-1 in VEGF-mediated stimulation of tumor development and progression.

The following studies were designed to explore the mechanism of VEGF autocrine signaling in breast cancer. My hypothesis is “NRP-1 is an essential component of VEGF autocrine action in breast carcinoma cells,” addressed by the following specific aims:

- 1. To identify possible neuropilin-1 co-receptors on breast cancer cells.**
- 2. To Determine the role of neuropilin-1 co-receptors in the autocrine effect of VEGF on breast cancer cells.**
- 3. To generate of transgenic mouse line that over-expresses a novel soluble isoform of neuropilin-1.**

## CHAPTER 2

### LITERATURE REVIEW

#### **Neuropilin-1 is a Vascular Endothelial Growth Factor Receptor**

Angiogenesis, the formation of new blood vessels from pre-existing vessels, is necessary for the growth of solid tumors beyond 1-2 mm (Folkman, 1990; Folkman, 1992). This process is believed to be essential for the progression and eventual metastasis of breast cancer. While multiple factors are known to regulate the complex process of neovascularization, vascular endothelial growth factor (VEGF) is well established as a potent inducer of angiogenesis (Ferrara, 1995). VEGF is responsible for many important steps in the angiogenic cascade, such as inducing the expression of various proteases by endothelial cells, inducing vascular leakage, and stimulating endothelial cell migration, proliferation, and survival (Alon et al., 1995; Ferrara, 1996). Our laboratory has previously shown increased VEGF messenger RNA and protein in malignant breast epithelial cells compared to non-neoplastic tissue (Yoshiji et al., 1997; Yoshiji et al., 1996). In fact, the importance of VEGF in the progression of breast cancer is emphasized by its use as a prognostic indicator of patient survival (Manders et al., 2002).

Alternative splicing of a single VEGF gene results in the generation of five different isoforms, which vary in the number of amino acid residues: VEGF<sub>121</sub>, VEGF<sub>145</sub>, VEGF<sub>165</sub>, VEGF<sub>189</sub>, and VEGF<sub>206</sub> (Ferrara et al., 1991). VEGF<sub>165</sub> is the most

potent endothelial cell mitogen of the VEGF isoforms and is the isoform most commonly expressed by both normal and transformed cells (Whitaker et al., 2001). Until recently, two endothelial cell-specific receptor tyrosine kinases for VEGF had been identified, namely the 180-kDa fms-like tyrosine kinase (Flt-1) and the 200-kDa kinase insert domain-containing receptor (KDR) (Neufeld et al., 1999). According to knockout studies, KDR is considered essential for endothelial growth and migration while the role of Flt-1 involves the general regulation of angiogenesis (Fong et al., 1995; Shalaby et al., 1995; Shalaby et al., 1997). Recent findings, however, demonstrate that a 140-kDa transmembrane glycoprotein identified as neuropilin-1 (NRP-1) can also act as an isoform-specific VEGF<sub>165</sub> receptor (Soker et al., 1998). NRP-1 has been shown to complex with KDR resulting in increased VEGF<sub>165</sub> binding affinity as well as enhanced mitogenicity and chemotaxis of endothelial cells (Soker et al., 2002). The effect of NRP-1 as a VEGF receptor on the surface of cells lacking KDR, such as breast cancer cell lines, remains unknown. However, in rat prostate carcinoma cells lacking both KDR and Flt-1, overexpression of NRP-1 enhanced tumor and endothelial cell proliferation and decreased apoptosis, which resulted in enlarged tumors with substantially enhanced tumor angiogenesis (Miao et al., 2000).

The first indication of the role of NRP-1 in VEGF signaling came from indirect evidence in transgenic animals generated to either over-express NRP-1 or NRP-1 knockout animals, which exhibit excessive vascular development and malformation of the heart and blood vessels, respectively (Kitsukawa et al., 1995a; Kawasaki et al., 1999). However, the mechanism of these effects, which are both embryonically lethal, only began to surface with the discovery of NRP-1 as a receptor for VEGF<sub>165</sub>. Early studies

have determined that the presence of NRP-1 on endothelial cells contributes to the angiogenic effects of VEGF, but the high expression of NRP-1 on certain breast cancer cells ( $1-2 \times 10^5$ /cell for MDA-MB-231 cells) supports the hypothesis that VEGF<sub>165</sub> is directly affecting tumor cells through binding of NRP-1 in association with a signal transducing protein that remains to be identified.

One study touched on the impact of NRP-1 in tumor progression using rat prostate carcinoma cells injected into rats, and allowed to form tumors (Miao et al., 2000). As a result of directly enhancing angiogenesis, these tumors were much larger than control tumors, and it is found that both endothelial cells, and tumor cells exhibit more proliferation in NRP-1 overexpressing tumors. In addition, there is an increase in the vasculature of these tumors, consistent with the increase in angiogenesis. However, these tumors exhibit evidence for an autocrine effect of VEGF through NRP-1, because it is found that the tumor cells, not expressing typical VEGF Receptors (KDR, Flt-1) show a decrease in apoptosis and in culture show an increase in migratory ability over control cells. Another study also indicates the importance of NRP-1 in VEGF- dependent prevention of apoptosis in breast cancer cell lines, where it was found that treatment of antisense VEGF in vitro results in a 4-fold increase of apoptosis in hypoxia, as compared to control samples (Bachelder et al., 2001). In this process they noted a decrease in PI3-Kinase activity. However, when they introduced recombinant VEGF-165 to antisense VEGF treated cells, they are able to inhibit apoptosis. NRP-1 was discovered to be crucial for the anti-apoptotic effect of VEGF in breast cancer cells, one reason for this is that only VEGF 165, not other VEGF isoforms, was able to prevent apoptosis. Further evidence for the importance of NRP-1 in this process is shown when cells negative for

both NRP-1 and traditional VEGF receptors (KDR, Flt-1) are transfected to overproduce NRP-1, they found an increase in the ability of recombinant human VEGF-165 to specifically inhibit apoptosis in VEGF-antisense treated cells. One downstream target identified in this response was the upregulation of phosphorylated Akt, a downstream target of PI3-Kinase. To understand how NRP-1 could be contributing to the autocrine effect of VEGF 165, we turned to what is known of NRP-1 in neurobiology.

### **Neuropilin-1 in neurobiology**

Neuropilin-1 was first identified in the optic tectum during screening for novel antigens. It was later found to be expressed in the human brain during neuronal development (Fujisawa et al., 1995; Fujisawa et al., 1997). During embryogenesis, attractive and repulsive signals direct the guidance of neuronal axons to provide appropriate innervation of the developing nervous system. Although the embryonic lethality of NRP-1 transgenic and knockout mice was attributed to the vascular malformation of these animals, these mice also show abnormal neuronal wiring, indicating the importance of NRP-1 in axonal guidance. Neuropilin-1 was found to act as a specific receptor for certain class III semaphorins, which belong to a large family of proteins that provide signaling clues for axonal growth cones to find their appropriate paths. As a specific receptor for the prototypical semaphorin, known as Sema3a, Neuropilin-1 also binds Sema E and Sema IV, while the other member of the Neuropilin family, Neuropilin-2, is closely related to NRP-1 but acts as a receptor for different semaphorin family members (Bagri and Tessier-Lavigne, 2002). Treatment with NRP-1 blocking antibodies *in vitro* abolishes the ability of Sema3a to repel sensory axons and

causes the collapse of growth cones (Kolodkin et al., 1997; He and Tessier-Lavigne, 1997). *In vitro* studies (Bagnard et al., 2001; Miao et al., 1999) have revealed that Sema3A can inhibit endothelial cell motility, capillary sprouting, and microvessel formation. This inhibition has been attributed to competition between VEGF<sub>165</sub> and Sema3A for the same binding site on NRP-1.

The specificity of NRP-1 and NRP-2 has been studied (Mamluk et al., 2002; Whitaker et al., 2001) for the different VEGF family members. NRP-1 is able to bind VEGF<sub>165</sub>, placenta growth factor (PlGF), PlGF-2, and VEGF-B, while NRP-2 can bind both VEGF<sub>165</sub> and VEGF<sub>145</sub>. The NRP-1 gene encodes for three different extracellular domains with homology to other known proteins. These are the **a** domain (a1/a2), which is homologous to components of the complement systems, the **b** domain (b1/b2), which is homologous to coagulation factors V and VIII, and the **c** domain which is homologous to mepin metalloproteinases. Both VEGF and Sema3a have been shown to bind to the same region in the **b** domain of NRP-1 (Mamluk et al., 2002). NRP-1 also contains a transmembrane domain with a short 40 amino acid cytoplasmic domain with no known direct signaling abilities. In fact, the intracellular domain of NRP-1 is not necessary for the repulsive effects of Sema3a on growth cones, as shown by deletion experiments (Nakamura et al., 1998). Because of the inability of NRP-1 to transduce signals, it is believed to provide binding specificity in molecular reactions while requiring a co-receptor for signal transduction to take place. In Sema3a signaling, the co-receptor responsible for growth cone collapse is Plexin-A1 (PXN-A1), which forms complexes with NRP-1 on the cell surface. Both Plexin-A1 and Sema3A mutant animals show equal

deficiency in the axonal guidance in motor and central nervous system axons (Takahashi et al., 1999).

### **Plexin-A1 Structure and Function**

Like NRP-1, Plexin was also identified in the optic tectum during a screen for novel antigens (Fujisawa, 2002). At the same time, Plexin-A1 was cloned independently (Tamagnone et al., 1999) in humans based on its homology with the protooncogene MET, the receptor for hepatocyte growth factor (HGF) that is often overexpressed in tumor cells. In fact, it is believed that the family of Plexins, MET, or so-called scatter-factor receptors, and Semaphorins themselves are derived from a common ancestor. The extracellular domain of all of these protein families contain a highly conserved region of approximately 500 amino acids named the “sema domain” in addition to an MRS, or Met Related Sequence, domain which is approximately 80 amino acids in length and rich in cysteine residues (Artigiani et al., 1999). The intracellular domain of plexins, however, is highly conserved among Plexin family members and is rather large. This observation suggests the importance of Plexins in signal transduction, but plexins show no significant homology to any traditional transmembrane signaling proteins. While it is known that the intracellular domain of Plexin-A1 can be phosphorylated, it does not exhibit intrinsic tyrosine phosphatase activity, but it does contain many alpha helices, which could possibly indicate protein-protein interactions (Tamagnone et al., 1999). The signal transduction pathway of plexins is only beginning to be unraveled. We can, however, deduce from studies of Sema3a activity that plexins signal to rearrange actin organization, which results in cell motility and migration. Based on evidence provided by

studies on the nature of NRP-1/Plexin-A1 complex formation, where NRP-1 acts to provide binding specificity, and Plexin-A1 to transduce signals, we hypothesized that VEGF has the ability to use this receptor complex in an autocrine manner to influence cell motility in breast carcinoma cells.

In studies on neurons treated with Sema3A, the laboratory of Strittmatter et al (Liu and Strittmatter, 2001; Takahashi and Strittmatter, 2001; Takahashi et al., 1999) identified the downstream targets directly responsible for signal transduction through PNX-A1. One clue to the Plexin signaling mechanism came from discovery of intracellular sequence homology with a family of GTPase-activating proteins (GAPs) (Bagnard, 2001). Upon further examination, it was shown (Rohm et al., 2000) that mutations in only two conserved amino acid residues of this region in plexins were able to abolish the growth cone collapse response to Sema3a. Before this discovery, the role of Rho family GTPase proteins, which contains Rho, Rac and Cdc42 subfamilies, has been well established as regulators of cell motility and shape through interactions with actin fibers (Nikolic, 2002). Early studies involving Sema3a signaling pointed to a direct role for Rho family proteins in the process (Liu and Strittmatter, 2001). The Rho family of proteins are found either in an “on” (GTP-bound) or “off” (GDP-bound) states, and the proteins that regulate these states are referred to as either guanine nucleotide exchange factors (GEFs) or GTPase activating proteins (GAPs), which the Plexin domain resembles. In the activated state, Rho family proteins are able to activate effector proteins, which signal the change in actin dynamics. Plexins have been shown (Swiercz et al., 2002) to bind both Rho, which is responsible for the contraction of actin and myosin filaments resulting in cell contraction, and Rac, which results in the formation of

lamellapodia through actin rearrangements. In addition, one downstream target of both Rho and Rac, known as LIM kinase (LIM domain containing kinase) has been shown (Aizawa et al., 2001) to be necessary for Sema3a signaling. The current understanding of this pathway is that Rac activates PAK (p21-activated kinase), which in turn activates LIM kinase through phosphorylation at threonine 508. This activated LIM kinase is then responsible for the phosphorylation of cofilin, a protein that covalently binds, depolymerizes, and severs actin filaments directly (Figure 1.2). However, phosphorylation of cofilin by LIM kinase results in inhibition of the ability of cofilin to depolymerize actin filaments. While the complex dynamics of cell movement is yet to be fully understood (Summarized in Figure 1.3), we expect to see opposite effects of Sema3a and VEGF165 in down-stream signal transduction with respect to these actin-targeting proteins. This speculation is based on the observation of exact opposite reactions of both neurons and endothelial cells to VEGF-165 and Sema3A, which is a result of binding NRP-1 (Miao et al., 1999; Bagnard et al.,2001).

Interestingly, in endothelial cells, Rac activation has been shown to be necessary and sufficient for VEGF-induced chemotaxis (Eriksson et al., 2003). While the mechanism of this action is not fully known, it seems reasonable to expect that due to the expression of both Plexin-A1 and NRP-1 on endothelial cells, this complex is contributing to the Rac activation required for VEGF chemotactic responses. Our goal in this study has been to assess the role of VEGF and its autocrine action on breast cancer cells, namely the motility and actin reorganizational control. We designed experiments to determine the role of the NRP-1/Plexin-A1 complex in LIM kinase / cofilin activation and effect on motility in breast cancer cells.

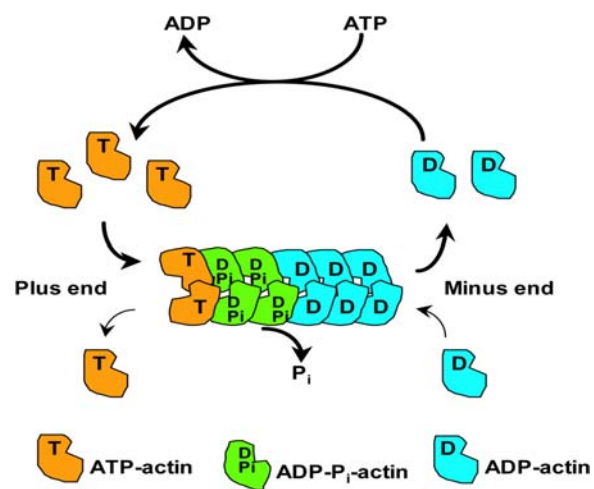


Figure 1.1: Actin polymerization and depolymerization (Gungabissoon and Bamberg, 2003). This image illustrates the importance of ATP/ADP states in determining the addition of actin to the “plus” end- the place of elongation, or the deletion of actin from the “minus” end. Cofilin is directly involved in facilitating removal of ADP-actin from the minus end.

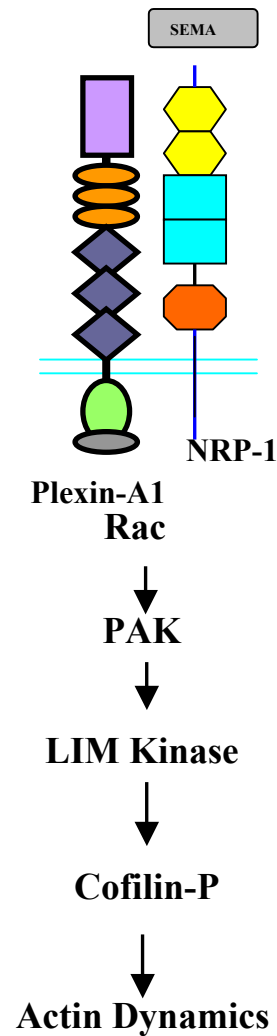


Figure 1.2: Signal transduction through Plexin-A1. Adapted from (Liu and Strittmatter, 2001). This figure emphasizes downstream targets of Plexin signaling as determined by Sema3A- ligand binding. Rac is a member of the Rho family proteins that is responsible for activation of PAK. PAK then activates LIM kinase, which causes the phosphorylation of cofilin. When phosphorylated, cofilin is unable to depolymerize actin.

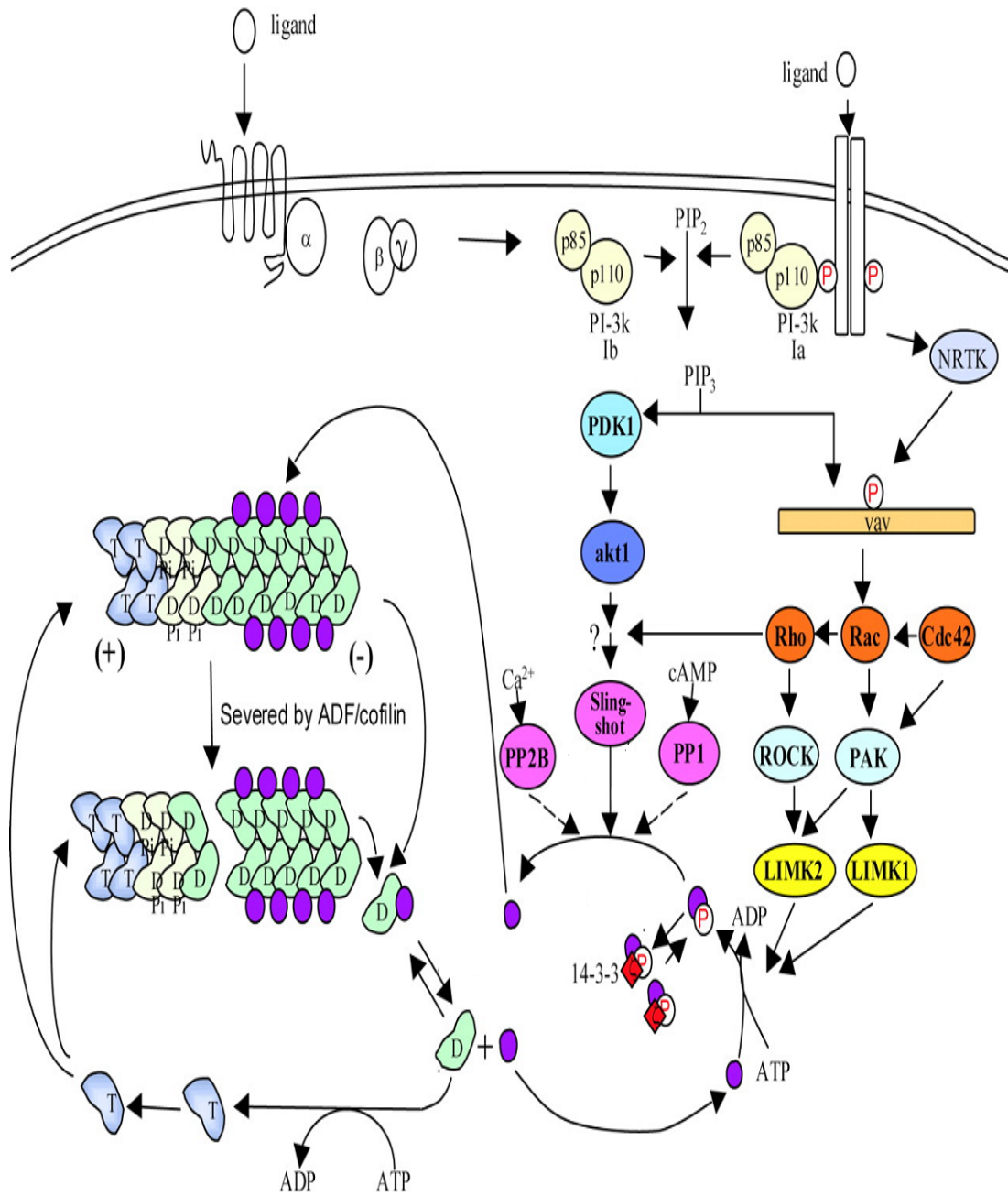


Figure 1.3 : Regulation of actin dynamics. Adapted from (Gungabissoon and Bamberg, 2003).

## L1-Cellular Adhesion Molecule Structure and Function

The cell adhesion molecule L1 (L1-CAM) was first identified in the early 1980's as a 200 kDa protein expressed in the nervous system. It is a member of the immunoglobulin (Ig) superfamily due to the structure of Ig-like domains in its extracellular domain (reviewed in (Crossin and Krushel, 2000)). As a member of the CAM family, L1-CAM falls into one of the four main types of structural adhesion receptors, mainly the CAMs, the cadherins, gap and tight junction proteins, and the integrins. L1-CAM is believed to be essential for cellular adhesion, but is also able to transduce signals through its highly conserved cytoplasmic domain which is found to be identical in mammals. The involvement of L1-CAM in neuronal development was established by linking mutations in L1-CAM to several X-linked neurological genetic disorders such as the MASA syndrome, ( mental retardation, aphasia, shuffling gait and adducted thumbs) (Ruiz et al., 1995; MacFarlane et al., 1997). L1-CAM involvement in neuronal development was believed to be a result of its ability to act in binding L1-CAM on other cells in addition to a many other cell surface proteins, such as axonin-1, conactin, DM1-GRASP,  $\alpha$ V $\beta$ 3 and  $\alpha$ 5 $\beta$ 1 integrins, and certain chondritan sulfate proteoglycans ((Kadmon et al., 1990), reviewed in (Crossin and Krushel, 2000)). In addition, analysis of L1-CAM knockout mice showed axonal malformations similar to those seen in Sema3a deficient animals (Castellani et al., 2000). This lead to the speculation that L1-CAM is required for Sema3A axon repulsion, which was indeed verified *in vitro*, using L1-CAM deficient neurons that where unable to respond to Sema3a, and by showing that L1-CAM associates with NRP-1 specifically (He, 2000; Castellani et al., 2000).

Our interest in L1-CAM arose from its role as a protein essential for NRP-1-mediated Sema3A signal transduction in axonal guidance. NRP-1 provides specificity for binding in axonal guidance (Van Vactor and Lorenz, 1999), but it requires interaction with other signal proteins (Plexin-A1 and L1-CAM in neurons, KDR on endothelial cells) in order to be functional. In addition, L1-CAM has been known to prevent apoptosis in neuronal cells by the upregulation of bcl-2 (Chen et al., 1999). Bcl-2 is an anti-apoptotic protein that has been shown to be a downstream target of VEGF-mediated survival effects in endothelial cells (Gerber et al., 1998). However, as our goal is to assess the role of VEGF autocrine signaling in tumor cells of epithelial origin, there is also a study suggesting that VEGF causes the upregulation of bcl-2 in primary murine mammary carcinoma cells (Pidgeon et al., 2001), which has been supported by experimental evidence in our laboratory (data not shown). Such evidence suggests that this aspect of VEGF signaling is not isolated to endothelial cells and the involvement of KDR. Therefore, we sought to determine if L1-CAM could be involved in survival effects of VEGF through NRP-1 in breast cancer cell lines. Additional support for our hypothesis comes from studies that demonstrate the overexpression of L1-CAM in various types of cancer, such as neuroectodermal tumor lines, carcinomas of the skin, lung and kidney, and in certain leukemias (Figarella-Branger et al., 1990; Li et al., 2002b; Miyahara et al., 2001a; Meli et al., 1999). Due to the ability of L1-CAM to act in an anti-apoptotic manner and its known association with NRP-1, L1-CAM is an attractive candidate for VEGF autocrine signal transduction in breast carcinoma cells.

