Integrin alpha4 promotes neuroblastoma metastasis

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

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by

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Co-Chair

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University of California, San Diego

2014
DEDICATION

This dissertation is dedicated to every family member, friend, and individual that supported, encouraged and pushed me along this journey. I appreciate every word and every smile. I would not have made it this far without you. To my parents, Lee and Nancy Roberson, thank you for supporting me through everything I have ever done or tried to do. Thank you for being amazing examples of hard work, integrity, and loving-kindness. To my sister, Joyce AsSadiq (Brotha), thanks for being my number one fan. I will always look up to you. To my church families, thanks for the love, laughter and personal growth. To my husband, Ray Young, Jr., thanks for being my rock.

I thank God for each of you. I pray that He shower his choice blessings upon you and continue to do exceedingly, abundantly above all you ask or think. Be blessed.
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<th>Definition</th>
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<tbody>
<tr>
<td>ALK</td>
<td>anaplastic lymphoma kinase</td>
</tr>
<tr>
<td>BARD1</td>
<td>BRCA1-associated ring domain 1 gene</td>
</tr>
<tr>
<td>BRCA1</td>
<td>breast cancer 1, early onset gene</td>
</tr>
<tr>
<td>BDNF</td>
<td>bone-derived neurotrophic factor</td>
</tr>
<tr>
<td>COG</td>
<td>Children’s Oncology Group</td>
</tr>
<tr>
<td>CS1</td>
<td>alternatively spliced region of type III connecting segment of fibronectin</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>GD2</td>
<td>disialoganglioside</td>
</tr>
<tr>
<td>GM3</td>
<td>monosialodihexosylganglioside</td>
</tr>
<tr>
<td>Grb2</td>
<td>growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>HSCT</td>
<td>hematopoietic stem cell rescue/transplantation</td>
</tr>
<tr>
<td>IAP</td>
<td>inhibitor of apoptosis</td>
</tr>
<tr>
<td>IGFR-1</td>
<td>insulin-like growth factor receptor 1</td>
</tr>
<tr>
<td>INRGSS</td>
<td>International Neuroblastoma Risk Group Staging System</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>INSS</td>
<td>International Neuroblastoma Staging System</td>
</tr>
<tr>
<td>LFA-1</td>
<td>lymphocyte function-associated antigen-1; αLβ2 integrin</td>
</tr>
<tr>
<td>MAdCAM-1</td>
<td>mucosal addressin cell adhesion molecule 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloprotease</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>manganese divalent cation</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>MYCN</td>
<td>myelocytomatosis viral oncogene neuroblastoma</td>
</tr>
<tr>
<td>NB</td>
<td>neuroblastoma</td>
</tr>
<tr>
<td>NBPF23</td>
<td>neuroblastoma breakpoint family member 23</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>pFN</td>
<td>plasma fibronectin</td>
</tr>
<tr>
<td>PHOX2B</td>
<td>paired homeobox 2B</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PML</td>
<td>progressive multifocal leukoencephalopathy</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>RGD</td>
<td>arginine-glycine-aspartic acid</td>
</tr>
<tr>
<td>RGDS</td>
<td>arginine-glycine-aspartic acid-serine</td>
</tr>
<tr>
<td>Sos</td>
<td>son of sevenless protein</td>
</tr>
<tr>
<td>TrkA</td>
<td>neurotrophic tyrosine kinase receptor, type 1</td>
</tr>
<tr>
<td>TrkB</td>
<td>neurotrophic tyrosine kinase receptor, type 2</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>VEGFR</td>
<td>vascular endothelial growth factor receptor</td>
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Professor Dwayne G. Stupack, Chair
Professor Judith A. Varner, Co-Chair

Neuroblastoma (NB) is a childhood cancer arising from neural crest cells. Despite aggressive therapy, NB with distant metastasis is associated with an overall survival rate of 30-50%. Thus, NB metastasis poses a significant obstacle in achieving remission. NB adhesion receptors in the integrin family, impact cell adhesion, migration, proliferation and survival. Integrin α4, a fibronectin and vascular cell adhesion molecule-1 (VCAM-1) receptor, is essential for neural crest cell motility during development. In adults, integrin α4 is primarily expressed on leukocytes and is critical for transendothelial migration. Accordingly, cancer cells that express this receptor may acquire an enhanced ability to extravasate into surrounding tissues, increasing their metastatic potential. The work described in this dissertation examines
whether integrin α4 plays a role in neuroblastoma malignancy. I found that
reconstitution of α4 expression in human and murine neuroblastoma cell lines
selectively enhances in vitro interaction with fibronectin and VCAM-1 and increases
migration. In vivo, α4 expression enhanced experimental metastasis in a syngeneic
tumor model, reconstituting a pattern of organ involvement similar to that seen in
patients. Accordingly, antagonism of integrin α4 blocked metastasis, suggesting
adhesive function of the integrin is required. However, adhesive function was not
sufficient, as mutants of integrin α4 that conserved the matrix-adhesive and
promigratory function in vitro were compromised in their metastatic capacity in vivo.
Clinically, integrin α4 is selectively associated with poor prognosis in non-MYCN
amplified neuroblastoma. These results reveal an unexpected role for integrin α4 in
neuroblastoma dissemination and identify α4 as a prognostic indicator and potential
therapeutic target.
CHAPTER 1:

Introduction
1.1 Neuroblastoma

Neuroblastoma (NB) is a neural crest-derived pediatric malignancy of the peripheral sympathetic nervous system. It is the most common extracranial solid tumor in children and accounts for more than 10% of childhood cancer deaths in the United States (Ishola and Chung, 2007). More than 600 new NB cases (U.S.) are diagnosed each year at a median age of 19 months (Brodeur and Maris, 2002). The clinical presentation and progression of NB is remarkably heterogeneous. Undifferentiated metastatic tumors may undergo spontaneous remission via differentiation into benign lesions, resulting in high survival rates, while other advanced stage tumors are fatal even with multi-modal treatment approaches (Lode et al., 1997; Sims et al., 2008). As a neuroendocrine tumor, NB most commonly develops in the adrenal medulla but may also develop in other areas of the sympathetic nervous system (Schwab et al., 2000). The disease spreads via hematogenous and lymphatic routes. Common metastatic sites include the bone, bone marrow, liver, lymph nodes and skin (DuBois et al., 1999). Approximately 70% of NB patients present with distant metastasis at the time of diagnosis (Brodeur et al., 1993). Due to diverse clinical progression, NB treatment regimens vary widely and are based on disease stage and risk stratification systems.

1.1.1 Staging and risk stratification

Until recently, NB staging has been defined by the International Neuroblastoma Staging System, which outlines 6 stages based on surgical and pathological observations such as the degree of tumor resectability and metastatic...
involvement (Table 1.1) (Brodeur et al., 1993). This system has been replaced by the International Neuroblastoma Risk Group Staging System (INRGSS) (Table 1.2), which uses radiologic image-defined risk factors (Table 1.3) and clinical criteria to assign disease stages (Cohn et al., 2009; Monclair et al., 2009). An approximate comparison of staging definitions between these systems is available (Table 1.4) (Bhatnagar and Sarin, 2012). A key difference between the two systems is that INSS-based staging is assigned via post-surgical analysis and is influenced by the degree of surgical resection and the expertise of the surgeon, whereas INRGSS-based staging relies on risk factors identified by pre-operation imaging. The INRGSS was developed to provide a system that could be utilized for risk-based clinical trials. Its developers anticipate that the increased objectivity and precision of this system will improve evaluation of therapeutic and disease risk.

Neuroblastoma is categorized into low-, intermediate- and high-risk groups. Currently, these groups are defined by the Children’s Oncology Group (COG) and are based on INSS stage, age, tumor histology and amplification status of the MYCN oncogene (Table 1.5). The assigned risk correlates with patient outcome. Five-year survival rates for children in low-, intermediate-, and high-risk groups are 95%, 80-90%, and 30-50%, respectively (Society, 2012). New risk classifications based on the INRGSS may soon replace the COG system.

1.1.2 Neuroblastoma treatment

Treatment of NB is determined by risk classification and generally includes surgery, chemotherapy, radiotherapy, hematopoietic stem cell rescue/transplantation
(HSCT), and immunotherapy. For some infants with presumed low-risk, localized
disease detected via prenatal or perinatal ultrasound, clinical observation without
surgery may be recommended in light of potential complications of surgery (Institute).
However, most low-risk patients undergo surgery to excise the primary tumor. For
intermediate-risk patients, surgery is supplemented with chemotherapy and radiation.
Approximately 40% of NB patients are classified as high-risk (Suenaga et al., 2009).
These patients are placed on a regimen that is divided into three phases: induction,
consolidation, and maintenance. The induction phase includes chemotherapy followed
by resection of the primary tumor. Common chemotherapeutic agents are cisplatin,
etoposide, cyclophosphamide, vincristine, and doxorubicin (Ishola and Chung, 2007).
The consolidation phase involves myeloablative chemotherapy followed by HSCT and
radiation. Last, the maintenance phase is a combination of differentiation therapy and
immunotherapy. Agents, such as 13-cis-retinoic acid, that promote cellular
differentiation are administered to treat any residual disease. Immunotherapy targeting
the GD2 disialoganglioside on NB tumor cell surfaces has been shown to improve
event free survival (Cheung et al., 2012; Yu et al., 2010). Despite this aggressive
regimen, many high-risk neuroblastoma are refractory to current therapies. Of the
patients with recurrent disease, less than 10% survive (Matthay et al., 2009). Novel
agents and treatment approaches are being actively researched and developed to
combat the therapeutic challenges of this disease.

1.1.3 Pathobiology: genetic and molecular characterization
More effective management of aggressive neuroblastoma requires a clearer understanding of the pathobiology of the disease. There has been continuous progress toward the identification of genes and signaling pathways that play a role in NB pathogenesis; however, there are still many undefined aspects of NB development and progression. NB most commonly develops sporadically; less than 2% of cases are familial. Both forms have similar clinical behavior yet distinct genetic abnormalities have been linked to the development of each type (Malis, 2013). Mutations in the anaplastic lymphoma kinase (ALK) and paired homeobox 2B (PHOX2B) genes are considered predispositions for familial NB. Single nucleotide polymorphisms in the BRCA1-associated RING domain 1 (BARD1) gene and copy number variations in the neuroblastoma breakpoint family member 23 (NBPF23) may play a role in sporadic NB tumorigenesis (Capasso and Diskin, 2010). There are several other genetic aberrations and molecular markers that are common to NB and that correlate with prognosis. One genetic model of NB proposes three NB subtypes. Subtype 1 is characterized by mitotic dysfunction and a triploid karyotype with increased expression of TrkA, a nerve growth factor (NGF) receptor. Subtypes 2 and 3 have specific chromosomal abnormalities (i.e. loss of heterozygosity in 1p, 11q and/or 14q or trisomy 17q), diploid or tetraploid karyotypes and commonly express the TrkB receptor and its ligand, brain-derived neurotrophic factor (BDNF) (Brodeur and Maris, 2002). Additionally, subtype 3 has amplification of MYCN, a transcription factor with roles in growth, survival, and differentiation and that is known to cause malignant transformation (Cole and McMahon, 1999). NB patients with TrkA expression have a more favorable prognosis than those with TrkB or elevated MYCN expression.
Of note, MYCN is one of the strongest indicators of disease progression and poor patient prognosis and is amplified in 20-25% of primary neuroblastomas (Ishola and Chung, 2007; Kelleher, 2013). Other common properties of NB tumor cells include loss of the extrinsic apoptosis initiator protein, caspase 8, and differential integrin expression (Ara and DeClerck, 2006; Teitz et al., 2000).

In order to improve NB survival rates, further genetic and molecular characterization of NB and identification of key players in NB metastasis is needed. This body of work explores the role of integrins in NB progression.

1.2 Integrins

In the body, cells are surrounded and supported by an intricate network of glycoproteins and proteoglycans that make up a complex extracellular matrix, or ECM. Many constituents, such as collagen, laminin, and fibronectin, are locally produced within the tissues, where they act as physical scaffolds, growth factor depots, and points of anchorage (Hynes, 2009). The local rigidity and composition of the matrix also provide environmental cues that govern cell behavior.

The ECM surrounding cells can be considered in two broad classes. On one hand, there exists a ‘physiologic’ ECM, present in all tissues, that aids in structuring and maintaining homeostasis. Typical ECM components include several collagens and laminins, as well as proteoglycans. On the other hand, there is a provisional ECM that is deposited during wounding, hemostasis and tissue remodeling. This ECM is typically deposited, digested and replaced in a very dynamic manner, and contains proteins such as fibronectin, fibrin, vitronectin and even residual fragments of collagen.
and laminin. This type of ECM promotes tissue remodeling as well as cellular survival, proliferation and invasion. In both types of ECM, however, the diversity in the type and quantity of each individual ECM component present determines the physical properties of these tissues. In so doing, this modulates the mechanical forces sensed by cells that bind to the ECM, and provides yet another layer of information relayed to cells. This ‘mechanosensation’ requires integrins, receptors that can transmit extracellular forces to the actin cytoskeleton.

Although many classes of receptors can interact with components of the ECM, the integrins are regarded as the principle receptors mediating anchorage and attachment to the ECM (Ingber, 1991). The name integrin was derived from initial observations that these receptors permitted a realignment of the actin cytoskeleton to match that of an underlying ECM. Integrins are transmembrane glycoprotein receptors that are composed of a heterodimer of α and β subunits (Hynes, 1987). There are 18 different α subunits and 8 β subunits, but there are a limited number of possible combinations that can form from these subunits. To date, at least 24 unique integrin complexes have been identified, each with its own binding specificity for different subsets of ligands (Fig. 1.1). Cells will generally express only a limited number of integrins, perhaps 10 of these combinations. The particular repertoire of integrins expressed by a given cell varies, but is typically closely tied to a cell’s particular extracellular microenvironment. Differences in integrin binding to a ligand can be subtle. For example, approximately one third of human integrins bind to an arginine-glycine-aspartic acid (RGD) sequence of amino acid residues, but this can be
profoundly conformation specific, and thus not all ‘RGD-binding’ integrins are capable of binding all RGD sequences with appreciable affinity.

1.2.1 Integrin structure and activation

Each integrin is composed of a large extracellular region of 600-1000 amino acids, as well as a single transmembrane domain. The extracellular regions can be broadly thought of in terms of a head and stalk (leg/thigh) region; the head is the critical site for ligand binding and divalent cation binding, as well as heterodimerization between the α and β subunits. Most integrins also have a small (~30-50 amino acid) cytosolic domain, with the singular exception being integrin β4, which has a large cytosolic domain that interacts with intermediate filaments (Stepp et al., 1990). Integrins are cysteine–rich proteins, and have extensive crosslinking within domains that stabilize domain structure. Thus, integrins appear at different sizes when analyzed on reducing and non-reducing gels, and detection of integrins by some antibodies may require either condition, depending upon the linearity or conformation dependence of an epitope.

The integrin extracellular domains are required for and sufficient to bind to ECM or to ‘receptor-ligands’ present on the surface of adjacent cells. However, the binding of integrins to their ligands is controlled by their conformation, which is influenced by the stalk and cytosolic regions of the molecule. Inactive integrins adapt a ‘folded back’ conformation at a region halfway up the stalk (at the ‘genou,’ or knee, between the thigh and leg). Active integrins are extended molecules with stalks separated, and intermediates between these states tend to have intermediate affinities.
Integrin-ligand binding requires the presence of divalent cations, with a typical preference for manganese, magnesium and calcium, although the relative preference for optimal affinity varies among the different heterodimers. These divalent cations, and Mn$^{2+}$ in particular, directly influence integrin conformation, stabilizing them in the extended and high affinity conformation (Fig. 1.2).

With the exception of circulating hematopoietic cells, which tend to maintain their integrins in an inactive conformation, most cells that have been examined express both active and inactive integrins. Active integrins tend to form higher order clusters on the cell surface, which promotes their localization to sites of ligation. There, the integrins are further stabilized by interaction with ligand. The accumulation of integrins in these sites creates a ‘Velcro-like’ effect, with groups of integrins (rather than individual molecules) collaborating to strengthen anchorage and to induce downstream signaling points of extracellular matrix contact. This clustering effect is called integrin ‘avidity’ regulation, which is distinct from affinity. This permits the stable interaction with the ECM required for sustained cellular anchorage and signaling via the assembly of a ‘focal adhesion complex’ that accumulates proximal to the membrane.

1.2.2 Integrin signaling

The focal adhesion complex that forms as a result of integrin clustering is multifunctional, and is capable of signaling directly, scaffolding additional or alternative signals, and engaging the actin/myosin system. Thus, despite the absence of intrinsic kinase or proteolytic activity, integrins transform mechanical and chemical
cues from the extracellular environment into intracellular signals that profoundly impact cell behavior and function.

The focal adhesion complex contains a complicated array of non-receptor kinases and adaptor proteins that mediate downstream signaling events. As will be discussed in more detail in chapter 2, integrin effectors in the focal adhesion include diverse signaling elements such as: focal adhesion kinase (FAK), src kinase, cytoskeletal elements including talin, paxillin and vinculin, phosphoinositide 3 kinase, and small GTPases of the Ras and Rho families and their effectors (Juliano et al., 2004; Schlaepfer and Hunter, 1998). Importantly, as part of the clustering process, integrins tend to undergo lateral associations with other cell surface receptors such as the receptor tyrosine kinases, EGFR and VEGFR, which are important for other global cellular signaling events. This type of signaling, in which the integrin ectodomain is ligated and transforms information from the extracellular environment into cues for cytosolic signaling events has been termed “outside-in signaling.”

However, in some cases, signals from inside the cell result in changes in integrin conformation. These are typically associated with cytosolic proteins binding to the cytosolic domains of the integrins. This type of regulation of integrin conformation is called “inside-out signaling.” Both types of signaling are important for understanding the role of integrins in normal tissues and in disease pathology.

