Neuropilin 2 expression and function in melanocytes and melanoma
NEUROPIGIN 2 EXPRESSION AND FUNCTION IN
MELANOCYTES AND MELANOMA

by

SALVATORE RIZZO
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First Reader

Karen Symes, Ph.D.
Assistant Dean of Student Affairs
Associate Professor of Biochemistry

Second Reader

Diane Bielenberg, Ph.D.
Assistant Professor of Surgery, Harvard Medical School
Research Associate, Boston Children’s Hospital, Boston, MA
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Neuropilin 2 (NRP2) is a transmembrane protein receptor originally discovered in neurons, and their precursors, neural crest cells (NCC), which are a transient, migratory precursor population derived from neural ectoderm. NRP2 serves as both a vascular endothelial growth factor (VEGF) co-receptor to initiate a pro-angiogenic signaling cascade and as a receptor for the class 3 semaphorin family molecules (primarily SEMA3F). Binding of SEMA3F induces a strong repulsive and antiangiogenic signal. NRP2 was discovered on melanocytes, which was a novel finding. NRP2-positive melanocytes were first identified in the hair follicles of Nrp2+/gfp transgenic mouse model. It is known that melanocytes reside in the bulb of the hair, where they provide pigment. Melanocyte stem cell populations are found in the bulge, a superficial structure also containing keratinocyte precursors. NRP2 has also been suggested as a potential biomarker in cases of malignant melanoma. The aim of this study was to elucidate the role of NRP2 three areas: in the growth and development of hair, in melanocytes, and in malignant melanoma. A greater understanding of the role of NRP2 in these locales may have significant clinical significance in disease states such as alopecia, vitiligo, and melanoma. It was discovered that Nrp2 expression was strongest within the bulge region of the hair follicle where melanocyte stem cells reside. Additionally, human and mouse primary melanocytes express NRP2, whereas keratinocytes do not. Neither melanocytes
nor melanoma express VEGFR2, indicating NRP2 may be serving as a SEMA3F receptor. The NRP2 receptor was functional in melanocytes as treatment with SEMA3F inhibited migration of both melanocytes and melanoma cultured lines. Melanoma cells downregulate expression of SEMA3F and upregulate the expression of NRP2. In the patient samples analyzed, the expression of NRP2 correlated with disease progression.
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<tr>
<td>AAALAC</td>
<td>Association for Assessment and Accreditation of Laboratory Animal Care</td>
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<td>ABC</td>
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<td>BM</td>
<td>Basement Membrane</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>DC</td>
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<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
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<td>EDTA</td>
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<td>FBS</td>
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<td>FFPE</td>
<td>Formalin fixed, paraffin embedded</td>
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<td>FGF</td>
<td>Fibroblast Growth Factor</td>
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<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<td>HIF1α</td>
<td>Hypoxia Inducible Factor 1 alpha</td>
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<td>HRP</td>
<td>Horseradish Peroxidase</td>
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<tr>
<td>IACUC</td>
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<td>KO</td>
<td>Knockout</td>
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<tr>
<td>LEC</td>
<td>Lymphatic Endothelial Cell</td>
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<td>McSc</td>
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<td>NC</td>
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<td>NIP</td>
<td>Neuropilin Interacting Protein</td>
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<tr>
<td>Abbreviation</td>
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<td>--------------</td>
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<tr>
<td>NRP</td>
<td>Neuropilin (human)</td>
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<td>Nrp</td>
<td>Neuropilin (mouse)</td>
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<td>NRP1</td>
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<td>NRP2</td>
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<tr>
<td>PIGF</td>
<td>Placental Growth Factor</td>
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<td>PBS</td>
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<td>PDAC</td>
<td>Pancreatic Ductal Adenocarcinoma</td>
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<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
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<tr>
<td>RIPA</td>
<td>Radio Immune Precipitation Assay</td>
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<td>SEA</td>
<td>Serine, Glutamic Acid, Alanine</td>
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<td>SEMA3F</td>
<td>Semaphorin 3F</td>
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<tr>
<td>TNB</td>
<td>Tris-NaCl blocking buffer</td>
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<td>TNS</td>
<td>Trypsin Neutralizing Solution</td>
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<tr>
<td>TEMED</td>
<td>Tetramethylethylethylenediamine</td>
<td></td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<tr>
<td>VEGFR2</td>
<td>Vascular Endothelial Growth Factor Receptor 2</td>
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<td>WT</td>
<td>Wild Type</td>
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INTRODUCTION

The Neuropilins: A Brief Comparison

Neuropilin (human: NRP; mouse: Nrp) is a transmembrane receptor glycoprotein, with a molecular weight between 130 and 140 k-Da (Bielenberg et al., 2006). Neuropilin 1 was first discovered in the midbrain of a frog 1987 by Fujisawa (Takagi et al., 1987), and was shortly thereafter found to exist in several other neuronal tissue across multiple species (Kawakami et al., 1995; Takagai et al., 1995). Neuropilin 2 was discovered a decade later by two independent labs (Chen et al., 1997; Kolodkin et al., 1997). The domain structure of NRP2 is identical to that of NRP1, and both share 45% base pair homology. The genes for the two molecules are located on separate chromosomes in humans and in mice, however, with NRP1 found on chromosome 10 and NRP2 on chromosome 2 (Klagsbrun, Takashima, & Mamluk, 2002). The two molecules have similar extracellular domains (a1/a2, b1/b2, and c), transmembrane and cytoplasmic domains (Chen et al., 1997).

Figure 1 | Structure of NRP: NRP in both mouse and human is composed of three binding domains, a transmembrane domain, and a cytoplasmic domain ending in the SEA motif. From Panigrahy et al., 2014.
Each of the three extracellular domains binds different ligands. The a1/a2 domain is the primary receptor for the class 3 semaphorin (SEMA3) family molecules, while the b1/b2 domain acts as a co-receptor for Vascular Endothelial Growth Factor (VEGF) binding. The c domain serves as a locale for NRP:NRP dimerization. The intracellular domain consists of a relatively short, 40 amino acid sequence. NRP1 and NRP2 share 55% homology in this domain, but both terminate in the same serine, glutamic acid, alanine (S-E-A) sequence (Bielenberg et al., 2006). This terminal sequence has been shown to interact with a cytoplasmic protein called neuropilin interacting protein (NIP), although no signaling pathway has been discovered associated with this interaction (Cai & Reed, 1999). Recent studies have shown that deletion of this SEA sequence in NRP1 disrupts Placental Growth Factor (PIGF) signaling in medulloblastoma lines, suggesting that the cytoplasmic domain may indeed have an important, if yet undiscovered, function (Snuderl et al., 2013).

Several isoforms of neuropilin 2 exist, all of which arise from alternative splicing of the NRP2 exons (Chen et al., 1997). In addition, there are soluble forms of NRP’s which may act as competitors for SEMA3 and VEGF binding (Rossignol, Gagnon, & Klagsbrun, 2000).

**Ligands of Neuropilin 2: the Semaphorin Family**

The first ligand of NRP was discovered in 1997 to be the semaphorin class III (SEMA3) family of molecules (Kolodkin et al., 1997). There are over 20 SEMA genes producing eight known classes of semaphorins, and the molecules are organized according to their structure and evolutionary origin. The class 3 family are the only
secreted semaphorins and there are seven members of the SEMA3 family, designated A-G. The seven molecules have similar molecular weights (approximately 100kDa) and are the only members of the semaphorin tree found to bind to NRP (Worzfeld & Offermanns, 2014). SEMA3 molecules bind two domains on NRP. The highly conserved “sema” region binds to the a1a2 domain, while the carboxy-terminus binds to the b1b2 domain of NRP (Bielenberg & Klagsbrun, 2007). The binding specificities of the members of the SEMA3 family relative to the neuropilins are as follows: SEMA3A only binds to NRP1, and SEMA3F and SEMA3G bind specifically and with high affinity to NRP2. The remaining SEMA3 molecules (save SEMA3E) can bind either NRP1 or NRP2 (Klagsbrun & Shimizu, 2010). In the context of SEMA3, both NRP1 and NRP2 act as essential coreceptors for their respective SEMA3 molecules; that is, the SEMA3/NRP complex is not sufficient to activate downstream signaling cascades (Puschel, 2002). In order to initiate a signaling cascade, SEMA3/NRP must also bind the primary semaphorin signaling family of receptors, the plexins. Of primary significance is the NRP2/SEMA3F relationship, which signals through Plexin A1 in both cancerous cells and in endothelial cells (EC) (Shimizu et al., 2008). SEMA3E is unique in that it does not require a NRP coreceptor, but rather binds directly to its receptor, Plexin D1, where it can initiate the signaling cascade (Gu et al., 2005; Klagsbrun & Shimizu, 2010).