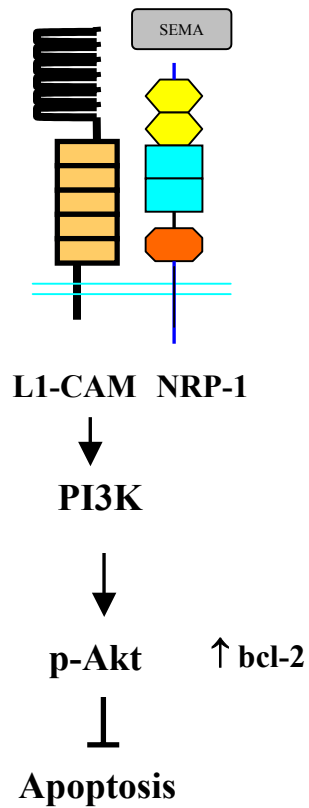


Figure 1. 4. Signal transduction through L1-CAM. (adapted from (Schmid et al., 2000). Activation of L1-CAM has been shown to result in activation of PI3Kinase, the protein upstream of Akt. When PI3 kinase phosphorylates Akt, there is an increase in resistance to apoptosis, and an increase in the anti-apoptotic bcl-2.

### **Isoforms of Neuropilin-1**

Since the discovery of NRP-1 as a VEGF receptor, the laboratory responsible for this finding has published findings on two different soluble isoforms of NRP-1. Full-length NRP-1 is encoded by 17 exons, and has been found to be located on chromosome 10, with 17 introns separating these exon regions (Rossignol et al., 1999; Rossignol et al., 2000). These isoforms retain the VEGF binding position of NRP-1, but lack the transmembrane and short cytoplasmic domains of the full-length NRP-1. Sequence analysis of the mRNA from these splice variants revealed that the first one identified, named s12NRP-1, retains the first 12 exons in its entirety, but after the 12<sup>th</sup> exon, contains part of the intron sequence of intron 12, which results in the generation of 3 unique amino acids to the protein before truncation of signal by a stop codon (Rossignol et al., 2000). In the second identified isoform, there is a similar pattern. In this isoform, named s11NRP-1, the message contains the first 11 exons as the full-length NRP-1, but at the end of exon 11, there are 246 base pairs taken from the sequence of intron 11, resulting in an additional 82 amino acids to the end of exon 11 encoding site (Rossignol et al., 2000). In this study, we describe the isolation of a novel NRP-1 isoform, named s10NRP-1, which, like the other isoforms characterized, contains the expected coding region of NRP-1, but at the end of exon 10 contains the coding sequence for 54 unique amino acids before a stop codon that is encoded by the 10<sup>th</sup> intron of the NRP-1 sequence. In addition to following the pattern of known NRP-1 isoforms, we have also verified the sequence end of exon 10 has a known GT 5' donor site and the 3' AG splice acceptor site in intron 10 at the junction region. Very interestingly, analysis of the structure of the full length NRP-1 shows that the amino acid corresponding to the end of

the VEGF and Semaphorin binding domains, known as the B1/B2 domain, ends at amino acid 587 (Rossignol et al., 2000), which is the last amino acid encoded by our splice variant. Therefore, unlike the other isoforms, which still retain linker regions of the full-length NRP-1, our soluble NRP-1 is able to act solely as a soluble VEGF-165 receptor that lacks any other protein binding abilities inherent in the longer NRP-1 isoforms. This isoform will allow us to study the direct effect of VEGF in the autocrine effect on breast cancer progression.

Gagnon et al. (2000) injected rat prostates with Dunning rat prostate carcinoma cells which overexpress the  $s_{12}$ NBRP-1 isoform in order to identify the effect of  $s_{12}$ NRP-1 in tumor progression. Tumors were collected after three weeks. Those tumors which overexpressed  $s_{12}$ NRP-1 exhibited hemorrhaging, an increase in apoptotic cells, and damaged interior blood vessels. While the Gagnon et al. explained these effects as inhibition of VEGF in angiogenesis, we also saw in our studies an increase in tumor cells themselves suggesting that there is a survival action of VEGF on the cancer cells, which is blocked with overexpression of a soluble NRP-1 isoform. We have designed various methods to use our novel  $s_{10}$ NRP-1 as a powerful tool to examine our findings of the autocrine effect of VEGF-165 on mammary tumor formation and progression both *in vitro* and *in vivo*.

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## CHAPTER 2

NEUROPILIN-1 CO-RECEPTORS PLEXIN-A1 AND L1-CELLULAR ADHESION  
MOLECULE ARE EXPRESSED IN HUMAN BREAST CANCER CELL LINES AND  
BREAST TISSUE SAMPLES<sup>1</sup>

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<sup>1</sup> Matheny, S.L., O.R. Bunce, and U. P. Thorgeirsson. To be submitted to *Cancer Research*

## ABSTRACT

Neuropilin-1 (NRP-1) was recently discovered as a novel isoform-specific vascular endothelial growth factor-165 (VEGF-165) receptor expressed on both endothelial and tumor cells. VEGF is known to be a potent inducer of angiogenesis through stimulation of tyrosine kinase receptors expressed by endothelial cells, resulting in enhanced mitogenicity, migration and inhibition of apoptosis. The discovery of NRP-1 on tumor cells gave strength to the argument for an autocrine effect of VEGF in tumorigenesis and tumor promotion. We have sought to examine this possibility in breast carcinoma cells. Due to the fact that NRP-1 has a short cytoplasmic domain without known signaling capabilities, we decided to look for possible NRP-1 co-receptors in cancer cells that could be involved in VEGF autocrine signaling. We found that the NRP-1 co-receptors involved in semaphorin signaling in neuronal development, Plexin-A1 (PXN-A1) and L1-Cellular Adhesion Molecule (L1-CAM) are expressed in a variety of breast cancer cell lines. We also sought to characterize the expression of NRP-1, Plexin-A1 and L1-CAM in human breast cancer samples, and found both Plexin-A1 and L1-CAM expression by RNA and protein analysis of human tumor samples. Further examination showed the colocalization of NRP-1 with its neuronal co-receptors in both cell lines and human breast tissue by immunostaining and immunoblotting. Here we present data that suggests the possibility that traditional neuronal NRP-1 co-receptors are associated with NRP-1 expression in breast cancer. While L1-CAM has been shown to be upregulated in certain tumor types, this is the first study to our knowledge to link L1-CAM and NRP-1 in cancer, and the first demonstration of Plexin-A1 expression in cancer.

Keywords: Neuropilin-1, Plexin-A1, L1-Cellular Adhesion Molecule, Vascular Endothelial Growth Factor, breast cancer.

## INTRODUCTION

Neuropilin-1 (NRP-1) was first identified as a protein required for correct innervation in the developing brain (Fujisawa et al., 1995; Fujisawa et al., 1997). However, knockout NRP-1 mice did not survive gestation due to vascular malformation. Transgenic mice which overexpressed NRP-1 also died in utero from excessive vascularization (Kitsukawa et al., 1995b; Kawasaki et al., 1999). This unexplained effect of NRP-1 began to be understood when the NRP-1 was identified as a receptor specific for vascular endothelial growth factor-165 (VEGF-165) (Soker et al., 1998). VEGF-165 is a potent inducer of angiogenesis and regulator of embryonic vascular development. VEGF-165 is a member of the VEGF family, which contains VEGF-A, -B, -C, and D (Ferrara, 1996; Robinson and Stringer, 2001). VEGF-165 is a splice variant of VEGF-A, which contains 165 amino acids and is the most potent of the VEGF-A splice variants. The explanation for the increased effectiveness of VEGF-165 can be explained by the ability of this isoform to bind NRP-1, which has been shown to increase signal transduction through VEGF receptor-2, a 200-kDA kinase insert domain-containing receptor (also known as KDR) (Soker et al., 2002). Because NRP-1 is expressed on both endothelial cells (like other VEGF receptors) and on tumor cells (unlike other VEGF receptors) we are interested in NRP-1 as a mediator of a VEGF-165 autocrine action on breast cancer cells.

What is known about NRP-1 comes mainly from studies of this protein as a semaphorin receptor in neurobiology. NRP-1 acts as a receptor for Sema3A, the prototypical semaphorin (Kolodkin et al., 1997 ; Chen et al., 1998; Kolodkin, 1998). Semaphorins act as either attractive or repulsive cues for neuronal guidance in the developing brain (Bagnard et al., 2000; Bagnard et al., 1998). NRP-1 is required for providing binding specificity of Sema3A signaling, but the transmembrane domain of NRP-1 is not required for this process (Nakamura et al., 1998), suggesting the need for other proteins to act as NRP-1 co-receptors for transduction of signals. The proteins Plexin-A1 (Liu and Strittmatter, 2001; Takahashi et al., 1999) and L1-Cellular Adhesion Molecule (L1-CAM) have been identified to be necessary for signal transduction of Sema3A through NRP-1 in neuronal development (Castellani et al., 2000; He, 2000). Plexin-A1 expression to our knowledge has not been examined in tumor samples or tumor cell lines of any kind. L1-CAM has been found to be overexpressed in the following tumor types: neuroectodermal tumor lines, carcinomas of the skin, lung and kidney, and in certain leukemias (Figarella-Branger et al., 1990). However, to our knowledge, this is the first evidence of characterization on L1-CAM expression in breast cancer. NRP-1 is a protein that provides binding specificity of specific ligands, such as VEGF-165 and Sema3a, to the surface of cells. However, other proteins are required to interact with such complexes as co-receptors in order for ligands to be able to transduce signals intracellularly. Therefore, in this study we sought to characterize the expression patterns of NRP-1, and its neuronally associated co-receptors, Plexin-A1 and L1-CAM in breast cancer cell lines and in human breast tumor tissues.

## MATERIALS AND METHODS

**Human Breast Tissue Samples.** All human breast tissue samples were obtained from the Cooperative Human Tissue Network (Philadelphia, PA). Samples were provided embedded in paraffin blocks, and sent to Histoserve (Gaithersburg, MD) to be cut into 5.0  $\mu\text{m}$  thick slices and applied to poly-l-lysine coated glass slides.

**Cell Culture and Analysis of Cell Proliferation.** All cell lines were obtained from the American Tissue Culture Collection (ATCC, Manasses, VA) and were grown in a humidified atmosphere of 5%  $\text{CO}_2$ , 95% air at 37 °C with change of medium every 2-3 days. Human breast cancer cell lines, MDA-MB-231 and the non-tumorigenic breast epithelial HBL-100 cell line were cultured in DMEM with 25mM HEPES (Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics. The MCF-7 breast carcinoma cell line was cultured in EMEM with non-essential amino acids (Biofluids) supplemented with 10% FBS, 2 mM glutamine, 0.2 U/ml insulin, 1.0 mM sodium pyruvate, and antibiotics. T47-D and BT-549 breast carcinoma cell lines were maintained in RPMI-1640 (Biofluids) supplemented with 10% FBS, and 0.2 U/ml insulin. In addition, T47-D media was supplemented with 0.2 mM L-glutamine, 4.5 g/L glucose, 10 mM HEPES, and 1 mM sodium pyruvate. Primary antibodies for NRP-1 (goat polyclonal IgG clones C-19, and N-18, and rabbit polyclonal IgG clone H-286), Plexin-A1 (goat polyclonal IgG clones Q-15 and S-16), and L1-CAM (goat polyclonal IgG clone C-20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Additional mouse monoclonal L1-CAM antibody (clone UJ127) was

obtained from Neomarkers (Fremont, CA). Secondary antibodies for Western blotting were obtained from Dako (Carpinteria, CA) and for fluorescence from Molecular Probes (Eugene, Oregon).

**RT-PCR.** For RT-PCR, total RNA was extracted from subconfluent cell cultures using RNeasy (Qiagen, Crawfordsville, IN) according to the manufacturer's protocol. After extraction, RNA was DNase treated for 30 minutes at 37° C and inactivated using DNase inactivating buffer (Ambion, Austin, TX). Total RNA was quantitated using a Quant II 280/260 nm spectrometer (Pharmacia Biotech), and 2.5 µg of total RNA was used to synthesize cDNA with the Superscript II kit (Invitrogen, Carlsbad, CA) with Oligo-dT priming according to the manufacturer's protocol. PCR was carried out with the following primers: 5' ACAACTATGATACACCTGAGCTGCG 3' and 5'GACCAGCTGATCG-TACTCCTCTGG3' for NRP-1 (Genebank Accession AF016050); 5' TGCTGTACTGCGCCATCAAGCAGC 3' and 5'CGCAGCATGCTCTCGTATCTGC3' for Plexin-A1 (Genebank Accession X837832); 5' GCGCCTGAAA AATGGTACAGTCTG3' upper and 5' CTCCAGAGTAGCCGATAGTGACC 3' for L1-CAM (Genebank Access X59847); 5'ACCACAGTCATGCCATCAC 3' upper and 5' TCCACCACCCTGTTGCTGTA3' lower for GAPDH control. All primers were synthesized by Bioserve Biotechnologies (Laurel, MD). PCR amplification was carried using PCR Platinum Supermix (Invitrogen, Carlsbad, CA) with 0.5 µl of 10 µM solutions of upper and lower primers, and 1 µl of cDNA generated by RT per each PCR reaction. Specificity of reactions for all cell line cDNA was determined by duplicate RT-PCR reactions performed without reverse transcriptase, and checked for

GAPDH expression by PCR to ensure samples were free of genomic DNA contamination (data not shown). All PCR reactions were performed in a Perkin Elmer 9600 Machine at 95°C for 5 min for one cycle, followed by 35 cycles at 95°C for 45 seconds, 65°C for 30 seconds, 72°C for 45 seconds, and final extension at 72°C for 10 min. The PCR product was then subjected to electrophoresis on a 1.0% agarose gel. Bands of NRP-1, Plexin-A1, and L1-CAM, KDR, Flt-1, and GAP were visualized on a UV light box and extracted with Wizard PCR Preps DNA Purification System (Promega Corporation, Madison, WI) for sequence confirmation and probe synthesis.

**Western blotting.** Subconfluent monolayers of MDA-MB-231, MCF-7, BT-549, T-47D, and HBL-100 cells were trypsinized, counted and seeded at  $2 \times 10^5$  cells/ 35mm dish and grown in 35 mm diameter culture for 48 hours. The cells were then washed three times in ice-cold PBS and lysed in 200  $\mu$ l of 2X SDS-PAGE buffer (Invitrogen, Carlsbad, CA). The cell lysates were transferred to Eppendorf tubes, sonicated for 15 seconds, rocked gently on ice for 15 min, and spun at 15,000 rpm for 15 min at 4°C to remove cellular debris. The protein lysates were boiled for 5 minutes and 40  $\mu$ l of the lysate was immediately subjected to SDS-PAGE analysis on 8% Tris-Glycine 1.0mm gels (Invitrogen, Carlsbad, Ca). Gels were run at 120V for approximately 1.5 hours and transferred to PVDF membranes (Invitrogen, Carlsbad, CA). After transfer, the membranes were blocked for 1 hour in 10% non-fat dry milk, rinsed with PBST, and immunoblotted with anti-NRP-1 antibody (C-19) overnight at 4°C with gentle rocking. The bound antibodies were detected using horseradish peroxidase-conjugated secondary antibodies (mouse anti-goat-HRP conjugated secondary antibody, DAKO, CA) and an

enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia, Piscataway, NJ). The blot was subsequently stripped and re-probed with antibodies against Plexin-A1 (Clone S-16), L1-CAM (Clone C-20) and  $\alpha$ -actin (Cell Signaling Technology Inc., Beverly, MA), which was used as a loading control for total protein. Additional positive controls for NRP-1 and L1-CAM were provided by Santa Cruz and Neomarkers, respectively.

**Immunohistochemistry.** Staining for human NRP-1, Plexin-A1, and L1-CAM was performed on paraffin sections of human breast tissues, using avidin-biotin immunoperoxidase staining. Briefly, slides were deparaffinized in xylene, followed by rehydration with graded washes in ethanol (100-70%), and finally washed in distilled water. Tissues were permeabilized in 1% Antigen Unmasking solution (Vector laboratories, Burlingame, CA) according to the manufacturer's protocol. Endogenous peroxidase was quenched using a 3% hydrogen peroxidase solution methanol, followed by incubation in 1x Power Block (Innogenex, San Ramon, CA) for 10 minutes at room temperature. Slides were incubated overnight at 4°C with goat polyclonal antibody for human NRP-1 (clone H-286), goat polyclonal antibody for Plexin-A1 (Q-15), or goat polyclonal antibody for L1-CAM (C-20) at 1:50 dilution in 1X power block. After incubation, the slides were rinsed in PBS. The secondary antibody reagents for detection of anti-NRP-1, anti-Plexin-A1, and anti-L1-CAM primary antibodies were supplied by Vectastain goat elite ABC kit (Vector Laboratories, Burlingame, CA). Secondary antibody incubation was performed for 1 hour at room temperature, and the slides were rinsed in PBS. The tissue sections were then incubated with chromogen 3-amino-ethyl-

carbazole for 10 min and counterstained with methyl green for 2 minutes. Slides were mounted in Permount (Vector Laboratories, Burlingame, CA) and visualized and photographed using a Nikon camera at magnifications listed in figures.

**Immunofluorescence.** Staining for human NRP-1, Plexin-A1, and L1-CAM was performed on paraffin sections of human breast tissues, following the procedure listed above with the following exceptions: a rabbit polyclonal antibody for human NRP-1 (clone H-286) and goat polyclonal antibody for Plexin-A1 (Q-15) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and mouse monoclonal antibody for L1-CAM (UJ127) from Neomarkers (Fremont, CA) were incubated overnight at 4°C at 1:50 dilution in 1X power block. For tissues triple-stained for NRP-1, Plexin-A1, and L1-CAM, the secondary antibodies for NRP-1, Plexin-A1, and L1-CAM were AlexaFluor donkey anti-rabbit 568, AlexaFluor donkey anti-goat 350, and AlexaFluor donkey anti-mouse 488, respectively (Molecular Probes, Eugene, OR). The triple stained section was not treated with DAPI. For double-receptor staining, the secondary antibodies used were as follows: for NRP-1/Plexin-A1 sections, AlexaFluor donkey anti-rabbit 488 and AlexaFluor donkey anti-goat 568 were used, and for NRP-1/L1-CAM stained sections, AlexaFluor donkey anti-rabbit 488 and AlexaFluor donkey anti-mouse 568 were used. After one hour incubation with the secondary antibodies, the slides were washed with PBS and incubated for 5 minutes with a 300nM solution of DAPI (Molecular Probes, Eugene, OR) for nuclear visualization, and mounted using ProLong Anti-Fade mounting media (Molecular Probes, Eugene, OR). The tissues samples were visualized using a Zeiss confocal microscope at various magnification.

**Cancer Profiling Array.** 200 matched tumor and normal adjacent tissue pairs were analyzed for NRP-1 expression from the following different tumor/normal tissue types: breast, ovary, lung, stomach, bladder, prostate, colon, kidney, uterus, rectum, cervix, small intestine, pancreas, rectum, thyroid, and small intestine using the cancer profiling array (Clontech, Palo Alto, CA). A cDNA probe for NRP-1 was created from MDA-MB-231 cDNA as described above. PCR fragments were subcloned into TOPOTA 2.1 vector (Invitrogen, Carlsbad, CA), grown in chemically competent *E. coli*, and plated on agar containing IPTG/X-Gal/AMP. White colonies were grown in LB broth containing 100 µg/ml ampicillin overnight, and DNA was extracted using the Qiagen Mini-Prep kit (Valencia, CA). DNA was sequenced by the NCI DNA Sequencing Core (Bethesda, MD) using M13 forward and reverse primers. After sequence confirmation, the original PCR fragment was quantitated and used as the NRP-1 probe. Briefly, the probes were randomly labeled with dCTP-P32 using the RediPrime Labeling Kit (Amersham Pharmacia). The cancer profiling array was hybridized according to the manufacturer's instructions, except the first wash buffer contained 0.5% SDS for a more stringent wash, and the membrane was exposed to Kodak BioMax film at -70°C for 1 day for analysis of hybridization. Densitometric analysis was performed using a LumiImager (Roche, Indianapolis, IN).

## RESULTS

### **Neuropilin-1, Plexin-A1, and L1-CAM are Expressed in Various Human Breast**

**Cancer Cell Lines.** RT-PCR analysis was performed on RNA isolated from MDA-MB-

231, MCF-7, BT-549, and T-47D human breast cancer cell lines, and the non-neoplastic HBL-100 cell line (Fig 3.1). NRP-1 transcripts were detected in all of these cell lines except the T-47D cells. Plexin-A1 and L1-CAM transcripts were detected in all cell lines. Western Blot analysis (Fig 3.2) on lysates from the cell lines detected NRP-1 in all lines except for T-47D, while Plexin-A1 was detected in each cell line. L1-CAM was found in all but the BT-549 cell line, which showed a faint positive band via RT-PCR. MDA-MB-231 and MCF-7 cell lines consistently expressed NRP-1, Plexin-A1, and L1-CAM, while there was also a consistent lack of NRP-1 expression in T-47D cells. Results presented are representative of three separate isolations of both RNA and protein for confirmation of results. In addition, due to high background when using the Plexin-A1 antibodies and a lack of certain positive controls, specificity in western blotting of Plexin-A1 binding was confirmed by successful competition of results with a Plexin-A1 blocking peptide (data not shown).

### **Immunolocalization of NRP-1, Plexin-A1, and L1-CAM in Human Breast**

#### **Carcinomas and Normal Breast Tissue.**

We found areas in many breast cancer tissue samples examined that stained intensely for NRP-1. It was apparent that the tumor cells themselves expressed NRP-1. In addition, we found many tumor samples which stained very intensely for both Plexin-A1 and L1-CAM. Shown are serial sections of two representative cases of infiltrating ductal carcinoma. In Figure 3.3, NRP-1 and L1-CAM positive staining was limited to the tumor cells (Fig.3.3a and 3.3b respectively), while Plexin-A1 appears to be mostly confined to the tumor stroma (Figure 3.3c). Similar tissue distribution of NRP-1, L1-CAM, and

Plexin-A1 is shown in Figure 3.4. VEGF was also found to be concentrated in the tumor nests of the invasive carcinoma. In addition, we also tested the expression patterns of NRP-1, Plexin-A1, and L1-Cam in normal breast tissue. We were able to find rather significant staining of NRP-1 in the lobules of normal breast tissue (Figure 3.5a). L1-CAM expression was extremely rare in the normal breast tissue, but we were able to find a few positive cells in smooth muscle cells surrounding the normal vasculature (Figure 3.5b). We found some expression of Plexin-A1 in normal lobules, but the staining was not nearly as pronounced in the normal tissues as the NRP-1 staining (Figure 3.5c). We then used confocal microscopy and immunofluorescence to further characterize the relationship between NRP-1 and co-receptors Plexin-A1 and L1-CAM in breast cancer tissues. We found areas where NRP-1 and Plexin-A1 (Fig 3.6) were definitely co-localized and appeared to form clusters on tumor cells (similar to what has been shown in neurons (Takahashi, 1999)). Staining for NRP-1 and L1-CAM also showed many areas of co-localization of these proteins at the cancer cell surface, and the NRP-1/L1-CAM complexes also were found in the cytoplasm (Fig 3.7). Interestingly, while the direct relationship between NRP-1, Plexin-A1, and L1-CAM remains to be determined biochemically, we were able to find instances of triple staining in human breast cancer tissues for all three proteins, where all three proteins appeared to co-cluster on isolated cells (Figure 3.8).