1.2.3 Integrins in cancer

Integrins play a role in a many physiological cellular processes. It follows that many diseases, including cancer, involve the dysregulation of integrin expression and
signaling. As cells are transformed from a normal to malignant state, their integrin expression is modulated to support pathologic behaviors. In primary tumors, integrin signaling can impact cell growth, differentiation, and vascular infiltration and continues to be important as the cancer progresses through the stages of metastasis (Fig. 1.3). The initial steps of the metastatic process involve the degradation and remodeling of extracellular matrix adjacent to primary tumor cells, facilitating cancer cell migration into recruited blood vessels. This process is termed invasion. Usually, for local invasion to begin, cells from the primary tumor shift from an epithelial or non-motile to a more mesenchymal phenotype. In addition, cells frequently create a pathway for themselves by inducing degradation of the matrix via enzymes such as matrix metalloproteases (Kähäri, 2000). Integrins can regulate MMP expression and/or activity. For example, integrin α2β1 is a positive regulator of MMP-1 expression (Riikonen et al., 1995; Znoyko et al., 2006).

For many types of cancer, metastasizing cells spread to a specific subset of secondary locations for establishment of metastatic nodules. This phenomenon, termed tissue tropism, has historically been explained by two major theories. The “seed and soil” hypothesis proposed by Stephen Paget in 1889 followed his observation of tissue-specific patterns of tumor metastasis in 735 breast cancer patients. Paget noted that the pattern of organs bearing metastases was not random, and suggested that certain tumor types preferentially metastasized to compatible environments (Paget, 1989). He proposed that “seeds” of tumors required compatible “soil” to take root and grow. An alternative theory, by Ewing, suggests that tissue tropism is simply due to mechanical forces and circulatory patterns (Ewing, 1928). These are not absolutely
exclusive theories, and it is reasonable that blood flow patterns are important for the initial distribution of circulating tumor cells, while the propensity to invade, grow and survive may be dependent on the presence of the appropriate integrin ligands as well as other pro-survival factors.

Since the metastatic cascade involves several steps, including local tumor invasion, intravasation, survival in the lymphatics/blood stream, extravasation, invasion into new tissue parenchyma and growth and establishment of metastatic nodules, there are many opportunities for integrins to facilitate this process. The role of integrins in local invasion is clear. Once cells gain entry into the vasculature, integrins are important for cell-cell and cell-platelet adhesion leading to increased formation of cell emboli and subsequent lodging in capillary beds (Ruoslahti and Giancotti, 1989). Integrins are also important for the endothelial transmigration that follows. At the site of distant metastasis, the microenvironment and composition of the extracellular matrix may be different from that of the native tissue of the invading tumor cells. Here, the balance of ligated and unligated integrins impacts cell behavior and survival, as discussed in chapter 2.

1.3 Integrin α4

Each integrin plays a distinct role (or set of roles) in cancer development and progression. This role depends on the cell type on which the integrin is expressed and the disease context (i.e. type of cancer, stage of cancer, microenvironment, etc.). The focus of this dissertation is the role of integrin α4 in neuroblastoma.
Integrin $\alpha 4$ is a 150 kDa subunit that constitutes the $\alpha 4\beta 1$ and $\alpha 4\beta 7$ heterodimers. Its ligands include vascular cell adhesion molecule-1 (VCAM-1), mucosal addressin cell adhesion molecule-1 (MadCAM-1), a region in the alternatively spliced type III connecting segment of fibronectin (CS1) (Pankov and Yamada, 2002), the heparin II domain of fibronectin, and osteopontin (Liu et al., 2000). Ligand specificities for the $\alpha 4$ heterodimers often overlap; however, one heterodimer may have a higher affinity for the ligand than the other. For example, $\alpha 4\beta 7$ has a higher affinity for MadCAM-1 than $\alpha 4\beta 1$ (Strauch et al., 1994).

### 1.3.1 Expression and physiological functions

Integrin $\alpha 4$ expression patterns vary based on developmental stage. $\alpha 4$ is expressed on neural crest cells during development and is critical for neural crest cell migration and proper embryonic maturation (Kil et al., 1998; Testaz and Duband, 2001). Mice null for $\alpha 4$ are embryonic lethal due to placental and cardiac defects (Yang et al., 1995). In adults, $\alpha 4$ is primarily expressed on leukocytes and has roles in hematopoiesis, homotypic cell-cell adhesion, cell homing to the bone marrow, and leukocyte development (Campanero et al., 1990; Hsia et al., 2005; Kumar and Ponnazhagan, 2007; Liu et al., 2000; Rose et al., 2002). In addition, the binding of $\alpha 4$ to its endothelial ligand, VCAM-1, is essential for adhesion, spreading and endothelial transmigration during leukocyte trafficking out of the vasculature and into surrounding tissues (Liu et al., 2000; Rose et al., 2002).

Integrin $\alpha 4$ signaling induces unique cellular behavior. Unlike other integrins, $\alpha 4$ opposes cell spreading and focal adhesion formation (Liu et al., 2000; Pinco et al.,
α4 also promotes enhanced cell migration in comparison with other α subunits (Chan et al., 1992). Protein interactions with the α4 cytoplasmic domain are essential for these properties. One such interaction is α4-paxillin binding. Besides integrin α9, α4 is the only α subunit whose cytoplasmic tail binds directly to the paxillin adaptor protein found in cell adhesion complexes. This interaction may explain some α4-related changes in biological responses (Liu et al., 2002; Liu et al., 2001; Liu et al., 1999). Src kinase activation is also a critical event in α4 signaling and is required for α4-mediated migration (Wu et al., 2008). Post-translational modifications also impact α4 signaling. Phosphorylation on serine 988 regulates α4-paxillin association and impacts cell spreading and migration (Han et al., 2001; Liu and Ginsberg, 2000). In addition to phosphorylation, α4 is cleaved into 70 kDa and 80 kDa fragments; however, this cleavage has no known functional relevance (Teixido et al., 1992).

1.3.2 Pathological roles

Integrin α4 plays an important role in a variety of diseases. Given its physiological involvement in leukocyte development and function, it is not surprising that many of these diseases are associated with inflammation. Pathological roles of α4 include involvement in inflammatory diseases such as asthma, multiple sclerosis, Crohn’s disease and rheumatoid arthritis (Davenport and Munday, 2007; Rose et al., 2002). In cancer, α4 expression has been noted on lymphoma, melanoma, sarcoma, ovarian and NB cells. Inappropriate expression of integrin α4 can lead to an aggressive migratory phenotype. Notably, α4 promotes motility in NB, melanoma and sarcoma
Integrin α4 is also expressed on tumor endothelium and can promote the growth of tumor associated blood vessels and lymphatics (Garmy-Susini et al., 2005; Garmy-Susini and Varner, 2008). In some cases, however, the role of α4 in cancer progression is controversial (Holzmann et al., 1998). Melanoma studies show that α4 expression is enhanced in human melanoma tumors and metastases but that α4 can decrease spontaneous lung metastasis in subcutaneous mouse models (Qian et al., 1994; Schadendorf et al., 1993). Importantly, the pulmonary vascular endothelial cells in these subcutaneous mouse experiments did not express the α4 ligand, VCAM-1. Other studies reveal that there may be an optimal range of α4 expression required to promote invasion and metastasis in vivo, as high levels of α4 can induce formation of cell aggregates and inhibit local invasion (Beauvais et al., 1995). In neuroblastoma, it is clear that α4 expression enhances motility in vitro, but additional studies are needed to determine the effect of α4 expression on other malignant properties and whether these in vitro behaviors translate to differences in aggressiveness in vivo.

1.4 **Rationale and significance**

Aggressive metastasis is a significant obstacle to neuroblastoma treatment. Approximately 40% of NB patients present with advanced high-risk disease at the time of diagnosis. High-risk patients are often refractory to current treatments; thus, their chances of survival are extremely low. Identification of key players in NB metastasis is critical for more effective management of this disease.
Integrins play a role in many aspects of cancer development and progression but are particularly suited for roles in tumor cell invasion and metastasis. Given the physiological roles of integrin $\alpha 4$, it was selected as a strong candidate for involvement in neuroblastoma malignancy. Neuroblastoma is a neural crest derived malignancy. During development, integrin $\alpha 4$ is expressed in neural crest cells and is important for their migration (Kil et al., 1998; Testaz and Duband, 2001). Many neuroblastoma cell lines also express integrin $\alpha 4$ and demonstrate enhanced $\alpha 4$-mediated motility in vitro (Wu et al., 2008). In adults, integrin $\alpha 4$ is expressed on leukocytes and is important for extravasation from the vasculature into surrounding tissues (Liu et al., 2000; Rose et al., 2002). Transformed neural crest cells that express this integrin might possess an increased ability to extravasate, enhancing their metastatic potential. Integrin $\alpha 4$ also promotes homing to the bone, the most common site of neuroblastoma metastasis (Kumar and Ponnazhagan, 2007; Matsuura et al., 1996). Furthermore, $\alpha 4$ expression impacts leukocyte proliferation and survival and may function similarly in other cell types (Hayashida et al., 2000; Koopman et al., 1994).

The work described in this dissertation investigated the role of integrin $\alpha 4$ in NB malignancy. I demonstrated, for the first time, that integrin $\alpha 4$ expression promotes NB metastasis. In mechanistic studies, antagonism of integrin $\alpha 4$ reduced NB metastasis suggesting that $\alpha 4$-mediated adhesion is important for this process. In addition, I determined that the cytoplasmic tail is required for $\alpha 4$-mediated metastasis. Interestingly, functional studies revealed that the identity of the $\alpha 4$ tail is not essential for adhesive and migratory functions in vitro. These findings highlight integrin $\alpha 4$ as a
potential therapeutic target in NB. They also shed light on mechanisms of α4-mediated migration and the growing realization of the distinct requirements for 2D versus 3D cell motility.

1.5 Acknowledgements

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### Table 1.1: International Neuroblastoma Staging System (INSS)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Localized tumor with complete gross excision, with or without macroscopic residual disease; representative ipsilateral lymph nodes negative for tumor microscopically (nodes attached and removed with the primary tumor may be positive)</td>
</tr>
<tr>
<td>2A</td>
<td>Localized tumor with incomplete gross excision; representative ipsilateral nonadherent lymph nodes negative for tumor microscopically</td>
</tr>
<tr>
<td>2B</td>
<td>Localized tumor with or without complete gross excision, with ipsilateral nonadherent lymph nodes positive for tumor. Enlarged contralateral lymph nodes must be negative microscopically</td>
</tr>
<tr>
<td>3</td>
<td>Unresectable unilateral tumor infiltrating across the midline, with or without regional lymph node involvement; or localized unilateral tumor with contralateral regional lymph node involvement, or midline tumor with bilateral extension by infiltration (unresectable) or by lymph node involvement</td>
</tr>
<tr>
<td>4</td>
<td>Any primary tumor with dissemination to distant lymph nodes, bone, bone marrow, liver, skin and/or other organs (except as defined for stage 4S)</td>
</tr>
<tr>
<td>4S</td>
<td>Localized primary tumor (as defined for stage 1, 2A, or 2B), with dissemination limited to skin, liver, and/or bone marrow (limited to infants &lt;1 year of age)</td>
</tr>
</tbody>
</table>
Table 1.2: International Neuroblastoma Risk Group Staging System (INRGSS)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>Localized tumor not involving vital structures as defined by the list of image-defined risk factors and confined to one body compartment</td>
</tr>
<tr>
<td>L2</td>
<td>Locoregional tumor with presence of one or more image-defined risk factors</td>
</tr>
<tr>
<td>M</td>
<td>Distant metastatic disease (except stage MS)</td>
</tr>
<tr>
<td>MS</td>
<td>Metastatic disease in children younger than 18 months with metastases confined to skin, liver, and/or bone marrow</td>
</tr>
</tbody>
</table>
### Table 1.3: INRG image-defined risk factors

<table>
<thead>
<tr>
<th>Category</th>
<th>Risk Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ipsilateral tumor extension within two body compartments</td>
<td>Neck-chest, chest-abdomen, abdomen-pelvis</td>
</tr>
</tbody>
</table>
| Neck                     | Tumor encasing carotid and/or vertebral artery and/or internal jugular vein  
                              | Tumor extending to base of skull  
                              | Tumor compressing the trachea                                                                                                                                 |
| Cervico-thoracic junction| Tumor encasing brachial plexus roots  
                              | Tumor encasing subclavian vessel and/or vertebral and/or carotid artery  
                              | Tumor compressing the trachea                                                                                                                                 |
| Thorax                   | Tumor encasing the aorta and/or major branches  
                              | Tumor compressing the trachea and/or principal bronchi  
                              | Lower mediastinal tumor, infiltrating the costo-vertebral junction between T9 and T12                                                                                                                                 |
| Abdomen/pelvis           | Tumor infiltrating the porta hepatis and/or the hepatoduodenal ligament  
                              | Tumor encasing branches of the superior mesenteric artery at the mesenteric root  
                              | Tumor encasing the origin of the coeliac axis, and/or of the superior                                                                                                                                 |
| Mesenteric artery        | Tumor invading one or both renal pedicles  
                              | Tumor encasing the aorta and/or vena cava  
                              | Tumor encasing the iliac vessels  
                              | Pelvic tumor crossing the sciatic notch                                                                                                                                 |
| Intraspinal tumor extension whatever the location provided that: | More than one third of the spinal canal in the axial plane is invaded and/or the perimedullary leptomeningeal spaces are not visible and/or the spinal cord signal is abnormal |
| Infiltration of adjacent organs/structures | Pericardium, diaphragm, kidney, liver, duodeno-pancreatic block and mesentery |
| Conditions to be recorded, but not considered IDRFs | Multifocal primary tumors  
                              | Pleural effusion, with or without malignant cells  
                              | Ascites, with or without malignant cells |
Table 1.4: INSS and INRGSS Comparison

<table>
<thead>
<tr>
<th>INSS Stage</th>
<th>INRGSS Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 &amp; 2</td>
<td>L1</td>
</tr>
<tr>
<td>3</td>
<td>L2</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
</tr>
<tr>
<td>4S</td>
<td>MS</td>
</tr>
</tbody>
</table>

Table 1.5: Neuroblastoma Risk Stratification

<table>
<thead>
<tr>
<th>INSS Stage</th>
<th>Age</th>
<th>Histology</th>
<th>DNA Ploidy</th>
<th>MYCN</th>
<th>Risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>Low</td>
</tr>
<tr>
<td>2A/2B</td>
<td>&lt;365d</td>
<td>--</td>
<td>--</td>
<td>Non</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>&gt;365d</td>
<td>--</td>
<td>--</td>
<td>Non</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>&gt;365d</td>
<td>--</td>
<td>--</td>
<td>Amp</td>
<td>High</td>
</tr>
<tr>
<td>3</td>
<td>&lt;365d</td>
<td>--</td>
<td>--</td>
<td>Non</td>
<td>Int</td>
</tr>
<tr>
<td></td>
<td>&gt;365d</td>
<td>Favorable</td>
<td>--</td>
<td>Non</td>
<td>Int</td>
</tr>
<tr>
<td></td>
<td>Any</td>
<td>--</td>
<td>--</td>
<td>Amp</td>
<td>High</td>
</tr>
<tr>
<td>4</td>
<td>&lt;365d</td>
<td>--</td>
<td>--</td>
<td>Non</td>
<td>Int</td>
</tr>
<tr>
<td></td>
<td>Any</td>
<td>--</td>
<td>--</td>
<td>Amp</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>&gt;547d</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>High</td>
</tr>
<tr>
<td>4S</td>
<td>&lt;365d</td>
<td>Favorable</td>
<td>Hyperploid</td>
<td>Non</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>&lt;365d</td>
<td>Unfavorable</td>
<td>Normal</td>
<td>Non</td>
<td>Int</td>
</tr>
<tr>
<td></td>
<td>&lt;365d</td>
<td>--</td>
<td>--</td>
<td>Amp</td>
<td>High</td>
</tr>
</tbody>
</table>

Abbreviations: -- = not applicable, d = days, Non = non-amplified, Amp = amplified, Int = Intermediate
Figure 1.1: Integrin heterodimers and their ligands

This diagram shows the 24 known heterodimers and their ligands. Integrin heterodimers are represented by an α and a β subunit connected by a black line. For example, the β1 subunit dimerizes with 12 different α subunits. The ligands for each heterodimer are written in purple.
Figure 1.2: Integrin structure and activation

Integrins are composed of a large extracellular domain and short intracellular tails (with the exception of the β4 tail). The extracellular domain comprises a head region and a stalk region, which includes the “thigh” and “leg” areas of the integrin. Ligand binding occurs at the head region and requires the presence of divalent cations such as manganese, magnesium, and calcium. Integrins on the cell surface can exist in a range of conformations that affect their affinity for ligand. In the low affinity conformation, the extracellular domain is folded back at the knee (between the thigh and leg areas) and the intracellular tails are clasped together. In the high affinity conformation the extracellular stalk is straight, the subunits are slightly separated and the tails shift apart as well. Conformations between these low and high affinity states confer intermediate affinity for ligand. Changes in conformation can be regulated by intracellular signaling events such as the binding of cytosolic proteins to integrin tails leading to integrin clustering focal adhesion formation and further interaction of cytoskeletal proteins.
Integrins play key roles in each phase of cancer progression. 1. Ligation of integrins promotes cell survival 2. Co-signaling with growth factor receptors impacts cell proliferation 3. Endothelial cell integrins are important for tumor angiogenesis 4. Integrins modulate the expression of proteolytic enzymes such as matrix metalloproteinases, which play a role in matrix degradation during tumor cell invasion 5. Integrins are required for migration during invasion and binding to endothelial cells during intravasation (entry into the vasculature) 6. In circulation, tumor cell interact with platelets and leukocytes via integrins and form cell emboli that can lodge in capillary beds of distant tissues 7. Binding of tumor cell integrins such as α4β1 to endothelial VCAM-1 can then promote extravasation of tumor cells into surrounding tissues.
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CHAPTER 2:

Neuroblastoma integrin
2.1 Integrins in early development

Neuroblastoma is a malignancy that arises during early development. It is unclear how the dysregulation of specific embryonic processes impacts neuroendocrine tumorigenesis and progression. The ability of cells to interact with their extracellular environment is crucial for most developmental processes. Consequently, it is perhaps not surprising that integrins, as mediators of the interplay between cells, the ECM and the microenvironment, have critical roles in early development and developmental or pediatric diseases. The early physiological relevance is evident in defects observed in murine genetic models lacking proper integrin function or expression. Overall, the loss of the β1, α5, and α4 subunits leads to an embryonic lethal phenotype. The loss of the αv or α3 subunits permits initial and subsequent development, but results in perinatal lethality. Other integrin subunits do not appear to be essential during development.