SEMA3F is a potent inhibitor of cell migration, reflective of its role as a guidance molecule. Several studies have demonstrated the inhibitory effects of SEMA3 on EC proliferation (Shimizu et al., 2008; Acevado et al., 2008). Additionally, it may have a critical role in angiogenesis and cancer metastasis as an angiogenesis inhibitor. For
example, A375SM melanoma cells transfected with SEMA3F demonstrated decreased adhesion and motility, while cell proliferation remained unaffected (Bielenberg et al., 2004) Additional studies suggest that the lack of adhesion and motility is due to the inhibitory effects of SEMA3F on Beta1 Integrin (Bielenberg et al., 2004). SEMA3F may also act to collapse f-actin stress fibers, effectively preventing cell membrane rearrangement and consequently, cell movement (Shimizu et al., 2008; Bielenberg et al., 2008).

**Ligands of Neuropilin 2: VEGF**

The second important ligand of NRPs is the VEGF family, a well-known group of molecules, particularly in the field of angiogenesis. VEGF has been described as a potent inducer of endothelial cell proliferation and migration, and well as a strong pro-angiogenic and permeability factor (Dvorak et al., 1995). The proteins are approximately 20 kDa, but are functionally active as dimers (Ferrara, 1996). The principal form of VEGF, VEGF-A, is subject to alternative splicing and post-translational modification yielding four distinct isoforms: VEGF$_{121}$, VEGF$_{165}$ (the most common isoform), VEGF$_{189}$, and VEGF$_{206}$ (Klagsbrun & D’Amore, 1996). The VEGF family can bind one of two classes of receptors, the neuropilins and the receptor tyrosine kinase (RTK) VEGF Receptor (VEGFR) family, which consists of VEGFR1 (FLT-1), VEGFR2 (KDR), and VEGFR3 (FLT-4). The VEGFR receptors are tyrosine kinase receptors, with the dimerization and autophosphorylation typical of such receptors. The three VEGFR have different affinities for the various members of the VEGF family. VEGFR1 binds VEGF-A, -B, and PlGF, another member of the VEGF family. VEGFR2 binds VEGF-A, -C, -D,
and –E. VEGFR3 binds VEGF-C and VEGF-D (Ruiz de Almodovar et al., 2009).

VEGFR2 is considered the primary regulator of VEGF-induced cell migration and proliferation due to its greater tyrosine kinase activity compared to the other two VEGFR (Ferrara et al., 2003).

The second family of receptors that bind VEGF are the neuropilins. Unlike the VEGFR family, binding to NRP by VEGF is not necessary or sufficient to induce an intracellular signaling cascade. Instead, NRP acts as a coreceptor for VEGFR2 (angiogenic) and VEGFR3 (lymphangiogenic), amplifying the response to ligand binding (Soker et al., 2001; Favier et al., 2006)(Figure 1). NRP1 associates with VEGF-A, -B, and –E. NRP2 binds VEGF-A, -C, and –D (Klagsbrun et al., 2002; Gaur et al., 2009).

**Figure 2 | Neuropilin 2 and its Ligands:** The two ligand families of neuropilin 2 (SEMA3 and VEGF) are shown above, along with their respective primary receptors. The lines between the free molecules and NRP2 demonstrate the binding sites for each. The C domain on NRP2 is used for dimerization (not shown).
**Neuropilin 2 Physiologic Expression**

While the neuropilins were initially discovered on neuropiles (hence the name), subsequent studies have revealed a much more robust expression throughout the body (Gagnon et al., 2000; Wild et al., 2012). Despite their similarities in structure, the two neuropilins are differentially expressed throughout the body, though their expression is not necessarily mutually exclusive. NRP are differentially expressed on neural crest (NC) cells, contributing to the polarity and location of these cells (Ruhrberg & Schwartz, 2010; Theveneau & Mayor, 2012). For another example, in the nervous system NRP2 is restricted to sympathetic neurons while NRP1 is found on both sympathetic and sensory neurons (Bagri & Tessier-Lavigne, 2002). Both neuropilins are expressed on endothelial cells. In the blood vasculature, NRP2 is clearly expressed in veins, while NRP1 is restricted to arteries during development, and while this definitive demarcation is lost in the mature organism, NRP2 is still heterogeneously expressed in capillaries, veins, and lymphatics (Herzog et al, 2001; Bielenberg et al., 2006). While expression of NRP2 is variable among lymphatic vessels, there is no evidence of NRP1 expression on these vessels (Yuan et al., 2002).

The neuropilins are not restricted to only migratory and guidance functions. NRP1 has a demonstrated function in the immune system, serving as a mediator for dendritic cell (DC)-T cell interactions. Inhibition of this interaction abolishes the DC-dependent T cell proliferation (Tordjman et al., 2002). Both NRPs have been found on leukocytes and macrophages as well, though complete comprehension of their role is still to be determined (Stepanova et al., 2007; de Paulis et al., 2006).
NRP2 is also expressed in developing bone and in visceral smooth muscle (particularly in the bladder and intestine) (Chen 1997; Bielenberg et al., 2012). NRP2 has also been implicated in bone homeostasis, as loss of Nrp2 results in trabecular bone loss mediated by enhanced osteoclast activity coupled with a decreased osteoblast population (Verlinden et al., 2013). While the mechanism of NRP2 function in many tissues is still yet to be determined, its extensive expression throughout the body’s tissues hints at its significance in normal physiological function.

**Nrp2 Transgenic Mice**

Yuan et al. were the first to describe the lymphatic phenotype of transgenic Nrp2 knockout mice in the early 21st century. They found that while viable into adulthood, homozygous Nrp2 mutant mice demonstrated a severe reduction of small lymphatic vessels and capillaries in the heart, lung, skin, and other tissues examined. This malformation of lymphatic capillaries was found to be due to a reduction in Nrp2 protein synthesis, and not from other factors like improper folding of the protein (Yuan et al., 2002). Interestingly, the Nrp2 knockout mice are not edematous. Heterozygotes express a phenotype similar to wild type mice. Homozygous mutant Nrp2 offspring are found to be present at a lower than expected Mendelian frequency, both in Yuan’s experiments and during the current study. Later studies involving double knockout genetic lines for NRP2/VEGFR3 (the primary receptor for VEGF-C) demonstrated the importance of proper NRP2 function in lymphangiogenesis, and has hinted at a key role in cancer metastasis (Xu et al., 2010). Additionally, mice lacking Nrp2 demonstrate a marked loss in neuronal function, particularly in cranial nerve, spinal sensory axon, and hippocampal
mossy fiber axon development (Chen et al., 2000). As mentioned previously, SEMA3F is critical for the proper migration of axons and NC cells, and these malformations can likely be attributed to lack of functional SEMA3F mediated repulsion.

The Bielenberg laboratory houses two lines of transgenic mice. The Nrp2<sup>+/gfp</sup> transgenic mice have a Green Fluorescent Protein (GFP) insert after exon 1, functionally “knocking in” GFP while “knocking out” Nrp2. The endogenous Nrp2 promoter remains untouched and functionally active, resulting in expression of GFP in place of endogenous Nrp2 in vivo. Thus, the GFP serves as a read-out of Nrp2 expression. Nrp2<sup>gfp/gfp</sup> (knockout) mice are viable through adulthood. Adult homozygous knockout mice are smaller in size than their wild-type littermates, although the heterozygotes display no noticeable difference in gross appearance. The second line of transgenic mice is the Nrp2<sup>+/LacZ</sup> mice, which serve a similar function as the GFP line; the LacZ protein is expressed in lieu of endogenous Nrp2. Like the GFP mice, the LacZ mice have an insert after exon 1 encoding the beta-galactosidase (LacZ) gene while maintaining the original Nrp2 promoter. Thus, LacZ is expressed <em>in vivo</em> in lieu of endogenous Nrp2. Staining with X-gal reagent reveals the location of LacZ; GFP must be observed with fluorescent microscopy. Unlike the GFP knock-in mice, however, Nrp2<sup>LacZ/LacZ</sup> mice are not viable and perish at birth for unknown reasons.

**The Melanocyte**

The skin can be divided into three distinct layers: the epidermis, the dermis, and the subcutaneous tissue. The cells of the skin are constantly exposed to a variety of damaging external conditions and carcinogens from which it needs protection. One of
these carcinogens is ultraviolet (UV) radiation, typically originating from the sun. UV radiation causes the formation of cyclobutane pyrimidine dimers in the DNA of exposed cells, and mutations accumulate when this damage is not repaired (Berneburg & Krutmann, 2000). Melanocytes, a small minority of specialized cells in the epidermis, confer protection to neighboring cells (primarily keratinocytes) from UV radiation through production and distribution of melanin, a photoprotective pigment (Tandler, Mosch, & Pietzsch, 2012; Lang et al., 2013). In the skin, melanocytes are typically found along the basal layer of the epidermis, where they are anchored to the basement membrane of the epidermis (Thingnes et al., 2012).