#### **Cancer Profile of NRP-1 expression.**

Using the cancer profiling array from Clontech, we were able to analyze 200 matched tumor and normal adjacent tissue pairs for the many different types of cancer to

examine relative expression levels of NRP-1 in both normal and cancerous tissues. Using desometric analysis, each tumor sample expression level was compared to the normal adjacent tissue expression level of NRP-1. Each matched pair was counted as either having higher NRP-1 expression in the tumor sample or in the normal adjacent tissue sample, which was then charted (Figure 3.9) for comparison between each tissue type.

## DISCUSSION

We describe for the first time the co-expression of the neuronal co-receptors of NRP-1, Plexin-A1 and L1-CAM in breast cancer cell lines. Due to the consistent and strong expression in breast cancer cell lines, we decided to test whether human breast cancer samples also expressed these signal-transducing proteins. We found co-localization of both NRP-1 and L1-CAM in various tumor samples by IHC, and strong stromal staining of Plexin-A1 in these same samples. This leads us to speculate that perhaps NRP-1 and Plexin-A1 could interact in a juxtacrine fashion, where Neuropilin-1 expressed on tumor cells could binds VEGF, but is still able to interact with Plexin-A1 expressed on the stromal surface. Plexins are known to act in such a fashion when binding cell-surface bound Semaphorins (Pasterkamp and Verhaagen, 2001). However, we are also able to show expression of both Plexin-A1 and L1-CAM co-localizing with NRP-1 in breast tumor tissue. This is the first direct evidence of either Plexin-A1 or L1-CAM associating with NRP-1 in non-neuronal tissue.

Due to the nature of NRP-1 function as a cell surface protein providing binding specificity to certain ligands, but requiring co-receptors for transduction of ligand signals,

we propose there is a high probability that both Plexin-A1 and L1-CAM are contributing to VEGF signal transduction in breast cancer cells. This theory is strengthened by the fact that both Plexin-A1 and L1-CAM are able to interact directly with NRP-1 independent of ligand binding. The specificity of the reaction is the responsibility of NRP-1, and the reaction to the signal is dictated by the co-receptors present. We are very interested in determining the result of VEGF binding NRP-1 in the presence of both Plexin-A1 and L1-CAM.

Analysis of expression of NRP-1 from the Cancer Profiling array indicates it is a highly expressed protein in many different tumor types, but it is also prevalent in normal tissue. As shown by immunohistochemistry of normal breast tissue, there is high expression of NRP-1 in normal mammary lobules. This could explain the equal number of cases of the breast cancer patients (23 cases in each group) that either over-expressed NRP-1 in the tumor tissue or in normal adjacent tissue. While the “normal adjacent tissue” (NAT) removed around tumor samples could be questioned as to how “normal” the tissue truly is, we suggest that this array data provides insight into the complexity of NRP-1 expression. NRP-1 has to date been shown to be involved in neuronal development, vascular development and maintenance, and most recently, involved in immune function, in addition to its role as a VEGF-165 specific receptor. The only tissue types in this array which show noticeable over-expression of NRP-1 in tumor vs. NAT are from stomach and rectal tumor patients (Figure 3.9, lanes 4 and 8). Perhaps the high NAT NRP-1 expression of other tissues can be explained by the high vascularization of the tissue types. The breast cancer patient samples are of particular interest, given ongoing research in our lab (LCCTP, TBCS, NCI, NIH) in which we find VEGF is

essential for normal development of the mammary gland in mice. Perhaps the high expression of NRP-1 in normal mammary tissue is indicative of VEGF regulation, or that NRP-1 is acting with other ligands for mammary gland maintenance and/or development.

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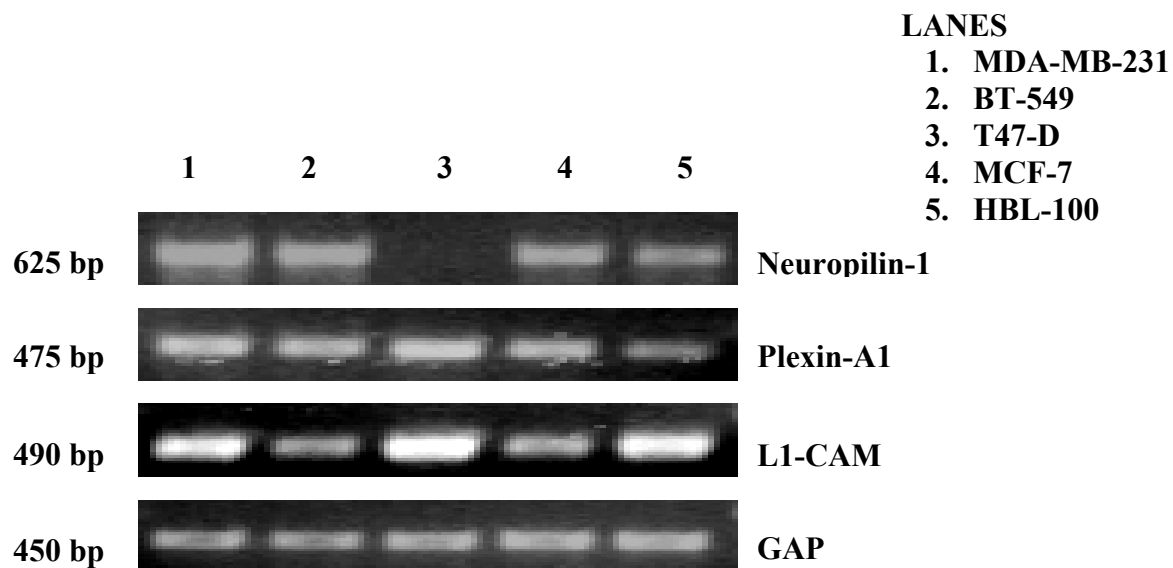


Figure 3.1 RT-PCR Expression of Neuropilin-1 and Co-Receptors Plexin-A1 and L1-CAM in Breast Cancer Cell Lines. Neuropilin-1 expression was found consistently in all cell lines tested except T47-D (lane 3) cells. Plexin-A1 and L1-CAM were found to be expressed in all cell lines tested. To show equal loading of PCR products, GAPDH reaction was also performed.

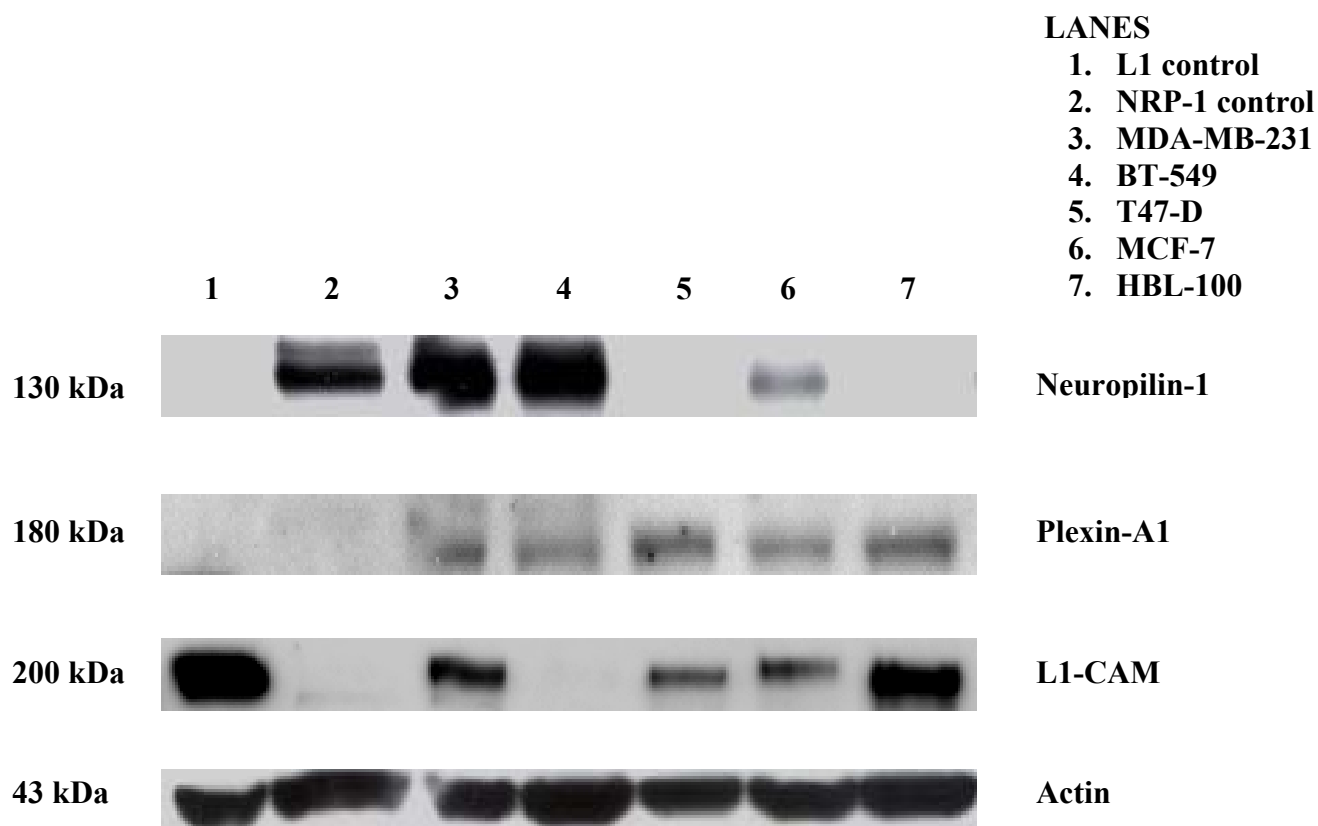


Figure 3.2. Western Analysis of Neuropilin-1 and Co-Receptors Plexin-A1 and L1-CAM Expression in Breast Cancer Cell Lines. Neuropilin-1 expression was found in all cell lines tested except T47-D cells (lane 5), as was shown in the RT-PCR analysis, but we also did not find protein expression in the HBL-100 cell line (lane 7). Plexin-A1 and L1-CAM were found to be expressed in all cell lines tested by RT-PCR, and we found protein expression of Plexin-A1 in all lines tested. L1-CAM, however, was not found in BT-549 cell lysates. This blot was first probed for NRP-1, stripped and then reprobed for Plexin-A1, L1-CAM, and actin respectively.

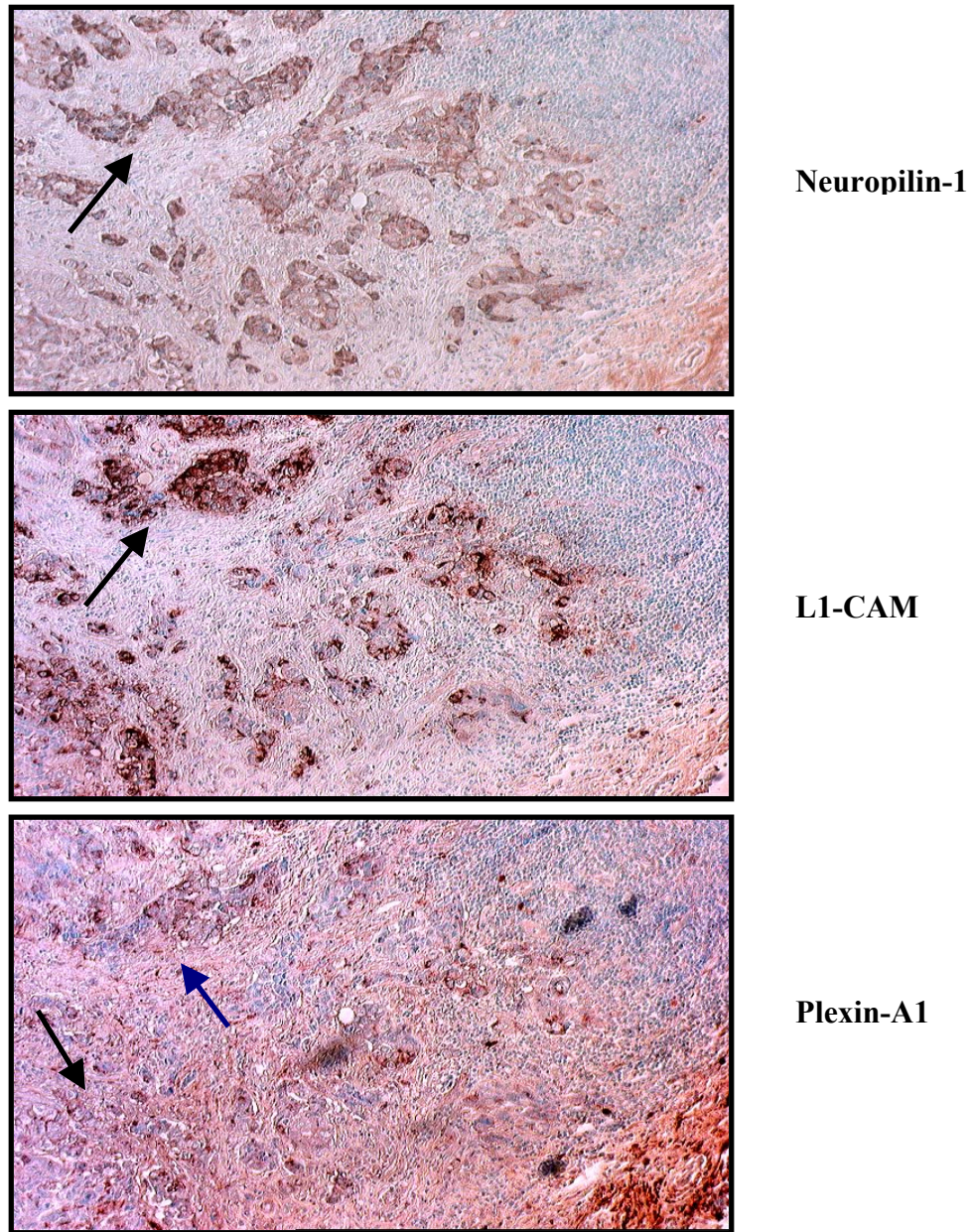


Figure 3.3. Immunohistochemistry Detection of Neuropilin-1, L1-CAM, and Plexin-A1 in human breast tumor tissue. Serial sections of human breast tissue were examined for the expression of NRP-1, Plexin-A1, and L1-CAM. We found strong staining for all three proteins, and obvious co-expression of NRP-1 and L1-CAM in tumor beds (black arrows). Plexin-A1 expression was predominantly expressed in stromal tissue (black arrow), but was also found in tumor beds to a lesser extent (blue arrow).

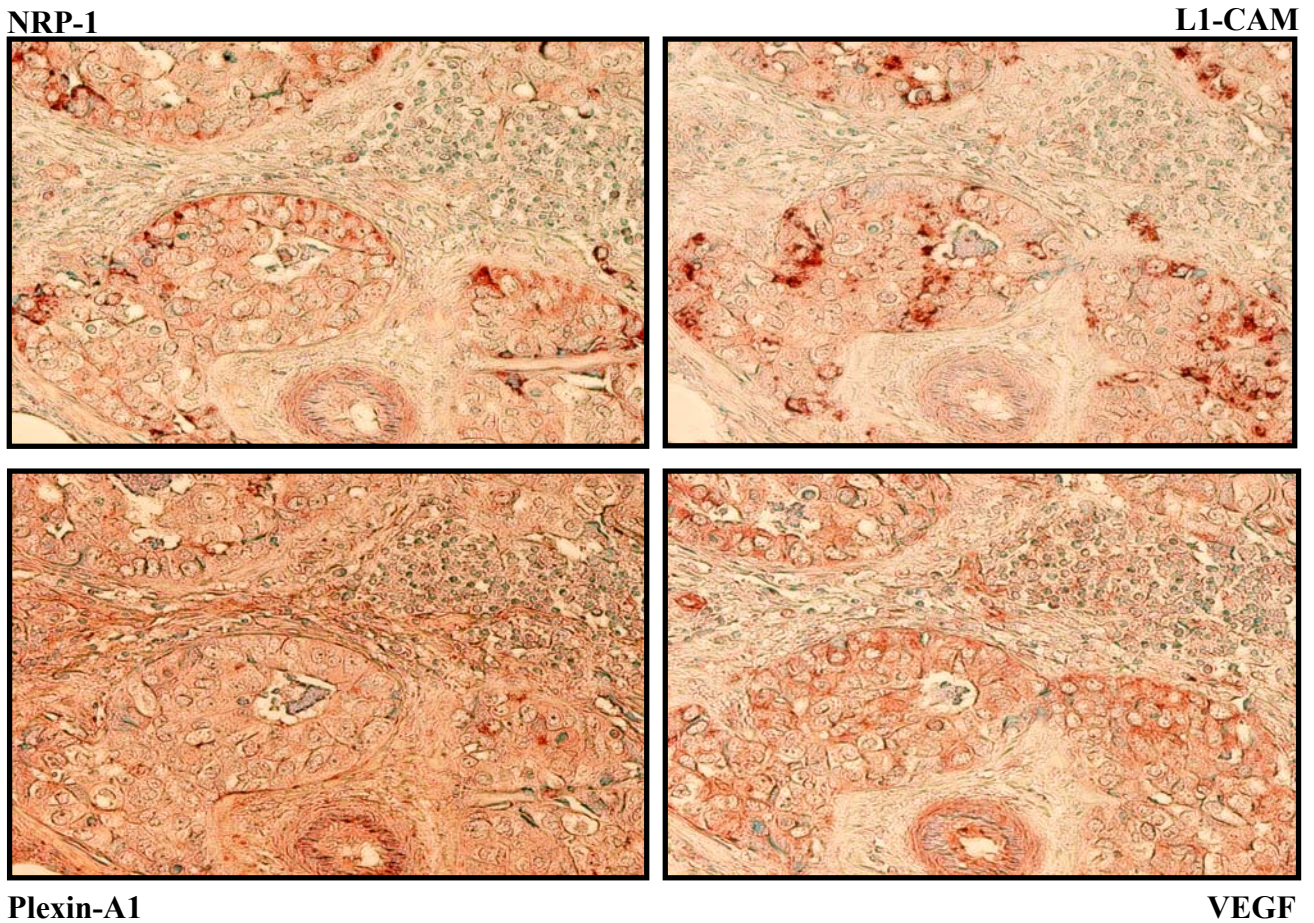


Figure 3.4. Immunohistochemistry Detection of Neuropilin-1, L1-CAM, and Plexin-A1 and VEGF in human breast tumor tissue. Serial sections of human breast tissue were examined for the expression of NRP-1, Plexin-A1, and L1-CAM and VEGF. Again, we found strong staining for NRP-1, L1-CAM and VEGF in the tumor beds, and high stromal expression of Plexin-A1, with some expression in the tumor beds. Magn 40X.

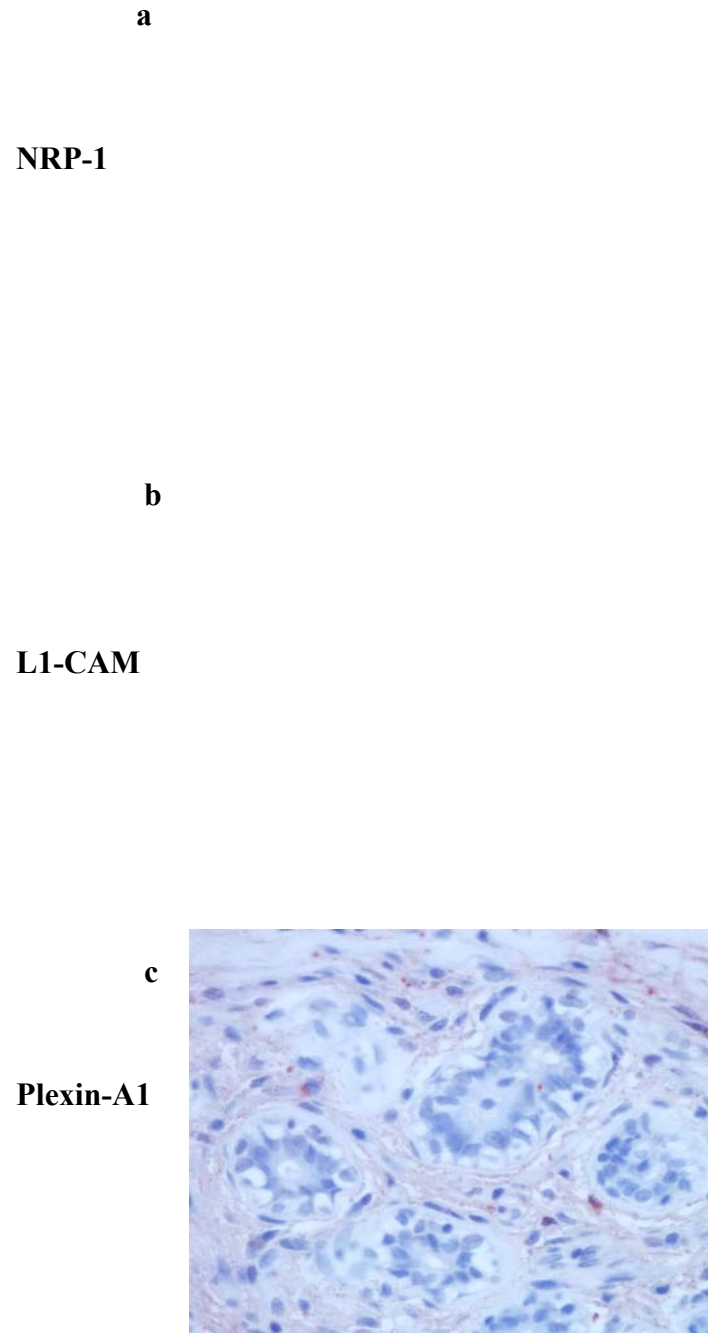


Figure 3.5. Immunohistochemistry Detection of Neuropilin-1, L1-CAM, and Plexin-A1 in normal breast tissue. NRP-1 (a) and Plexin (c) were taken at 40X, while the L1-CAM (b) image was taken at 10X to illustrate how little staining was found.