Nonetheless, loss, misregulation, or improper function of integrins can lead to abnormalities and diseases (Beauvais-Jouneau and Thiery, 1997) (Table 2.1).

2.1.1 Integrins in nervous system development

The development of the nervous system is dependent on integrin function, in part, because it involves extensive migration of neuronal precursors, which is mediated by integrins. During the process of neurulation, the neural crest forms in the region of the neural plate border. Upon formation of the neural tube, neural crest cells undergo an “epithelial-to-mesenchymal-like transition” which permits them to move along migratory tracks. These tracks lead cells to a variety of destinations where they
differentiate and help to form several different tissue types. During development, collagens, laminins, fibronectin and vitronectin are expressed along these migratory pathways (Erickson and Perris, 1993). Disruption of integrin-ligand binding inhibits neural crest cell migration and results in impaired function in the peripheral nervous system. Following the initial gross exodus of neurons from the neural crest, integrins also play other key roles in the development of the peripheral nervous system, including the establishment of Schwann cell polarity (Bartlett Bungee, 1993), neurite outgrowth (Kuhn et al., 1995; Luckenbill-Edds et al., 1995) and myelination (Fernandez-Valle et al., 1994).

In addition to the requirement for integrins to support migration, integrins are also important for arresting migration at the proper time and place. In the central nervous system, for example, the presence of the α6 and β1 subunits appears to serve as stop signals for neuronal cells when they reach a laminin rich region. This is critical for cortical plate formation. In the absence of these integrins, neuronal precursors migrating outward to the outermost layer of the cortical plate overshoot their destination and disrupt the cortical plate structure (Georges-Labouesse et al., 1998; Graus-Porta et al., 2001).

2.1.2 Integrin expression in the dorsal root ganglion and in neuroblasts

Neuroblastoma is a tumor that is considered to arise from ganglion or pre-ganglion cells. To begin to understand the pathological roles of integrins in this disease, it is helpful to be familiar with the normal expression patterns of these receptors in neural crest cells and how that expression changes over time. Neural crest
cells express subsets of integrins that allow them to adhere to the fibrillar proteins that line their migratory pathways. Truncal neural crest cells, which give rise to dorsal root ganglia, sympathetic ganglia, and the adrenal medulla express receptors for vitronectin ($\alpha v \beta 1$, $\alpha v \beta 3$, and $\alpha v \beta 5$) ([Delannet et al., 1994], laminin ($\alpha 1 \beta 1$, $\alpha 3 \beta 1$) (Duband et al., 1992; Kil et al., 1998), and fibronectin and associated molecules ($\alpha 4 \beta 1$, $\alpha 5 \beta 1$, $\alpha 8 \beta 1$, $\alpha v \beta 1$ and $\beta 8$ integrin) (Kil et al., 1998; Testaz et al., 1999). Antibody blockade of any one type of these integrins is unable to completely abolish cell migration, consistent with a multi-receptor and complex ligand system. However, in studies on avian truncal neural crest cells, the $\alpha 3 \beta 1$, $\alpha 4 \beta 1$, and $\alpha v$ integrins appear to be the most crucial to maintain migration (Testaz et al., 1999). In particular, inhibition of the interaction between $\alpha 4 \beta 1$ and its ligands via blocking antibodies or ligand-mimicking peptides, leads to a marked reduction in neural crest cell migration (Kil et al., 1998).

As neural crest cells reach their target tissues and differentiate, their integrin expression changes. For example, neural crest cells do not express detectable levels of $\alpha 6 \beta 1$ until they differentiate into a peripheral nervous system cell type such as a Schwann cell precursor (Bronner-Fraser et al., 1992). Conversely, neural crest cells express $\alpha 1 \beta 1$ but Schwann cell precursors do not (Perris, 1997; Stewart et al., 1997). This induction of expression of one class of integrins while another is eliminated is not well understood, however, and further study will be required to elucidate additional neuroblast-specific integrin expression and function.

2.1.3 Integrins in vascular system development
Similarly, the formation of the vascular system relies heavily on integrin function. During vasculogenesis, or de novo formation of blood vessels, and angiogenesis, the growth of new vessels from pre-existing vasculature, integrins play essential roles in endothelial cell migration, adhesion to basement membranes and cell survival. Endothelial cells are known to express a large number of β1 integrin heterodimers including the α1 through α6 subunits as well as integrins α6β4, αvβ5, and αvβ3. The expression of different subsets of these integrins is dependent on the activation state of the endothelial cells. For example, integrins αvβ3 and α4β1 are primarily expressed on activated or angiogenic endothelial cells (Stupack and Cheresh, 2004). Knockout of integrin αv leads to perinatal lethality due to vessel malformation (Fässler et al., 1996) and studies on the αvβ3 heterodimer show that it is essential for the survival of angiogenic endothelial cells (Brooks et al., 1994). In addition, knockout of integrin α4 in mice is embryonic lethal by day 14.5 due to placental and cardiac defects (Yang et al., 1995), likely due to a lack of binding to the α4 ligand, vascular cell adhesion molecule-1 (VCAM-1) which is present on endothelial and smooth muscle cells.

The formation of the vasculature, and angiogenesis in particular, is of interest to scientists who study neuroblastoma, which is typically a highly angiogenic disease. Although a focus has been placed on the roles of integrins in development of the neuronal and vascular systems, the ability of integrins to regulate such a large array of cellular functions renders them essential for most, if not all, developmental processes. Their roles may be directly associated with their adhesion and motility-related
functions, or with the ability of integrins to indirectly enhance the efficiency of other signaling pathways (Bökel and Brown, 2002).

2.2 Integrin expression during tumorigenesis and tumor progression

Integrin expression and signaling impacts each stage of cancer progression – from the initial development of the primary tumor through each step of the metastatic cascade. These effects can be due to integrin expression on tumor, stromal, vascular or other cells. Examples of direct effects include the involvement of tumor cell integrins in growth, differentiation, invasion and endothelial transmigration.

In addition to impacting cancer progression and metastatic potential via regulation of migration, invasion, and extravasation, integrin expression influences the location of metastatic lesions (tissue tropism). This occurs via regulation of the adhesion and survival of cells invading into foreign tissue microenvironments at secondary sites. Though there have been no studies specifically linking integrins to site-specific metastasis in neuroblastoma, integrins have been shown to play a role in tissue tropism. The primary sites of neuroblastoma metastasis are bone marrow, bone, lymph node and liver. In general, certain integrins have been linked to metastasis to these sites. For instance, integrin $\alpha_4\beta_1$ can promote homing to the bone (Kumar and Ponnazhagan, 2007) and has been shown to enhance bone metastasis in melanoma (Matsuura et al., 1996). This effect may be due to expression of VCAM-1 on bone marrow stromal cells. Integrin $\alpha_4\beta_1$ may also promote lymphatic metastasis by enhancing binding to VCAM-1 present on lymphatic endothelial cells (Rebhun et al., 2010). Integrin $\alpha_2\beta_1$ is associated with enhanced liver metastasis. This is potentially
due to its binding to collagen type IV expressed in liver sinusoids (Yoshimura et al., 2009).

Integrins may also play indirect roles in cancer. It is worth noting that at any phase of tumor progression, cancer cells must evade the immune system. Some T-cell lysis mechanisms are dependent on integrin expression. For instance, binding of T-cell integrin LFA-1 (αLβ2) to its ligand, ICAM-1, on tumor cells is important in CD3-mediated T-cell lysis (Anichini et al., 1990; Braakman et al., 1990). Of note, ICAM expression on neuroblastoma cells is associated with increased susceptibility to lymphokine-activated killer (LAK) cell lysis following interferon gamma treatment (Naganuma et al., 1991).

Finally, the shedding of gangliosides also impacts neuroblastoma metastasis. Gangliosides are glycosphingolipids with one or more sialic acids linked to them. In circulation, gangliosides are associated with lipoproteins. There are several different types of gangliosides that are classified based on the number of associated sialic acids. Some of these gangliosides, such as G_{M3}, are normally present in circulation. Conversely, elevated levels of circulating G_{D2}, a disialoganglioside, have been found in neuroblastoma patients and its concentration is inversely related to progression-free survival. Shedding of gangliosides enhances integrin α2β1-dependent platelet activation, leading to platelet aggregation, and increased adhesion to vascular basement membranes (Jabbar et al., 2006). These events can enhance tumor cell embolization impacting the occurrence of cells lodging in capillary beds and invading into surrounding tissue.
2.2.1 Trends in integrin expression with neuroblastoma stage and grade

Since integrins impact cell differentiation and invasion, there has been an interest in linking the expression of subsets of integrins with a particular tumor stage, or more appropriately, with tumor ‘risk.’ Key risk predictors to date have been established by the Children’s Oncology Group, and include status of the MYCN gene, the pathology of the tumor according to guidelines established by Shimada (Shimada, 2003), and in some cases the relative ploidy of the tumor. Since integrins are associated with neuronal cell developmental stages and activities, it is reasonable that integrin expression could offer insights into tumor activities.

In pioneering studies, using 45 clinical samples, Favrot et al. showed that the α2 and α6 subunits were associated with low grade, well-differentiated neuroblastoma samples. The finding is consistent with observations of normal ‘neural crest cell to neuronal’ differentiation. The β1 subunit was expressed on all samples while the α5 subunit was not expressed on any samples examined. Samples expressing the α4, αv, β3, and β4 subunits revealed no N-Myc amplification, and were associated with a good prognosis. In addition, expression of α4 and β4 subunits was found selectively on Schwannian stromal cells (Favrot et al., 1991).

Conversely, more recent studies have found that many neuroblastoma cell lines express integrin α4 and that α4 expression is associated with increased tumor stage (stages 3 and 4) in clinical samples (Wu et al., 2008). At least on cell lines, integrin α5β1 also appears to be expressed (Kähäri, 2000) and integrin αvβ3 has been described to be present on some malignant neuroblastomas (Gladson et al., 1996). In addition, by flow cytometry, our lab consistently observes low levels of integrin αvβ5
on established neuroblastoma cell lines, although whether this is a tissue culture adaptation or reflects actual expression *in situ* remains unclear. Indeed, neuroblasts exhibit significant plasticity, and although integrins may be associated with specific stages of neuroblastoma, or specific developmental states where transformation of the neuroblast initially occurred, an alternative hypothesis is that neuroblastoma may retain the capacity to alter their relative integrin expression, and that this type of plasticity may itself be a malignancy factor.

Neuroblastomas fall into three common morphological/adhesive categories when grown *in vitro*: S (Substrate adherent), N (Neuroblastic), and I (Intermediate) types (Ciccarone et al., 1989; Ross and Spengler, 2007). These different types are sometimes ascribed to a particular cell line, though in many cases a cell line may contain cells of all three types. Studies using tissue culture cell lines have shown that, relative to S-type, N-type neuroblastomas exhibit decreased expression of β1 integrin and greater expression of αvβ3, and are more migratory *in vitro*. However, the expression of αvβ3 on these cells is still relatively low, at least when one compares with tissues well known to express αvβ3, such as angiogenic endothelium or melanoma. N-type cells also form more colonies in soft agar and are more tumorigenic when implanted in mice than S-type, which are rarely able to form xenograft tumors (Spengler et al., 1997). S-type cells express fibronectin; it is therefore not surprising that they represent the group of neuroblastoma that express α5β1 integrin, the fibronectin receptor (Meyer et al., 2004).

The third type of cells is the ‘intermediate cells.’ Noted as potential ‘cancer stem cells’ as early as 1989 by Ross and colleagues, these cells look like an
intermediate between the N and S types via diverse measures including phase contrast microscopy, intermediate filament expression, tyrosine hydroxylase activity, and norepinephrine uptake (Ciccarone et al., 1989). Consistent with being a tumor stem-like cell (or tumor initiating cell), I-type cells are by far the most tumorigenic in mice and in \textit{in vitro} surrogate assays of tumor formation. Treatment with 13-cis retinoic acid or 5-bromo-2’-deoxyuridine can differentiate I-type cells into N-type or S-type, respectively. Retinoic acid has significant effects on integrins, consistent with changes seen during neuronal differentiation, and can differentiate some neuroblastomas into a benign growth-arrested state (Hadjidaniel and Reynolds, 2010). Clinically, retinoic acid has also been demonstrated to improve event free and overall survival in a long-term follow-up on a large cohort of neuroblastoma patients (Matthay et al., 2009).

2.3 Signaling by integrins

In addition to key roles in cell anchorage and migration, integrin-mediated ligation of the extracellular matrix results in the initiation of signaling events exerting both local and cellular effects. Thus, the extracellular matrix encodes information via the local milieu of cell surface or diffusible factors presented to the cell (Fig. 2.1). Most of these signals have been studied in rigorously defined systems \textit{in vitro} with cell lines, rather than primary \textit{in vivo} investigation.

2.3.1 Integrin ligation promotes the activation of the non-receptor tyrosine kinases FAK and Src
Signaling that follows the ligation of integrins by extracellular matrix components can be studied by introducing suspended neuroblastoma cells to a surface coated with an extracellular matrix component, such as fibronectin. This results in cell attachment and spreading. Concurrent with these events, phosphorylation is observed on cytosolic nonreceptor tyrosine kinases like FAK (tyrosine residue 397) and Src (tyrosine residue 418), which indicate activation of the tyrosine kinases. At least some of this activity is physically present in the integrin associated focal adhesion complex, and these kinases can be co-purified with integrins from this complex.

FAK and Src can associate with each other and with an array of cytosolic adaptor proteins and other effectors. For example, FAK can associate with the cytoskeletal adaptor protein talin, which also binds to integrins. The adhesion of NB7 neuroblastoma cells to fibronectin or collagen has been shown to promote co-association of these molecules together in a complex with the protease calpain. Calpain in turn cleaves talin in a cell-adhesion dependent manner, which facilitates more rapid turn over of the focal adhesion, and promotes neuroblastoma cell migration. The same cleavage is observed in other neuroblastoma cells, including NB5 and NB16, suggesting it may be a conserved pathway (Barbero et al., 2009).

FAK also associates with Grb2 and SoS (Schlaepfer et al., 1994), key regulators of Ras-GTP mediated activation of the Raf/MEK/ERK pathway of MAP kinase signaling. This pathway helps to drive proliferation of the tumor cells, and may account for adhesion-based induction of cyclin E in neuroblastoma (and other) cells (Hulleman et al., 1999). FAK is perhaps best known for its capacity to support and promote integrin-mediated cell migration on an ECM, and performs this function in
neuroblastoma cells as well, although this appears to be integrin specific (Wu et al., 2008). For example, integrin α5β1 activates FAK and uses this kinase for migration, while integrin α4β1 migration is dependent upon the non-receptor kinase Src. Both integrins can bind to a fibronectin substrate, thus the particular integrin ligated can have an impact on the cells' response. Other effects of specific integrin ligation have been reported in non-neuroblastoma cell lines, such as the FAK and α5β1-induced expression of the pro-survival gene Bcl-2 (Matter and Ruoslahti, 2001). Thus, signals from FAK can play a role in regulating cell survival in an ECM and integrin-dependent manner.

2.3.2 Integrin activation of the phosphoinositide 3’ kinase signaling axis

Integrins stabilized and ligated to correct ECM promote signaling via class I phosphoinositol-3 kinases (PI3K). PI3K’s are a family of lipid bound kinases found at the cell membrane or intracellular endosomes, and can promote cell motility, intracellular trafficking and survival. Among the four class I PI3K’s, neuroblastoma tend to express P110α and p110β, with the latter more likely to be associated with N-Myc expressing tumors. Nonetheless, P110γ and p110δ are also sometimes detected (Spitzenberg et al., 2010). Activation of the PI3K signaling axis promotes malignancy in numerous cancer cell lines and models of human cancer (Osaki et al., 2004). PI3K signaling also enhances turnover of pro-mitochondrial apoptotic proteins like Bad and promotes downstream pro-survival pathways, such as AKT and mTOR (Scott et al., 1998). PTEN, a suppressor of PI3K, is frequently lost in cancer, although studies in neuroblastoma have shown a lesser degrees of loss, in the range of ~5% for
homozygous deletion (Muñoz et al., 2004). Mutations of PI3K that enhance kinase activity have been reported in other cancers (Hafsi et al., 2012), yet they have been proven to be infrequent in neuroblastoma (Dam et al., 2006). Thus, the activity of PI3K appears to frequently depend upon extrinsic regulatory factors, mediated by receptor tyrosine kinases (eg., IGFR-1, ALK) and integrins.

Given the lack of effective therapies for malignant neuroblastoma, it is perhaps not surprising that the PI3K pathway is being pursued for pharmacological intervention (Fulda, 2009). In neuroblastoma, inhibition of PI3K has been demonstrated to decrease migration and survival of tumor cells in vitro, and inhibit tumor growth in vivo (Opel et al., 2011; Peirce et al., 2011). The efficacy of pharmacological PI3K inhibition may be enhanced by combining a pro-drug with an RGDS peptide to target the agent to tumor sites (Peirce et al., 2011). The relative affinity for this linear peptide for integrin, however, is quite low, and it is improbable that enhanced efficacy is due to direct action on integrins, rather, it is likely due to improved pharmacokinetics associated with the targeting peptide.