**Figure 3 | Melanocyte and Melanocyte Stem Cell Development:** Both melanocytes and melanocyte stem cells (McSc) originate from melanoblasts derived from Neural Crest (NC) cells. Mature melanocytes proliferate and populate the epidermis, as well as the bulb of hair follicles. McSc reside in the bulge and migrate to the bulb at the beginning of a new hair cycle, where they differentiate into melanocytes as the new hair begins to grow. From Li et al., 2014.

Melanocytes originate during development from the ectoderm-derived neural crest, and migrate and differentiate into three distinct subpopulations (Uong & Zon, 2009). Trunk neural crest cells (NC cells) can progress through either of two pathways: the ventral pathway (forming neuronal tissue) and the dorsolateral pathway (giving rise to
melanoblasts, the precursor cells of melanocytes) (Serbedzija et al., 1990). These melanoblasts then migrate to their final destinations before differentiating into mature melanocytes or melanocyte stem cells (McSc) (Figure 2) (Li, 2014). NC cells migration is directed by a variety of environmental cues, which repel NC cells from forbidden locations and allow the colonization in appropriate locales (Theveneau & Mayor, 2012). One of these repellents is Semaphorin. NC cells express the semaphorin receptors, the neuropilins, which aid in NC cell migration (Lumb et al., 2014).

In addition to forming the aforementioned cutaneous melanocytes, melanoblasts also migrate to the hair follicle and the iris (Boissy, 1988). In the hair follicle, mature melanocytes are localized in the bulb, where they secrete melanin into the matrix of the hair. McSc are found in the bulge, a structure superficial to the bulb (Li, 2014).

**Hair Cycle**

The animal model of interest is the murine model. Thus, it is important to note the differences between the murine and human skin and hair cycle. Both mouse and human consist of the same three layers of skin (epidermis, dermis, and hypodermis) and adhere to a morphologically similar defined cycle of hair growth and loss (Wong et al., 2011; Kligman, 1959). The three stages are as follows: anagen, catagen, and telogen (Figure 3). The anagen phase is characterized by rapid growth of the hair follicle due to a robust blood supply (Muller-Rover et al., 2001). In the catagen phase, the hair follicle regresses, driven by keratinocyte apoptosis (Lindner et al., 1997). The final phase, telogen, is a resting stage characterized by relative quiescence and lack of a blood supply to the follicle (Muller-Rover et al., 2001). Where human and mouse differ in regards to
these stages is in the timing. In mice, the coat of hair progresses sequentially through the hair cycle in wavelike manner from cranial to caudal; in humans, each hair cycles individually, so that three hairs picked at random may be in three different stages of growth (Wong et al., 2011). Upon depilation, the hair cycle in mice resets, and all depiled follicles will start the cycle anew in sync (Muller-Rover et al., 2001).

**Figure 4 | The Murine Hair Cycle:** The hair cycle consists of three phases, a) anagen, b) catagen, and c) telogen. Melanocytes are lost during catagen and are replaced by McSc during the telogen phase. Murine hair cycles through this process in waves, moving cranial to caudal; human hair cycles on an individual basis. Removal of the hair follicle starts the cycle anew. Taken from Muller-Rover et al., 2001.

**Neuropilin 2 Pathologic Expression**

The extensive physiological expression of neuropilin is mirrored in pathological conditions, particularly in cancer. The first reported tumors expressing neuropilin were the PC3 prostate and MDA-MB-231 breast cancer cell line (Soker et al., 1998). Since then, many types of tumors have been shown to express at least one neuropilin, although it is not uncommon to observe dual expression. Generally, carcinomas express higher levels of NRP1; NRP1 has been found in bladder, pancreatic, renal, colon, ovarian, and
lung carcinomas (Wey, Stoeltzing, & Ellis, 2004; Neufeld et al., 2005; Bielenberg et al., 2006) (Figure 4).

<table>
<thead>
<tr>
<th>Carcinoma</th>
<th>Non-carcinoma</th>
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<td>Prostate</td>
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<td>Breast</td>
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**Figure 5 | Expression of NRP in Various Human Cancers:** The above PCR demonstrates the differing expression of both NRPs in various human cancer cell lines. Expression is not mutually exclusive, but a clear trend emerges between the two. NRP1 is highly expressed in carcinomas, while NRP2 is favored by cancers of neural crest derived cells. Cell lines used for RT-PCR (from left to right): PC3, MDAMB231, 253JB5, SN12PM6, A375SM, U87MG, Sy5Y, HUVEC. From Bielenberg et al., 2006

Reflecting their physiological expression, NC derived tissues and sarcomas upregulate NRP2 (Bielenberg et al., 2006). One exception to the non-carcinoma-NRP2 guideline is pancreatic ductal adenocarcinoma (PDAC), which highly expresses NRP2. Normal pancreatic ductal cells do not express any NRP1 and only lowly express NRP2 (Fukahi et al., 2004).

The discovery of NRP in so many tumors has raised questions into its potential role in cancer. Early research on malignant prostate carcinoma biopsies showed a marked upregulation of NRP1 when compared to normal prostate tissue (Miao et al.,...
These samples were negative for VEGFR, but displayed classic signs of angiogenesis, including high VEGF levels and increased vessel density. This finding ruled out the possibility of autocrine VEGF signaling, while simultaneously positing a function for NRP. The VEGF sequestration hypothesis suggests that NRP may be binding VEGF not to initiate a signaling cascade, but rather to act as a reservoir. In this way, the tumor is able to achieve higher total VEGF concentration in the tumor microenvironment than would be possible if the VEGF was free. This VEGF–rich environment then promotes angiogenesis (Miao et al., 2000). NRP2-expressing tumors may act in the same manner, in addition to promoting lymphangiogenesis. A study conducted in 2008 further hints at the importance of NRP in tumors. NRP2 knockdown in PDAC was achieved using shRNA (Dallas et al., 2008). Without NRP2, the PDAC cells displayed a 70% decrease in migration and a similar reduction in invasion and in vivo growth capabilities; proliferation in vitro was unaffected. These NRP2 knockdown tumors in vivo were up to 95% smaller than the control tumors and exhibited significant decreases in blood vessel diameter (Dallas et al., 2008). These data demonstrate that direct inhibition of NRP2 can inhibit tumorigenicity and progression, as well as tumor-induced angiogenesis.

Additionally, it is known that in many types of cancer, the increased expression of NRP2 or the downregulation of SEMA3F correlates with tumor progression (Bielenberg et al., 2004). Studies have demonstrated the important role the SEMA3F-NRP2 relationship has in cancer. SEMA3F mRNA levels were found to be decreased in highly metastatic tumors relative to those cell lines with lower metastatic potential, both in vitro
and in vivo (Bielenberg et al., 2004). When A375SM cells transfected to overexpress SEMA3F were implanted \textit{in vivo}, the resulting tumors were found to have large voids consisting of apoptotic cells in the center of the mass. SEMA3F transfected tumors also showed an inability to metastasize to lung and lymph node, while 21/22 control mice had tumors in those locations. SEMA3F induces an encapsulated tumor phenotype and inhibits tumor angiogenesis, characteristic of benign neoplasms, demonstrating its clinical potential as an anti-tumor agent (Bielenberg et al., 2004).

\textit{The Origin of a Cancer Cell}

Cancer cells originate from the accumulation of abnormal characteristics in the body’s own cells. Healthy cells must accumulate at least six essential malignant characteristics before the devastatingly limitless dividing capabilities of a tumor can be unlocked. The “Hallmarks of Cancer” first outlined by Hanahan & Weinberg at the turn of the century defined these “acquired capabilities” a cell needs to obtain in order to transform into a malignant tumor cell (Figure 5). It may take many generations of cells before enough mutations accumulate to transform a benign cell into a malignant one, and these features need not be obtained sequentially. In addition, the mechanisms by which cells acquire these malignant capabilities may vary between from tumor to tumor. All requirements must be satisfied if a tumor is to develop, however, let alone metastasize (Hanahan & Weinberg, 2000).
Figure 6 | Acquired Capabilities of Tumor Cells: The six hallmarks of cancer cells, as described by Hanahan & Weinberg. A tumor, not necessarily one cell, must acquire these traits in order to become truly malignant. The tumor at the center depicts this heterogeneous composition of a tumor. Taken from Hanahan & Weinberg, 2000.