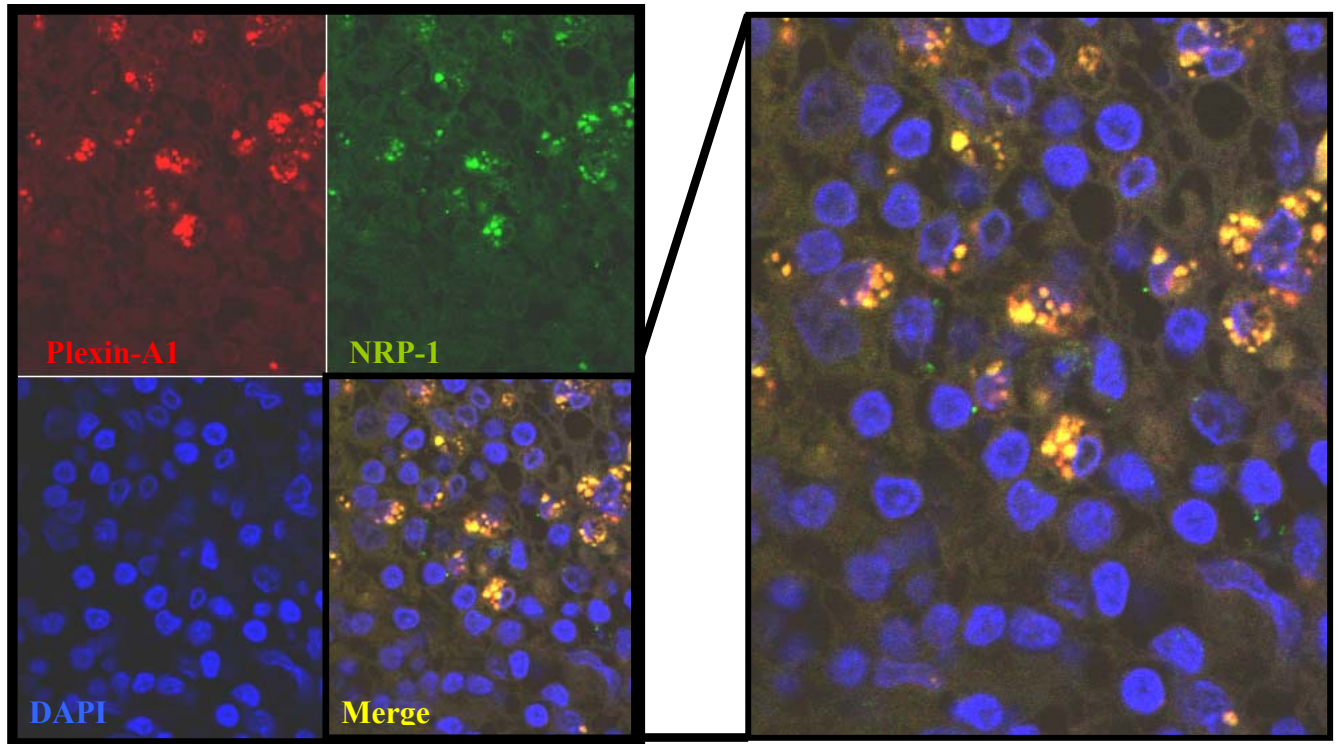


Figure 3.6. Immunofluorescent Detection of Neuropilin-1 and Plexin-A1 co-localization in human breast tumor tissue. Isolated pockets of tumor cells showed co-localization of Neuropilin-1 (green) and Plexin-A1 (red) as illustrated in the merged image that is enlarged on the right. DAPI was used to stain nuclei. Magn 40X.

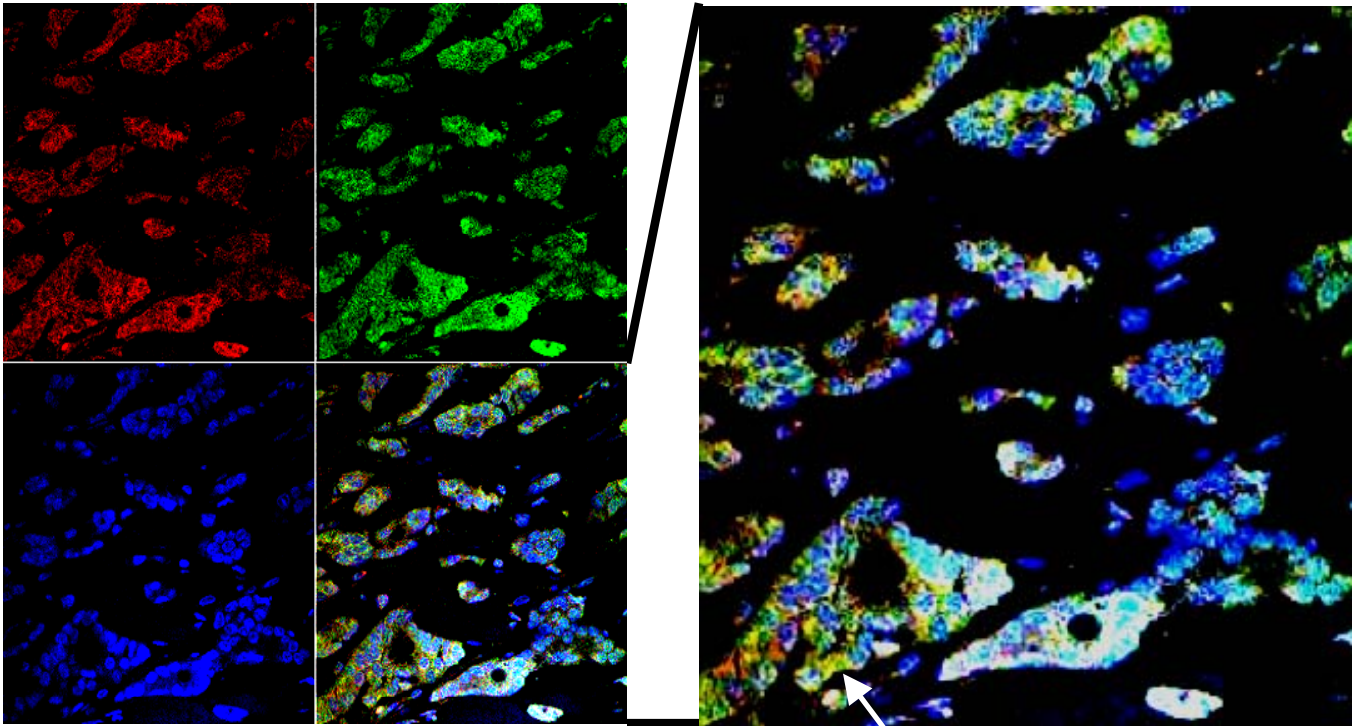


Figure 3.7. Immunofluorescent Detection of Neuropilin-1 and L1-CAM co-localization in human breast tumor tissue. Large areas of tumorous tissue beds showed co-localization of Neuropilin-1 (green) and L1-CAM (red) as illustrated in the merged image (yellow areas) that is enlarged on the right. DAPI was used to stain nuclei. Magn 25X.

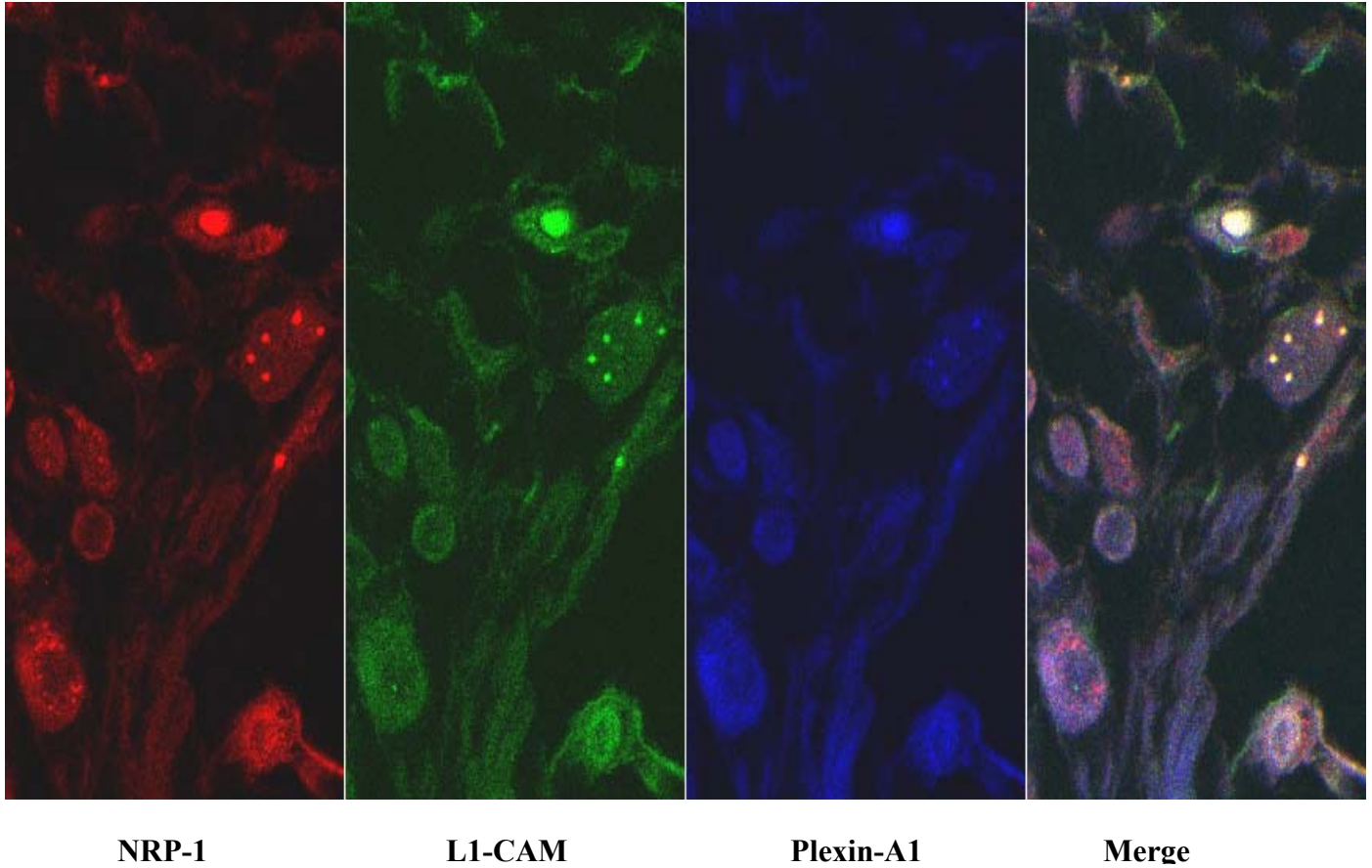


Figure 3.8. Immunofluorescent detection of NRP-1/L1-CAM/Plexin-A1 co-clustering in human breast cancer. Confocal microscopic detection of all three proteins in clusters on isolated tumor cells, with nuclear localization of NRP-1/L1-CAM/Plexin-A1 in a neighboring cell. Neropilin-1 staining is in red, L1-CAM in green, and Plexin-A1 staining in blue. The merged image illustrates white regions of triple co-localization.

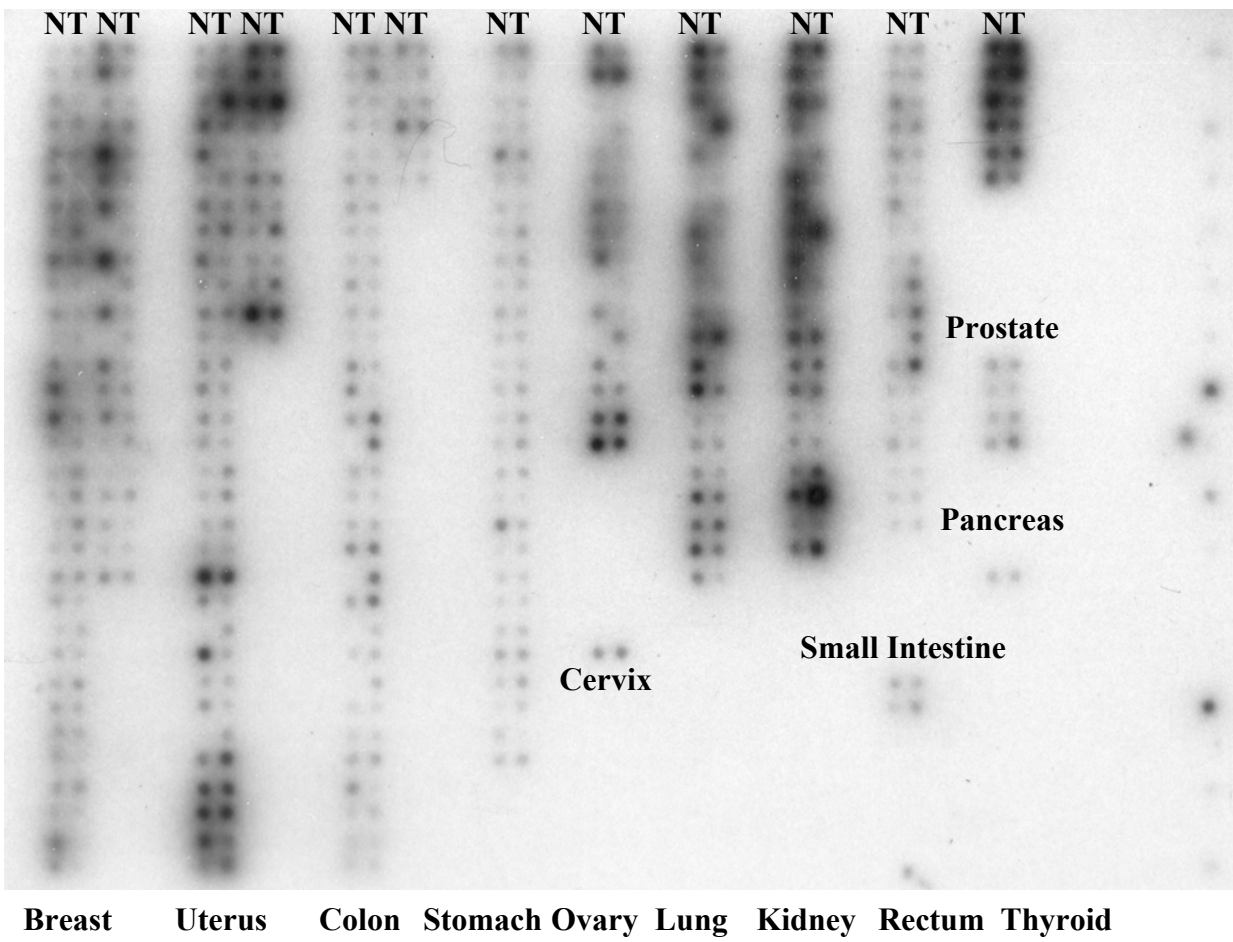


Figure 3.9- cDNA expression of NRP-1 in a variety of human cancers and normal adjacent matching tissue. Using the Cancer Profiling Array (Clontech) we were able to quantitate the expression of NRP-1 in matched tumor (T) /normal (N) tissue types from multiple organs.

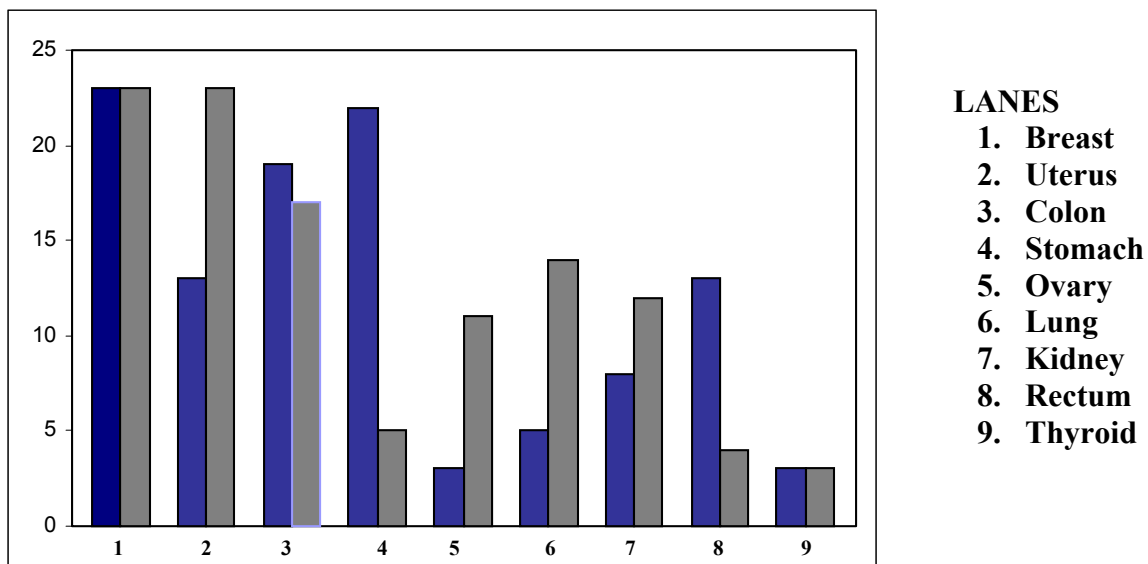


Figure 3.10. Analysis of NRP-1 expression in tumor and normal adjacent tissue of matched samples. Densimetric analysis was performed on the Cancer profiling array, hybridized with a probe specific for NRP-1. Each tumor sample was compared to its matched normal tissue pair, and it was determined whether the expression of NRP-1 was higher in the tumor or normal tissue. This graph represents number of pairs in which the tumor sample exhibited higher expression than the normal adjacent tissue (blue columns) or the normal adjacent tissue exhibited higher NRP-1 expression than the matched tumor tissue (grey columns).

## CHAPTER 4

EVIDENCE FOR THE ROLE OF NEUROPILIN-1 CO-RECEPTORS PLEXIN-A1  
AND L1-CELLULAR ADHESION MOLECULE IN THE AUTOCRINE EFFECT OF  
VASCULAR ENDOTHELIAL GROWTH FACTOR ON BREAST CANCER CELLS<sup>1</sup>

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<sup>1</sup> Matheny, S.L., O.R. Bunce, S. Yanagi, and U.P. Thorgeirsson. To be submitted to *Cancer Research*

## ABSTRACT

NRP-1 is an isoform-specific receptor of VEGF-165 that is found on both endothelial cells and breast cancer cells. In this study we sought to examine the role of NRP-1 in the autocrine function of VEGF inhibition of apoptosis and its effect on migration in breast cancer progression. Specifically, we sought to establish if the NRP-1 co-receptors Plexin-A1 and L1-CAM, previously believed to be functionally isolated to neuronal development, were involved in the autocrine effect of VEGF on breast cancer cells. Here we have demonstrated the co-immunoprecipitation of NRP-1 with both L1-CAM and Plexin-A1 in the MDA-MB-231 breast cancer cell line that expresses high levels of endogenous VEGF. In addition, using a HA-tagged recombinant Plexin-A1 construct, we showed that overexpression of Plexin in MDA-MB-231 and MCF-7 cells in the presence of recombinant VEGF-165 resulted in an immediate decrease in phosphorylation of LIM-kinase and down-regulation of phosphorylated cofilin, suggesting the involvement of Plexin-A1 in VEGF-mediated motility of these cells. In addition, when MCF-7 cells were treated with a recombinant L1-Fc chimera protein, which is known to activate L1-CAM/NRP-1 complexes, there was an increase in VEGF-mediated anti-apoptotic effects under hypoxic conditions. Mechanistic studies indicate an up-regulation of phosphorylated Akt in MCF-7 cells treated with L1-Fc. This upregulation could be reversed in the presence of a VEGF-165 blocking antibody. We show here for the first time, evidence suggesting the importance of neuronal co-receptors of NRP-1, Plexin-A1 and L1-CAM, in the autocrine effect of VEGF165 on breast cancer cells.

## INTRODUCTION

Vascular endothelial growth factor (VEGF) is a major stimulator of angiogenesis, is secreted by many tumors, and is a negative prognostic factor in breast cancer patients (Ferrara, 1995; Manders et al., 2002). VEGF is known to bind tyrosine kinase receptors expressed on endothelial cells (VEGFR-1, and -2), resulting in increased migration, resistance to apoptosis, and proliferation of endothelial cells, thus stimulating angiogenesis (Neufeld et al., 1999). With such a profound effects on endothelial cells and a lack of traditional VEGF receptors on non-endothelial cells most studies on VEGF function to date have focused on the angiogenic effect of VEGF. The belief that VEGF is affecting signaling in tumor cells themselves is a relatively new concept (Klagsbrun et al., 2002). With the discovery in 1998 of a VEGF-165 specific receptor found on tumor cells, which was later identified as Neuropilin-1, evidence for the autocrine ability of VEGF in tumor biology has increased (Soker et al., 1998). However, since this discovery, only a few papers have addressed the effect of VEGF on cancer cells directly (Miao and Klagsbrun, 2000; Miao et al., 2000; Pidgeon et al., 2001a; Bachelder et al., 2001). NRP-1 has a short cytoplasmic domain, and is believed to require co-receptors to induce signals. These results are demonstrated in studies in neuronal development, where it has been shown that the cytoplasmic domain of NRP-1 is not required for axonal guidance responses to Semaphorin 3A (Sema3A) cues (Nakamura et al., 1998). However, it has been found that the transmembrane proteins Plexin-A1 and L1-CAM are required for NRP-1 mediated responses to Sema3A (Takahashi et al., 1999; Castellani et al., 2000). It

has been determined that NRP-1 acts to ensure binding-specificity, and that NRP-1 requires co-receptors to transduce signals, reviewed in (Van Vactor and Lorenz, 1999). Studies of NRP-1 on endothelial cells show that NRP-1 interacts with VEGFR-1 (KDR), using KDR as a co-receptor, and the result of NRP-1 in this complex is to enhance the binding of VEGF, resulting in increased chemotaxis (Miao et al., 1999).

We have shown for the first time (Chapter 2), the expression of previously neuronally isolated Plexin-A1 and L1-CAM in breast cancer cell lines and that these proteins are in fact interacting with NRP-1. While the interaction between NRP-1, Plexin-A1, and L1-CAM is not perfectly clear, it is known that all three proteins are required for the response to *Sema3A* in neuronal development (discussed in (He, 2000)). Independent studies have revealed certain down-stream targets involved in signaling events of L1-CAM and Plexin-A1. Plexin-A1 is believed to mainly affect the migratory ability by signaling through G-proteins and eventually cause rearrangement of actin fibers (Liu and Strittmatter, 2001; Artigiani et al., 1999; Bagnard, 2001). Downstream targets of Plexin-A1 signaling, known from *Sema3A* signaling studies, include the activation of Rac, followed by phosphorylation of PAK which phosphorylates LIM kinase, which then serves to inactivate cofilin by phosphorylation. Activated cofilin (dephosphorylated) is a protein directly responsible for the depolymerization and severing of actin fibers by binding these fibers irreversibly in a one-to-one ratio (Aizawa et al., 2001). Using a Plexin-A1 expression construct (Mitsui et al., 2002), we were able to determine the effect of overexpressing Plexin-A1 in the presence of recombinant VEGF-165 in breast cancer cell lines.

L1-CAM was initially associated with diseases as a result of X-chromosome linked genetic mutations resulting in neurological disorders (reviewed in (Crossin and Krushel, 2000). After extensive research into this molecule, although L1-CAM has been shown to be upregulated in a variety of different tumor types (Figarella-Branger et al., 1990; Li et al., 2002a; Miyahara et al., 2001b; Meli et al., 1999 ), it remains to be seen what role this molecule directly plays in tumor progression. L1-CAM knockout mice implicated the role of L1-CAM in signal-transduction of Sema3a in neuronal guidance, and it has been confirmed that L1-CAM and NRP-1 biochemically associate for Sema3A signal transduction (Castellani et al., 2000). After piecing together many different studies which have focused on the role of L1-CAM in neuronal development, and given the strong and consistent expression of L1-CAM in breast cancer cells lines under study, we sought to determine the effect of L1-CAM on VEGF-165 signaling. Studies involving the direct role of L1-CAM in neurons found that activation has been shown to prevent apoptosis of neuronal cell death. By culturing neurons either on plates coated with an L1-Fc chimera protein, or by treating neurons with the soluble L1-Fc chimera, L1-CAM is activated and apoptosis is prevented (Chen et al., 1999; Castellani et al., 2002). Here we present data showing the involvement of L1-CAM in VEGF-mediated signaling in breast cancer, where we find that activation of MCF-7 cells with the L1-Fc chimera results in increased resistance to apoptosis in hypoxic conditions.