2.3.3 Interplay between integrins and signature neuroblastoma signaling pathways

N-Myc is a transcription factor normally expressed during early lymphocyte development and in embryonic brain and kidney tissues (Hurlin, 2005), and is critical for survival of neural crest-derived neurons (Sawai et al., 1993). Amplification of greater than ten copies of the MYCN gene has long been recognized as a strong negative prognostic indicator of outcome in neuroblastoma (Brodeur et al., 1984).
Myc interacts with integrins in an antagonistic manner; while N-Myc seems to increase expression of FAK, it has also been shown to down-regulate the expression of integrins such as α3β1 and α1β1 (Judware and Culp, 1995; Judware and Culp, 1997a; Judware and Culp, 1997b; Judware et al., 1995). Transcriptional analysis of the β3 and αv promoters have revealed negative transcriptional regulatory elements in their promoters by the closely related c-Myc (Muñoz et al., 2004), suggesting why αvβ3 is not highly expressed in neuroblastoma relative to other tumors. In fact, the loss of integrin expression may be important for survival in specific circumstances, particularly among tumors that retain intrinsic apoptotic capacity, as discussed below.

ALK is a tyrosine kinase that is expressed largely during development within the nervous system. ALK belongs to the ‘insulin-like tyrosine kinase’ family of receptors that is frequently upregulated or subject to oncogenic mutation in neuroblastoma (Azarova et al., 2011). Signaling by tyrosine kinases generally requires integrin ligation (Schlaepfer and Hunter, 1998), activating downstream targets (such as FAK, Src, PI3K etc.). This suggests that there is an intrinsic requirement for ECM adhesion to permit a tumor to ‘leverage’ amplified ALK. However, mutant forms of ALK also exist, particularly a F1174 mutation that drives neuroblastoma malignancy cooperatively with MYCN. In this case, it is unclear whether integrin-mediated adhesion is actually required for cell proliferation, although it is likely to enhance signaling in keeping with the rationale described above. MYCN also leads to increased expression of a close ALK relative, insulin-like growth factor I receptor (IGF-IR). In this case, crosstalk between IGF-IR and integrins is also observed (Zheng and Clemmons, 1998).
2.3.4 Integrins and cell survival signaling

Cells that lose anchorage for extended periods of time will typically undergo apoptosis. This phenomenon encompasses one aspect of anoikis (gr., homelessness), a phenomenon wherein a cell that finds itself in an inappropriate environment is signaled to undergo apoptosis. However, there is no ‘central cell death pathway’ associated with anoikis, and in fact many different pathways have been validated in the literature. This underscores the critical need for cell adhesion. One anoikis pathway is focused on the activation of caspase-9. Although many neuroblastomas lose expression of one copy of caspase-9 (as many are LOH1p21), this does not appear to impact the capacity of caspase-9 to activate (Teitz et al., 2001). Antagonism of β1 integrins on differentiated neuroblastoma, but not undifferentiated, promotes this apoptotic pathway (Bonfoco et al., 2000).

Integrin-mediated death is an anoikis pathway in which the presence of unligated, or antagonized, integrins on the cell surface promote cell death via the activation of caspase-8. Neuroblastoma avoid this death pathway via several mechanisms. First, the amplification of MYCN can lead to an overall decrease in integrin expression, which lowers the capacity of the pathway to trigger. Secondly, stage III and IV neuroblastoma tend to methylate, delete, or disrupt the caspase-8 gene (Fulda et al., 2006; Teitz et al., 2000), preventing the triggering of the apoptotic pathway, and this results in a survival advantage in vitro and a metastasis advantage in vivo. Finally, neuroblastoma that are seeded as individual cells in an ‘inappropriate’ three dimensional matrix will tend to either die or, within only a couple days, find each other and form small cell clusters. These islands of cells promote their own
survival and can persist, although they are sometimes surrounded by apoptotic bodies as errant progeny try to migrate away from the original cell mass.

Opposing the induction of death by unligated or antagonized integrins, it is worth noting that a cell that has a robust interaction with the ECM is more resistant to certain insults than others, and integrin ligation has been linked to chemo and radiation resistance. Mechanistically, this is likely to result from remodeling of the ECM, combined with transcriptional alterations of survival promoting genes such as Bcl-2 family members, IAPs and others. However, direct effects, such as maturation-inhibiting phosphorylation of procaspase-8, cannot be excluded from contributing to this effect (Cursi et al., 2006; Keller et al., 2010).

2.4 Specific integrins in neuroblastoma progression

2.4.1 Integrin αvβ3

Integrin αvβ3 is the most ‘promiscuous’ member of the integrin family, in that it binds a variety of different RGD conformations, and thus binds to ligands that include vitronectin, fibronectin, fibrinogen, von Willebrand factor and others. Gladson et al. found that αv was present in all tumors they examined regardless of stage. While αvβ1 and αvβ5 heterodimers were found in normal adrenal tissues and ganglioneuroblastomas which exhibit lower levels of dissemination, the αvβ3 integrin was found to be expressed in highly metastatic, undifferentiated neuroblastomas (Gladson et al., 1996). By contrast, we observe only very low levels of integrin αvβ3 on our neuroblastoma specimens relative to melanoma or cultured endothelial cells, which express robust levels of αvβ3. However, it remains possible that the techniques
originally used by Gladson were simply very sensitive and detected this modest but
important level of integrin expression. Indeed, $\alpha v \beta 3$ is, in some systems, a stem cell
marker, and this may reflect the advanced stage and poor prognosis of her positive
cohort.

In addition, on a variety of tumor cells, $\alpha v \beta 3$ expression has been
demonstrated to promote tumor progression by its ability to bind to a wide array of
different ligands, facilitating anchorage and invasion. Integrin $\alpha v \beta 3$ also stimulates
MMP activity, promotes the activation of receptor and non-receptor tyrosine kinases
including src, and the release of growth factors such as TGF that promote tumor
response. This vascularization provides the growing tumor with the nutrients it needs
and brings tumor cells proximal to vessels, which may facilitate invasion and
metastasis. As previously mentioned, $\alpha v \beta 3$ is also expressed on angiogenic endothelial
cells where it promotes cell survival and migration. One study showed that there is
higher $\beta 3$ expression on invasive and metastatic melanomas than on noninvasive
melanomas (Albelda et al., 1990), although the levels demonstrated in these cases
appear to be logarithmically higher than those seen on neuroblastoma cell lines
(Stupack et al., 2006).

2.4.2 Integrin $\alpha 4 \beta 1$ and tumor spread

Integrin $\alpha 4 \beta 1$ is primarily known as a trafficking integrin, as it is present on
most leukocytes. Binding to its ligand VCAM-1, present on activated endothelial cells,
enhances the transendothelial migration of white blood cells into surrounding tissues.
Cancer cells that express $\alpha 4 \beta 1$ acquire this same enhanced trafficking potential and
show increased tumor cell arrest in circulation and increased extravasation and colony formation. $\alpha 4 \beta 1$ may also enhance invasion and metastasis through promotion of angiogenesis and lymphangiogenesis (Garmy-Susini et al., 2005; Garmy-Susini and Varner, 2008). In (Albelda et al., 1990), $\alpha 4 \beta 1$ expression was found on 40% of invasive and metastatic melanomas, although not on non-malignant melanocytes.

It is important to note that, though the expression of $\alpha 4 \beta 1$ can indeed promote extravasation, the overall role of integrin $\alpha 4 \beta 1$ in tumor progression and metastasis is highly controversial and is dependent on the level of expression and the phase of tumor progression. For example, high $\alpha 4 \beta 1$ expression in some primary tumors can enhance homotypic cell-cell adhesion (Qian et al., 1994), preventing cells from breaking away from the tumor and invading into surrounding tissues (Beauvais et al., 1995). In addition, $\alpha 4 \beta 1$ expression can lead to a reduction in MMPs and impair the ability of the cells to degrade the matrix and create a pathway for invasion (Huhtala et al., 1995). If cells do successfully metastasize to distant sites, $\alpha 4 \beta 1$ expression may promote or inhibit metastatic growth depending on the microenvironment.

2.5 Drugs that target integrins

The involvement of integrins in multiple stages of tumor progression makes them attractive therapeutic targets. Inhibition of integrin signaling can be achieved using several approaches including blocking ligand binding, preventing the formation of functional focal adhesion complexes and disrupting integrin association with the cytoskeleton. Because the structure of integrins has been extensively studied and because having an extracellular target eliminates the challenges of intracellular
delivery, the most common approach has been to target the integrin ligand-binding site. This has been accomplished using blocking antibodies, cyclic and ligand-mimicking peptides, small molecule antagonists and disintegrins (Millard et al., 2011) (Table 2.2)

2.5.1 Integrin αv

The primary rationale for targeting integrin αvβ3 in cancer is to reduce primary tumor growth and metastasis via nutrient deprivation due to inhibition of tumor angiogenesis. Several αvβ3 antagonists have gone to clinical trials with the most notable being cilengitide. Cilengitide is a cyclic peptide containing the RGD integrin-binding motif. It inhibits both αvβ3 and αvβ5. Cilengitide produces both anti-angiogenic and anti-tumor effects through inhibition of VEGF stimulation and FAK-Src and Erk signaling, respectively (Oliveira-Ferrer et al., 2008). In vitro, cilengitide reduces cell growth and survival and inhibits endothelial and tumor cell migration. In clinical trials, cilengitide has been evaluated as a single agent and in combination with radiation, DNA-alkylating agents and gemcitibine. Importantly, cilengitide in combination with radiotherapy and temozolomide (a DNA-alkylating agent) has reached phase III trials in glioblastoma multiforme patients. Other small molecule antagonists are in development for noncancer indications.

2.5.2 Integrin α4

The integrin α4 subunit is predominantly expressed in lymphocytes and leukocytes and supports endothelial transmigration of these cells via binding to
VCAM-1. Consequently, α4 is important for immune function and has been targeted in diseases such as multiple sclerosis (MS), Crohn’s disease and asthma that are characterized by excessive inflammation or an improper immune response. Natalizumab, the only FDA approved α4 antagonist, is a humanized mouse monoclonal antibody that binds both α4 heterodimers. The use of natalizumab was successful in clinical trials in MS (Dalton et al., 2004; Havrdova et al., 2009) and Crohn’s disease (Targan et al., 2007) with the exception of rare cases of progressive multi-focal leukoencephalopathy (PML) caused by reactivation of latent JC virus associated with immunosuppression (Lindå et al., 2009). Unfortunately, this side effect was detrimental enough to lead to limitation of the use of natalizumab to patients who are unresponsive to other treatments. Other α4 antagonists under clinical evaluation include MLN-00002 (human α4β7 antibody), firategrast and IVL745 (small molecules) (Millard et al., 2011). Though the rationale for the use of most α4 antagonists is to reduce excessive infiltration of immune cells, these therapies have the potential for use against cancer cells that exploit α4 for tumor cell extravasation. The success of targeting α4 in cancer will depend on the ability to minimize immunosuppression or to indirectly impair α4 function via downstream targets.

2.5.3 Integrin αIIbβ3

Integrin αIIbβ3 is also a frequently targeted integrin. This heterodimer is expressed selectively on platelets and megakaryocytes and is mostly known for its role in blood coagulation. Antagonists of this receptor are primarily employed in diseases such as stroke, sickle cell anemia and acute coronary syndromes (Millard et al., 2011).
2.6 Summary and considerations

Integrins are a unique group of receptors that provide anchorage, mediate cell migration and invasion, and signal via cell survival and proliferation pathways. Aptly named, integrins integrate extracellular cues with intracellular signaling and serve to regulate many cellular processes that are mediated by other receptors, such as receptor tyrosine kinases. The importance of integrins in cancer development of the nervous system is well established; it seems inevitable therefore that they play a major role in neuroblastoma progression. In fact, integrin expression has been linked to malignancy in neuroblastoma, possibly due to alterations in invasiveness and the ability to evade cell death in foreign tissue environments. Aggressive disease may modulate integrin expression (i.e. N-Myc).

Targeting integrins has shown great clinical promise. By inhibiting ligand binding, many antagonists successfully disrupt cellular connections to the extracellular environment and pro-survival pathways that are necessary for tumor progression. As we continue to learn more about the downstream signaling activity of integrin receptors, we can also explore more therapeutic avenues against these targets, attacking the problem from both sides. However, the logical use of integrin antagonists in complex, multi-agent regimens is lacking. Given the synergy of integrins with signaling through receptor tyrosine kinases and in the induction of susceptibility to apoptosis, this is where one would suspect that these relatively non-toxic agents would have their greatest impact.

Though clinical studies of integrin-targeted drugs in neuroblastoma have not been performed, *in vitro* antagonism has been shown to decrease cell survival,
migration and invasion. Despite these characteristics, integrin-targeted drugs are well tolerated. Given that current treatment for neuroblastoma still has a significant failure rate, the addition of new, low toxicity adjuncts to current treatment regimens seems a logical step forward. In the future, an increased understanding of the roles of specific integrins in neuroblastoma has the potential to provide better prognostic information regarding disease course, while targeting integrins, perhaps in combination with other targeted therapies as a cocktail addition to standard chemotherapy approaches, may lead to increased effectiveness in managing this disease.

2.7 Acknowledgements

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2.8 Figures and tables

Table 2.1: Effects of integrin deletion in murine models

<table>
<thead>
<tr>
<th>Integrin subunit</th>
<th>Genetic Defect (KO)</th>
<th>Expressed in NB</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1</td>
<td>Viable</td>
<td>Yes</td>
<td>Normal; (Gardner et al., 1996)</td>
</tr>
<tr>
<td>α2</td>
<td>Viable</td>
<td>Yes</td>
<td>Abnormal mammary branching morphogenesis; (Chen et al., 2002)</td>
</tr>
<tr>
<td>α3</td>
<td>Perinatal lethality</td>
<td>Yes</td>
<td>Abnormal kidneys; (Kreidberg et al., 1996)</td>
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<tr>
<td>α4</td>
<td>Lethal, by E14.5</td>
<td>Yes</td>
<td>Abnormal placenta and heart formation; (Yang et al., 1995)</td>
</tr>
<tr>
<td>α5</td>
<td>Lethal, E11</td>
<td>Yes</td>
<td>Abnormal mesoderm morphogenesis; (Yang et al., 1993)</td>
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<tr>
<td>α6</td>
<td>Perinatal lethality</td>
<td>Yes</td>
<td>Skin blistering; (Georges-Labouesse et al., 1996)</td>
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<tr>
<td>α7</td>
<td>Viable</td>
<td>Yes</td>
<td>Muscular dystrophy; (Mayer et al., 1997)</td>
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<tr>
<td>α8</td>
<td>Perinatal lethality</td>
<td>No*</td>
<td>Abnormal kidneys and lungs; (Fässler et al., 1996; Hartner et al., 2002)</td>
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<td>α9</td>
<td>Perinatal lethality</td>
<td>No</td>
<td>Bilateral chylothorax; (Huang et al., 2000b)</td>
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<tr>
<td>α10</td>
<td>Viable</td>
<td>No</td>
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<td>α11</td>
<td>Viable</td>
<td>No</td>
<td>Dwarfism; (Srichai and Zent, 2010)</td>
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<tr>
<td>αL</td>
<td>Viable</td>
<td>No</td>
<td>Impaired leukocyte recruitment; (Srichai and Zent, 2010)</td>
</tr>
<tr>
<td>αM</td>
<td>Viable</td>
<td>No</td>
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<td>αE</td>
<td>Viable</td>
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<tr>
<td>αIIb</td>
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<td>Impaired platelet aggregation; (Srichai and Zent, 2010)</td>
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<td>β1</td>
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<td>Abnormal mesoderm morphogenesis; (Fässler and Meyer, 1995)</td>
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<tr>
<td>β2</td>
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<td>No</td>
<td>Impaired leukocyte recruitment; (Wilson et al., 1993)</td>
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<td>β3</td>
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<td>Yes</td>
<td>Glanzmann’s thrombasthenia; osteosclerotic; (McHugh et al., 2000)</td>
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<td>β4</td>
<td>Perinatal lethality</td>
<td>No</td>
<td>Skin blistering; (Dowling et al., 1996)</td>
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<tr>
<td>β5</td>
<td>Viable</td>
<td>Yes</td>
<td>No apparent phenotype; (Huang et al., 2000a)</td>
</tr>
<tr>
<td>β6</td>
<td>Viable</td>
<td>No</td>
<td>Macrophage infiltration in skin and lungs; (Huang et al., 1996)</td>
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<tr>
<td>β7</td>
<td>Viable</td>
<td>No</td>
<td>No gut-associated lymphoid tissue; (Wagner et al., 1996)</td>
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<td>β8</td>
<td>Lethal, E12 - birth</td>
<td>No*</td>
<td>Abnormal placenta; defects in neurovascular homeostasis; (Mobley et al., 2009)</td>
</tr>
</tbody>
</table>

*Subunit found on neural crest cells but not yet reported on NB tumor cells
The continued survival of a single cell and its progeny in the wrong environment can disrupt the homeostasis of the tissue that contains them. Thus, the impetus of individual cells to live or die is critical for the continued homeostasis of an organism. Recognition of compatible ECM promotes stable ligation and clustering of integrins, as well as assembly of the heterogeneous and dynamic focal adhesion complex. Signaling from integrins and focal adhesion-associated receptor tyrosine kinases (RTK) leads to downstream pro-survival signaling pathways such as the PI3K/AKT and Erk axes. By contrast, the presence of an incompatible ECM or of unligated or antagonized integrins promotes cell death via anoikis pathways, including integrin-mediated death. N-Myc exerts pleiotropic effects via transcription (or inhibition thereof) of many downstream genes, enhancing proliferation and survival, and attenuating the expression of integrins, and therefore decreasing anoikis signaling.
### Table 2.2: Drugs that target integrins

<table>
<thead>
<tr>
<th>Target</th>
<th>Antagonist</th>
<th>Type</th>
<th>Clinical Development</th>
</tr>
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Abbreviations: PCI= percutaneous coronary intervention; GBM = glioblastoma multiforme; SCCHN = small cell carcinoma of the head and neck; NSCLC = non-small cell lung carcinoma
2.9 References


CHAPTER 3:

Integrin α4 enhances metastasis and is associated with poor prognosis in MYCN\textsuperscript{low} neuroblastoma.
3.1 Abstract

High-risk neuroblastoma is associated with an overall survival rate of 30-50%. Neuroblastoma-expressed cell adhesion receptors of the integrin family impact cell adhesion, migration, proliferation and survival. Integrin α4 is essential for neural crest cell motility during development, is highly expressed on leukocytes, and is critical for transendothelial migration. Thus, cancer cells that express this receptor may exhibit increased metastatic potential. We show that α4 expression in human and murine neuroblastoma cell lines selectively enhances in vitro interaction with the alternatively spliced connecting segment 1 of fibronectin, as well as vascular cell adhesion molecule-1 and increases migration. Integrin α4 expression enhanced experimental metastasis in a syngeneic tumor model, reconstituting a pattern of organ involvement similar to that seen in patients. Accordingly, antagonism of integrin α4 blocked metastasis, suggesting adhesive function of the integrin is required. However, adhesive function was not sufficient, as mutants of integrin α4 that conserved the matrix-adhesive and promigratory function in vitro were compromised in their metastatic capacity in vivo. Clinically, integrin α4 is selectively associated with poor prognosis in non-MYCN amplified neuroblastoma. These results reveal an unexpected role for integrin α4 in neuroblastoma dissemination and identify α4 as a prognostic indicator and potential therapeutic target.