In physiologic conditions, a cell must receive an external growth signal input in order to progress from a quiescent state to a mitotically active one. While normal cells cannot proliferate without these stimulatory signals, tumor cells have freed themselves from the influence of exogenous signaling. This may be accomplished by altering the external signal itself, by a mutation in the receptor for the signal, or by modifying the signaling pathway of the receptor, among others (Hanahan & Weinberg, 2000). Not every tumor cell in the neoplasm needs to act by the same mechanism. There is also evidence that a thriving tumor is composed of several unique malignant subpopulations, rather than of one continuously dividing clone. Thus, the tumor microenvironment is heterogeneous, not homogenous (Fidler, 2003). Furthermore, the tumor architecture is complex and
often differs remarkably from the surrounding tissue (both normal and neoplastic) (Heppner, 1984). These two facts are important to consider when discussing the development and progression of any tumor. For example, one subpopulation can evolve a mutation to produce Fibroblast Growth Factor (FGF) when it should not; neighboring tumor cells that lack the capability to produce FGF may take up this tumor-derived growth factor for its own survival and proliferation (Jouanneau et al., 1994).

Tumor cells similarly are able to liberate themselves from regulation by inhibitory anti-growth signals. Cells in healthy tissue are subject to such signals, which arrest the cell in the non-proliferative phase (G₀), returning them to or maintaining quiescence. The inactivation of tumor-suppressor genes (e.g. SEMA3F), an area of great research, is one method by which tumor cells can accomplish this (Hanahan & Weinberg, 2000). Another highly productive field of research focuses on the ability of malignant cells to evade normal signals for apoptosis; the process is highly regulated primarily by the caspase family of proteins (Khan et al., 2014). Caspase dysregulation, among other mechanisms (i.e. p53 inactivation) is highly prevalent among malignant cancers (Hanahan & Weinberg, 2000). In addition to the regulatory cellular signals mentioned above, every cell has finite number of replicative divisions, regulated by the repetitive nucleotide sequences at each end of the chromosome called telomeres. These sequences serve to protect the DNA from deterioration during mitosis. Tumor cells, however, are able to grant themselves infinite reproductive capabilities. Telomerase, normally inactive in mature cells, is reactivated in tumor cells, contributing to their increased mitotic abilities (Blasco, 2002; Hanahan & Weinberg, 2000). There is also evidence that it is
possible to obtain this limitless dividing potential even without the activation of telomerase (Seger et al. 2002). The final two acquired capabilities a tumor must obtain are sustained angiogenesis and tissue invasion, both of which will be discussed at greater length below.

**Physiological and Pathological Importance of Angiogenesis**

One of the most important organ systems of the body, the vascular system is typically a stable and quiescent tissue. The unit of this system, the endothelial cell (EC), lines all blood and lymph vessels. Under normal physiologic conditions EC’s rarely divide (Folkman 1974). Under certain physiologic and pathologic conditions, however, EC’s can proliferate remarkably, forming new vessels for the body’s fluids. This process of neovascularization is called angiogenesis (Auerbach & Auerbach, 1994). More specifically, angiogenesis is the formation of new vessels from previously existing ones, as opposed to vasculogenesis, which is the creation of new vessels from angioblasts, the mesoderm-derived progenitor cells of the endothelium (Noden, 1989). Angiogenesis is vital during growth and proliferation of the body’s normal tissues, i.e. during development of the fetus and embryo, and abnormal tissues, i.e. tumors (Chung, Lee, & Ferrara, 2010). This fact is important both in understanding the development of the human body and the growth and eventual metastasis of tumors.

Physiological and pathological (specifically tumor) angiogenesis are similar in that both begin in response to hypoxia associated proteins like Hypoxia Inducible Factor 1 alpha (HIF1α) and VEGF, often in response to ischemia. Nevertheless, there are several important distinctions to note. Under physiological conditions, the signals
promoting angiogenesis subside once the hypoxic tissue has been perfused. Tumor neovasculature persists even in normoxic conditions, however, largely due to the aberrant production of pro-angiogenic factors by tumor cells (Chung, Lee, & Ferrara, 2010).

There is a limit to the maximum size of an avascular tumor, i.e. one that has not developed its own blood supply. The seminal paper by Dr. Judah Folkman noted that this distance is approximately 1–2 mm. Without adequate blood supply, tumor cells cannot thrive. In addition to its importance in tumor survival, angiogenesis is an essential step in the process of tumor metastasis (Folkman, 1971).

**The Angiogenic Process**

In both physiologic and pathologic conditions, angiogenesis begins with EC response to hypoxia. In response to low oxygen (O₂) concentration, cells upregulate expression of HIF1α, which in turn upregulates expression of VEGF-A and vascular endothelial growth factor receptor 2 (VEGFR-2). VEGF-A is a potent stimulator of angiogenesis (Chung, Lee, & Ferrara, 2010). The increased binding and signaling of VEGF-A leads to the selection and migration of the tip cell, a highly polarized and specialized EC. This tip cell is the beginning of the new vessel, and other EC proliferate (stalk cells) and migrate behind it (Gerhardt et al., 2003). In order to progress through the extracellular matrix (EM), the tip cell and stalk cells must secrete matrix metalloproteases (MMP), proteins which serve to degrade the surrounding collagenous basement membrane. This degradation of EM proteins by MMP’s allows the growing vessel stalk to migrate up the gradient of growth factors into the perivascular stroma (Effert & Strohmeyer, 1995). It is important to note that throughout this process, the
endothelium is kept intact. Distal cell migration is supported by proliferation of the proximal EC, ensuring that the fluids of the vessel are always contained. Following migration and proliferation, the endothelium undergoes reorganization to form a mature capillary (Auerbach & Auerbach, 1994).

Angiogenesis is a tightly regulated process in normal tissue involving a complex interplay between proangiogenic and antioangiogenic molecules (Hanahan & Folkman, 1996). A surplus of proangiogenic molecules encourages the local formation of new blood vessels; a surplus of angiogenesis inhibitors prevents the development of new vessels. This sensitive balance can be tipped in one direction or the other by a variety of molecules. Pathological conditions, like a hypoxic cell or a tumor microenvironment, can introduce a variety of angiogenesis promoters like fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), angiopoietin (Ang-1, Ang-2), platelet-derived growth factor (PDGF), transforming growth factor alpha and beta (TGF-α, TGF-β), and interleukin-8 (IL-8), to name a few (Longo, 2012). Introduction of these molecules, possibly in conjunction with a downregulation of antiangiogenic factors like semaphorin (SEMA), endostatin, angiostatin, or thrombospondin may also contribute to the generation of an imbalance in the angiogenic switch, kickstarting the process of neovascularization (Ribatti, 2009; Wang et al., 2015).

**The Process of Cancer Metastasis**

Metastasis is a complex biological process, consisting of many interrelated steps. At each step exists the possibility of failure, and a single misstep can derail the entire process. In 2003, Fidler equated a successful metastatic cell to “a decathlon champion
who must be proficient in all ten events, rather than just a few” (Fidler, 2003). A successful metastasis depends not only on the correct characteristics of the tumor cells ("the seed"), but also on an accommodating environment of the target tissue ("the soil") (Paget, 1889). There are two primary avenues for tumor metastasis: through the cardiovascular vessels or through the lymphatic vessels. Regardless of entry method, tumor cells must reach the blood circulation before settling in a novel organ (Tobler & Detmar, 2006). Robert Weinberg proposed a seven step process necessary for the metastasis (Valastyan & Weinberg, 2011). These steps were developed with the progression through the blood vasculature of epithelial-derived tumors, or carcinomas, in mind. The seven steps are as follows (Figure 6):

1. Local invasion of the tumor, first by breaking through the basement membrane and then spreading into the stroma, often as a result of MMP upregulation
2. Intravasation of tumor cells into the lumen of blood vessels and/or lymphatic vessels of the stroma, frequently as a result of neoangiogenesis
3. Survival in the blood and lymphatic circulation, which exposes the tumor cells to a wider spectrum of tissues
4. Arrest at a distant organ site, whereby the tumor cell(s) adhere to the wall of the vessel; this typically occurs in the microvasculature, i.e. capillary beds
5. Extravasation into the parenchyma of distant organ
6. Initial survival in the foreign microenvironment and subsequent micrometastasis formation
7. Proliferation of micrometastases, progressing to colonization of the new organ or tissue
Figure 7 | The Process of Cancer Metastasis: Tumor cells (in red) must embark on a complex and difficult journey before appearing as a clinically detectable metastasis. Cells must invade the nearby tissue for access to a blood supply. If this supply is reached, tumor cells must then intravasate and survive in the systemic circulation. Once the cells arrive at a suitable site (often the capillary bed of a distant organ), they must extravasate and successfully evade the host defenses long enough to colonize the new organ. Taken from Fidler et al., 1998.

After observing these guidelines, Weinberg concluded that metastasis is an inefficient process (Valastyan & Weinberg, 2011).