## MATERIALS AND METHODS

**Cell Culture and Materials.** All cell lines were obtained from the American Tissue Culture Collection (ATCC, Manasses, VA) and were grown in a humidified atmosphere

of 5% CO<sub>2</sub>, 95% air at 37 °C with change of medium every 2-3 days. The human breast cancer cell line MDA-MB-231 were cultured in DMEM with 25 mM HEPES (Biofluids, Rockville, MD) and supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics. MCF-7 cells were cultured in EMEM with non-essential amino acids (Biofluids) supplemented with 10% FBS, 2 mM glutamine, 0.2 U/ml insulin, 1.0mM sodium pyruvate, and antibiotics. Transfection experiments were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. An HA-Plexin construct was generously provided by Dr. Shigeru Yanagi and has been previously described (Mitsui et al., 2002). L1-Fc chimera and IgG-Fc control chimera were purchased from R&D Systems. Recombinant human VEGF-165, and VEGF-165 neutralizing antibodies were purchased from R& D Systems (Minneapolis, MN). For hypoxia experiments, cells were cultured in low serum media (DMEM with 0.5% FBS) and maintained in either normoxic (5% CO<sub>2</sub>, 20% O<sub>2</sub>, 75% N<sub>2</sub>) or hypoxic (5% CO<sub>2</sub>, 3%O<sub>2</sub>, 94.5% N<sub>2</sub>) conditions for indicated time periods. The following antibodies were used for this study: anti- NRP-1 (clone C-19), Plexin-A1 (clone Q-15), and L1-CAM (N-100) all from Santa Cruz Biotechnologies (Santa Cruz, CA), L1-CAM clone (UJ127) from Neomarkers (Fremont, CA). Phosho-specific cofilin, total cofilin, phospho-specific LIM kinase, total LIM kinase, HA-tag antibody , phospho-specific Akt (specific for phosphorylation as Ser473) and total Akt, were all from Cell Signaling Technologies (Beverly, MA).

**Transfection of Cells.** Transient and stable transfections of MDA-MB-231 cells and transient transfection of MCF-7 cells were performed using the HA-Plexin-A1 construct

on 90% confluent cells according to the manufacturer's protocol (Lipofectamine 2000, Invitrogen, Carlsbad, CA). Control experiments were performed to ensure validity of the transfections using the Lipofectamine treatment in tandem without introducing DNA (data not shown). Stable clones of MBA-MD-231 cells were selected in DMEM with 25 mM HEPES (Biofluids, Rockville, MD), and supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, antibiotics and 500  $\mu$ g/mL G418 for several weeks. Selected clones were transferred to individual dishes and maintained in selective media described above.

**Immunofluorescence.** Staining for human NRP-1 was performed on MDA-MB-231 cells grown on glass slides. Cells were treated with either 100 ng/ml recombinant human VEGF-165 or with PBS/0.5% BSA negative control. Recombinant proteins and negative controls were added to low serum DMEM (DMEM with 0.5% FBS) and treated for the time indicated in the figures in a humidified atmosphere of 5% CO<sub>2</sub>, 95% air at 37 °C. After treatment, slides were rinsed with PBS and fixed in 4% paraformaldehyde for 10 minutes. After fixation, cells were rinsed in PBS, and permeabilized with 0.1% Triton X-100 for 10 minutes, followed by a 10 minute incubation in methanol. After PBS washes, non-specific binding was blocked using a 30 minute incubation in 1 mM glycine, followed by a ten minute incubation in 1X Power Block (Innogenex, San Ramon, CA). A rabbit polyclonal antibody for human NRP-1 (clone H-286) was incubated overnight at 4°C at 1:50 dilution in 1X Power Block solution. After washing in PBS, the slides were incubated in secondary antibody solutions using the AlexaFluor donkey anti-rabbit 488 in a 1:100 solution of PBS. After a one hour incubation with the secondary antibodies, the

slides were washed with PBS and incubated for 5 minutes with a 300 nM solution of DAPI (Molecular Probes, Eugene, OR) for nuclear visualization, and mounted using ProLong Anti-Fade mounting media (Molecular Probes, Eugene, OR). The tissues samples were visualized using a Zeiss confocal microscope at indicted magnifications.

**Western blotting.** All cells were washed in 1X PBS and lysed directly using 2x SDS loading Buffer (Invitrogen); and cell lysates were transferred to eppendorf tubes, sonicated for 15 seconds, rocked gently on ice for 15 min , and spun at 15,000 rpm for 15 min at 4°C to remove cellular debris. The protein lysates were boiled for 5 minutes and 20 µl of the lysate was immediately subjected to SDS-PAGE on 8% Tris-Glycine 1.0mm gels or 10-20% Tris-Glycine 1.0 mm gels (Invitrogen, Carlsbad, Ca). Gels were run at 120V for approximately 1.5 hours and transferred to PVDF membranes (Invitrogen, Carlsbad, CA). After transfer, the membrane was blocked for 1 hour in 10% non-fat dry milk, rinsed with PBST, and immunoblotted with antibodies specific for the protein of interest (phosphorylated (p)- Akt, p-cofilin, p-LIM Kinase) overnight at 4°C with gentle rocking. The bound antibodies were detected using horseradish peroxidase-conjugated secondary antibodies (mouse anti-goat-HRP, goat anti-mouse-HRP, and mouse anti-rabbit-HRP conjugated secondary antibodies depending on source of primary antibody from DAKO, NY) and an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia). The blot was subsequently stripped and re-probed with antibodies which were used as a loading control for total protein (total Akt, total cofilin or total LIM kinase) respectively.

**Immunoprecipitation.** Cells were washed with cold PBS and immunoprecipitation experiments were performed using the Catch and Release Reversible Immunoprecipitation kit™ (Upstate, Waltham, MA) according to the manufacturer's protocol. Briefly, cells were washed with ice-cold PBS and immediately lysed using 1X lysis buffer (Upstate) supplemented with protease inhibitor Complete™ tablets (Roche, Indianapolis, IN). 500 µl of cell lysate was incubated with 10ug of antibodies specific for the desired target (see Figure 4.2). Added to the lysates was 10µl of the Antibody Capture Affinity Ligand, as indicated in the manufacturer's protocol. Samples were incubated at room temperature for 20 minutes and then loaded into Catch and Release spin tubes and centrifuged at 1,500 x g for 10 minutes. Flow-through was discarded and the columns were washed four times with 500µl of 1X Lysis/Wash buffer and centrifuged at 2,000 x g. After washes, 30ul of Elution buffer (Upstate, Waltham, MA) was added to each sample, spun at 500g, and to this eluate an equal volume of 2X SDS loading buffer (Invitrogen, Carlsbad, CA) was added. The samples were immediately boiled and subjected to SDS-PAGE analysis as described above.

**Migration Assay.** MDA-MB-231 parental cells and stable HA-Plexin transfected cells were used in invasion assays to determine if Plexin overexpression was affecting invasive ability MDA-MB-231 breast cancer cells. Cells were subjected to the Cell Invasion Assay Kit from Chemicon (Temecula, CA) according to the manufacturer's protocol. Briefly, test (HA-Plexin) and control (parental) cells were trypsinized, counted in a Coulter counter, and resuspended in serum-free media at a concentration of  $0.5 \times 10^6$  cells/ml. DMEM containing 10% FBS was added to the bottom chamber of the invasion

well to act as a chemoattractant for the cells. The invasion chambers containing 8  $\mu\text{m}$  pores were covered with extracellular matrix components (ECMatrix, Chemicon, CA), which blocks non-invasive cells from migrating through the pores. After 72 hours in culture, invasion inserts were removed. Cells that had migrated through the membrane and plated on the bottom chamber were rinsed in PBS and stained for 20 minutes using the stain supplied in the kit. Stained cells were rinsed in water, and cells were photographed using a phase-contrast microscope (Figure 4.5).

**Apoptosis Induction and Analysis.** Apoptosis was induced in cell lines by incubation under hypoxia (5% CO<sub>2</sub>, 3% O<sub>2</sub>, % N<sub>2</sub>). Controls experiments were treated exactly as experimental counterparts with the exception that they were incubated under normoxic (5% CO<sub>2</sub>, 20%O<sub>2</sub>, 75% N<sub>2</sub>) conditions. Cells were seeded at  $2 \times 10^5$  cells/well in 6 well culture plates in normal media and 2 days after seeding washed once with PBS. The cells were then incubated in either hypoxic or normoxic atmospheres in low serum DMEM (DMEM with 0.5% FBS) with either 2.5  $\mu\text{g}/\text{mL}$  IgG-Fc fusion chimera, 2.5  $\mu\text{g}/\text{mL}$  L1-Fc fusion chimera, or 2.5  $\mu\text{g}/\text{mL}$  L1-Fc fusion chimera with 5X excess (weight/volume) of VEGF-165 blocking antibody (R&D Systems MAB293). After 24 hours, cells were analyzed by flow cytometry or by Western analysis (described above) for apoptosis. The Vybrant Apoptosis Assay Kit #2 with Alexa Fluor 488 annexin V / propidium iodide kit (Molecular Probes, Eugene, OR) was used for flow cytometric analysis. Cells were trypsinized, spun down, washed with cold PBS, and cell numbers were determined using a Coulter counter. Cells were resuspended at a concentration of 500,000 cells in 100  $\mu\text{l}$  of Binding buffer (Molecular probes, Eugene, OR) with 5ul AnnexinV 488 nm

labeled stain (Molecular Probes, Eugene, OR) and incubated at room temperature for 15 minutes. After incubation, the cells were kept on ice, protected from light and 400  $\mu$ l of binding buffer with 5  $\mu$ l of a 100  $\mu$ g/ml Propidium Iodide solution was added to each sample for nuclear staining of dead or dying cells. The number of apoptotic cells in each sample was counted using a FACS Calibur Flow cytometer, calibrated with untreated, unstained MCF-7 cells.

## RESULTS

### **Treatment of MDA-MB-231 cells with VEGF results in translocation of NRP-1 from the cytoplasm to the cell surface.**

Here we show, for the first time to our knowledge, visual evidence for an autocrine effect of NRP-1 in breast cancer cells. MDA-MB-231 cells were grown on glass slides, and treated with 100 ng/ml of human recombinant VEGF-165 or with a negative vehicle control for 10 minutes. Cells were then stained for NRP-1 and visualized with fluorescent secondary antibodies and confocal microscopy. NRP-1 was consistently localized in the cytoplasm of untreated MDA-MB-231 cells. Following treatment with recombinant human VEGF-165, NRP-1 translocated to the cell surface to where the cell edge was outlined by NRP-1 positive clusters. This effect was observed as early as 2 minutes after treatment with VEGF-165, and provides direct visual evidence of the autocrine effect of VEGF in human breast cancer cells. (See figure 4.1).

### **Plexin-A1 Over-Expression in MDA-MB-231 Cells Results in Decreased Phosphorylation of Cofilin in the Presence of VEGF.**

To determine how Plexin-A1 is contributing to the autocrine effect of VEGF in breast cancer cells the full-length construct of Plexin-A1 with a HA- tag for detection (of the transfected Plexin-A1) was transfected into MDA-MB-231 and MCF-7 cells. Characterization of this construct has been previously described (see (Mitsui et al., 2002)). MDA-MB-231 cells were transfected as described in Methods, and 72 hours after transfection, the cells were washed and treated with the following concentrations of human recombinant VEGF<sub>165</sub> in duplicate: control (PBS/BSA- 500 ng/ml), 25 ng/ml, 50 ng/ml, 100 ng/ml, 250 ng/ml and 500 ng/ml. Ten minutes after treatment, the cells were washed once with cold PBS and used in either direct Western Blot analysis or for co-immunoprecipitation (coIP) experiments. Using an anti-HA specific antibody to pull down proteins associated with the HA-Plexin, NRP-1 was detected by Western Blot in the CoIP experiment. (Figure 4.2a). Using an anti-NRP-1 specific antibody to pull down associated proteins in these samples, L1-CAM was detected in each treatment group by Western analysis (Figure 4.2b). In addition, we found a drastic decrease in phosphorylated cofilin in the MDA-MB-231 cells treated with 50ng/ml that was also seen in the cells treated with higher doses (Figure 4.3 a). Equal loading of these blots was confirmed by stripping and reprobing with an antibody specific for total cofilin (Figure 4.3 b). These results were duplicated. This is the first indication of VEGF affecting actin filament rearrangement in cancer cells. Also, this is the first indication for the direct role of Plexin-A1 in VEGF signaling effects.

In support of these findings, preliminary data suggests that stable MDA-MB-231 Plexin-A1 overexpressing clones are significantly less invasive than parental MDA-MB-

231 cells through an extracellular matrix membrane. We consider this data preliminary because we have yet to decipher the role of VEGF165 in this process, however MDA-MB-231 cells overexpressing Plexin-A1 show a drastic decrease in their ability to migrate through extracellular matrix components in comparison to parental MDA-MB-231 cells. For this invasion assay, 10% FBS was used as the chemoattractant. (Figure 4.5).

#### **Plexin-A1 Over-Expression in MCF-7 Cells Results in Decreased Phosphorylation of Cofilin and Decreased Phosphorylation of LIM Kinase in the Presence of VEGF.**

We transiently transfected MCF-7 cells as described above with the HA-tagged Plexin-A1 construct. This time we treated the cells with 100ng/ml of recombinant human VEGF165 for different incubation times as follows: 0 minutes, 3 minutes, 5 minutes, 10 minutes, 15 minutes, and 30 minutes. Cells were lysed immediately after incubation and subjected to Western analysis. In the untreated samples (0 minutes), the phosphorylation status of LIM kinase, the protein responsible for deactivation (phosphorylation) of cofilin, was relatively high (Figure 4.4). Three minutes after VEGF-165 treatment, a drastic decrease in the phosphorylation status of LIM kinase was observed. Phosphorylation gradually increased over the 30 minutes observation time. Densitometric analysis was performed on phosphorylated and total LIM kinase to compare the amount of phosphorylated Lim kinase to the total amount of LIM kinase loaded .

#### **Treatment of MCF-7 Cells with L1-Fc Chimera Results in Inhibition of Apoptosis; a Suggested Role in VEGF Autocrine Signaling.**

We sought to also determine the role of L1-CAM in the VEGF autocrine effect on breast cancer cells. Using hypoxia as a cause of apoptosis, due to its direct relevance to the tumor environment, we treated MCF-7 cells with an L1-Fc Chimera protein that has been shown to stimulate L1-CAM signal transduction. As a negative control, cells were also treated with an IgG-Fc chimera protein. To assess the relevance of VEGF-165 in this process, we also treated cells with both the L1-Fc chimera and 5-fold excess of the VEGF-165 functional blocking antibody. All experiments were performed in tandem with cells receiving the exact same treatments, but in normoxic conditions to ensure hypoxia as the cause for apoptosis and not a side effect of treatments. After 24 hours of incubation, cells were isolated for flow cytometric quantitation of apoptosis and also for Western analysis for possible mechanism assessment (Figure 4.6). We found that treatment with the L1-Fc chimera resulted in a decrease in apoptosis in MCF-7 cells in hypoxic conditions. Analyses of the protein lysates from cells indicate that there is an increase in phosphorylated Akt as compared to total Akt in L1-treated samples, another indication of protection from apoptosis (Fig 4.7). We also found apoptosis to be increased compared to the IgG-Fc control in both normoxic and hypoxic conditions when the L1-Fc chimera was added to cells in combination with the VEGF-blocking antibody, (Figure 4.6- bottom panel), which indicates a direct role of VEGF in this process. Likewise the protein lysates indicate a decrease in phosphorylation of Akt in comparison of total Akt in the samples treated with this antibody (Figure 4.7).

## DISCUSSION

In this study, we show for the first time, visual evidence of the VEGF autocrine effect on breast cancer cells, which results both in translocation of NRP-1 to the cell surface and clustering of NRP-1 at the cell surface upon stimulation with recombinant human VEGF-165 (Figure 3.1). While the autocrine effect of VEGF-165 has been detected in breast cancer cell lines and is known to involve NRP-1, this is the first time to our knowledge of direct visual evidence of VEGF-165 treatment effecting positioning of any of its receptors in such a manner. As soon as two minutes after treatment with VEGF-165, NRP-1 immunofluorescence staining shows that NRP-1 translocates from compartments near the nucleus to the cell surface. By ten minutes after treatment with VEGF-165, outlines of the treated cells are well defined, and the NRP-1 staining can be seen as well-defined clusters (Figure 3.1d note arrow). This experiment was performed 3 times with representative samples shown.

Shown for the first time in cancer, the known neuronal co-receptors of NRP-1, Plexin-A1 and L1-CAM, are capable of interacting with NRP-1, as shown by immunoprecipitation experiments (Figure 3.2). While the interaction between NRP-1, Plexin-A1, and L1-CAM is not perfectly clear, it is known that all three proteins are required for the response to Sema3A in neuronal development. These results illustrate what has previously been shown in neuronal development, but to our knowledge, this is the first direct evidence of the capability of NRP-1 to co-immunoprecipitate with both Plexin-A1 (Figure 3.2.a) and L1-CAM (Figure 3.2b) in breast cancer. Such results indicate that these proteins could be contributing to the ability of VEGF to transduce signals in an autocrine manner.

The signal transduction mechanisms of Plexins are only beginning to be understood. Downstream targets of Plexin-A1 signaling, known from Sema3A activation, include the activation of Rac, followed by phosphorylation of PAK, which leads to the activation of LIM kinase, which then serves to phosphorylate and inactivate cofilin, a protein directly responsible for the depolymerization of actin fibers by binding these fibers irreversibly in a one-to-one ratio (Aizawa et al., 2001). What is seen in the presence of Sema3a is the eventual increase in phosphorylation of cofilin, resulting in decreased depolymerization of actin filaments. Although not fully understood, the repulsive action of Sema3a, which binds NRP-1 and activates Plexin-A1 signaling, can be reversed in the presence of increased amounts of Sema3a (Bagnard et al., 1998). In fact, the importance of NRP-1 in this event is illustrated by the fact that the exact opposite effect is seen in both endothelial cells and neuronal cells that are stimulated with VEGF vs. Sema3a (Bagnard et al., 2001; Miao et al., 1999). Biochemically, this can be explained by studies (Miao et al., 1999) that show that VEGF165 and Sema3a compete for the same binding site on NRP-1. Specifically, VEGF inhibits neuronal apoptosis, while Sema3A enhances this process. These effects are dependant on NRP-1 expression (Bagnard et al., 2001). Additionally, VEGF enhances neuronal migration, while Sema3A repulses or inhibits neuronal and endothelial migration and chemotaxis. Again, note that these effects are dependant on NRP-1 binding. While it remains to be elucidated precisely how the concentration of Sema3a is able to act as both a repulsive and attractive clue, the best explanation involves the clustering of NRP-1 co-receptors as a result of ligand concentration. This clustering of co-receptors results in higher cellular levels of cGMP, which is the direct result of GTPase activity (Song et al., 1998). Certain downstream

targets have been identified, namely, the transmembrane domain of Plexin-A1, which is structurally able to be involved in protein-protein interactions. Some Plexin family members have been shown to directly bind small G-proteins in these regions. Most recently, however, Plexin-A1 activation has been shown ((Yu and Kolodkin, 1999), discussed in (Rohm et al., 2000b)) to result in the activation of Rac, which is one such protein. To date, all of the data on Plexin-A1 signaling comes from studies involving stimulation by Sema3A in relation to neuronal effects. We set out in this study to determine if there is any contribution of Plexin-A1 in VEGF signaling through NRP-1. Given the opposing effects of Sema3A and VEGF in both neuronal and endothelial cells, we expected to see opposite effects by VEGF in cancer cells. Instead of increased phosphorylation of LIM Kinase (as seen with Sema3A) (Aizawa et al., 2001), we expected a decrease in phosphorylation of LIM Kinase upon VEGF stimulation. In fact, we do show an decrease of LIM Kinase activation in MCF-7 cells transfected to overexpress Plexin-A1 in the presence of VEGF-165. Also, downstream of LIM Kinase, with Sema3A stimulation, is a known increase in phosphorylation of cofilin, causing less actin depolymerization in response to ligand binding. Therefore in the presence of VEGF, we would expect to see a decrease in the phosphorylation of cofilin, and thus increased activity of cofilin in the presence of VEGF. In fact we are able to show in the MDA-MB-231 cell line a drastic decrease in a dose-dependant manner with VEGF treatment in these cells overexpressing Plexin-A1. This is the first evidence that we are aware of to implicate Plexin-A1 in tumor biology signaling and also to provide a deeper understanding of the role of all of these proteins in neurobiology.

Piecing together many studies in many different cell types, we find that treatment with an L1- Fc chimera molecule results in activation of the known signaling linked to L1-CAM, a protein that is stimulated by homophilic interaction, binding of various different ligands including integrins and growth factor (PIGF) stimulation, but also a protein that its signal transduction pathway is not completely understood. What we do know is that activation of L1-CAM has been shown to prevent apoptosis of neuronal cell death. Here we present data showing the involvement of L1-CAM in VEGF-mediated signaling in breast cancer, where we find that activation of MCF-7 cells with the L1-Fc chimera results in increased resistance to apoptosis in hypoxia. Analysis of these cells shows an increase in phosphorylated Akt, a protein showed to be upregulated in response to VEGF in other breast cancer cells, which was shown to be dependant on NRP-1 involvement. We suggest that L1-CAM could act as the signal transducing co-receptor of NRP-1 in VEGF induced suppression of apoptosis in breast cancer cells, as previously described (Bachelder et al., 2001).

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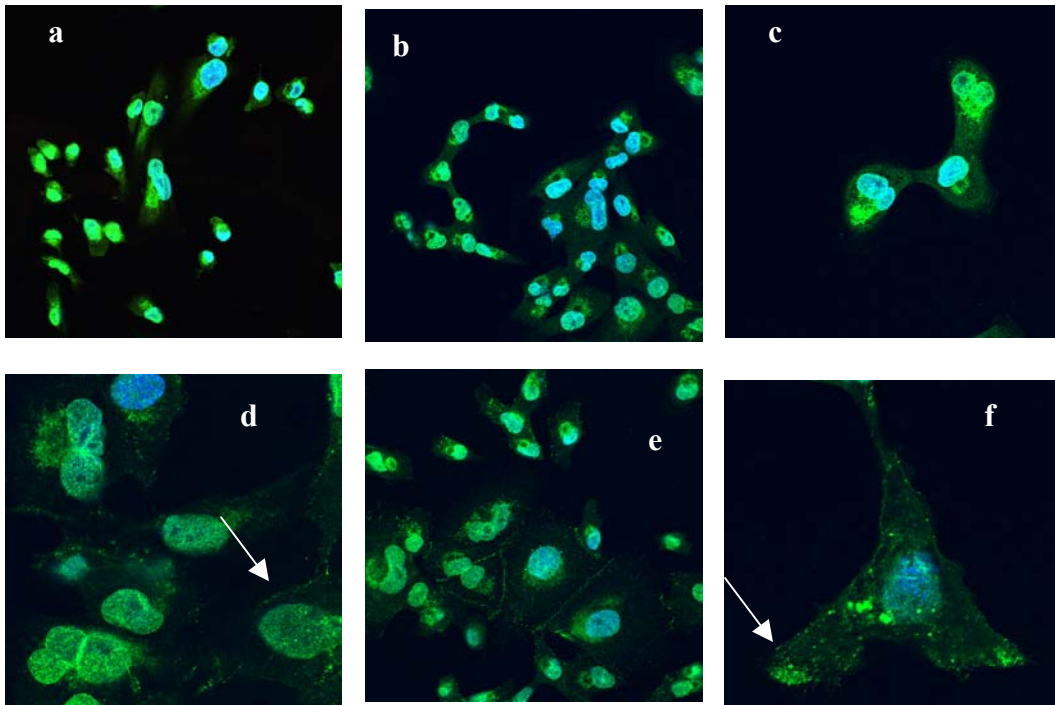


Figure 4.1 Immunolocalization of NRP-1 in MDA-MB-231 Cells treated with either vehicle control (PBS/BSA) or with VEGF 165. The top panel illustrates treatment with control; and the bottom panel contains cells treated with 100 ng/ml of VEGF 165 after 10 minutes incubation. Arrows in the bottom panel indicate translocation of NRP-1 to the cells surface with VEGF treatment, which is consistently lacking in such marginalization of the NRP-1 protein. Panels a, b, e at 25X, c and d at 40X, and f at 100X.