3.2 Introduction

Neuroblastoma (NB) is a highly metastatic childhood cancer that accounts for up to 15% of deaths among pediatric cancer patients (Ishola and Chung, 2007). This
malignancy most commonly develops sporadically in the adrenal medulla from neuroectodermal cells of neural crest cell origin and spreads to the bone marrow/bone, liver, and lymph nodes (DuBois et al., 1999). Approximately 70% of NB patients present with disseminated disease at the time of diagnosis (Ara and DeClerck, 2006; DuBois et al., 1999). NB patients with aggressive disease have an overall survival rate of 30-50%. Amplification of the MYCN oncogene is perhaps the strongest indicator of an aggressive tumor, however, for tumors without MYCN amplification, such as stage IV disease in children older than 18 months, markers are poor (Brodeur and Maris, 2002; Lode et al., 1997; Matthay et al., 1999). Thus, identification of key players in NB metastasis is critical for more effective management of this disease.

Metastasis is an extremely complex multi-step process that requires tumor cells to invade local tissues, access and survive in circulation, exit the circulation at a distant site, and adapt and grow in a new tissue microenvironment. This requires orchestrated actions of a variety of cell surface molecules. Notably, the integrin family of cell surface adhesion receptors is involved in each step of this process and is an important determinant of metastatic ability (Varner and Cheresh, 1996). Integrins facilitate binding to the extracellular matrix and other cell surface proteins and translate mechanical and chemical cues into intracellular signals that regulate cell behavior. To date, 18 α and 8 β subunits have been identified and are expressed as 24 unique α-β heterodimers (Hynes, 2002). Each heterodimer can have a distinct impact on cancer progression that is dependent upon a variety of cellular, molecular, and microenvironment-related factors (Varner and Cheresh, 1996).
Among these, integrin α4 is a 150 kilodalton subunit that constitutes the α4β1 and α4β7 heterodimers. Integrin α4 facilitates the extravasation of leukocytes from circulation into surrounding tissues via binding to its endothelial ligand, vascular cell adhesion molecule-1 (VCAM-1) (Liu et al., 2000; Rose et al., 2002). Integrin α4 also binds surface-exposed alternatively spliced forms of fibronectin (CS1). During development, α4 is important for neural crest cell motility (Kil et al., 1998; Testaz and Duband, 2001). In transformed neural crest-derived cells, α4 expression may enhance NB adhesion to endothelium, leading to transendothelial migration and increasing NB metastatic potential. In addition, α4 expression promotes leukocyte proliferation and survival and may function similarly in other cell types such as NB (Hayashida et al., 2000; Koopman et al., 1994).

The role of integrin α4 in cancer is controversial and is dependent on expression levels, cellular context, and stage of tumor progression (Holzmann et al., 1998). However, there is evidence to suggest that integrin α4 plays a significant role in NB malignancy. NB cells that express α4 demonstrate enhanced cell motility on select substrates in vitro, and α4 expression was associated with increased tumor stage in a small sample of clinical cases (Wu et al., 2008). Thus, the role of α4 in NB metastasis is implied but has not been validated in vivo. Signaling mechanisms downstream of α4 are also unclear.

Our studies here show that α4 expression enhanced experimental NB metastasis, reconstituting a pattern of organ involvement similar to that seen in patients. In addition, we show that α4-mediated malignancy is dependent upon the presence of the α4 cytoplasmic tail. Importantly, integrin α4 tended to be expressed
more frequently in primary tumor samples from patients lacking MYCN amplification and is associated with poor prognosis in these patients. These results implicate integrin α4 as a target for the control of NB metastasis. Clinical approaches to target α4 in inflammatory diseases might therefore be considered for this aggressive malignancy (Dalton et al., 2004; Havrdova et al., 2009; Targan et al., 2007).

3.3 Materials and methods

Ethics Statement

The Institutional Animal Care and Use Committee of the University of California San Diego (UCSD) has approved all animal studies (Protocol #S05356). The UCSD Institutional Review Board has approved use of all human tissue samples used (071729).

Antibodies and reagents

Mouse anti-human α4 (P1H4), anti-β1 (P4C10), anti-β5 (P1F6), anti-Src (GD11), and anti-phosphotyrosine (4G10) antibodies were purchased from Millipore. Mouse anti-human integrin α1 (FB12), α2 (P1E6), α5, and α6 antibodies were from Chemicon International. Anti-integrin β7 (FIB504) and anti-FAK antibodies were from BD. Anti-pFAK [Y397] was from Invitrogen and anti-pSrc [Y416] was from Cell Signaling. The production of the GST-CS1 fibronectin has been previously described (Hsia et al., 2005). Unless otherwise noted, all other reagents were from Sigma.
**Human tissues and Cell lines**

De-identified human neuroblastoma primary tumor samples were obtained under IRB approval (protocol 071729). Human NB5 and NB8 cells and murine C1300 cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 1% MEM non-essential amino acids, 1mM sodium pyruvate, and 1% antibiotic-antimycotic solution from Mediatech. HEK-293T cells were cultured in Hi-Glucose DMEM (Mediatech) and supplemented with 10% FBS and 1% MEM non-essential amino acids. All cell lines were grown in a 37°C incubator containing 5% CO₂ and a humidified atmosphere.

**Integrin α4 constructs, lentivirus production and establishment of stably expressing cell lines**

Enhanced green fluorescent protein (eGFP), α4-GFP, and α4 Δcyto-GFP (Pinco et al., 2002) were shuttled into pCDH lentiviral constructs. The chimeric α4-GFP fusion construct contains a human extracellular domain and a mouse intracellular domain (See Fig. 3.2A). Constructs were verified by DNA sequencing. Integrin α4 constructs lacking the GFP fusion were developed by inserting (via QuikChange PCR) a stop codon immediately after the termination of the α4 sequence.

For lentivirus production, HEK-293T cells were transfected with a pCDH1 eGFP or an integrin α4 construct and CMV-VSVG envelope vector, RSV-Rev, and pMDL g/p RRE using Lipofectamine 2000 (Invitrogen). Lentiviral particles were harvested 48 hours after transfection. Target cells were transduced in the presence of 8
ug/ml polybrene (Sigma). Expression of GFP, or integrin α4 constructs was verified via flow cytometry.

*Flow cytometry*

To examine or isolate cells stably expressing integrin α4 constructs, cells were placed in blocking buffer (2% BSA-PBS) for 15-30 min followed by incubation with 5 ug/ml anti-α4 (P1H4) or primary antibody for 30-45 min on ice. Cells were washed with blocking buffer and incubated with 1 ug/ml secondary antibody (Alexa Fluor 647, Invitrogen) for 30 min on ice. Cells were washed, resuspended in PBS and analyzed/sorted for α4 and GFP expression.

*Cell proliferation and soft agar growth*

To measure cell proliferation, cells were seeded in a 6-well tissue culture-treated plate and incubated at 37°C for up to 6 days. At various time points, cell number and viability (trypan blue exclusion) was assessed using the Vi-Cell XR (Beckman Coulter). For soft agar assays, 48-well dishes were coated with a 0.5% bottom layer of Difco Agar Noble (BD Biosciences) in complete RPMI and allowed to solidify. Cells were then seeded in 0.3% agar-RPMI. Once solidified, complete RPMI was added to the top of each well. Growth was monitored daily. For quantification, colonies were stained with 0.005% crystal violet in methanol, imaged and counted.

*Cell adhesion and migration*
For cell adhesion, 48-well non-tissue culture treated plates were coated with 150 µL of PBS or substrate overnight at 4°C. Plates were blocked with 2.5% BSA for 1 hour at 37°C. Cells were trypsinized and treated with a 1:1 ratio of trypsin neutralizing solution (Lonza) and resuspended in adhesion medium (serum-free DMEM). Cells were held in suspension for 30 minutes in a 37°C water bath. 1 x 10^5 cells in 200 µL were seeded per well in triplicate and allowed to adhere for 30 minutes at 37°C. Wells were washed gently with adhesion medium to remove unattached cells. Cells were then stained for 10 minutes with 0.1% crystal violet in methanol and washed with water. The crystal violet was then reabsorbed with 100 µL of methanol and transferred to a 96-well plate for analysis. Absorbance was measured at 600nm using a spectrophotometer (BioTek).

For haptotaxis cell migration assays, cells were starved for 16-18 hours in 0.5% FBS in DMEM. Following trypsinization and quenching with TNS, 1 x 10^5 cells in 300 µL of migration medium (0.5% BSA-DMEM) were seeded into transwells (6.5mm diameter, 8um pore size; Fisher Scientific) coated on the bottom side of the membrane with substrate. Cells were pre-incubated with 10 ug/ml antibody where indicated. Cells were allowed to migrate for 3hrs at 37°C. Cells were then fixed with 0.1% crystal violet in methanol and all cells on the upper side of the filter were removed. Migration was quantified via spectrometry of reabsorbed crystal violet or by counting the cells using light microscopy.

For wound healing studies, 24-well non-TC plates were coated with 300 µL of the desired substrate over night at 4°C. Plates were blocked for 1 hr in 2.5% BSA then cells were seeded at confluence for 3hrs in 10% FBS complete RPMI. A wound was
created in each well using a 10 µL pipette tip. Wells were washed and cells were allowed to migrate in 1% FBS RPMI in a heated chamber supplemented with CO₂. Timelapse microscopy on the Olympus IX51 was used to capture migration every 20 minutes for 18 hours. Migration was quantified by analyzing the pixel area of the initial and final wound (Adobe Photoshop).

In vivo cell lodging

For sensitive detection, C1300 eGFP or α4-GFP cells were stained in suspension with CellTracker Red CMPTX (Molecular Probes) according to the manufacturer’s instructions. 1 x 10⁶ cells were injected into the tail vein of A/J mice. Tissues were harvested 24, 48, and 72 hours after injection. Livers were gently minced with a razor blade and flattened between slides. Cells arrested in the liver were visualized using the OV-100 imaging system. Livers from mice injected with unstained cells were used as a control. Quantification of cellular arrest (area of fluorescence) in the liver was performed using the thresholding and measure functions in ImageJ.

Animal Studies

Syngeneic A/J mice (Jackson Labs) were used for C1300 in vivo studies. For tail vein injections, 1x10⁶ cells were injected into 8-week-old mice in 100µL saline. Cells were pre-incubated in anti-α4 (P1H4) or IgG control antibody where indicated. Mice were sacrificed after 18-24 days and examined for metastatic lesions. Macroscopic liver lesions were counted after Bouin’s staining. For subcutaneous
studies, 0.5-1x10^6 cells were injected into the flank region in 50 - 100µL saline. Tumors were harvested after 10-14 days. For adrenal gland injections, mice were anesthetized with isofluorane (Med-Vet International). Buprenorphine was administered as a pre- and post-operative analgesic. The surgical area was depilated and disinfected. 2 x 10^5 cells in 2 µL saline were injected directly into the adrenal gland through a small suprarenal incision. The peritoneum was sutured closed and the skin was closed with wound clips. Mice were monitored for 2 weeks then euthanized and examined for tumors.

**Immunohistochemistry (IHC)**

For IHC on human primary neuroblastoma samples, tissue sections were cut from blocks of formalin-fixed paraffin tumor tissue. Four-micron tissue sections were stained with an antibody to integrin alpha 4 (1:150; Cell Signaling) or CD45 (1:250; Cell Marque). Antigen retrieval was performed using Cell Conditioning 1 (CC1) for 48 minutes at 95°C. Primary antibody was visualized using DAB as a chromagen using the UltraMap system (Ventana Medical systems) followed by hematoxylin as a counterstain. Slides were rinsed, dehydrated through alcohol and xylene and coverslipped.

For IHC on tissues from animal studies, sections from tissues frozen in OCT medium were fixed in ice-cold acetone for 10 minutes, rehydrated for 5 minutes in wash buffer (0.5% BSA-PBS) and blocked for 15 – 30 minutes in 1.25% normal goat serum (NGS) in PBS. Tissues were washed and incubated with an anti-α4 antibody (1:200; Millipore) overnight followed by washing and incubation with a fluorophore-
conjugated secondary antibody for 30 minutes. Following the final washes, tissues were coverslipped using Dako Fluorescent Mounting Medium. Hematoxylin and eosin (H&E) staining was performed by the UCSD Moores Cancer Center Histology & Immunohistochemistry Core. For H&E sections, tissues were fixed in 10% zinc formalin (Fisher). Following deparaffinization with xylene and rehydration, tissues were stained with Gill II Hematoxylin and eosin (Surgipath). Slides were then rinsed, dehydrated through alcohol and Citrisolv (Fisher) and coverslipped.

**Cell lysis and immunoblotting**

For immunoblotting, cells were starved in suspension in serum-free DMEM for 2hrs (with agitation every 20 minutes), replated on the indicated substrate and allowed to adhere for the indicated time period or held in suspension on ice. Cells were lysed using radioimmunoprecipitation assay (RIPA) buffer composed of 100mM Tris, 150mM NaCl, 1mM EDTA, 1% Deoxycholic Acid, 1% Triton-X, 0.1% SDS, 50mM NaF, 1mM sodium orthovanadate, and complete protease inhibitor cocktail (Roche). When necessary, PVDF membranes were stripped using 0.2M NaOH and reprobed. Quantification of blots was achieved by analyzing the density of protein bands using the analyze gels function in ImageJ.

**Database Analyses**

Expression array data were evaluated using the R2: Genomics analysis and visualization platform ([http://r2.amc.nl](http://r2.amc.nl)) developed within the Department of Oncogenomics of the Academic Medical Center (AMC) in Amsterdam, the
Netherlands. The Seeger dataset used included gene expression and patient relapse data. The ITGA4 (213416_at) probe was used. The query parameter was relapse-free survival. For the Kaplan-Meier analysis of the Seeger dataset, the cut-off value was 38.2. Raw and Bonferroni p significance values were calculated via the website interface.

Statistical Analysis

Unless otherwise noted, values for adhesion, haptotaxis, and proliferation are means ± standard deviation (s.d.) from a representative experiment performed at least three times in triplicate. Values for soft agar growth assays are means ± s.d. from a representative experiment performed at least two times with 6 replicates each. Differences between groups were assessed via a two-tailed Student’s t-test for all in vitro assays and via the Wilcoxon Rank Sum Test/Mann Whitney U test for in vivo assays. P<0.05 was set as the level of statistical significance.

3.4 Results

3.4.1 Integrin α4 is associated with poor prognosis in non-MYCN amplified patients

We previously demonstrated that cultured neuroblastoma cell lines quite commonly express integrin α4, which can contribute to signaling via non-receptor tyrosine kinases following ligation of fibronectin in MYCN-amplified tumor cell lines, such as NB8 (Wu et al., 2008). To begin to evaluate the incidence of integrin α4 expression in tumors, we sought to analyze α4 expression in a panel of clinical
samples. Over the course of 2 years, we performed troubleshooting of staining protocols using multiple integrin α4 antibodies (P1H4, Millipore; H210, Santa Cruz; anti-α4, ProSci-Inc.) and fluorescent and chromogenic secondary antibodies with no success. These difficulties were primarily attributed to the poor quality of α4 antibodies available for staining paraffin sections. We tested various conditions for antigen retrieval via citrate buffer. However, the α4 signal remained weak and inconsistent. To overcome this obstacle, we located a new α4 antibody and collaborated with the UCSD Human Tissue Technology Core (Tissue Core), which tested a matrix of conditions and employed automated staining using Ventana Medical Systems multiplex equipment. Staining was moderately weak, but was considered consistent and interpretable. A panel of 28 clinical samples processed by the Tissue Core was then analyzed for the expression of integrin α4 and the incidence of CD45, a marker of leukocytes. It was important to evaluate serial sections from α4-positive samples stained with anti-CD45 to validate whether tumor cell expression of α4 was present or whether α4 was simply present on leukocytes. Integrin α4 expression in tissues was heterogeneous (Fig. 3.1A), and the incidence of positive cells was somewhat lower in patients than in cell lines. Integrin α4 expression was observed on nearly 50% of tumors, but was principally present on inflammatory cells. Nonetheless, there was putative tumor cell (CD45-, CD49d+) expression in 23% of the α4-positive cases (Fig. 3.1B).

Interestingly, α4 was expressed more frequently in samples from patients lacking MYCN amplification (54.5% vs 16.7% of α4-positive samples) (Fig. 3.1C). In general, the presence of α4 expression on any cell type in the tumor correlated with
worse prognosis in the MYCN\textsuperscript{low} tumors (30\% vs 50\% recurred/deceased). To extend these results, we analyzed α4 gene expression among non-MYCN amplified patients. Kaplan Meier analysis revealed a significantly decreased probability of relapse-free survival among patients expressing high levels of integrin α4 (Fig. 3.1D). While MYCN status is sufficient to predict poor outcome in 20-25\% of primary neuroblastoma (Ishola and Chung, 2007; Kelleher, 2013), those tumors lacking amplification of this oncogene are usually classified into low or intermediate risk groups (Brodeur et al., 1993). The expression of integrin α4 may therefore serve as an additional prognostic marker to further delineate therapeutic regimens for these patients.