While still inefficient, metastasis via the lymphatic system may be slightly easier than in its counterpart system. In fact, for many tumor types, the lymphatic system is the primary mode of dissemination. Certain inherent differences between lymphatic vessels and cardiovascular vessels may explain why tumor cells move more freely through the
lymphatic system. Lymphatic capillaries are characterized by a wider and more irregular lumen than their cardiovascular counterparts, have incomplete or non-existent BM, have fewer tight junctions and adherens junctions between the endothelial cells lining the lumen, and are more tightly associated with the adjacent interstitial tissue (Leak, 1970).

Taken together, these characteristics of lymphatic vessels may result in a truncated version of the stepwise process of metastasis outlined above. For example, intravasation from the vessel is not necessarily required in for tumor cells in the lymphatic circulation (Sleeman, 2000). Instead, tumor cells in the lymphatic system may enter the bloodstream after colonizing a nearby lymph node (via the efferent lymphatic vessels) or through the thoracic and right lymph ducts, which return lymph to the systemic circulation (Tobler & Detmar, 2006). Metastasis may be inefficient, but it still accounts for 90% of deaths associated with solid tumors like melanoma (Gupta & Massague, 2006).

**Lymphangiogenesis and Metastasis**

As noted above, angiogenesis is necessary for a tumor to grow larger than 1–2mm in diameter, which roughly translates to $10^6$ cells. The proliferation of vessels is usually associated with a tumor that metastasizes to secondary sites in the body.

Lymphangiogenesis is to lymphatic vessels as angiogenesis is to blood vessels; that is, both processes result in the formation of new vessels from previously existing ones. The lymphatic system is a blind-ended network of vessels that serves to drain the interstitium of excess fluid, and works in concert with the immune system to provide an efficient system of lymphocyte transportation and surveillance (Swartz & Skobe, 2001).
Lymph is moved through the vessels by several, primarily local, means. Pressure arising from the interstitium (both hydrostatic pressure and mechanical strain of the ECM) and contraction of neighboring skeletal muscle are the most prominent sources of driving force. Additionally, the larger lymphatic collecting vessels are enveloped by smooth muscle cells and like veins, contain valves. Unfortunately, lymphatic vessels also provide tumor cells with an alternate route for migration, and one that is often used in clinically assessing the metastatic potential of the tumor (Swartz & Skobe, 2001).

It is well known that tumors secrete pro-lymphangiogenic factors, which in turn provide the growing tumor with access to a larger lymphatic network (Stacker et al., 2002). In addition to these growth factors, tumor lymphangiogenesis also depends on the tumor’s anatomical location. Tumors derived from tissues that are naturally closer to smaller lymphatic vessels, which are more lymphangiogenic, demonstrate a greater affinity for metastasis than those farther away (Shayan et al., 2013). Lymphatics associated with tumors and tumor cell metastasis are most likely to derive from small lymphatics. For example, a melanoma with a higher ratio of peripheral to centrally-located lymphatic vessels is more likely to metastasize than one with a lower ratio (Shayan et al. 2013).

The Development and Progression of Melanoma

Tumors arising from melanocytes are called melanomata (singular: melanoma). Together, melanomata account for 5% of all malignant skin cancers, but compromise a stunning 90% of skin cancer deaths (Tandler, Mosch, & Pietzsch, 2012). While primary tumors are easily excised, melanomata that have progressed and spread are not so
successfully treated, primarily due to the resistance of the tumors to chemotherapeutic and radiotherapeutic agents (Uong & Zon, 2009). Due to this, early identification and treatment of a melanoma is critical in ensuring positive outcomes. Risk factors include family history, a fair complexion, and exposure to UV radiation, particularly having had multiple severe sunburns in childhood (Lo & Fisher, 2014).

Melanomas must progress through the previously outlined steps in order to achieve malignancy and metastasis, but also encounter another hurdle along the path to malignancy. Melanocytes are normally subject to regulation by keratinocytes and must escape this control in order to progress through the stages of cancer (Haass & Herlyn, 2005). This is accomplished through a five step molecular process, outlined below.

1) Downregulation of E-cadherin, P-cadherin, and desmoglein, which connect melanocytes and keratinocytes
2) Upregulation of Mel-CAM, N-cadherin, and other proteins that allow for melanoma cell-melanoma cell and melanoma cell-fibroblast communication
3) Deregulation of morphogens, which regulate cell fate
4) Downregulation of basement membrane anchoring proteins
5) Increased production of MMP

Once melanomata have freed themselves from control by keratinocytes (and have accumulated the requisite hallmarks of cancer), they then begin to progress through a series of well-defined stages (Laga & Murphy, 2010) (Figure 7). The first step in the process is abnormal proliferation of noninvasive, nontumorigenic cells. This initial growth eventually proliferates and expands radially through the epidermis. This is called the radial growth phase and is rarely metastatic. The third step in the progression is characterized by a propensity for vertical growth, which often presents superficially as an
elevated growth on the skin. The transition from the radial growth phase to the vertical growth phase correlates with VEGF accumulation and increased protein expression in the tissue surrounding the tumor (Streit & Detmar, 2003). More importantly, however, is the deep growth of the tumor and its subsequent breaching of the basement membrane. This third development, called the vertical growth phase, is necessary for the progression to the final step, metastasis to distant lymph nodes and organs (Laga & Murphy, 2010).

Figure 8 | Progression of Melanoma: Melanomata progress through a series of distinct stages. The tumor grows first radially, then vertically, before it penetrates the basement membrane. Here, it has access to the blood and lymph vessels needed for successful metastasis. Once the tumor has metastasized, it is exceedingly lethal. From Melanoma New Zealand (melanoma.org.nz)

Clinically, this process is identified and labelled according to the system developed by Alexander Breslow, eponymously named the Breslow depth. Increased Breslow depth correlates to a greater likelihood of metastases, and consequently a worse
prognosis (Breslow, 1970). Perhaps not coincidentally is the finding that NRP2 expression levels correlate with Breslow depth and increases from nevi to primary melanomas to metastases. Other measures have been shown to have more clinical relevance however. Tumor lymphatic vessel density is a better predictor of metastasis than Breslow depth (Dadras & Detmar, 2004). Unsurprisingly, an increase in NRP2 expression correlates with probability of developing the metastatic disease. Expression of NRP2 mRNA was found to be higher in thicker melanomas than in thin tumors, implicating NRP2 as a potential biomarker for malignant melanoma (Rossi et al., 2014).

Primary tumor location has a significant influence on the metastatic potential of a tumor. In one study, murine tumors located in the skin metastasized more readily than those growing in the body wall. This occurred due to the natural anatomic access a tumor growing in the skin has to smaller lymphatic vessels, which have greater lymphangiogenic potential. This fact explains the tendency for melanomata to travel via the lymphatic system versus the cardiovascular system (Shayan et al., 2013). Due to the high metastatic potential of melanoma, and the difficulty in treating the tumor once it has metastasized, the identification of a biomarker that can accurately and precisely discern between those tumors with high vs. low metastatic potential would be revolutionary in the management of the disease.

This study hopes to elucidate the physiological role of NRP2 in melanocytes, where it may function as a regulator of migration, and pathological location of NRP2 in melanoma. In melanoma, the upregulation of NRP2 accompanied with a decrease in SEMA3F concentrations may play a role in tumor growth and progression.
METHODS

Cell Culture

Human epithelial melanocyte cells (neonate) (HeMn) were purchased from Cascade. SKMel28 human melanoma cells, A2058 human melanoma cells, and WM266-4 human melanoma cells were purchased from American Type Culture Collection. MMAN human melanoma cells and A375SM human melanoma cells were obtained from Isaiah Fidler (MD Anderson Cancer Center, Houston, TX). Human keratinocytes (HaCaT) were obtained from Michael Detmar (Switzerland), and human fibroblasts (Milo) were obtained from Deborah Freedman (Boston Children’s Hospital). Once thawed, using a 37°C water bath, the cells were deposited in 100mm culture plates accompanied by 10mL of media. Each cell line requires a different medium to flourish. The media used were as follows: RPMI (MelaC), Medium 254 (HeMn), DMEM (all melanoma lines, HaCaT, milo). Each medium was supplemented with 10% Fetal Bovine Serum (FBS) and 1% glutamine-penicillin-streptomycin (GPS). Once plated, cells were incubated in humidified 37°C incubators at the appropriate CO₂ concentrate (either 5% or 10%). Media was aspirated and replaced every three days. Once plates reached approximately 90% confluence, the cells were assigned one of several fates based on the needs of the lab at the time.