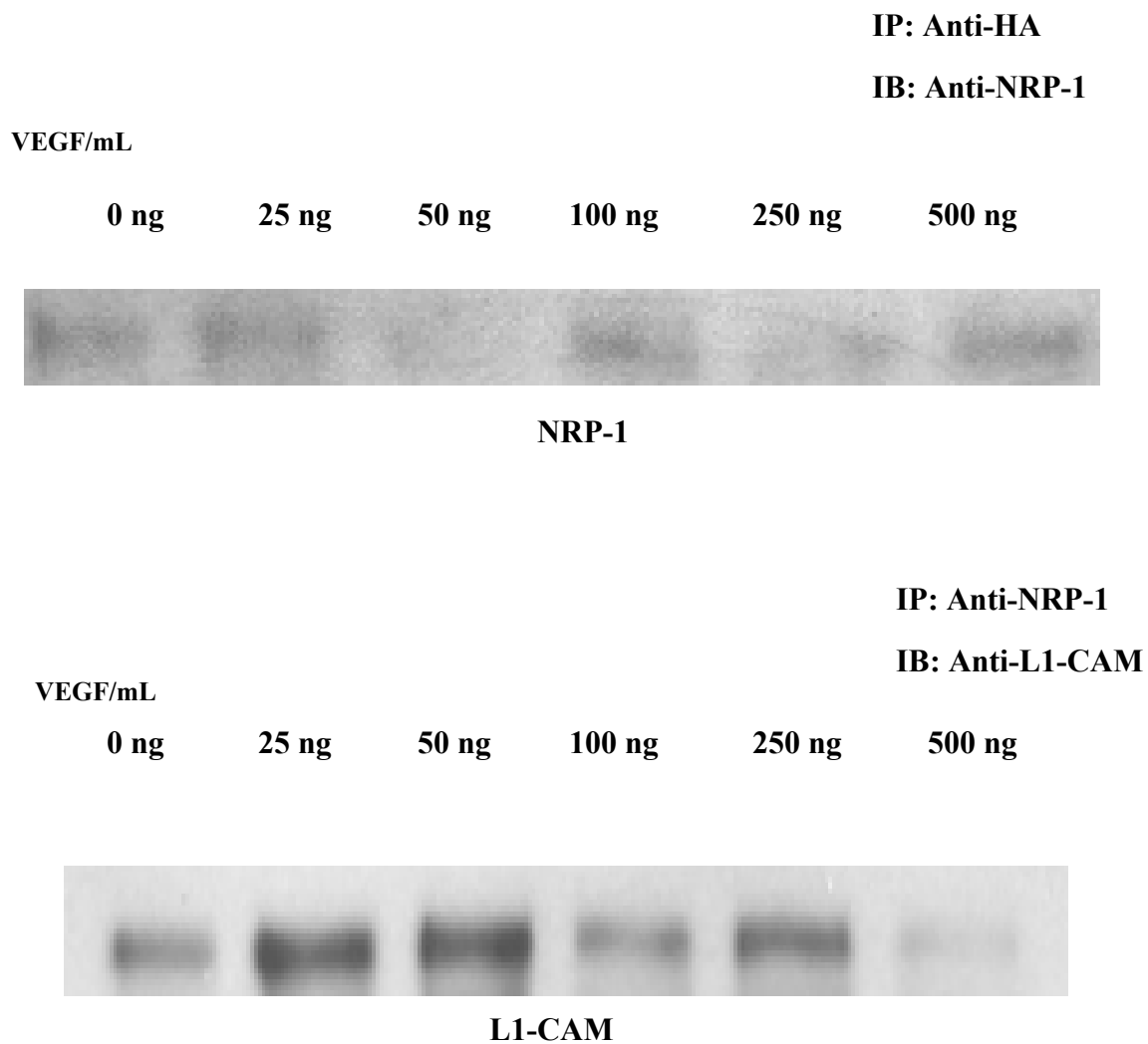


Figure 4.2. Analysis of Co-Immunoprecipitation of Plexin/NRP-1 and NRP-1/ L1-CAM in MDA-MB-231 cells overexpressing Plexin-A1 with increasing concentrations of recombinant human VEGF 165. While not a quantitative assay, we do show the ability of NRP-1 to co-immunoprecipitate with both Plexin-A1 and L1-CAM in the presence of rVEGF-165.

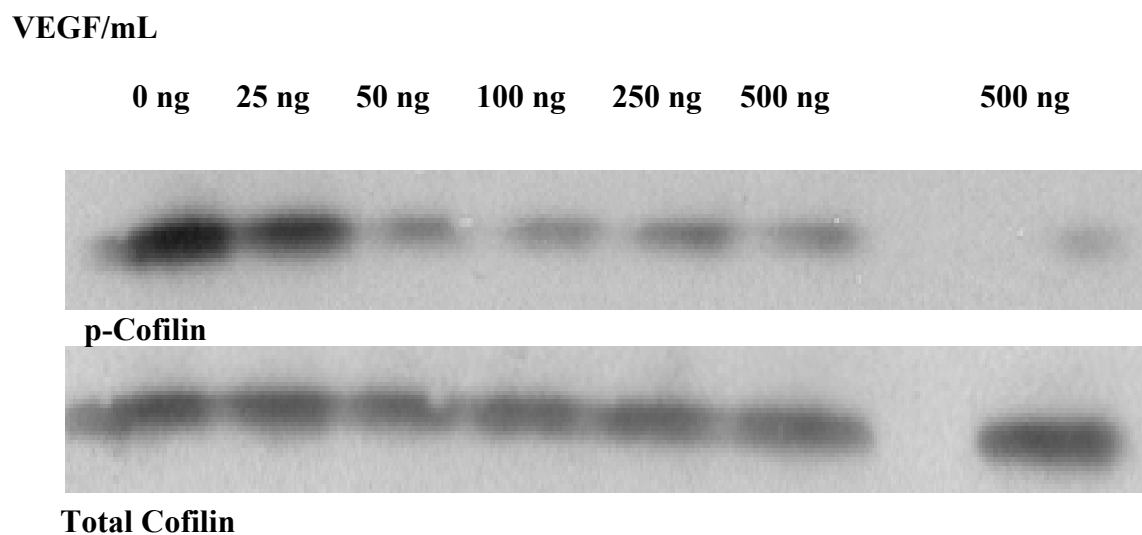


Figure 4.3 Analysis of Phosphorylation of Cofilin in MDA-MB-231 cells overexpressing Plexin-A1 with increasing concentrations of recombinant human VEGF 165. The last lane contains a repeat of the sample treated with 500 ng/ml VEGF to ensure consistent transfer of protein across the blot.

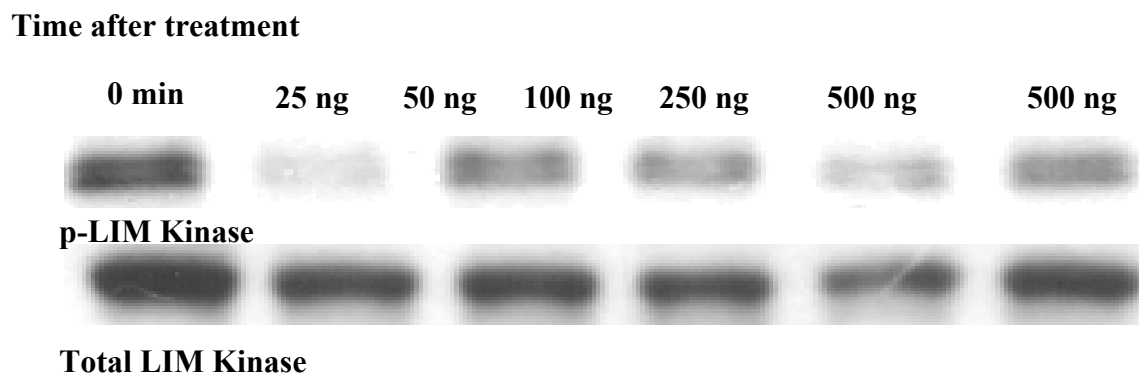


Figure 4.4 Analysis of Phosphorylation of LIM Kinase in MCF-7 cells overexpressing Plexin-A1 treated with 100 ng/ml VEGF 165 and incubated for varying times, indicated above each lane. Note the drastic decrease in LIM Kinase immediately following VEGF treatment.

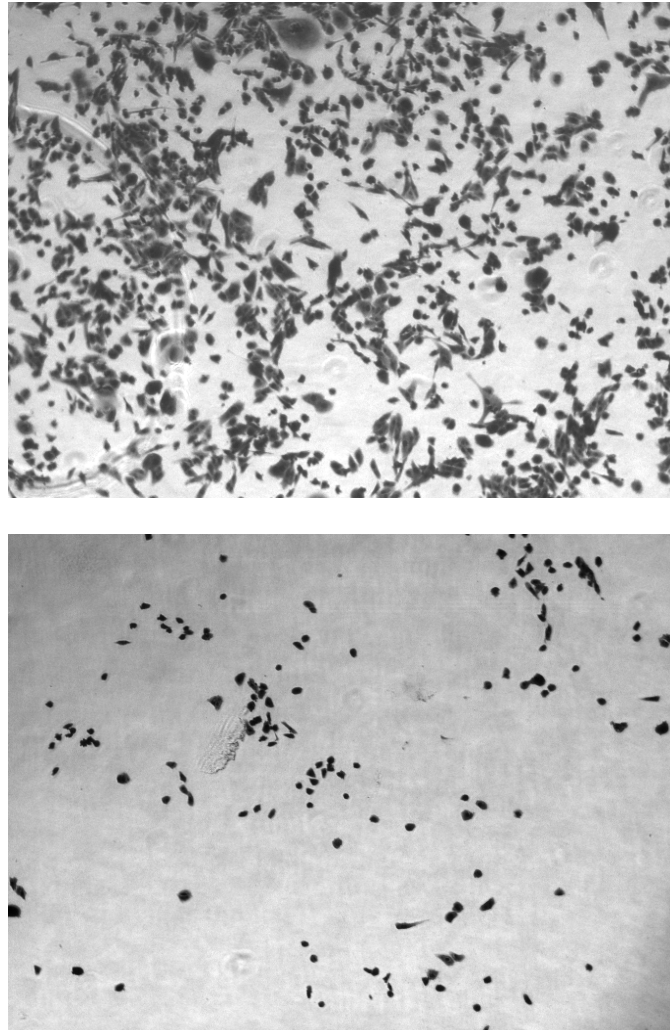


Figure 4.5. MDA-MB-231 Cells Overexpressing Plexin Show Decreased Invasive Ability. Representative samples of parental MDA-MB-231 (top picture) and MDA-MB-231 transfected to overexpress Plexin-A1 (bottom panel) were tested for invasive ability after 72 hour incubation with 10% FBS used as a chemoattractant in the Chemicon Cell Invasion Assay. Shown are cells that migrated through the ECM membrane and plated on the bottom chamber.

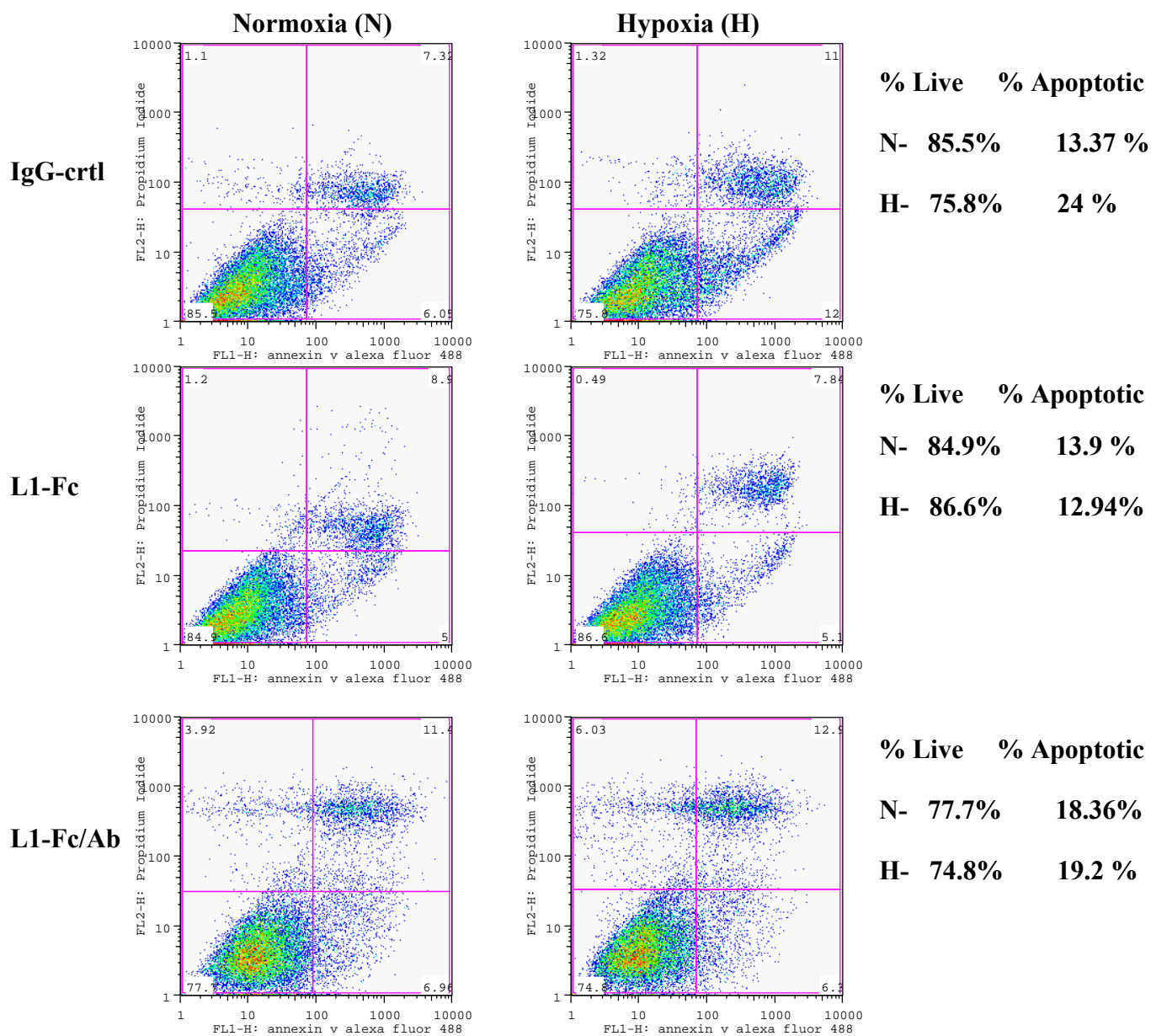


Figure 4.6. Flow cytometry data of MCF-7 cells incubated in either normoxia (control) or hypoxia to induce apoptosis. Cells were treated with IgG-Fc (control- top panel), L1-Fc (middle panel) and L1-Fc with 5-fold excess VEGF-165 functional blocking antibody (bottom panel). Numbers in quadrants represent percent of total cells in quadrant. Lower left –live cells, lower right- early apoptotic, upper left- apoptotic, upper right- dead cells.

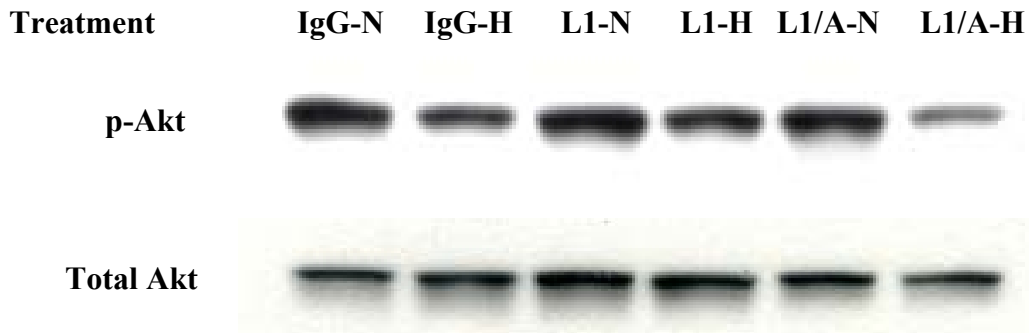


Figure 4.7 Analysis of Phosphorylation of Akt in MCF-7 cells treated with either IgG- Fc Chimera control (IgG) control, L1-Fc alone (L1) or L1-Fc with VEGF blocking antibody (L1/A). N indicates normoxic conditions, and H indicates samples grown in hypoxia. Samples were taken after 24 hours in culture.

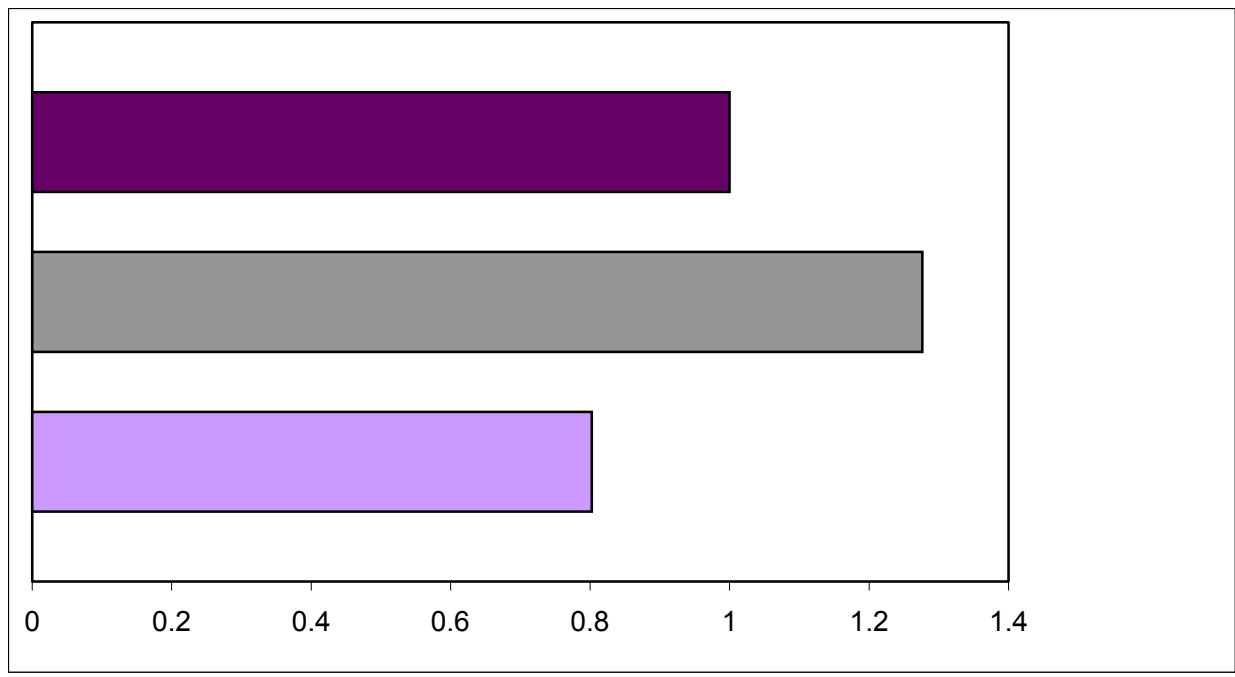


Figure 4.8 Analysis of phosphorylated Akt to total Akt ratio performed by densitometric analysis of samples exposed to hypoxia for 24 hours. Results are densitometric comparison from two separate experiments of MCF-7 cells treated with IgG-Fc chimera (dark purple), L1-Fc chimera (grey), and L1-Fc chimera with VEGF blocking antibody (light purple). Results of each experiment were compared to the IgG control, and the ratio of pAkt/total Akt was given an arbitrary value of 1 for analysis.

CHAPTER 5  
GENERATION OF TRANSGENIC MICE OVER-EXPRESSING A NOVEL  
SOLUBLE ISOFORM OF NEUROPILIN-1 <sup>1</sup>

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<sup>1</sup>Matheny, S.L., O.R. Bunce, and U.P. Thorgeirsson. To be submitted to *Genomics*

## ABSTRACT

Neuropilin-1 (NRP-1) was discovered to be an isoform-specific vascular endothelial growth factor VEGF -165 (VEGF-165) receptor that is expressed both on endothelial cells and tumor cells. To date two different soluble isoforms of NRP-1 have been reported, and one of the soluble NRP-1 isoforms, (s<sub>12</sub>NRP-1) has been shown to act as a soluble VEGF receptor and to inhibit tumor growth of carcinoma cells overexpressing this isoform. In this study, we describe a novel soluble NRP-1 isoform (named s<sub>10</sub>NRP-1), which contains the complete VEGF-binding region of the full-length NRP-1 and contains a unique 54 amino acid 3' region derived from a coding sequence from intron 10 of NRP-1. Analysis of expression in tumor cell lines and multiple human tissues suggests this NRP-1 soluble factor to be a regulator of vascularization under non-pathological conditions. However we recognized the importance of using this soluble NRP-1 isoform to act both in vitro and in vivo as a soluble receptor of VEGF-165. We describe both the generation of recombinant s<sub>10</sub>NRP-1 that will be used in future mechanistic studies of the autocrine effect of VEGF-165 in breast cancer. We also describe the generation of transgenic mice that overexpress s<sub>10</sub>NRP-1 under the direction of the muscle creatine kinase (MCK) promoter. Under this promoter, cells generate high circulating levels of s<sub>10</sub>NRP-1, thus generating an ideal model for the study of the effect of VEGF-165 signaling in the primary growth and progression of mammary tumors.

## INTRODUCTION

Neuropilin-1 was recently found to be a VEGF165 isoform specific receptor expressed on both cancer cells and endothelial cells (Soker et al., 1998). VEGF165 is an important mediator of angiogenesis and regulation of vascularization in tumor promotion and development, respectively (Ferrara, 1995; Neufeld et al., 1999). The structure of NRP-1 consists of three specific extracellular domains, the a1/a2, b1/b2, and c domains which exhibit homology to specific protein families, namely the complement components C1r/C1s (the a1/a2 domains), coagulation factors V and VIII (b1/b2 domains) and meprin proteases (domain c) (reviewed in (Klagsbrun et al., 2002)). The full-length NRP-1 sequence also contains linker regions between these extracellular domains, a transmembrane, and a short cytoplasmic domain. The NRP-1 gene is located on chromosome 10 (Rossignol et al., 1999), and its genomic organization has been recently described as containing 17 exons and 17 intron regions (Rossignol et al., 2000).