3.4.2 Integrin α4 promotes motility of neuroblastoma cells

The negative prognostic value of α4 integrin expression in microarray analysis (Fig. 3.1D) may indicate a robust inflammatory component rather than the tumor component. Moreover, since dorsal root ganglion neurons transiently express integrin α4 during outgrowth (Testaz et al., 1999), the capacity to express integrin α4 on some or all cells in NB tumors may simply indicate tumor plasticity. Thus, it was not immediately clear whether tumoral integrin α4 would provide any cell-intrinsic metastatic advantage.

Therefore, to directly test whether neuroblastoma with low expression of MYCN derive a biological advantage from α4 integrin expression, we established stable cell lines ectopically expressing integrin α4 with a C-terminal GFP-tag in the MYCN\textsuperscript{low} (NB5) NB cell line (Teitz et al., 2000; Tekautz et al., 2006; Thiele, 1998).
Parental NB5 cells lack this integrin. Integrin α4 expression was detected via flow cytometry (Fig. 3.2B). An increase in integrin β1 expression was observed concordant with α4-gfp expression, but no other significant changes associated with α4 reconstitution were observed (Fig. 3.3).

We next undertook functional assays, including adhesion and haptotaxis toward an α4-specific ligand, the connecting segment 1 (CS1) of fibronectin, and to plasma fibronectin (pFN), a ligand that includes both CS1 and arginine-glycine-aspartic acid (RGD) sites bound by other integrins (Fig. 3.2C and 3.2D). Integrin α4 expression enhanced adhesion on, and haptotaxis toward, the CS1 domain. There was no difference in adhesion to pFN, consistent with the expression of other fibronectin-binding integrins on these cells (Fig. 3.3).

To evaluate whether the increased migration was due to the expression of integrin α4, we tested whether antagonism of integrin α4 impacted the ability of α4 to promote haptotaxis. Antibodies to integrin α4 significantly reduced migration of the NB5-α4-GFP cells, while antagonism with antibodies against a non-CS1 binding integrin had no significant effect on cell migration in the modified Boyden chamber assay (Fig. 3.2E).

Haptotaxis is tightly associated with adhesion, and may not reflect the dynamic turnover of complexes necessary for sustained cell migration. Therefore, we also evaluated the effect of α4 expression in a two-dimensional wound closure assay. Cells expressing α4 had significantly faster wound closure on fibronectin (Fig. 3.2F and 3.2G) suggesting that the effects of integrin α4 were extended beyond haptotaxis to sustained migration as well. Importantly, α4 integrin expression had no significant
impact on 2D cell proliferation (Fig. 3.2H), suggesting that proliferation during the migration interval was insufficient to explain the rapidity of wound closure.

Similar results were obtained in a MYCN-amplified tumor cell line (NB8) with endogenous α4 expression (Fig. 3.4). Adhesion and migration on CS1 were significantly reduced in a NB8 subline selected for the α4 negative population (SAN) (Wu et al., 2008) and reconstituted with a GFP control vector (SAN+GFP). However, reconstitution with integrin α4 (SAN+α4-GFP) rescued α4-mediated adhesion and migration, as we have previously shown (16). Together, these results support the concept that integrin α4 expression in human NB cells promotes migration.

3.4.3 Integrin α4 expression does not impact primary tumor growth

To extend these studies to an in vivo mouse model, we established a new cell line that stably expressed the α4-GFP fusion protein (Fig. 3.5A) in C1300 mouse neuroblastoma that lacked endogenous expression of integrin α4 and lack MYCN amplification (Beltinger and Debatin, 2001). Mirroring the results in the human cells, α4 expression promoted adhesion and haptotatic migration of C1300 cells towards the α4 ligand, VCAM-1 (Fig. 3.5B and 3.5C). As in the human system, α4 had no effect on proliferation (Fig. 3.5D).

Although integrin α4 did not impact cell proliferation in vitro, it remained unclear whether it would influence primary NB tumor growth in vivo. To evaluate this, we first tested soft agar colony growth (Fig. 3.6A), and found no difference in C1300 cell survival associated with integrin α4. Then, we injected C1300 cells subcutaneously into the flank of syngeneic A/J mice (Fig. 3.6B). In this case, we
observed no significant differences in tumor growth between the α4-GFP and eGFP controls.

Since the principle ECM component in skin is highly enriched for collagen, a non-ligand for integrin α4, and since we previously documented the negative impact of unligated integrins on NB survival, the subcutaneous model might not reflect the potential advantage offered by expression of the α4 receptor. The most common site of origin for NB tumors is the adrenal gland (Ishola and Chung, 2007). Therefore, control NB or NB cells expressing α4 were seeded into the adrenal gland of A/J mice and orthotopic tumors allowed to develop over two weeks. Perhaps surprisingly, orthotopic tumor growth was observed to be consistent with subcutaneous growth (Fig. 3.6C), validating the observation that integrin α4 expression had no significant impact on primary tumor growth. Importantly, no macroscopic spontaneous metastases were observed in this model. However, it would be challenging to detect metastases after only fourteen days. We did attempt this. Detection of metastases via RT q-PCR from RNA-later stored tissues was attempted using tyrosine hydroxylase transcript as a target (Lode et al., 1997). Despite amplification from cell culture cells, we were unable to detect the presence of cells in other tissues.

3.4.4 Integrin α4 promotes neuroblastoma metastasis

A principle role of integrin α4 is in leukocyte trafficking (Rose et al., 2002). To evaluate the effect of integrin α4 expression directly on NB colonization of distant tissues, we characterized the capacity of tumor cells to form metastases as a function of α4 expression. Initial experimental metastasis studies were attempted using the
human NB5 cells in nude mice. $2 \times 10^6$ cells were injected into the tail vein of 8-week-old nude mice. No microscopic or macroscopic metastases were observed after up to 10 weeks.

However, the syngeneic C1300-A/J model reproduced in mice a common clinical vignette in patients; metastases including lymph nodes and liver (Fig. 3.7A). The probability of lymph node metastases was slightly elevated in tumors expressing $\alpha_4$; moreover, the expression of this integrin was associated with metastases detected in kidney/adrenal regions that were not observed in tumors lacking $\alpha_4$. While there was no significant difference in the overall number of mice presenting with hepatic lesions, the number of individual lesions counted on the livers of mice injected was significantly (four fold) higher (Fig. 3.7B and 3.7C). Together, the results suggest that the expression of $\alpha_4$ significantly promotes metastasis.

We anticipated that the expression of integrin $\alpha_4$ might increase metastasis simply as a byproduct of increased tumor cell enlodgement in the liver capillaries. Examination of the tumor cells present at 24, 48 and 72 hours failed to show a significant difference in the number of tumor cells lodging as a function of $\alpha_4$ integrin expression (Fig. 3.8). Thus, events that occur following cell arrest may account for the success of the $\alpha_4$-expressing NB at colonization of the liver.

### 3.4.5 The $\alpha_4$ cytoplasmic tail is dispensable for adhesion and migration \textit{in vitro}

To begin to address the mechanisms by which $\alpha_4$-expressing cells might show increased metastasis, we evaluated the requirement for the $\alpha_4$ integrin cytoplasmic domain (‘tail’). Integrin tails are essential for a number of integrin-mediated effects...
(Chan et al., 1992; Wegener and Campbell, 2008). C1300 cells stably expressing truncated α4 with a C-terminal GFP tag (α4 Δcyto-GFP) (Fig. 3.9A and 3.9B) attached to α4 substrates similar to cells expressing the wild-type integrin (Fig. 3.9C). Similar results were obtained with NB5 cells expressing GFP-tagged serial truncations of the α4 cytoplasmic domain (Fig. 3.10). Initially, this result was somewhat surprising, and the cause of numerous repeated studies, since it appeared to be in contrast to prior studies in MYCN-amplified NB8 cells. In the case of the NB8 cells, the cytosolic domain of this integrin was found to be critical for mediating cell migration (Wu et al., 2008). Nonetheless, it was previously shown that the presence of a surrogate cytosolic domain could rescue some α4 integrin functions (Kassner et al., 1994), and in this case, the surrogate tail was the GFP tag.

In fact, while our tagged α4 truncation was able to mediate strong adhesion and migration (Fig. 3.9C and 3.9D), these were both compromised in cells expressing truncated α4 lacking the GFP tag fusion (α4 Δcyto), which terminate in a simple KAGFFKR sequence (Fig. 3.9C and data not shown). Similar results were obtained for adhesion and migration of human NB with or without amplified MYCN although migration was somewhat compromised in the MYCN amplified NB8 cells (Fig. 3.4B and 3.4C; Fig. 3.11), consistent with prior observations of MYCN-mediated downregulation of integrin function (van Golen et al., 2003). In our studies, the expression of truncated α4 (α4 Δcyto-GFP) had no effect on cell proliferation (Fig. 3.9E) or clonogenic growth (Fig. 3.9F). Thus, we observed no dominant effect associated with expression of the tagged integrin mutant.
3.4.6 Integrin α4 adhesive function and cytoplasmic tail are critical for α4 integrin-enhanced metastasis

To test whether the matrix adhesive function of the integrin tail was required for metastasis, C1300 α4-GFP cells were pre-incubated with a function-blocking antibody to integrin α4 prior to tail vein injection. Blockade of integrin adhesive function resulted in dramatically decreased metastasis (Figure 3.12A) implying that integrin adhesive function was required. As a complementary approach, we next tested metastasis formation in cells in which integrin function was compromised by deletion of the α4 cytoplasmic domain, or rescued by expression of the GFP tag. The loss of adhesive function in the α4 truncation (α4 Δcyto) was associated with decreased metastasis, as expected (Fig. 3.12B and C). Unexpectedly, the α4 Δcyto-GFP cells in which these functions were rescued were also deficient in metastasis formation (Fig. 3.12C). The results raise the interesting possibility that post-ligand binding events modulated by the alpha integrin tail may influence the eventual success of the tumor.

3.5 Discussion

Given the role of integrin α4 in the arrest, recruitment and extravasation of leukocytes (Alon et al., 1995; Rose et al., 2002), it is perhaps not surprising that its expression might be associated with increased tumor dissemination. However, there is limited data to support this concept, and in fact the amplification of this integrin is not commonly observed in the TCGA database. Except for a small number of uterine, ovarian and prostate tumors, the principle modification of the integrin appears to be
mutation. Nonetheless, the role for $\alpha_4$ as a potentiator of tumor cell metastasis is supported by studies in select cancer models where $\alpha_4$ promotes metastasis (Okahara et al., 1994; Slack-Davis et al., 2009). The reason for this apparent paradox is not clear, but possibly the integrin causes conflicts in cells that do not normally express it.

However, the observation may provide one explanation for the selective expression in MYCN$^{\text{low}}$ tumor cells, since expression of integrins without appropriate ligands may promote apoptosis in MYCN amplified cells (Stupack et al., 2006; van Golen et al., 2003). Nonetheless, like MYCN, the expression of integrin $\alpha_4$ is shown to be an indicator of poor prognosis, and might thus be useful as a target, or a simple diagnostic when choices between more and less aggressive treatment are made.

The mechanism by which integrin $\alpha_4$ promotes metastasis is dependent upon its adhesive function, and provides strong support for the idea that leukocyte mimicry and extravasation are critical factors permitting this integrin to impact neuroblastoma malignancy. Nonetheless, it appears that adhesive function was insufficient, since truncation mutants that were adherent and migrated were nonetheless only slightly more capable of dissemination than completely disabled integrins. The results suggest that the cytoplasmic domain of integrin $\alpha_4$ was critical for tumor metastasis. The reasons for this are not clear, but it is interesting that among the 182 mutations documented in the TCGA database for integrin $\alpha_4$, only 2 fall in the cytosolic domain, and both are semi-conservative changes (D1031N in TCGA-ER-A42K, and F1004L in TCGA-BS-A0UF). Overall, we noted no differences in cell growth in two and three dimensional culture systems, or primary tumors, but only observed differences in metastases. The data support the notion that the membrane proximal sequence
(KAGFFKR), which is commonly used for deletions (Kassner et al., 1995; Kassner et al., 1994) has significant mobility in the membrane (Kassner et al., 1994). The results suggest that chimeras, tagged constructs or point mutants be implemented as preferred probes of integrin function (Liu et al., 1999).

Besides its migratory effects, α4 integrins have roles in cell survival (Haack and Hynes, 2001). For example, α4 can activate critical pathways downstream of integrins, including Src, FAK, Akt and ERK (Wu et al., 2008). We found no deficiencies in activation of these proteins in cells expressing truncated α4 (Fig. 3.13 and data not shown). It is not yet clear if these signaling patterns are representative of 2D, as well as 3D growth, since different signaling patterns and requirements govern 2D versus 3D cell migration and survival (Hung et al., 2013). In other mechanistic studies, examination of cell signaling by full-length versus truncated α4 showed that the α4 tail is important for downstream signaling following integrin ligation. Three proteins of approximately 125, 140, and 180 kDa in size showed a significant difference in tyrosine phosphorylation (Fig. 3.13). Further study is needed to determine the identity of these potential mediators of α4 signaling and whether they play a functional role in α4-mediated metastasis.

Neuroblastoma prognosis frequently depends upon ploidy, loss of heterozygosity, pathology, and MYCN expression. Although MYCN amplification is likely the strongest predictor of disease progression, α4 may serve as a novel marker for patients lacking this primary indicator. Notably, the cell lines used in this study had low or no amplification of MYCN, directly supporting these clinical observations. Our finding that integrin α4 expression in patient tumor samples was indicative of
poor outcome in MYCN negative tumors (including lower grade tumors) provides an additional tool for the group of tumors with the most ambiguous prognosis, and may serve to inform choice of treatment.

3.6 Acknowledgements

The contents of Chapter 3, in part, have been submitted for publication as Young, SA; McCabe, KE; Bartakova, A; Delaney, J; Pizzo, DP; Newbury, RO; Varner, JA; Schlaepfer, DD; Stupack, DG. Integrin α4 enhances metastasis and is associated with poor prognosis in MYCN\textsuperscript{low} neuroblastoma. The dissertation author was the primary investigator and author of this material.
3.7 Figures and tables

**Figure 3.1: Integrin α4 expression is associated with worse prognosis in MYCN<sup>low</sup> NB**

Twenty-eight human primary neuroblastoma samples were stained for integrin α4 (HRP). Serial sections of α4 positive samples were stained for CD45. (A) Example of NB patient samples with tumor cell and leukocyte α4 staining (N13) or leukocyte α4 only (N73) Black arrows indicate α4-positive tumor cells (CD45<sup>-</sup>). (B) Integrin α4 status of human NB samples. (C) For the 28 samples analyzed, patient status was stratified by MYCN amplification and integrin α4 expression then grouped according to their last known disease status. (D) Kaplan-Meier curve of relapse-free survival for non-MYCN amplified patients with high or low α4 gene expression (raw p = 4.4 x 10<sup>-5</sup>; bonf. p = 3.8 x 10<sup>-3</sup>). Analysis was performed using the Seeger dataset from the R2: Genomic analysis and visualization platform.
A

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<tr>
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<tr>
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B

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D

Non-MYCN Amplified Neuoroblastoma

![Graph showing survival probability over follow-up months]
**Figure 3.2: Integrin α4 promotes human NB cell adhesion and migration**

(A) Map of the cytoplasmic domain of the chimeric integrin α4-gfp fusion protein. (B) Flow cytometry analysis of NB5 cells stably expressing a full-length integrin α4-GFP fusion construct (shaded peak) or an eGFP control vector (open peak) (α4 antibody; P1H4). (C) Adhesion to 5 μg/ml GST-CS1 FN (p<0.00001) or pFN after 30 minutes. (D) Transwell haptotaxis to 5 μg/ml GST-CS1 FN for 3 hours (p<0.001). (E) Haptotaxis to GST-CS1 FN of C1300 eGFP and α4-GFP cells pre-treated with 10 μg/ml of antibodies against integrins α4 (P1H4) (p<0.01), β1 (P4C10) (p<0.05) or β5 (P1F6). (F) Scratch wound healing on 5 μg/ml pFN. (G) Quantification of wound closure in E (p<0.001). (H) Proliferation (by live cell count) over 6 days. Values for wound healing migration are means ± s.d. (n=6) for a representative experiment (of three).
Figure 3.3: NB5 integrin expression profile

Flow cytometry analysis of integrin expression on NB5 parental cells or cells stably expressing eGFP or full-length α4-GFP fusion protein.
Figure 3.4: Integrin α4 promotes adhesion and migration in the NB8 model

(A) Flow cytometry analysis of integrin α4 expression (shaded peaks) in human NB8 parental cells or in cells sorted for the α4 negative population (SAN) and stably reconstituted with GFP, α4-GFP, or Δcyto-GFP. Geometric mean intensity of α4 positive cells is shown in parentheses. Open peaks represent the secondary only control. Adhesion (B) and haptotaxis (C) of NB8 cells to 5 μg/ml GST-CS1 FN.
Figure 3.5: Integrin $\alpha 4$ promotes mouse NB cell adhesion and migration

(A) Flow cytometry analysis of C1300 cells stably expressing a full-length integrin $\alpha 4$-GFP fusion construct (shaded peak) or an eGFP control vector (open peak) ($\alpha 4$ antibody; P1H4). (B) Adhesion of cells that were untreated ($p<0.001$) or pre-treated with an anti-$\alpha 4$ antibody (P1H4) to 2 ug/ml recombinant mouse (rm) VCAM-1 after 30 minutes ($p<0.001$). (C) Haptotaxis to 2 ug/ml rm-VCAM-1 after 3 hours ($p<0.01$). (D) Proliferation (by live cell count) over 6 days.
Figure 3.6: Integrin α4 does not drive NB tumor growth

(A) Soft agar colony growth of C1300 eGFP or α4-gfp cells after 4 days. (B) C1300 eGFP or α4-GFP cells were injected subcutaneously (SQ) into 8-week-old A/J mice (n=7). Tumors were harvested and weighed 11 days later. Each filled circle represents one mouse. Gray lines denote the means of each group. (C) C1300 eGFP or α4-GFP cells were injected into the adrenal gland (orthotopic site) of 8-week-old A/J mice (n = 10 eGFP, 8 α4-GFP). After 2 weeks, tissues were harvested and examined for macroscopic tumors.
Figure 3.7: Integrin α4 promotes NB metastasis