If no current experiments required new cells, the confluent plate would be split into two or more new 100mm plates. Media was aspirated and the cells were washed with 3mL 0.05% trypsin ethylenediaminetetraacetic acid (EDTA). Once the trypsin was
applied, plates were incubated for five minutes to maximize effectiveness of the trypsin. At the conclusion of this period, plates were struck by hand several times to loosen cells from the bottom of the plate. After confirming via microscope that cells had lifted off the plate, the trypsin was neutralized using either trypsin neutralizing solution (TNS)(Gibco) or, most often, fresh media containing serum. Cells were added to as many new plates as desired (1mL suspended cells per new plate) and once again incubated.

**Protein Isolation**

The second course of action for a nearly confluent plate was to lyse the cells in order to isolate and collect protein. This following process was carried out on ice or in a cold environment for as many steps as possible. This was accomplished by first removing the media and washing the plate with phosphate buffered saline (PBS). PBS was removed and 500µL protein lysis buffer was added. Protein lysis buffer was created by dissolving one ULTRA mini tablet protease inhibitor in 10mL radio immune precipitation assay (RIPA) buffer (Roche). Plates were scraped using a specialized sterile tool and the liquid was collected in an aliquot. After allowing the tube to rest for 20 min (on ice), the tube was centrifuged at 14,000 rpm for 10 min. This resulted in a pellet accruing at the bottom of the tube. Supernatant was collected and frozen in a new, labeled aliquot, and the pellet was discarded.

**Protein Analysis**

Cell lysates were analyzed via the VERSAmax microplate reader (Molecular Devices) at a wavelength of 575nm. Samples require a specific preparation protocol before they could be analyzed accurately. For each sample, two 5µL aliquots were
pipetted into a 96 well plate. A Bovine Serum Albumin (BSA) standard was serially
diluted and pipetted in triplicate alongside the lysate samples. Next, 20µL Protein Assay
Solution S (Bio-Rad) was added to 1mL Protein Assay Solution A (Bio-Rad). 25µL of
this A+S solution was added to each well containing lysate or standard to be tested.
Finally, 200µL Protein Assay Solution B (Bio-Rad) was added to the same wells that
received the A+S solution. Once each well was prepared, samples were allowed to rest at
room temperature for five minutes before being read by the VERSAmax reader. The
standard readings and their known concentrations were plotted in a line graph using
Microsoft Excel and a best fit formula was calculated. This formula was then used to
calculate the protein concentrations of each experimental sample.

**Casting the SDS-Page Gel**

All Western blots were gels created by the same formula, which is as follows.
The resolving layer, which separates the protein in each sample, was made by mixing
3.75mL of 30% acrylamide/0.8% bis-acrilamyde (National Diagnostics) with 3.75mL 4x
1.5M tris-HCL/0.4% SDS pH 8.8 (resolving buffer) (National Diagnostics), 7.5mL sterile
double distilled water, 50µL 10% ammonium persulfate (APS), and 10µL
tetramethylethylethylenediamine (TEMED). The stacking layer, which condenses each
sample so as to ensure an even run across samples down the resolving layer, was made by
mixing 0.65mL acrylamide/0.8% bis-acrilamyde (National Diagnostics) with 1.25mL
0.5M tris-HCl/0.4% SDS pH 6.8 (stacking buffer) (National Diagnostics), 3.05 mL sterile
double distilled water, 25µL APS, and 5µL TEMED. The resolving layer was pipetted
first into a casting mold (Bio-Rad) and consequently topped with a thin layer of methanol
to eliminate any bubbles. This ensured a level, uninterrupted surface onto which the stacking layer was placed. After approximately thirty minutes, once the resolving layer had polymerized, the methanol was poured out and the stacking layer added. For each layer, the TEMED was introduced immediately before pipetting into the mold. A ten well comb measuring 1.5mm in thickness was inserted into the still-liquid stacking layer. Any bubbles that may have formed between the teeth of the comb were removed. Once the gel had polymerized, it was removed from the plastic cast and either was used immediately or wrapped in damp paper towels and saran wrap to be stored at 4°C.

**Running the Western Blot**

Based on the previously run protein analysis, cell lysate samples were diluted accordingly with distilled water. Each sample was diluted such that there was 36µg of protein per well. A colored protein ladder was including alongside the samples for molecular weight reference. In addition to the cell lysate and water, each well also contained 5µL 6x-reducing SDS Sample Buffer. All samples (including the reference ladder) were boiled for five minutes and centrifuged prior to loading. While the samples were boiling, the SDS-Page gels were submerged in 1X running buffer (25mM Tris, 192mM glycine, 0.1% SDS, pH 8.3), and the ten well comb was removed. Salt was removed from the wells by pipetting running buffer into the submerged well until clear. Once the samples were prepared, the protein ladder and each sample was loaded in its entirety into a unique well, with care taken to record the identity of each sample. The lid was connected to the apparatus and to the power supply. Gels were allowed to run at 100V for two hours.
Following separation by electrophoresis, proteins were similarly allowed to transfer to a nitrocellulose membrane (Bio-Rad). The gel/membrane was then submerged in 1X transfer buffer (25mM Tris, 192mM glycine, pH 8.4) and subjected once again to electrophoresis. The apparatus was allowed to run overnight at 4°C and 75mA or for two hours on ice and at 300 mA.

Once the transfer was complete, the gels were discarded, and Ponceau S solution (Sigma) was introduced to the nitrocellulose membrane to ensure a successful transfer. Ponceau was removed by washing with Tris Buffered Saline (10 mM Tris-HCl, 150mM NaCl, pH 7.4) with 1% Tween-20 (TBS-T). The membrane was then blocked with 5% blotting-grade blocker non-fat dry milk (Bio-Rad) in TBS-T for one hour at room temperature. After blocking, the membrane was rinsed once briefly with TBS-T. The primary antibody was prepared using anti-NRP2 antibody (H-300, Santa Cruz) at a 1:1000 ratio in TBS-T. The membrane was then incubated with the primary antibody for two hours at room temperature with mild agitation. Following primary antibody incubation, the membrane was again washed with TBS-T (3x15 min). Horseradish peroxidase (HRP) conjugated anti-rabbit antibody was used as the secondary antibody at a concentration of 1:3000. The secondary antibody solution was added to the membrane and allowed to incubate for one hour with agitation at room temperature. Once again, the membrane was washed with TBS-T three times for fifteen minutes each after secondary antibody incubation. An enhanced chemiluminescence (ECL) solution was prepared by mixing Oxidizing Reagent with Enhanced Luminol Reagent from the Western Lighting Plus-ECL kit (Perkin Elmer) in a 1:1 ratio. The solution was poured on the membrane,
which was allowed to rest at room temperature for five minutes before being placed in a cassette for transportation to the dark room for exposure. Initially the membrane was exposed for one minute, and the exposure time was increased or decreased as needed.

**Migration Assays**

Cells were trypsinized and removed from culture. Each cell sample was spun down in a centrifuge for five minutes at 1000 rotations per minute. After centrifuging, the existing media was aspirated and replaced with the appropriate serum-free media. In order to count the cells, 10 µL of suspended cells in serum-free medium was loaded into a hemocytometer. The hemocytometer is then observed with light microscopy at 100x magnification. Cells are counted in the four demarcated corner squares, making sure to only count cells on the lines of two sides of the square. This ensures that no cell is counted twice. The average count for the four squares is then multiplied by 10,000 to obtain the density of cells in suspension, measured in cells/mL. Once the cells have been counted, the migration plate is prepared.

The migration plate used is a standard 24 well plate with twelve plastic inserts. At the bottom of each insert is an 8-micron pore cell-permeable membrane. An assembled migration apparatus consists of two wells separated by the barrier. The bottom well was filled with 600 µL of culture medium with 10% FBS. In the top well, the suspended cells were plated in known quantity. In the SEMA3F experiment, the SEMA3F was added to the top chamber at graduated concentrations. The inserts (and suspended cells) were placed in the bottom wells, ensuring that no bubbles were present between the two chambers. The entire 24-well plate was then placed in the incubator for
sixteen hours.

After allowing sufficient time for the cells to migrate, the 24 well plate was removed from the incubator. The top well inserts were removed and each one was fixed and stained with the DiffQuik kit. First, each insert was placed in a fixative for five minutes. Once fixed, the inserts were placed in the first staining solution for five minutes and a second staining solution for ten minutes. After fixing and staining, the inserts were allowed to rest in double distilled water for five minutes. At the end of this wash, the cells remaining inside the insert (i.e. those that did not migrate) were removed with cotton swab. The inserts were observed under a light microscope at 200x magnification and the cells were counted. Eight fields were averaged for each condition.