To date, two isoforms of NRP-1 have been described that lack the transmembrane and cytoplasmic regions, thus acting as soluble NRP-1 isoforms (Rossignol et al., 2000). Genomic analysis of these isoforms reveals truncation of the full length construct at the end of exons 12 and 11, with additional coding sequences 3' of this region coming from introns 12 and 11, named s<sub>12</sub>NRP-1 and s<sub>11</sub>NRP-1 respectively. Both of these isoforms retain the VEGF binding domains (b1/b2) and *in vitro* studies demonstrate the ability of the NRP-1 isoforms to bind VEGF165 (Mamluk et al., 2002; Lee et al., 2003). Lacking transmembrane and cytoplasmic domains, soluble NRP-1 isoforms are able to prevent binding of VEGF to both tumor and endothelial cells, and prevent phosphorylation of

VEGFR-1 (KDR) in endothelial cells (Gagnon et al., 2000). By overexpressing s<sub>12</sub>NRP-1 in rat prostate carcinoma cells and injecting in rats, tumors exhibited increased hemorrhaging, resulting from a lack of regulation of angiogenesis in these tumors. In addition, these tumors also showed damaged vessels, another indication of deregulation of angiogenesis. The tumor cells themselves displayed an increase in apoptosis, evidence for the role of the autocrine effect of VEGF-165 in survival of tumor cells. Soluble NRP-1 isoforms retaining the VEGF-binding domains of the full-length NRP-1 are able to act as natural antagonists of VEGF signaling, and the expression of these isoforms in highly vascularized tissue suggests they may serve a regulatory role in vascular regulation and maintenance. They also serve as powerful tools for studying the function of VEGF165, both in vitro and in vivo. In this study we describe the isolation and characterization of a novel NRP-1 soluble isoform. We named it s<sub>10</sub>NRP-1, because it follows the pattern of other described NRP-1 isoforms, by containing the exact sequence of the full-length NRP-1 to exon 10, with the 3' sequence corresponding to a portion of intron 10.

## MATERIALS AND METHODS

### **Isolation of a Novel NRP-1 Isoform.**

A lung cDNA library was purchased from Clontech (Palo Alto, CA). A probe for NRP-1 was generated by PCR amplification of cDNA generated from MDA-MB-231 cells with primers specific for amplification of the fragment from 108-814 bp of NRP-1 (Accession #AF016050). The library was screened by BioServe Biotechnologies (Laurel, MD). The library was plated on 100mm dishes, and once grown, the plaques were lifted

to filters and screened with the NRP-1 probe. Nine positive plaques were identified, cloned into pcDNA3- Uni vector (Invitrogen), and sequenced for identification. One clone contained 1749 bp matching the 5' end of NRP-1, but contained another 900 bp at the 3' end that was not found in the full-length NRP-1 coding sequence. BLAST searches of this unique 3' region revealed high similarity to the region (bp 59306-60208 of chromosome 10 clone RP11-342D11 #HSA342D11) in a clone of chromosome 10.

### **Cell Culture and RNA Isolation and Northern Analysis.**

Cells were grown in ATCC recommended media in a standard incubator in a humidified atmosphere of 5% CO<sub>2</sub>, 95% air at 37 °C with change of medium every 2-3 days. The following cell lines were tested for the presence of the s<sub>10</sub>NRP-1 isoform: Tera2a cell line established from a lung metastases of a testes cancer, A549, lung cancer cell line, HBL-100, MCF-7, BT-549, MDA-MB-231, and MDA-MB-435 cell lines. RNA was obtained by phenol-chloroform isolation for cell lines. Briefly, cells were grown until 80-90% confluent, and washed with PBS. Cells were treated with RNAzol B (Tel-Test, Friendswood, TX) and 200ul/ml of chloroform was added to each tube. Lysates were incubated on ice until spun at 15,000g for 15 minutes at 4°C. Following centrifugation, the aqueous layer was transferred to a fresh tube, and an equal volume of isopropanol was added to the sample, followed by centrifugation at 15000g for 10 minutes at 4°C. The RNA pellet was washed in ice-cold 75% EtOH, and repelleted at 8500g centrifugation for 10 minutes at 4°C. EtOH was aspirated from samples, which were allowed to air-dry for 10 minutes. RNA was DNAase treated using DNA-free kit from Ambion (Austin, TX). RNA quantity and purity was then assessed by OD 280/260

readings on a Spectrometer (Gene Quant II, Phamacia Biotech). 10 ug of total RNA was subjected 1% formaldehyde agarose gel electrophoresis and transferred to a nylon membrane (Schleicher and Schuell, Keene,NH). Membranes were rinsed in 2X SSC, partially dried, cross-linked, and stored at -20°C until hybridized with a probe for s<sub>10</sub>NRP-1. RNA from cell lines was transferred to a nylon membrane (Schleicher and Schuell, Keene,NH). s<sub>10</sub>NRP-1 expression was detected using a 708 bp fragment using the following upper 5' ACAACTATGATACACCTGAGCTGCG 3' from the full-length NRP-1 and the lower 5' CAGTGACATTATCCCAGAAGCAAT 3' primers from the 3' end of the s<sub>10</sub>NRP-1 transcript. The probes were randomly labeled with P<sup>32</sup> using RediPrimeII (Amersham Biosciences, Piscataway, NJ) and hybridized in Express Hybridization solution (Clontech, Palo Alto, CA). The resulting bands were visualized by exposure to Hyperfilm-MP (Amersham Biosciences, Piscataway, NJ) at -70°C. For the analysis of human tissue, the Multiple Tissue Northern Blot (Clontech, Palo, Alto, CA) was used with the same probe and following the same procedures described above for Northern detection.

#### **Generation of s<sub>10</sub>NRP-1 Constructs.**

The coding sequence of s<sub>10</sub> NRP-1 was generated by RT-PCR using cDNA generated from MDA-MB-231 cell RNA. Briefly, total RNA was extracted from subconfluent cell cultures using RNazolB (TEL-TEST Inc., Friend Wood, TX) as described above. After extraction, RNA was DNase treated for 30 minutes at 37° C and inactivated using DNase inactivating buffer (Ambion, Austin, TX). Total RNA was quantatated using a Spectrometer (Gene Quant II, Phamacia Biotech), and 2.5 µg of total

RNA was used to synthesize cDNA with the Superscript II kit (Invitrogen, Carlsbad, CA) with Oligo-dT priming according to the manufacturer's protocol. PCR was carried out with the following primers: upper 5'GGAGAATGGAGAGGGGGCTGC 3' located at the start site for full-length NRP-1, and lower primer 5'CGA AGGCAATCTAGTCCC CAGG 3' containing the TAG stop site of the s<sub>10</sub>NRP-1 novel isoform. The expected 2.5 kB PCR product were cloned into TOPO-TA2.1 and subcloned for sequence verification (DNA-Sequencing Core, NCI, Bethesda, MD) using M13 forward and reverse primers, and internal primers. This sequence was used to generate a Gateway (Invitrogen, Carlsbad, CA) entry vector to ease problems with conventional cloning due to lack of required restriction sites. Briefly, we performed transformation and midpreps of the empty pCCLMCK-II vector and TOPO TA2.1 clone containing the full s<sub>10</sub>NRP-1 construct. The empty pCCLMCK-II vector was digested with Hind III, blunt-ended with T4 DNA Polymerase, treated with alkaline phosphatase to dephosphorylate the blunt ends, and ligated into a Gateway reading frame A conversion cassette. This construct was then transformed into DB3.1 cells, and selected for both ampicillin (from the pCCLMCK backbone and chloramphenicol (from the conversion cassette). Colonies were picked, grown, and DNA was extracted by midprep, and digested with Hind III, Bgl II, and Sal I to determine orientation. In parallel, the following primers were used to amplify the s<sub>10</sub>NRP-1 engineered to contain attB1 5' GGGGACAACCTTTGTACAAA AAAGTTGGCCA TGGAGAGGGGGCTGCCG CTCCTCTG 3' and attB2 sites lower primer 5'GGGGACAACCTTTGTACAAGAAAGTTGGCTACAGATTCGCATCCAG GG AGCAG 3' using the TOPO TA s<sub>10</sub>NRP-1 construct as template for 10 cycle PCR to amplify your gene and incorporating attB1 and B2 sites. The PCR product was then

recombined according to the manufacturer's protocol into the pDonr201 vector (Invitrogen, Carlsbad, CA).

### **Generation of Transgenic Mice Overexpressing s10NRP-1 under the direction of the Muscle Creatine Kinase Promoter**

At this point, the entry clone containing s<sub>10</sub>NRP-1 construct was sequenced for correct orientation and sequence verification and the verified entry clone with the s<sub>10</sub>NRP-1 construct into the converted destination vector with the pCCLMCK backbone following the manufacturer's protocol (Invitrogen, Carlsbad, CA). The resultant expression clone was restriction mapped using Hind III, Bam HI, and Bgl I to confirm proper insertion of the s<sub>10</sub>NRP-1 gene, the MCK promoter, and the CAT gene (See figure 4.5). The entire 7.8 kb promoter- transgene construct was isolated from the pSK prokaryotic vector background with Sal I and Not I digestion. This linearized fragment was microinjected into the pronuclei of a normal FVB mouse embryos using standard transgenic technology (Hogan, et al, 1994) by the NCI transgenic facility (Fredrick,MD).

### **Analysis of Transgenic Animals overexpressing s10 NRP-1 under the promotion of MCK.**

Possible founders were identified by Southern blot analysis. Briefly, genomic DNA was extracted from tail clippings and digested with BamHI. The linearized transgene construct was randomly primed using P<sup>32</sup>, and the digested genomic DNA was subjected to Southern Blot analysis. The positive control was the linearized fragment

digested with BamH I, generating a 4.8 kb and 2.5 kb fragments (Figure 4.4). A total of 7 founder animals were identified and bred with FVB mice for generation of F1 offspring. Offspring of founder animals were analyzed for the transgene by genomic PCR for the CAT gene using the following upper 5' GCTCTGGAGTGAATACCACGAC 3' and lower 5' GACGGCATGATGAACCTGAATCGC 3' and using GAPDH 5' ACCACAGTCCATGCCATCAC 3' upper and 5' TCCACCACCCTGTTGCTGTA 3' for positive control (Figure 4.4). We used this information to set up further breeding pairs to generate both homozygous and heterozygous lines. To determine which founder was producing the highest levels of s10NRP-1, we also looked at s10NRP-1 levels using protein analysis of serum samples, and RT-PCR analysis of skeletal muscle cDNA. For analysis of serum samples, mice were euthanized and blood was separated in a serum separation tube and either flash frozen in liquid nitrogen, and stored at -70°C, or mixed directly with 2x SDS loading buffer, boiled for 5 minutes and 40ul of the lysate was immediately subjected to SDS-PAGE on 8% Tris-Glycine 1.0mm gels (Invitrogen, Carlsbad, CA). Gels were run at 120V for approximately 1.5 hours and transferred to PVDF membranes (Invitrogen, Carlsbad, CA). After transfer, the membrane was blocked for 1 hour in 10% non-fat dry milk, rinsed with PBST, and immunoblotted with anti-NRP-1 antibody (C-19) or anti-NRP-1 antibody (N-18) overnight at 4°C with gentle rocking. The antibodies recognize the C-terminus and N-terminus respectively (Figure 5.6). For analysis by RT-PCR, RNA was obtained from skeletal muscle of euthanized mice, which was flash frozen following extraction. Total RNA was extracted from homogenized tissue using RNeasy (TEL-TEST Inc., Friend Wood, TX) according to the manufacturer's protocol. After extraction, RNA was DNase treated for 30 minutes at 37° C and inactivated using DNase

inactivating buffer (Ambion, Austin, TX). Total RNA was quantitated using a 280/260 nm spectrometer, and 2.5 µg of total RNA was used to synthesize cDNA with the Superscript II kit (Invitrogen, Carlsbad, CA) with Oligo-dT priming according to the manufacturer's protocol. PCR was carried out with the following primers specific for the s<sub>10</sub>NRP-1 transgene. (Figure 5.5).

## RESULTS

### **Identification of a Novel NRP-1 Isoform**

In this study we describe for the first time the isolation of a novel isoform of NRP-1, which we named s<sub>10</sub>NRP-1. Screening of a lung cDNA library led to the discovery of a novel NRP-1 isoform. Sequence analysis of this isoform reveals that s<sub>10</sub>NRP-1 matches exactly the NRP-1 amino acid sequence up to aa587, the end of exon 10, which is also the end of the B1/2 domains that encode the VEGF- binding domains of NRP-1 (MacFarlane et al., 1997; Lee et al., 2003). The 3' end of this isoform also contains an additional 900 base pairs which was discovered to be part of the intron sequence of intron 10, and this was confirmed as a likely true splice variant with verification of donor and acceptor sites. (See figure 5.1). This phenomenon follows descriptions of the other NRP-1 isoforms found to date, which are encoded by the full length NRP-1 until exons 12 or 11 with the remaining sequences coming from introns 12 and 11, respectively.

### **Expression Analysis of s<sub>10</sub>NRP-1 in tumor cell lines and Human Tissue Samples.**

We generated a probe that spanned both the traditional NRP-1 region to the 3' end

of our novel clone. We then used this probe in Northern analysis of both cell lines and various human tissues under non-pathological conditions in order to characterize the general expression of this clone in both immortalized and normal conditions. We sought to determine the distribution of this splice variant in cell lines using both breast cancer (MCF-7, T-47D, MDA-MB-231, and MDA-MB-435 cells) and non-tumorigenic immortalized breast epithelial cells (HBL-100 cells), and the lung cancer cell line (A549 cells) and a secondary lung metastases cell line from a primary testicular cancer (Tera2 cells). We chose these cell lines to study based on our interest in breast cancer, but included the lung cancer cell lines due to the original isolation of this isoform from a lung cDNA library. We found expression of our novel clone by Northern analysis in the following cell lines: A549, MCF-7, BT-549, MDA-MB-231, MDA-MB-435 and slight expression in HBL-100 cell lines (Figure 5.1). In addition to the 2.4 kB band representing our novel NRP-1 clone, due to the overlap of our probe with the full-length NRP-1 sequence, we also found a 6.0 kB band representing NRP-1.

To further characterize the expression of this clone in normal human tissue, we performed Northern blot analysis on a Multiple Tissue Northern blot from Clontech (Palo Alto, CA) using the same probe (Figure 5.2). Expression of this novel clone was found in the following tissues: placenta, kidney, skeletal muscle and heart. This expression is consistent with the expression profile of the other NRP-1 isoforms, which are also expressed in highly vascular tissues (Gagnon et al., 2000).

### **Generation of s10NRP-1 Mice**

Due to difficulty with traditional cloning of s10NRP-1 into the pCCLMCK-II expression vector, we decided to convert both vectors into donor and acceptor vectors

using Gateway cloning technology. This process involves incorporation of phage lambda recombination sites and subsequent treatment LR Clonase for the recombination reaction. This procedure generated the desired expression construct, which was then digested with Hind III, Bam HI, and Bgl I to confirm proper insertion of the s10NRP-1 gene, the MCK promoter, and the CAT gene (See figure 4.5). We then linearized the fragment to remove the pSK backbone for microinjection.

### **Analysis of Transgenic Mice**

In order to determine which animals were carrying the s10NRP-1 transgene, we performed Southern Blot analysis on genomic DNA isolated from all possible founders. After two separate injections with over 60 animals screened, we identified from Southern analysis 7 founder mice (representative blot shown in Figure 4.4). The founder animals were crossed with FVB mice, and genomic DNA of offspring were analyzed using PCR for detection of CAT sequences. We chose the CAT PCR as a primary screening option for offspring because, although the sequence is out of frame in the transgenic construct, thus not transcribed as a message, it is present only in offspring that have the transgene in their genomic DNA. Once positive offspring were identified, we euthanized positive animals at age of 6 weeks to determine expression of the construct. We took skeletal muscle for RT-PCR analysis for s10NRP-1 (Figure 5.5) and serum samples (Figure 5.6). At this time, we have identified at least one of the seven founders producing high enough levels of the transgene to detect in the serum of offspring.

## DISCUSSION

In this study, we describe the isolation and characterization of a novel isoform of NRP-1 that we have named s10NRP-1. We found expression of the s10NRP-1 isoform in certain breast cancer cell lines and in highly vascularized human tissues. Due to the retention of the VEGF-165 binding domain in our NRP-1 isoform and the localization of this isoform in vascular non-pathological tissues, we suspect this isoform can act in a regulatory manner in maintenance of vasculature. Also, due to retention of the VEGF binding domain, we decided to use this isoform to generate a transgenic mouse model overexpressing s10NRP-1 in order to be used in mammary carcinogenesis studies, with the ultimate goal being the investigation of the VEGF-165 autocrine effect in breast cancer progression. These transgenic animals over-express this isoform of NRP-1 under the direction of the muscle creatine kinase (MCK) promoter. We chose this transgenic construct in order to be able to generate high levels of circulating s<sub>10</sub>NRP-1 (as seen in transgenic animals using the same promoter constructs (Kawaguchi, et al, 2002)), which we are able to verify in the F1 generation offspring. These transgenic animals showed no gross abnormalities. We are in the process of creating heterozygous and homozygous lines to be cross-bred with mouse mammary tumor virus (MMTV) polyoma middle T-antigen (MT) mice which form spontaneous mammary tumors. Crossing the soluble NRP-1 transgenic with the MT mice will allow us to determine the direct response of VEGF-165 antagonism in an original system. This transgenic line will offer direct in vivo insight into the effectiveness of VEGF-165 blocking treatment, which is currently being investigated in clinical trials by only blocking traditional VEGF receptors (namely KDR). However, with the ability of NRP-1 to interact with many different co-receptors to

transduce signals, such as Plexin-A1 and L1-CAM, this transgenic line offers us the unique advantage of blocking VEGF-165 mediated signaling in not only endothelial cells, but also in cancer cells themselves. To further characterize the mechanism of VEGF-165 autocrine signaling in breast cancer cells, we have also generated the recombinant s<sub>10</sub>NRP-1 isoform in vitro to confirm results found in vivo by treatment in vitro. This novel transgenic line will provide valuable insight into the therapeutic importance of blocking VEGF-165 signaling in mammary tumor initiation and progression.

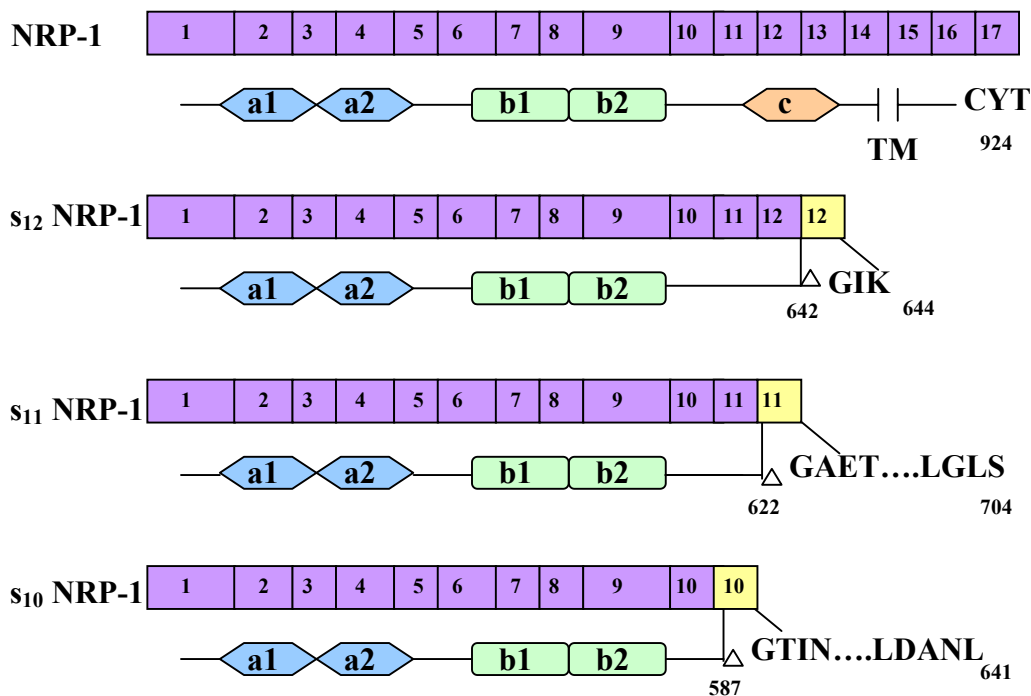


Figure 5.1. Neuropilin-1 and NRP-1 isoforms: genomic organization. (Adapted from (Rossignol et al., 2000) to include s10NRP-1.) NRP-1 exons are shown in purple with the introns encoding their 3' sequences noted in yellow. The full-length NRP-1 contains 924 amino acids encoding the a1/a2, b1/b2, and c domains, plus a transmembrane and cytoplasmic region. All three splice variants lack the transmembrane and cytoplasmic domains of the full length NRP-1, but retain the VEGF binding domains encoded by the b1/b2 domains, that terminate at aa 587. The numbers listed at the splice site indicate the last aa shared with the full-length NRP-1, followed by the ending aa sequence and the number of aa encoded by the isoforms before transcriptional stop sites.

MERGLPLLCAVLALVLPAGAFRNDKCGDTIKIESPGYLTSPGYPHSYHPSEKCEWLIQAPDP  
 YQRIMINFNPHFDLEDRDCKYDYVEVFDGENENGHFRGKFCGKIAPPPVSSGPFLFIKFVSD  
 YETHGAGFSIRYEIFKRGPECSQNYTTPSGVIKSPGFPEKYPNSELECTYIVFAPKMSEIILEFES  
 FDLEPDSNPPGGMFCRYDRLEIWDGFPDVGPHIGRYCGQKTPGRIRSSSGILSMVFFYTDSAI  
 AKEGFSANYSVLQSSVSEDFKCMEALGMESGEIHSQITASSQYSTNWSAERSRLNYPENGW  
 TPGEDSYREWIQVDLGLLRFTAVGTQGAISKETKKKYYVKTYKIDVSSNGEDWITIKEGNK  
 PVLFGQNTNPTDVVAVFPKPLITRFVRIKPATWETGISMRFEVYGCKITDYPCSGMLGMVS  
 GLISDSQITSSNQDRNWMPENIRLVTSRSGWALPPAPHSYINEWLQIDLGEEKIVRGIIHGG  
 KHRENKVFMRFKFKIGYSNNGSDWKMIMDDSKRKAASFEGNNNYDTPELRTFPALSTRFIRIY  
 PERATHGGLGLRMELLGCEVEGTINTDQECPCARALAKDAKKKKRWADSTHCRCVRSIHG  
 EMWSSGKGVCSLDANL\*



Figure 5.2. Amino Acid sequence of s10NRP-1. Predicted amino acid sequence of s10NRP-1 isoform, color coded to represent domains: Signal (**BROWN**), a1 domain (**RED**), a2 domain, (**BLUE**), b1 domain (**GREEN**), b2 domain (**PURPLE**), and unique to s10NRP-1 (**ORANGE**). The arrow indicates the point of diversion with full-length NRP-1.

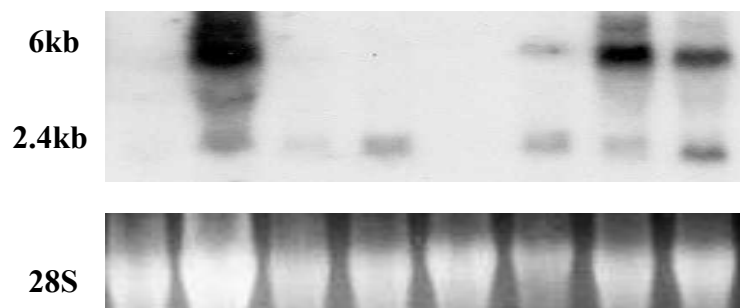


Figure 5.3. Northern blot analysis of s10NRP-1 expression in different cell lines. Using a probe that detected both full-length and s10NRP-1, we found the 2.4 kb transcript for

s10NRP-1 in the following cell lines: A549, McF-7, BT-549, MDA-MB-231, and MDA-MB-435.