(A) C1300 egfp or α4-gfp cells were injected into the tail vein of 8-week-old A/J mice. After 18-24 days, tissues were harvested and examined for macroscopic lesions. (B) The number of macroscopic metastatic liver lesions was assessed by counting after Bouin’s staining (n=10, p<0.05). Gray lines denote the means of each group. (C) Images of Bouin’s, hematoxylin and eosin, or human integrin α4 stained tissues.
**Figure 3.8: Integrin α4 expression does not affect C1300 cellular arrest**

(A) C1300 eGFP or α4-GFP cells were stained with CellTracker Red CMPTX and injected into the tail vein of A/J mice. Tissues were harvested 24, 48, and 72 hours after injection and cells arrested in the liver were visualized using the OV-100 imaging system. (B) Quantification of cellular arrest (area of fluorescence) in the liver (left) using ImageJ.
Figure 3.9: The α4 cytoplasmic tail is dispensable for mouse NB adhesion and migration in vitro

(A) Sequences of full-length and truncated α4 cytoplasmic domains. (B) Flow cytometry analysis of C1300 cells stably expressing full-length or truncated α4 and α4-GFP fusion protein (α4 antibody; P1H4) (shaded peaks) compared to secondary only control (open peaks). C1300 cell adhesion (C) and haptotaxis (D) to 2 ug/ml rmVCAM-1. (C) GFP-fusion restores C1300 cell adhesion to wildtype levels (p<0.0001). (E) C1300 cell proliferation over 5 days. (F) C1300 colony formation in soft agar after 4 days. Values for proliferation are means ± s.d. (n=3) of a representative experiment (of two). Values for the soft agar growth assay are the means ± s.d. (n=6) of a representative experiment.
Figure 3.10: GFP c-terminal fusion functions as a surrogate \( \alpha 4 \) tail in human NB cells

(A) Sequences of the cytoplasmic domains of \( \alpha 4 \)-GFP truncation mutants. Adhesion (B) or haptotaxis (C) of NB5 cells to 5 ug/ml GST-CS1 FN
Figure 3.11: The α4 cytoplasmic tail is dispensable for human NB cell adhesion and migration in vitro

(A) Flow cytometry analysis of NB5 cells stably expressing full-length or truncated α4-GFP fusion protein (α4 antibody; P1H4). Adhesion of NB5 α4-GFP and Δcyto-GFP cells to 5 μg/ml GST-CS1 FN after 30 minutes (p<0.01). (C) Scratch wound healing of NB5 α4-GFP or α4 Δcyto-GFP cells seeded on 5 μg/ml pFN or CS1.
Figure 3.12: The cytoplasmic tail is important for α4-mediated NB metastasis

(A) Number of metastatic liver lesions in mice receiving tail vein injection of C1300 α4-gfp cells pre-treated with 10 ug/ml anti-α4 antibody (P1H4) or control IgG. Tissues were harvested and examined for metastases after 21 days (n=4, p<0.05). 1 x 10^6 C1300 full-length or truncated α4 cells were injected into the tail vein of 8-week-old A/J mice (n = 8 α4-GFP, 6 Δcyto-GFP, 4 Δcyto). Tissues were harvested after 21 days. (B) The number of macroscopic metastatic lesions in tissues harvested. (C) Number of macroscopic liver lesions (p<0.01). Gray lines denote the mean of each group (A, C).
Figure 3.13: The α4 cytoplasmic tail mediates NB downstream signaling

(A, C) Western blotting of C1300 cells replated on 5 ug/ml GST-CS1 FN for the indicated time. (B) Quantification of FAK (top) and Src (bottom) activation as measured by the ratio of the relative density of pFAK (Y397) or pSrc (Y416) to total FAK or Src. (D) Quantification of downstream signaling as measured by the ratio of the relative density of phosphotyrosine (pY) to actin. Quantification values (B,D) represent the mean values from three independent experiments. Error bars are standard error of the mean.
3.8 References


preferentially in childhood neuroblastomas with amplification of MYCN. Nat Med. 6:529-535.


CHAPTER 4:

Integrin α4 in ovarian cancer
4.1 Introduction

The research previously described in this dissertation demonstrates a role for integrin α4 in neuroblastoma metastasis. To examine the effect of tumor-cell α4 expression in another cancer model, I investigated the role of integrin α4 in ovarian cancer. Ovarian cancer is a relatively uncommon cancer with an estimate of approximately 22,000 new cases in 2014 as compared to over 220,000 cases each in breast and lung cancer (Society, 2014). However, ovarian cancer accounts for 5% of cancer deaths in women and has a higher mortality rate than any other cancer of the female reproductive system. The lack of accurate methods for early detection is a major obstacle to effective management of this disease. Over 60% of ovarian cancer patients have distant metastases present at diagnosis resulting in a 5-year survival rate of 30% (Society, 2014).

Epithelial ovarian cancer is a heterogeneous disease that is categorized into two tumor types, type I and type II (Bast et al., 2009; Kurman and Shih Ie, 2010; Lengyel, 2010). Type I tumors include low-grade serous and endometrioid, mucinous and clear cell carcinomas. Type II tumors include high-grade serous and endometrioid, and undifferentiated carcinomas. Type II tumors represent 75% of epithelial ovarian cancers (Kurman and Shih Ie, 2010). The origin of these tumors is controversial. Potential sites of origin include the ovarian surface epithelium, the fallopian tube and the mesothelium lining the peritoneal cavity (Lengyel, 2010). Disease progression is dependent upon tumor type with type I tumors exhibiting slow growth and type II tumors exhibiting aggressive metastatic behavior. Unlike most cancers, which metastasize hematogenously, ovarian cancer primarily spreads by direct extension to
nearby organs and shedding into the abdominal cavity. The steps of ovarian cancer metastasis include an epithelial-to-mesenchymal transition, detachment from the primary tumor, dispersion throughout the peritoneal cavity, adhesion to mesothelial cells lining peritoneal tissues, invasion through the extracellular matrix and a reversion to an epithelial phenotype followed by continued growth of the metastatic nodules (Lengyel, 2010).

Vascular cell adhesion molecule-1 (VCAM-1), a ligand for integrin α4, is preferentially expressed on mesothelial cells of ovarian cancer patients when compared to cancer-free patients (Slack-Davis et al., 2009). Mesothelial cells form the mesothelium, which is a monolayer that lines internal organs and serous cavities (Mutsaers, 2004). Cell attachment to and invasion of the mesothelium are key steps in ovarian cancer metastasis. Studies by Slack-Davis, et al. investigated the role of VCAM-1 in the mesothelial invasion step of ovarian cancer metastasis. These studies demonstrated that anti-VCAM-1 treatment delayed ovarian cancer progression and increased survival in an orthotopic (intraperitoneal) mouse model of ovarian cancer. *In vitro*, function-blocking antibodies against VCAM-1 and integrin α4 reduced mesothelial transmigration of α4-positive SKOV-3 human ovarian cancer cells. This study implicates integrin α4 in ovarian cancer transmesothelial migration and metastasis; however, no ovarian cancer studies directly examine the role of tumor-cell integrin α4 *in vivo*. In addition, it is unclear whether α4 plays a role in other steps of ovarian cancer metastasis.

The studies described here examine the effect of α4 expression on ovarian tumor cell properties *in vitro* and the impact on ovarian tumor growth.
4.2 Materials and methods

**Antibodies and reagents**

Mouse anti-human α4 (P1H4) and anti-β5 (P1F6) antibodies were purchased from Millipore. The production of the GST-CS1 fibronectin has been previously described (Hsia et al., 2005). Rat-tail Collagen I was from BD Biosciences. Unless otherwise noted, all other reagents were from Sigma.

**Cell lines**

Hey human ovarian cancer cells were cultured in low-glucose DMEM (Mediatech) supplemented with 10% fetal bovine serum (FBS), 1% MEM non-essential amino acids, and 1% antibiotic-antimycotic solution from Mediatech. Cells were grown in a 37°C incubator containing 5% CO$_2$ and a humidified atmosphere.

**Integrin α4 constructs, lentivirus production and establishment of stably expressing cell lines**

Enhanced green fluorescent protein (eGFP), and α4-GFP (Pinco et al., 2002) were shuttled into pCDH lentiviral constructs. The integrin α4 construct lacking the GFP fusion was developed by inserting (via QuikChange PCR) a stop codon immediately after the termination of the α4 sequence. Constructs were verified by DNA sequencing.

For lentivirus production, HEK-293T cells were transfected with a pCDH1 eGFP or an integrin α4 construct and CMV-VSVG envelope vector, RSV-Rev, and pMDL g/p RRE using Lipofectamine 2000 (Invitrogen). Lentiviral particles were
harvested 48 hours after transfection. Target cells were transduced in the presence of 8 ug/ml polybrene (Sigma). Expression of GFP or integrin was verified via flow cytometry.

Flow cytometry

To examine or isolate cells stably expressing integrin α4, cells were placed in blocking buffer (2% BSA-PBS) for 15-30 min followed by incubation with 5 ug/ml anti-α4 (P1H4) or primary antibody for 30-45 min on ice. Cells were washed with blocking buffer and incubated with 1 ug/ml secondary antibody (Alexa Fluor 647, Invitrogen) for 30 min on ice. Cells were washed, resuspended in PBS and analyzed/sorted for α4 expression.

Soft agar growth

For soft agar assays, 48-well dishes were coated with a 0.5% bottom layer of Difco Agar Noble (BD Biosciences) in complete RPMI and allowed to solidify. 5 x 10^3 cells were then seeded in 0.3% agar-RPMI. Once solidified, complete RPMI was added to the top of each well. Growth was monitored daily. For quantification, colonies were stained with 0.005% crystal violet in methanol, imaged and analyzed using the ImageJ colony counter.

Cell adhesion and migration

For cell adhesion, 48-well non-tissue culture treated plates were coated with 150 µL of PBS or substrate overnight at 4°C. Plates were blocked with 2.5% BSA for
1 hour at 37°C. Cells were trypsinized and treated with a 1:1 ratio of trypsin neutralizing solution (Lonza) and resuspended in adhesion medium (serum-free DMEM). Cells were held in suspension for 30 minutes in a 37°C water bath. 1 x 10^5 cells in 200 µL were seeded per well in triplicate and allowed to adhere for 30 minutes at 37°C. Wells were washed gently with adhesion medium to remove unattached cells. Cells were then stained for 10 minutes with 0.1% crystal violet in methanol and washed with water. Plates were allowed to dry. The crystal violet was then reabsorbed with 100 µL of methanol and transferred to a 96-well plate for analysis. Absorbance was measured at 600nm using a spectrophotometer (BioTek).

For haptotaxis cell migration assays, cells were starved for 16-18 hours in 0.5% FBS in DMEM. Following trypsinization and quenching with TNS, 1 x 10^5 cells in 300 µL of migration medium (0.5% BSA-DMEM) were seeded into transwells (6.5mm diameter, 8um pore size; Fisher Scientific) coated on the bottom side of the membrane with substrate. Cells were pre-incubated with 10 µg/ml antibody where indicated. Cells were allowed to migrate for 3hrs at 37°C. Cells were then fixed with 0.1% crystal violet in methanol and all cells on the upper side of the filter were removed. Migration was quantified via spectrometry of reabsorbed crystal violet.

Animal Studies

Female nude mice (UCSD Animal Care Program) were used for Hey in vivo studies. For intraperitoneal injections, 2.5x10^6 cells were injected into 10-week-old mice in 800µL saline with or without 250ng of tumor necrosis factor α (TNFα). Mice were sacrificed after 17-19 days and examined for macroscopic tumors.
Statistical Analysis

Unless otherwise noted, values for adhesion, haptotaxis, and soft agar colony growth are means ± standard deviation (s.d.) from a representative experiment performed at least three times in triplicate. Differences between groups were assessed via a two-tailed Student’s t-test for adhesion, migration, and soft agar colony area. The Wilcoxon Rank Sum Test/Mann Whitney U test was used for soft agar colony number and in vivo assays. P<0.05 was set as the level of statistical significance.

4.3 Results

4.3.1 Integrin α4 promotes adhesion and motility in ovarian cancer cells

My previous work identified integrin α4 as a promoter of neuroblastoma metastasis. To begin to evaluate the role of tumor-cell α4 in ovarian cancer, I examined the effect of α4 expression on ovarian cancer cell properties in vitro. I established stable cell lines ectopically expressing integrin α4 (without the C-terminal GFP tag) in the Hey cell line originally isolated from a human serous papillary cystadenocarcinoma of the ovary (Buick et al., 1985) (Fig. 4.1A). Parental Hey cells lack integrin α4. Integrin α4 expression promoted adhesion toward the α4-specific ligand, connecting segment 1 (CS1) of fibronectin, and to plasma fibronectin (pFN), a ligand that includes both CS1 and arginine-glycine-aspartic acid (RGD) sites bound by other integrins (Fig. 4.1B). Incubation of Hey α4 cells with a function-blocking antibody to integrin α4 completely inhibited adhesion to CS1 indicating that adhesion was mediated by α4 expression (Fig. 4.1C). Integrin α4 also enhanced haptotatic migration toward CS1 (Fig. 4.1D). Together, these results support the concept that
integrin α4 expression promotes adhesion and migration of ovarian cancer cells and, therefore, has the potential to enhance mesothelial adhesion and transmigration.

4.3.2 Integrin α4 promotes ovarian cancer growth in vitro

Another critical aspect of ovarian cancer metastasis is growth and survival while suspended in the peritoneal cavity. Expression of unligated integrin α4 could lead to integrin-mediated death in suspended cells (Stupack et al., 2001). Conversely, α4-mediated homotypic cell adhesion may promote survival. To determine the effect of α4 on these properties, I suspended cells in soft agar and examined growth over approximately 2 weeks (Fig. 4.2A). Integrin α4 had no significant impact on colony number but tended to increase colony size (Fig. 4.2B and 4.2C). Additional studies are needed to determine whether this result is due to α4-mediated proliferation, survival or both.

4.3.3 Integrin α4 does not significantly impact ovarian intraperitoneal tumor growth

Integrin α4-enhanced adhesion, migration and colony growth in vitro indicate possible roles for α4 in various steps of ovarian cancer metastasis. To test the effect of α4 expression on intraperitoneal (IP) tumor growth, I injected Hey egfp or α4 cells into the peritoneal cavity of female nude mice and monitored the mice over approximately 3 weeks. Surprisingly, integrin α4 expression did not significantly impact tumor burden. (Fig. 4.3A and 4.3B).
Ovarian cancer is often characterized by the production of pro-inflammatory cytokines by immune cells and the cancer cells themselves (Maccio and Madeddu, 2012; Szlosarek et al., 2006). In melanoma, mice injected with the inflammatory cytokine TNFα increased endothelial VCAM-1 expression and enhanced α4-mediated metastasis (Okahara et al., 1994). To mimic the ovarian cancer inflammatory environment, mice were given intraperitoneal injections of Hey cells in 250ng of TNFα. Despite the presence of this cytokine, α4 had no significant effect on IP tumor growth (Fig. 4.3C and 4.3D). Interestingly, the presence of TNFα tended to reduce tumor burden in the eGFP control group.

4.4 Discussion

Ovarian cancer metastasis is uniquely characterized by the shedding of cells from the primary tumor surface followed by attachment and growth on the mesothelium. Given that VCAM-1 is expressed on the mesothelium, it is reasonable that integrin α4 might play a role in ovarian cancer metastasis. Surprisingly, α4 expression did not significantly impact tumor growth in the Hey intraperitoneal mouse model. Studies verifying endogenous and TNFα-induced VCAM-1 expression on mesothelial tissues are needed to assess whether these results are due to a lack of α4 ligand on these tissues. In addition, since TNFα co-injection tended to decrease tumor burden in Hey eGFP cells, it is possible that α4 expression protects cells from TNFα-induced death or anti-proliferative effects. The Hey cells should be tested for TNFα receptor expression and any direct effects on proliferation and survival.
Figure 4.1: Integrin α4 promotes ovarian cancer cell adhesion and migration

(A) Flow cytometry analysis of Hey cells stably expressing integrin α4 (filled peak) or and eGFP control vector (open peak) (α4 antibody; P1H4). (B) Adhesion of cells to 10 ug/ml substrate after 30 minutes. (C) Adhesion of Hey α4 cells that were untreated or pre-treated with 10 ug/ml of anti-α4 or anti-β5 antibody to CS1 after 30 min. (D) Haptotaxis to CS1 after 3hrs.
Figure 4.2: Integrin α4 promotes growth of ovarian cancer cells *in vitro*

(A) Soft agar colony growth of Hey eGFP or α4 cells after 12 days. Quantification of colony number (B) and size (C). Values are representative of one experiment with three replicates per group.
Hey eGFP or α4 cells were injected into the peritoneal cavity of 10-week old female nude mice (n=7, eGFP; n=8, α4). Tumors were harvested 2.5 weeks later. (A) Average total tumor weight in mice injected with cells in saline only. (B) Histogram of tumor weights in (A). (C) Average tumor weight in mice injected with cells in 250ng of TNFα. (D) Histogram of tumor weights in (C).
4.6 References


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CHAPTER 5:
Conclusions and Discussion
Amplification of the MYCN oncogene is the strongest predictor of neuroblastoma (NB) disease progression and is associated with poor prognosis (Ishola and Chung, 2007; Kelleher, 2013). Patients with MYCN amplification are considered high-risk and receive a complex combination of treatments. Despite a multi-modal treatment approach, patients with high-risk NB (40%) (Suenaga et al., 2009), have an overall survival rate of 30-50% (Society, 2012). Patients lacking MYCN amplification are classified as low- to intermediate risk and have a significantly higher survival rate (80-95%). However, there is still a question as to whether we can predict the 5-20% that do poorly. Currently, there are no strong prognostic indicators for patients lacking MYCN amplification. Identification of prognostic markers for this group of patients will allow for more effective modulation of treatment regimens by indicating the need for aggressive treatment of patients with markers of poor prognosis while sparing patients with a good prognosis the side effects and added concerns associated with aggressive therapy (Society, 2014). In search of additional molecular targets that contribute to or indicate NB malignancy, I examined the role of integrins in NB progression.