**Immunohistochemistry**

Formalin fixed, paraffin embedded (FFPE) tissue sections were submerged in xylene (first for four minutes, then for three) in order to remove the paraffin. The tissue was then progressively rehydrated by means of a six step ethanol ladder, consisting of two immersions in 100% ethanol, two in 95% ethanol, one in 70% ethanol, and finally one in 50% ethanol. Each submergence in the ethanol ladder lasted two minutes. Antigen retrieval was performed if necessary; the exact methods varied based on the primary antibody used. For NRP2, antigen retrieval consisted of submersion in 10mM sodium citrate buffer, pH 6, and heated for five minutes in a microwave. Once citrate reached a temperature of 95°C, the microwave was turned off and the tissue was allowed to cool gradually in citrate until it reached approximately 50°C. Slides stained for S100β required no antigen retrieval. Once the tissue had cooled to a safe temperature for
handling, each slide was removed from the citrate buffer and a hydrophobic ring was
drawn around each sample with a pap pen. Each slide was washed with 3 times for 3
minutes per wash with PBS. To block endogenous peroxidase activity, and to promote
permeability of the tissue, 3% hydrogen peroxide in methanol was added to the tissue and
allowed to incubate at room temperature for 12 minutes. Following the peroxide block,
each section was once again washed with PBS (3 x 3 min). Tissues were then subjected
to a protein block using Tris-NaCl blocking buffer (TNB) for at least thirty minutes.
Primary antibody was then pipetted to the slides at the appropriate concentration (in
TNB) and allowed to incubate at 4°C overnight.

The subsequent day, slides were washed of primary antibody (3 x 3min) with PBS
before application of the corresponding biotinylated secondary antibody. Secondary
antibody was also prepared in TNB at a concentration of 1:200 and, once applied to the
slide, allowed to incubate for one hour at room temperature. After this step, the signal
was amplified using an Avidin/Biotinylated Enzyme Complex (ABC) kit (Vector). This
step required preparation of the solution in PBS at least thirty minutes before application.
Before application of the ABC solution, the slides were washed with PBS (3 x 3min).
DAB Chromagen Kit (Vector) was prepared in distilled water and was used to detect
bound antibody. Prior to addition of chromagen, the slides were washed one last time,
twice with PBS (3 min each) and once with distilled water (3 min). DAB was then
applied and allowed to incubate at room temperature for up to twenty minutes. Brown
precipitate, indicating a bound antibody and thus a positive result, was observed using
light microscopy. After 20 minutes, the sections were washed with distilled water and
counterstained with hematoxylin and Tacha’s Bluing Solution (BioCare Medical), which turned the counterstain blue instead of purple. Slides were allowed to dry overnight before being mounted the next day with Permount (Fisher).

**Mice**

Mice were housed in a pathogen-free environment in the Boston Children’s Hospital animal facility, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). The standards and regulations set forth by the US Department of Agriculture, Department of Health and Human Services, NIH, and the Institutional Animal Care and Use Committee (IACUC) of Boston Children’s Hospital were carefully followed at all times when handling the animals.

The primary genetic line of interest was the previously purchased Nrp2^{+/gfp} line of transgenic mice (Nrp2^{+/gfp}/MomJ, Stock #006700). The mice were bred to yield viable homozygous knockouts, which were used to elucidate the physiological location and function of Nrp2.

After three weeks of age, mice were genotyped to determine its genetic classification. First, ear samples were collected by anesthetizing the mice and removing the ear with sterile equipment. The ears were placed on ice and observed under a fluorescent microscope. The knock-in of GFP for Nrp2 allowed one to discern two subcategories of mice based on the presence of GFP in the hair follicles. Wild-type (WT) mice had no knock-in of GFP and thus did not present with GFP positive hair follicles. Heterozygous (het) and knock-out (KO) both displayed green hair follicles, and as such, no conclusion could be drawn as to the identity of the mice belonging to this group. The
ear samples of the GFP positive group were packaged and sent to the mail-order genotyping service Transnetyx, which returned PCR results within a week.
RESULTS

*Melanocytes Express NRP2*

In order to test whether NRP2 was expressed in melanocytes, human epidermal melanocytes – neonate (HeMn) and murine melanocytes (MelaC) were cultured and lysed for analysis by western blot. The samples were run through a 7.5% SDS-Page gel, transferred to a nitrocellulose membrane, and immunoblotted with specific antibody in order to quantify relative neuropilin 2 expression. Based on these experiments, it was found that both human and murine melanocytes expressed NRP2, a novel finding. Human protein levels were detected using the Santa Cruz H300 antibody against NRP2, while murine samples were subjected to analysis with a rabbit polyclonal antibody for Nrp2 (D39A5) manufactured by Cell Signaling Technology.

HeMn western blots were run with HaCaT (human keratinocytes) and milo (human fibroblasts) cell lines, representing the most common cells present in the skin. Porcine Aortic Endothelial cells transfected to overexpress NRP2 (PAE NRP2) or VEGFR2 (PAE KDR) were added as control lines. Mouse primary melanocytes were run with mouse primary keratinocytes (MK) isolated from 3-day-old neonates in the Bielenberg lab and transformed mouse fibroblasts (NIH 3T3). Malignant cell lines (B16F10 – a mouse melanoma, and EOMA – a mouse endothelioma) as well as control samples from mouse skin and brain were also run in the gel. Neither human (HaCaT) nor mouse keratinocytes (MK), displayed any significant expression. Interestingly, human and mouse fibroblasts were found to express NRP2 on western blot but not with IHC.
HeMn were also compared to five melanoma lines (A2058, A375SM, WM 266-4, SKMel 28, MMAN), each of which also was found to express NRP2 (Figure 8). This finding was expected and confirms prior research (Bielenberg et al., 2004). Equivalently, MelaC cells were run with B16F10 mouse melanoma cells, which also tested positive for Nrp2.

**Figure 9 | Melanocytes Express NRP2:** a) Mouse primary melanocytes express Nrp2, as do fibroblasts and melanoma cells. Keratinocytes do not, but this may be due to a loading error; b) human melanocytes express NRP2, as do human fibroblasts; c) all five human melanoma lines tested express NRP2.
Melanocytes do not Express VEGFR2

Neither Western blot nor immunohistochemistry can reveal the function of the protein of interest. Additional experiments are needed in order to elucidate the protein’s function in vivo. As mentioned previously, NRP2 is involved primarily in the binding for two ligands: VEGF and SEMA3F, and successful binding of VEGF (i.e. one leading to induction of signaling cascade) and NRP2 requires the presence of a VEGF receptor. The western blot membranes used previously for NRP2 testing were incubated with an antibody for VEGFR2, which binds NRP2 ligands VEGF-A and VEGF-C. It was found that the HeMn did not express VEGFR2 on either of the two blots, even though the positive control cell line (PAE KDR) did. Additionally, none of the five human melanoma lines displayed any VEGFR2 expression (Figure 9).

![Figure 10 | Neither Melanocytes nor Melanoma Express VEGFR2: Western demonstrating the lack of VEGFR2 by all cell lines except the positive control (PAE KDR). NRP2 is shown as a loading control.](image)

MelaC cells did not test positive for VEGFR2 either, although this finding must be repeated on a blot with a control cell line present in order to consider this a true result.
This finding suggests that NRP2 is not acting as a VEGF coreceptor for either melanocytes or melanoma lines, but rather in some other capacity.

**Melanocyte Migration is Inhibited by the Presence of SEMA3F**

In order to test whether NRP2 on melanocytes was in fact acting as a receptor for SEMA3F, the mouse melanocytes (MelaC), along with B16F10 melanoma cells, were subjected to a migration assay. Cells were cultured and counted before plating on the migration wells. First, it was necessary to determine the optimal number of cells required for observable migration. Both cell lines were plated in duplicate for each condition; each line was tested at 10,000, 20,000, and 40,000 cells. B16F10 cells showed meaningful migration starting at 20,000 cells, while MelaC cells required at least 40,000 cells before any meaningful migration could be observed. In order to minimize confounding variables, it was determined that 40,000 cells would be plated for both cell lines in the experimental condition.

The migration assay was run again, using 40,000 cells per well. B16F10 cells and MelaC cells were once again plated in duplicate and assigned to one of four conditions: a control group, which received no treatment (plated in the same environment as the previous study), and three groups receiving some concentration of SEMA3F. The SEMA3F groups were treated with 150ng/mL, 300ng/mL, or 600 ng/mL, which was administered in the top well after combining the top and bottom chambers. After 16 hours, the cells were stained and counted using ImageJ software. It was determined that SEMA3F treatment significantly reduced migration of both melanocytes and melanoma cells when compared to the control group (Figures 10, 11).
Figure 11 | Melanoma Migration is Inhibited by SEMA3F: (top) Increased dose of SEMA3F resulted in decreased melanoma migration. (bottom) Images from the migration assay
Figure 12 | Melanocyte Migration is Inhibited by SEMA3F: (top) Increased dose of SEMA3F resulted in decreased melanocyte migration. (bottom) Images from the migration assay
**Antibody Optimization**

There are several NRP2 antibodies available on the market, and the Bielenberg laboratory is in possession of three of them: the C-9 antibody (Santa Cruz), an antibody from Sigma-Aldrich, and the H300 antibody (Santa Cruz). In order to determine the optimal antibody for IHC, three formalin-fixed, paraffin-embedded (FFPE) slides known to contain NRP2 were deparaffinized and prepped for IHC. All three were submerged in citrate buffer and heated to 95°C; this served to increase the amount of antigen available for antibody binding. After primary antibody incubation (all at a 1:200 dilution), ABC amplification, and subsequent chromagen application, each slide was observed based on intensity and specificity of staining. After this evaluation, it was determined that the H300 antibody was most effective.