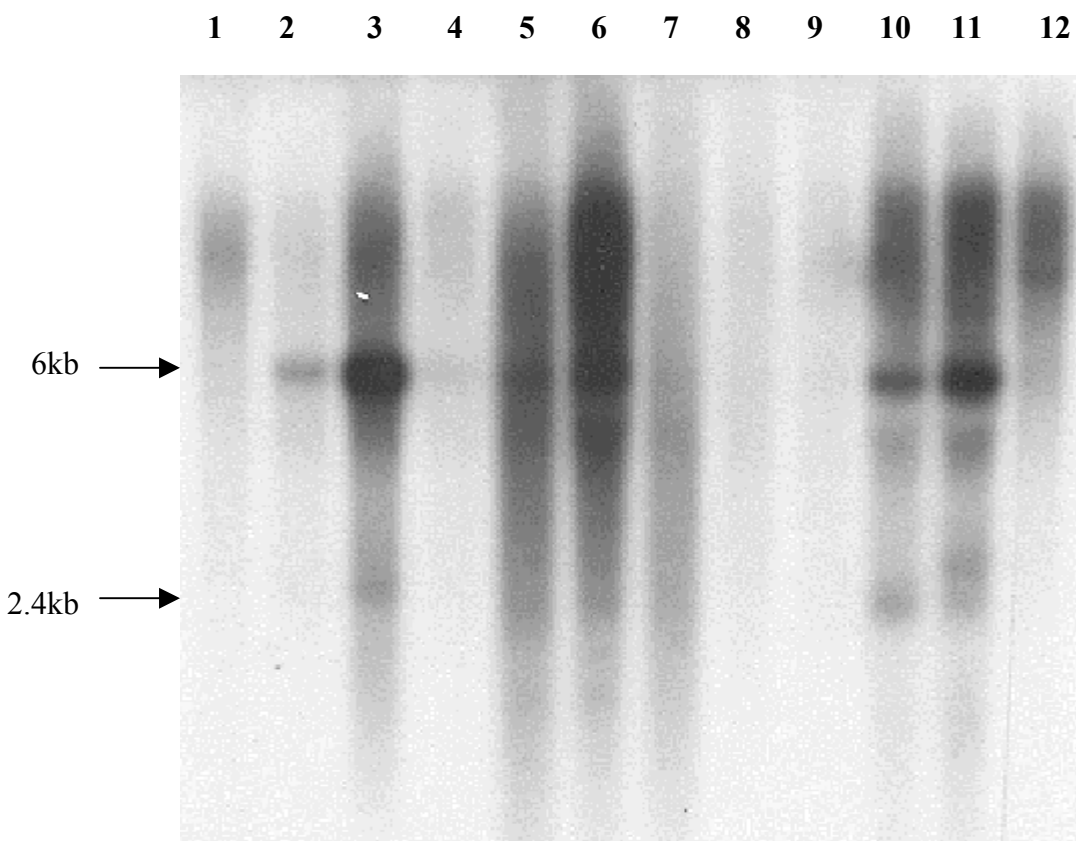


Figure 5.4. Northern blot analysis of s10NRP-1 expression in different human tissues.

Using a probe that detected both full-length and s10NRP-1, we found the 2.4 kB transcript for s10NRP-1 in the following tissues: placenta (3), liver (5), kidney (6), skeletal muscle (10), and heart (11). The following tissue types showed negligible staining: leukocyte (1), lung (2), sm intestine (4), spleen (7), thymus (8), and brain (12).

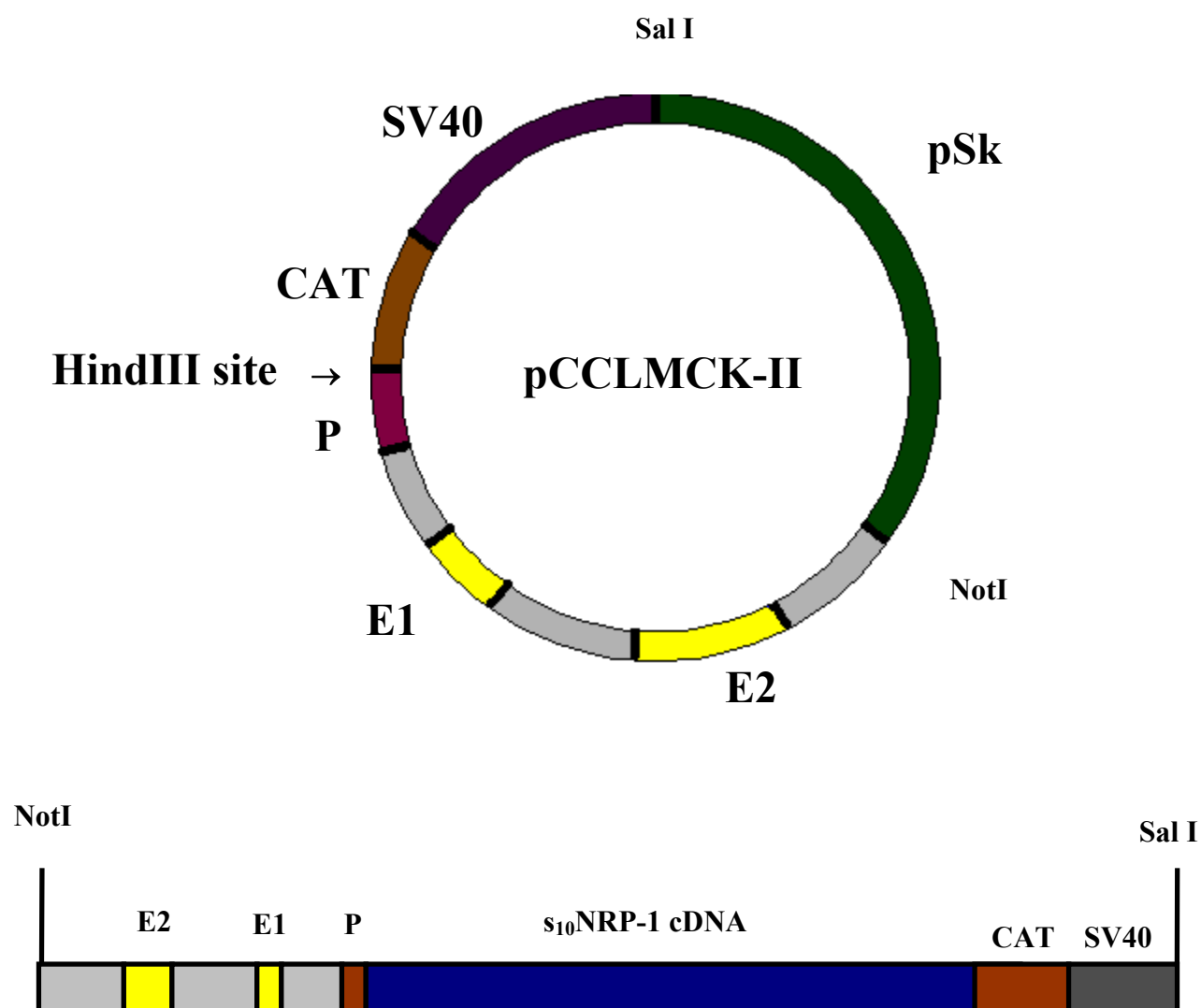


Figure 5.5. Description of transgene construct. The construction of the pCCLMCK-II construction was described previously (Lee et al., 1993). The s<sub>10</sub>NRP-1 cDNA was

cloned into the Hind III site. The pSk backbone was excised using Sal I and Not I. E1 and E2 are enhancers for the MCK promoter (P).

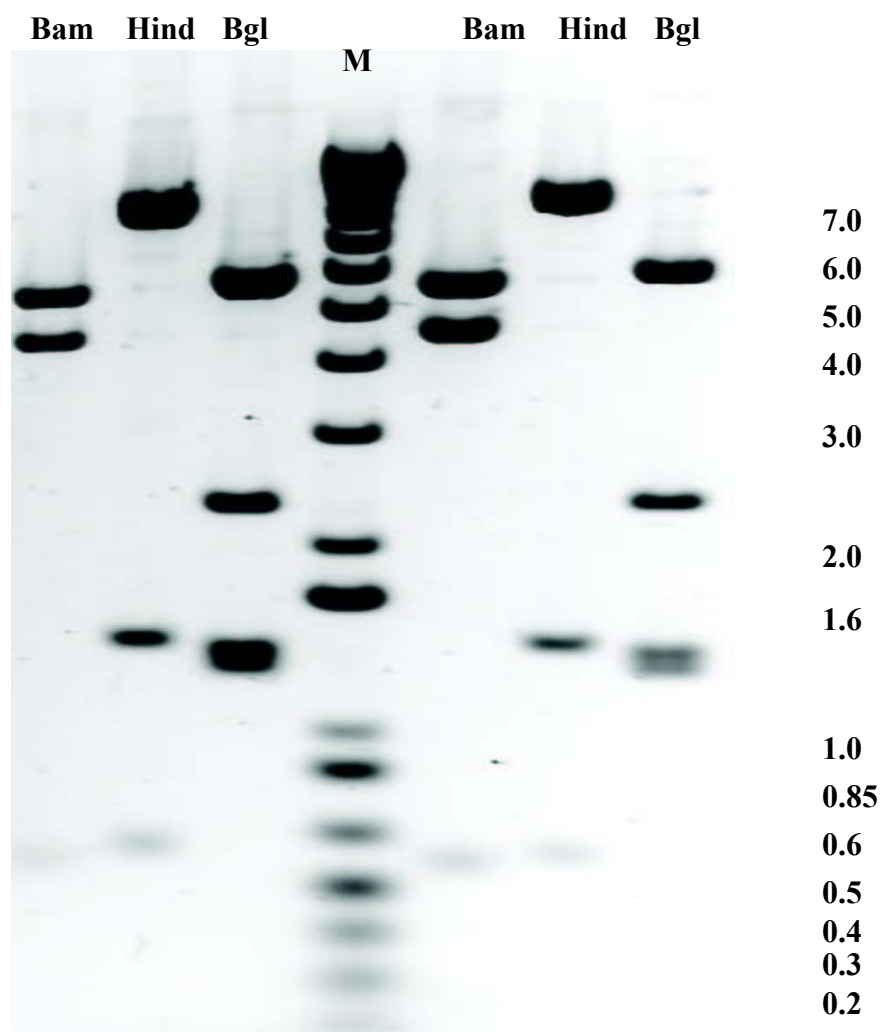


Figure 5.6. Restriction analysis of MCK-s10NRP-1 expression construct. Two independent clones were digested with BamHI, Hind III, or BglI. The middle lane is a 1 kB Marker. The expected sites for correct orientation were as follows: Bam HI-5.5, 4.4,

0.55, and 0.25 kB. For Bgl I: 5.8, 2.3, 1.33, and 1.27 kB. For Hind III: 8.7, 1.38, and 0.60 kB.

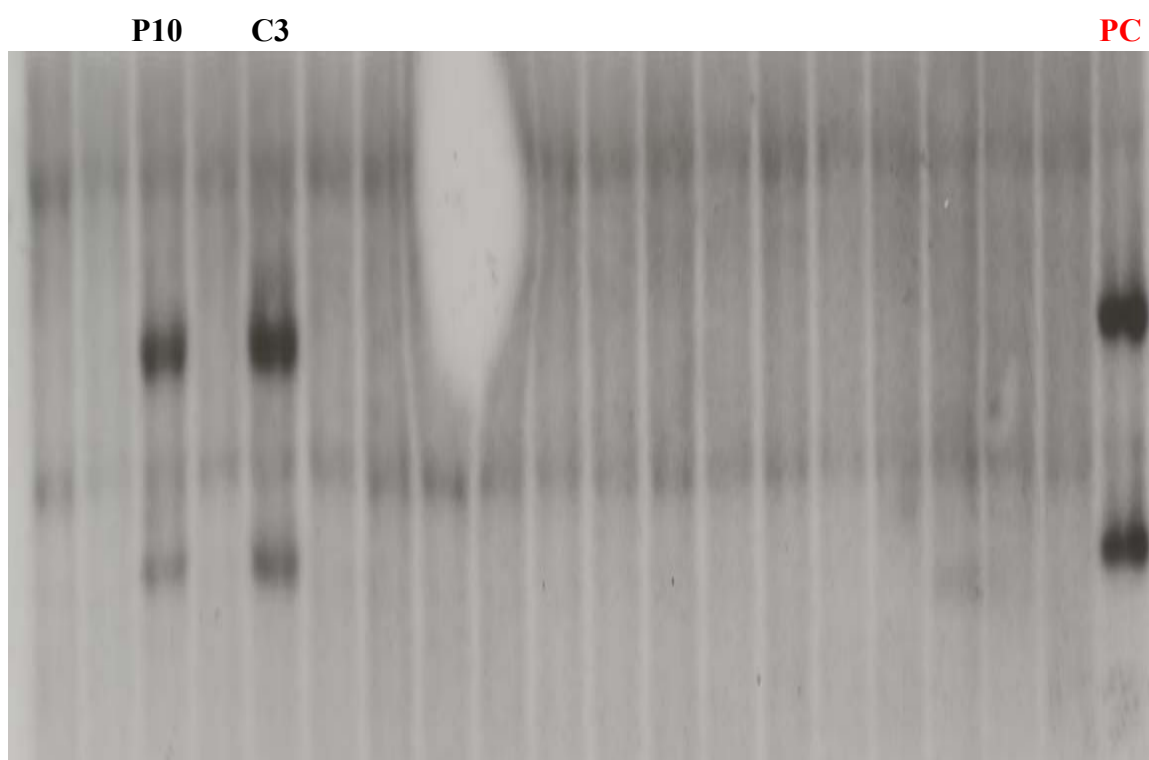


Figure 5.7. Southern Blot Analysis to Detect the s10NRP-1 Transgene in Genomic DNA. Genomic tail DNA was digested with BamHI and subjected to Southern Blot analysis using the P<sup>32</sup> randomly labeled transgenic construct as the probe. Animal designation is

listed above the 2 positive animals (P10 and C3) of the 19 screened in this blot. The last lane contains the positive control (PC).

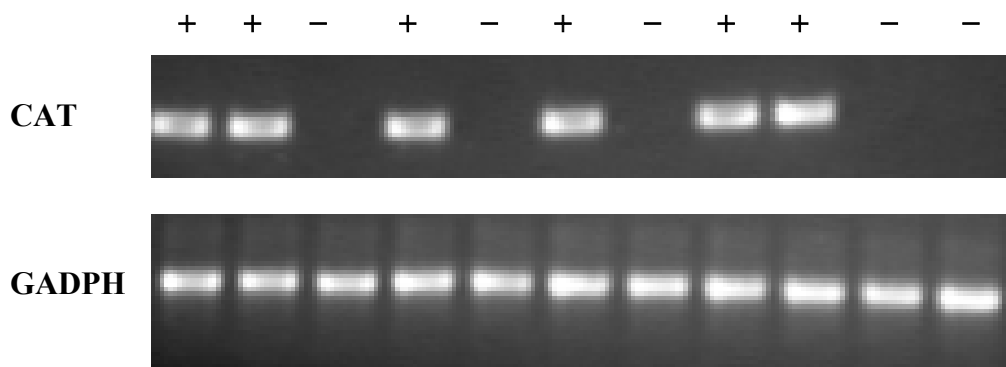


Figure 5.8. Genomic PCR detection of CAT gene in Transgenic F1 Generation. Offspring of founders were subjected to PCR for both the presence of CAT in their genome, with GAPDH used as a positive control. In this litter, we found 6 positive and 5 negative mice.

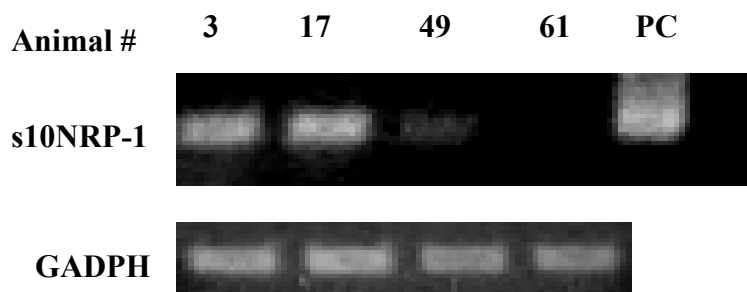


Figure 5.9. RT-PCR Detection of s10NRP-1 transcript from F1 generation skeletal muscle. Animal numbers are listed above each lane. Each animal was born of a different founder. Animals 3 and 17 were positive for the s10NRP-1 expression. The positive control is the RT-PCR of the original s10NRP-1 construct in the TOPO-TA2.1 vector. RT-PCR was also performed for GAPDH on skeletal muscle cDNA as a positive control.

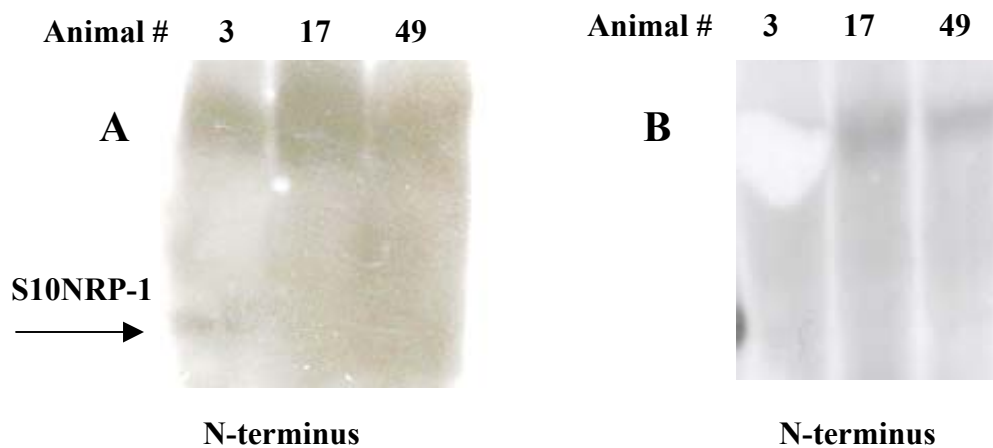


Figure 5.10. Western analysis of serum from transgenic F1 generation. SDS-PAGE was performed on serum collected from F1 mice. Serum samples were either incubated with NRP-1 antibodies recognizing either the N-terminus or the C-terminal domains. While the N-terminus specific antibody should recognize the full-length and s10NRP-1 isoforms, the C-terminal specific antibody will only recognize the full-length NRP-1. Note in animal 3, we find a band at the size predicted for s10NRP-1 protein (72kDa), using the N-terminal specific Ab.

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## CHAPTER 6

### SUMMARY AND CONCLUSIONS

VEGF is a popular protein in the field of cancer research due to its ability to act as a potent inducer of angiogenesis. Angiogenesis is important in the growth and survival of solid tumors. Only recently, with the discovery of NRP-1 as a VEGF-165 specific receptor expressed on tumor cells, has the dogma surrounding the role of VEGF begun to expand to include roles independent of its angiogenic effects. In these studies we provided evidence that previous neuronally-isolated NRP-1 co-receptors, Plexin-A1 and L1-CAM, are expressed in human breast cancer cell lines and in human breast tumor tissue. The expression patterns of NRP-1, Plexin-A1, and L1-CAM indicate that these proteins are capable of interacting in tumor cells, and that the interaction of these proteins is by no means limited to neuronal development. This is the first indication of NRP-1 and both Plexin-A1 and L1-CAM interaction in any cancer type, which we show in breast cancer cell lines and breast tumor samples via immunolocalization and immunoprecipitation.

Further examination into the specific involvement of Plexin-A1 in VEGF signaling yielded both expected and interesting results. When Plexin-A1 is overexpressed in two different breast cancer cell lines, the presence of VEGF-165 elicits results opposite to known effects of Sema3A. While other studies have shown opposing effects of VEGF and Sema3A in endothelial cells and neurons, to our knowledge this is the first direct evidence of VEGF influencing the phosphorylation status of both LIM kinase and cofilin

in any cell type. In addition, the results of these experiments point to the role of Plexin-A1 in VEGF autocrine signaling. Ongoing research is focusing on further understanding the role of endogenous Plexin-A1 in breast cancer cell actin dynamics by transfections with dominant negative Plexin-A1 constructs lacking signaling capabilities. While there has been a great increase in understanding Plexin signaling, there are many remaining areas to decipher. However, our data suggests that through NRP-1 binding, VEGF-165 may be using non-traditional receptors such as Plexin-A1 to send signals intracellularly. Analysis of NRP-1 expression in a variety of tumor and normal adjacent tissue samples revealed the almost universal presence of NRP-1 in each sample. Due to the function of NRP-1 as almost a “tether” for specific ligands to the surface of cells, and the ability of NRP-1 to act promiscuously with many different co-receptors, this expression pattern begins to make more sense. In future studies, we plan to do similar array analysis to establish expression profiles of NRP-1 co-receptors. Perhaps these studies will be more indicative of what is altered in normal vs. tumor tissue, which could be helpful for design of targets for intervention. The high presence of NRP-1 in the variety of different normal tissue samples of this array implies a role for NRP-1 in maintenance of normal environments. However, without a clear picture of which co-receptors (or ligands) could be acting to transduce signals in these tissues, many questions still remain.

While L1-CAM has been linked to various neuronal disorders, we sought to determine if activation of endogenous L1-CAM on breast cancer cells had any effect on cell survival in hypoxic conditions. Hypoxic conditions occur in the tumor environment, and result in the production of hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ). HIF-1 $\alpha$  causes the direct up-regulation of VEGF expression among other proteins which prepare cells for

survival in conditions with low oxygen. By adding L1-Fc to cells in hypoxia and normoxia, we sought to determine the role of L1-CAM activation in cell survival. We found that the addition of L1-Fc reduces the number of apoptotic cells in hypoxia. While this does not directly link L1-CAM to NRP-1, we were able to abolish the protective effect of the L1-Fc when cells were also incubated with a VEGF-functional blocking antibody. Although the mechanism of L1-CAM involvement in VEGF signaling is not yet clear, we do offer the first evidence that endogenous L1-CAM activation results in protection from apoptosis in breast cancer.

In addition to our interest in the role of Plexin-A1 and L1-CAM in the autocrine effect of VEGF, we also describe a novel NRP-1 isoform in this study. By generating transgenic mice that overexpress this novel, soluble NRP-1 isoform, we have generated the ideal tool for study of the autocrine effect of VEGF in development of mammary carcinogenesis and progression. Crossing these transgenic mice with MT mice will offer an *in vivo* look at the therapeutic advantage of soluble VEGF receptors in cancer development, growth, and progression.

These studies implicate Plexin-A1 and L1-CAM as novel signal-transducing proteins in VEGF signaling. We also found widespread expression of NRP-1 in a variety of tissues. While many questions remain unanswered mechanistically as to how these proteins interact, we will continue to address these questions using the s10NRP-1 transgenic mice in tumorigenesis studies. The discovery of the VEGF-165 receptor NRP-1 has expanded the possibilities of VEGF signaling from angiogenesis to neurobiology to the tumor cell surface. In this study, we have shown these connections to be more interrelated than previously believed.