Once the H300 antibody was chosen, it was necessary to determine the optimal dilution for IHC. Four tissue samples were used at three different dilutions, with the last serving as a control. It was determined that optimal dilution for the purposes of this study was 1:100.

**NRP2 Expression Correlates with Melanoma Progression**

Fifty eight human samples were stained for NRP2 (Santa Cruz) and S100B. Samples were analyzed by light microscopy and given an overall score ranging from 1 to 9. To determine the overall score, each slide was judged on two factors, intensity of staining and area of staining and given a score from 1 to 3, with a score of 1 categorizing the lightly stained or smallest area stained, and a 3 signifying most intense staining or largest area stained. The two scores were then multiplied to obtain the final overall score.
The intensity of NRP2 staining was compared to the intensity of S100 staining, and it was found that NRP2 expression correlated with melanoma progression. Tumor cells in lymph nodes were most positive for NRP2, while nevi were the most lightly stained. Primary melanoma samples fell between these two extremes (Figure 12).

Figure 13 | NRP2 Expression Correlates with Melanoma Progression: Samples taken from metastatic tumors display more intense staining of NRP2 than control (human nevi). Primary melanoma samples fall between these two extremes.
DISCUSSION

The foundation of this study began with the genotyping of the transgenic mice models. Once we discovered that hair follicles expressed NRP2, we wanted to know which cells were responsible. The two primary cells of the hair are keratinocytes and melanocytes, and we believed the melanocytes accounted for the distinct pattern we observed, largely based on their NC cell origin.

Neuropilin 2 continues to be observed in novel tissues, and in many cases, its function remains unknown. Only recently has its importance in its earliest known locales been elucidated, and much research is still needed to fully understand its physiologic and pathologic role. The presence of NRP2 in melanocytes is a novel finding, and, taken together with the fact that these cells also lack VEGFR2, suggests that NRP2 is not acting as a VEGF coreceptor. The “VEGF reservoir” theory that was described earlier is a poor conceptual fit for the melanocyte model, although it may have merit when considering the upregulation of NRP2 in the melanoma lines tested. Thus, the most plausible theory is that which considers NRP2 a functional SEMA3F receptor for melanocytes, but why should these cells need a directing force? Melanocytes are typically located at or near the basement membrane under normal conditions. Non-proliferating melanocytes adhere and are anchored to the basement membrane, but proliferating melanocytes lose these adhering properties (Danen et al., 1996). This loss of BM adhesion allows the melanocytes to migrate and weave dendrites through the neighboring keratinocytes (Haass & Herlyn, 2005). It is known that keratinocytes mediate melanocyte growth via cell-cell interactions, primarily via E-cadherin, which is expressed on both melanocytes...
and keratinocytes (Tang et al., 1994; Hsu et al., 1996) (Figure 13).

Figure 14 | Melanocyte-Keratinocyte Interactions: Melanocytes downregulate a number of regulatory proteins during mitosis, resulting in freedom from keratinocyte control. SEMA3F may play a supplemental role in maintaining keratinocyte influence over melanocytes through its receptor, NRP2. Adapted from Haass & Herlyn, 2005.

NRP2 may be another method by which keratinocytes can regulate the growth and migration of epidermal melanocytes. It has been shown in other epithelial tissue that there is a stratification of SEMA3F production by the superficial keratinocytes. In skin, this may serve to prevent melanocyte migration toward the surface of the skin (Figure 15).

Figure 15 | Expression of SEMA3F and NRP2 Varies in Bladder: SEMA3F is found in the outermost layers of the bladder epithelium, while NRP2 is found deeper in the tissue. This layout may be mirrored in skin, where keratinocytes in the outer layers produce SEMA3F to contain NRP2-expressing melanocytes in the inner layers. From Bielenberg, unpublished.
SEMA3F may also restrict melanocytes from straying during proliferation, when melanocytic downregulation of BM adhering proteins. Once melanocytes have completed mitosis, SEMA3F may act as a guiding force, repositioning melanocytes where they are most needed.

In order to investigate effects of NRP2 on melanocytes in vivo, further testing can center around a transgenic mouse line. The Jackson Laboratory (JAX) is in possession of a tyrosinase-cre mouse line; the cre lines are useful for selectively knocking out genes. Tyrosinase is the enzyme responsible for the production of melanin. Therefore, a tyrosinase-cre mouse can be bred with the NRP2 flox transgenic line already housed by the Bielenberg laboratory to selectively knock out NRP2 in melanocytes. In vivo studies could then be carried out on both wild type mice and those without NRP2-expressing melanocytes to further elucidate the role of NRP2. Additionally, by breeding inducible tyrosinase-cre mice with NRP2 floxed mice, one can observe the potential effects of NRP2 loss of function after birth. Knocking out NRP2 after birth may prevent compensatory measures from kicking in, allowing for a truer sense of the protein’s function. This has already been done in the Bielenberg lab, knocking out NRP2 in the smooth muscle of the bladder (Bielenberg et al., 2012). Additionally, the Bielenberg lab has recently purchased SEMA3F KO mice, and future studies on melanocyte location and function can be pursued with this model.

Previous research on melanoma cells and VEGFR2 has yielded mixed results. Several studies detected VEGFR2 expression on melanomata, both by IHC and by Western blotting, with reports claiming as prevalence as high as 89% (Salven et al., 1997;
Gitay-Goren et al., 1993; Pisacane & Risio, 2005). The results of this study run contrary to these findings, but are not novel. Others have raised similar doubts about VEGFR2 expression (Molhoek et al., 2011). There are several plausible explanations for this lack of VEGFR2. Rather than signal through the VEGF pathway, melanomata may use alternative growth pathways (like scatter factor for example). Melanomata may sequester VEGF via NRP2, establishing a strong gradient that encourages angiogenesis (Figure 16).

Figure 16 | VEGF Sponge Theory: Tumors expressing high levels of NRP2 (without concurrent VEGFR2 expression) may use NRP2 to sequester VEGF in tumor adjacent tissue. This creates a gradient of VEGF leading to the tumor, which promotes EC proliferation and angiogenesis.
Another possibility, which was explored recently with NRP1 and VEGFR2 on endothelial cells, is that the tumor expresses NRP2 in order to amplify VEGF signaling in nearby endothelial cells (Figure 17). This trans complex (NRP2 on melanoma, VEGFR2 on endothelial cells) can induce a signal and increase EC proliferation in tumor-adjacent tissue, inviting angiogenesis and subsequently metastasis (Koch et al., 2014).

**Figure 17 | Trans Signaling:** A schematic of trans, or juxtacrine, signaling. A tumor cells expressing NRP2 can amplify the response to VEGF by an adjacent EC, promoting further EC proliferation. This is one alternative possibility to the function of NRP2 on melanomata. Taken from Miao & Klagsbrun, 2000.
During the course of this thesis, a paper was published indicating that NRP2 mRNA levels correlate with disease progression (Rossi et al., 2014). The results of this thesis not only confirm those found recently, but also confirm that protein expression is increased. Further studies are needed to elucidate the role (if any) of NRP2 in melanomata, and to determine whether NRP2 can serve as an accurate biomarker for more aggressive tumors. A biomarker like this is greatly needed, as there is currently no reliable method for predicting which melanoma will metastasize.
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CURRICULUM VITAE
Salvatore A. Rizzo
YOB: 1991 | 36 Richmond St. | Islip, NY 11751
PHONE: 631-541-5599 | EMAIL: sal.rizzo21@gmail.com

EDUCATION

M.S. Medical Sciences
(anticipated) May 2015, Boston University, Boston, MA

B.A. Psychology
May 2013, Johns Hopkins University

RESEARCH EXPERIENCE

Graduate Student, Boston Children’s Hospital, Boston, MA
Studied Physiological and Pathological Function of Neuropilin 2 using the following techniques:
SDS-Page and Western Blotting
Immunohistochemistry
Sterile Culture of cell lines
In vivo studies with transgenic mice

POSTERS


AWARDS

Leading for Social Change Grant (2013) – Johns Hopkins University
Johns Hopkins University Dean’s List (2012)