Integrins play critical roles in early development, and their dysregulation or inappropriate expression leads to a variety of abnormalities and diseases (See Table 2.1) (Young et al., 2013). Of note, integrin α4 is expressed by neural crest cells and is important for their emigration from the neural tube to areas of the developing sympathetic nervous system (Kil et al., 1998). Integrin α4 is also known for its role in leukocyte transmigration and trafficking which has long served as a paradigm for hematologic metastasis (Rose et al., 2002). It is therefore, perhaps, not surprising that
the expression of $\alpha 4$ has been associated with an aggressive migratory phenotype in other cancers such as melanoma (Edward, 1995; Schadendorf et al., 1993).

Previous studies demonstrated that $\alpha 4$ expression promotes migratory behavior in NB cells in vitro (Wu et al., 2008); however, there was no in vivo work to suggest a bona fide role for $\alpha 4$ in NB progression. I evaluated the clinical significance of $\alpha 4$ by examining its expression in human tumor samples and correlation with patient prognosis and outcome. I used both human (NB5 and NB8) and murine (C1300) NB cell models to test the impact of $\alpha 4$ expression on NB cell behavior in vitro, in order to compare the behavior of these cells to those previously reported and to establish a baseline reference of cellular behavior. NB5 and C1300 cell lines lack endogenous expression of integrin $\alpha 4$. In addition, these cell lines are non-MYCN amplified (C1300) or MYCN-low (NB5) and usually exhibit a low-metastatic phenotype (Barbero et al., 2009; Fowler et al., 1995; Lode et al., 1997). As a counter approach, I studied NB8 cells, which exhibit endogenous $\alpha 4$ expression. Notably, these cells are MYCN-amplified and are more aggressive and metastatic in the chick chorioallantoic membrane metastasis model (Stupack et al., 2006). These complimentary models allowed me to evaluate whether alteration of integrin $\alpha 4$ expression universally affects the malignant properties of NB cells.

To extend this work in vivo, I used the syngeneic mouse C1300 NB model to determine whether $\alpha 4$ expression affects NB tumor growth and metastasis. C1300 neuroblastoma arose from a spontaneous neuronal tumor near the spinal cord of strain A mice (Ziegler et al., 1981). In general, the use of syngeneic models is important because they mimic physiological environments more accurately than
immunocompromised models by accounting for factors such as tumor immunogenicity. Since most immune cells also express α4, it may be important to more accurately mimic a tumor microenvironment that could potentially have [1] competition for common ligands, and [2] the presence of cells with α4 that may well co-express additional ligands for α4, such as fibronectin (McFadden et al., 2011) and osteopontin (Sangaletti et al., 2014).

Mechanistically, I performed studies using truncated integrin α4 constructs and examined α4 signaling pathways to elucidate mechanisms associated with α4-mediated effects. The conclusions and discussion of the work performed are discussed in the following sections.

5.1 Integrin α4 does not impact neuroblastoma proliferation and primary tumor growth

Cell growth and proliferation are regulated, in part, by integrin-mediated adhesion and signaling (Schwartz and Assoian, 2001). However, I found that integrin α4 had no discernable effect on cell proliferation in NB5 and C1300 cells (See Fig. 3.2 and Fig. 3.5). This differs from one study in melanoma, another neuroectodermal-derived tumor type, in which the expression of α4 decreased proliferation but supported metastasis (Rebhun et al., 2010), and from results in lymphoma cells, where, *in vivo*, the expression of this integrin compromises expansion (Bittner et al., 1998). Conversely, a second study in mouse melanoma showed no difference in melanoma proliferation (Qian et al., 1994). Given the inconsistent results, it is possible that the effect of α4 is highly cell specific, or alternatively, that some of the
reports of α4 suppression of proliferation effects may be clonal artifacts. In accord with *in vitro* observations, integrin α4 had no effect on C1300 NB tumor growth in subcutaneous or orthotopic models (See **Fig. 3.6**). In preliminary studies, MYCN-amplified NB8 cells exhibited increased soft agar growth compared to α4-null cells (data not shown). This was not observed in the MYCN-low NB5 and C1300 cells. This effect may be attributed to amplification of MYCN which is known to regulate proliferation and survival (Luscher and Larsson, 1999). Due to time constraints, no *in vivo* studies were performed using NB8 cells.

### 5.2 Integrin α4 promotes neuroblastoma cell migration *in vitro*

Previous studies showed that integrin α4 is expressed in many NB cell lines and promotes migration on select substrates such as CS-1 (Wu et al., 2008). *In vitro*, α4 integrin expression promoted NB cell (NB5 and C1300) adhesion to and migration towards substrate (haptotaxis) (See **Fig. 3.2 and Fig 3.5**). Haptotaxis is tightly associated with adhesion and may not reflect the dynamic turnover of complexes necessary for sustained cell migration. Therefore, I suspected that the impact of integrin α4 expression might differ in a monolayer wound assay. Interestingly, despite similar adhesion to plasma fibronectin (pFN) for α4-GFP and control cells, α4 significantly improved wound closure on this substrate (See **Fig. 3.2**). This enhanced wound healing could be due alterations in the expression of other fibronectin binding integrins. However, when the surface expression of other integrins was examined by flow cytometry, no significant changes were observed following stable expression of α4 (See **Fig. 3.3**). In particular, the expression of fibronectin binding integrin αvβ5
was unchanged and the cells expressed little to none of the other RGD-binding integrins (α5β1 and αvβ3). Alternatively, the increased cellular migration effect is likely due to α4 binding to pFN. Though cellular FN has more universal expression of the IIICS (CS1) regions, this alternatively spliced domain of fibronectin is also typically present in one FN polypeptide in each dimer of pFN, thus permitting α4β1 binding (Mao and Schwarzbauer, 2005). Similar migratory effects were observed in the human NB8 cell line. In agreement, adhesion and migration were significantly reduced in the NB8-derived subline NB8-SAN+GFP, which was developed by repeatedly sorting cells to isolate an α4 integrin-negative population (See Fig. 3.4).

5.3 Integrin α4 promotes neuroblastoma dissemination

An increase in migration in vitro often translates to an enhanced migratory and metastatic phenotype in vivo. In subcutaneous and orthotopic tumor models, no spontaneous metastases were observed. However, this was not unexpected, as other investigators have reported a lack of spontaneous metastases in the C1300 model (Lode et al., 1997). The latter is possibly due to the abbreviated time frame of these (10-14 days) and prior (19 days) experiments, and may not reflect tumor malignancy over a period of months. In contrast, in experimental metastasis (tail vein injection) studies, α4 expression enhanced NB dissemination, and the organ colonization followed a pattern similar to that observed in patients (See Fig. 3.7). Notably, the liver was the most common site of metastasis in the A/J mouse model despite the fact that the lungs are the first major capillary bed encountered by cells following tail vein injection (Ewing, 1928). Both the metastatic incidence and number of lesions on the
lungs were low compared to the liver suggesting that factors intrinsic to the liver facilitate the survival of NB cells during metastasis in mice and in children.

Why the liver is a preferred metastatic site in both human and mouse NB is not completely clear. However, C1300 neuroblastoma is known to elicit an immune response in its host (Ziegler et al., 1979). One theory is that the liver produces immunoregulatory factors that can alternatively increase tumor immunogenicity, leading to the spontaneous progression observed in stage 4S patients, or decrease immunogenicity and thereby foster tumor growth and malignancy (Ziegler et al., 1981). Preferential liver metastasis has also been suggested to occur due to expression of the α4 ligand, VCAM-1. In one large-cell lymphoma study, VCAM-1 was constitutively expressed on hepatic sinusoidal endothelial cells (HSECs). Blockade of VCAM-1 or α4 significantly reduced adhesion of α4-positive RAW117 lymphoma cells to HSECs (Papadimitriou et al., 1999). We detected VCAM-1 expression on the livers of A/J mice. However, the identity of the cell type expressing the VCAM-1 was not determined. Future studies investigating the potential for α4-mediated immune evasion and HSEC VCAM-1 expression in the A/J mouse model may shed light on site-specific development of NB metastasis.

Altogether, these findings demonstrate that α4 expression plays a pro-metastatic role in the stages of NB metastasis following intravasation. The impact of α4 on earlier stages of tumor progression such as invasion, intravasation and survival in circulation was not examined. A melanoma study by Qian et al. found that α4 expression can promote homotypic cell adhesion and impair the ability of cells to detach from the primary tumor and invade surrounding tissues (Qian et al., 1994).
contrast, α4-mediated homotypic cell adhesion may promote formation of tumor cell emboli thereby increasing lodging in the vasculature and metastatic potential. Thus, the overall impact of α4 in NB may depend on the stage of tumor progression that α4 is expressed, the presence of its ligand, VCAM-1, on vascular endothelium and other factors such as immune regulation and evasion.

5.4 **Mechanisms of α4-mediated metastasis**

5.4.1 **Integrin α4-mediated adhesion**

The trafficking of immune cells from the vasculature into surrounding tissues involves a variety of steps including initial tethering to endothelial cells, stable arrest and spreading, and transendothelial migration. Integrin α4 can participate in each of these steps (Alon et al., 1995; Rose et al., 2002). I hypothesized that integrin α4 expression would impact the initial lodging of NB cells. To test this hypothesis, I injected C1300 α4-GFP or eGFP control cells into the tail vein of A/J mice and looked for the presence of tumor cells in the liver after 24-72 hours. Interestingly, there was no difference in lodging of control and α4 cells (See Fig. 3.8). These studies suggest that, *in vivo*, α4 exerts its effect following initial cellular arrest. Tethering and initial adhesive steps in these cells may be mediated by other molecules such as selectins (McEver et al., 1995).

To further examine the role of α4-mediated adhesion in NB metastasis, C1300 α4-GFP cells were pre-treated with an α4 antagonist and injected into the tail vein of mice. The function-blocking antibody used (P1H4) is specific for human α4 as it did not recognize mouse α4 in prior *in vitro* studies. Therefore, any effects observed
should be due to the effect on C1300 cells expressing chimeric integrin α4 with a human extracellular domain. Blockade of α4 significantly reduced metastasis, indicating a requirement for α4 adhesive function (See Fig. 3.12A). Thus, α4 may play a role in later stages of NB adhesion and/or transmigration. Additional in vivo trafficking studies, in which the vasculature is labeled, could be used to directly examine the effect of α4 on NB transendothelial migration.

5.4.2 The role of the α4 cytoplasmic domain

Integrin cytoplasmic domains (tails) are important for integrin-mediated effects including migration, invasion, proliferation and survival (Chan et al., 1992; Wegener and Campbell, 2008). Previous studies by Kassner et al. found that truncation or substitution of the α4 cytoplasmic domain results in a significant reduction in cell migration (chemotaxis and haptotaxis to FN40) when compared to full-length α4 (Kassner et al., 1995). Indeed, cells expressing a complete cytoplasmic truncation (ending in the KAGFFKR sequence, where R is amino acid 974) showed impaired adhesion and migration (See Fig. 3.9C and data not shown). However, using NB cells expressing the α4 cytoplasmic truncation with a C-terminal GFP fusion, I showed, for the first time, that a surrogate integrin tail is sufficient to fully restore both adhesive and migratory abilities (See Fig. 3.9). Similar results were obtained for migration of Hey ovarian cancer cells expressing the α4 truncation with the GFP tag or a random amino acid C-terminal linker (AAAQQA) (data not shown). Thus, the restoration of α4 function is not specific to the GFP tag and any linker or tag of adequate length may
serve as a surrogate tail. Therefore, in cases where the α4 tail is adequately anchored, a functional β1 subunit may be sufficient for migration.

Notably, the GFP-tagged α4 truncation only partially restored adhesion and migration in the MYCN-amplified NB8 cell line (See Fig. 3.4). This result may be attributed to N-Myc-mediated effects on integrin function (van Golen et al., 2003), particularly β1 integrin. N-Myc expression has been shown to compromise β1 integrin expression and binding activity in SHEP NB cells and may also impact function of other integrins (van Golen et al., 2003). If β1 is limiting in the α4 Δcyto-GFP cells, then alterations secondary to MYCN expression would be predicted to have greater impact on these cells.

Mechanistically, truncation of integrin α4 did not affect cell growth and proliferation in the C1300 Δcyto-GFP cell line, suggesting that the expression of the truncation construct may not have exhibited dominant or artifactual effects on these tumor cells.

In *in vivo* studies of the α4 tail, I found that metastasis was significantly reduced in fully truncated α4 Δcyto cells (See Fig. 3.12C). Surprisingly, despite their ability to adhere and migrate *in vitro*, metastasis of Δcyto-GFP cells was only slightly better than cells with the full truncation. Thus, the α4 cytoplasmic domain is required for metastasis. These results highlight the existence of differential requirements for *in vitro* and *in vivo* migration. Studies by Hung et al., late during the course of my studies, demonstrated that α4 integrins may use distinct signaling mechanisms in unconfined (2D) and confined (3D) cell migration and provide some precedent for this. Unconfined migration was more dependent on Rac1 activity whereas confined
migration relied on myosin II (Hung et al., 2013). Thus, the presence of the α4 tail may be more important for regulating myosin II-driven migration. As all of the studies here were of the ‘unconfined’ type, I may have selectively studied the Rac-mediated pathways.

The requirement for the α4 cytoplasmic domain in vivo may well suggest a requirement for the ‘confined’ type of migration. While we did not have the appropriate chips to perform these assays, they may not be necessary. Prior studies in NB8 cells using 3mm pore sizes did demonstrate a requirement for the cytosolic domain of integrin α4. While there is no way to know if this represents ‘confined’ migration, these pores are roughly one third the size of the nucleus, and it is reasonable that this might be considered, in some way, a ‘confined’ form of migration, or at a minimum, one requiring significant mechanical force to deform and translocate the cell nucleus.

Integrin α4 is also implicated in neuronal cell survival and can activate critical downstream pathways including Src, FAK, Akt and ERK (Haack and Hynes, 2001; Wu et al., 2008). Interestingly, cells with truncated α4 tended to have lower FAK activation. However, I found no significant deficiencies in activation of Src or FAK when I replated cells expressing full-length or truncated α4 on CS1 fibronectin (See Fig. 3.13). FAK can impact survival through the PI 3-kinase/Akt pathway (Sonoda et al., 1999). However, no consistent differences were observed for Akt activation. It is not clear whether these signaling patterns (from 0 to 60 minutes) are truly representative of long-term signaling or of 3D growth.
When extending these studies, I found that cells with full-length $\alpha_4$ tended to have higher levels of downstream signaling (indicated by global tyrosine phosphorylation) than cells with truncated $\alpha_4$ (See Fig. 3.13). Notably, three proteins of approximately 125, 140, and 180 kDa in size showed the most striking differences. A plethora of tyrosine phosphorylated proteins fall within this range that include cytosolic effectors (such as p130Cas), neurotrophic tyrosine kinase receptors (such as Trk A, Trk B and Trk C), and other receptor tyrosine kinases (such as insulin growth factor 1 receptor $\alpha$). Further study is needed to determine the identity of these proteins and evaluate whether they truly play a functional role in $\alpha_4$-mediated metastasis.

5.5 Model: Function of integrin $\alpha_4$ in neuroblastoma metastasis

The data presented in this dissertation suggest that integrin $\alpha_4$ promotes the later stages of neuroblastoma metastasis following the entry of cells into the vasculature (Fig. 5.1). Surprisingly, the presence of $\alpha_4$ has no impact on initial cell lodging. However, $\alpha_4$-mediated adhesion is required for metastasis as blockade of $\alpha_4$ ligand binding and disabling $\alpha_4$ adhesive function via cytoplasmic truncation inhibits metastatic outgrowth. Thus, initial lodging and tethering may be mediated by other molecules such as selectins. Stable adhesion to endothelial VCAM-1 may provide a metastatic advantage for $\alpha_4$ expressing cells.

Mechanistically, full-length $\alpha_4$ can bind $\alpha_4$ ligand, is expected to have functional integrin trafficking (internalization and recycling) and can promote downstream signaling. Cells expressing truncated $\alpha_4$ with a GFP C-terminal tag ($\Delta$cyto-GFP) retain adhesive and migratory abilities. In fact, $\Delta$cyto-GFP cells
demonstrated enhanced adhesion in NB5 cells, suggesting that these cells may have a slightly higher basal level of integrin activation. This may be caused by forced separation of the integrin tails by the GFP-tag. Despite the ability to adhere and migrate, metastasis is significantly reduced in these cells. Thus, the α4 cytoplasmic domain is required for metastasis. In addition, the α4 tail is critical for downstream signaling and may affect integrin trafficking. Truncation of the α4 tail may prevent the formation of key signaling complexes that regulate cell proliferation and survival.

Moreover, it may impair integrin trafficking, which affects the ability of cells to move in three-dimensional spaces (Bridgewater et al., 2012). Impaired integrin trafficking can also indirectly affect the trafficking of other cell surface receptors such as growth factor receptors. Complete truncation of the α4 cytoplasmic domain (Δcyto) results in impaired adhesion and migration. Whether the fully truncated α4 subunit maintains a transmembrane topography is not clear. This mutant may be inactive as an artifact of no longer crossing the membrane.

The specific cytoplasmic regions critical for α4-mediated effects remain unclear. It will be important to further clarify the role of the cytoplasmic domain and key α4 signaling partners. In addition, it will be necessary to verify that the mechanisms identified via any in vitro studies are representative of events that occur in a complex 3D cellular microenvironment.

5.6 Integrin α4 in alternative cancer models

In cancer research, it is important to consider whether a finding is a general phenomenon or specific to the cancer type, cell line, or entity examined in the study. I
have shown that integrin α4 promotes NB metastasis. Prior works in other cancer models support this pro-migratory and pro-metastatic role for α4. For example, in melanoma, Schadendorf et al. found that there was a significant increase in α4 expression in metastatic versus primary melanoma (Schadendorf et al., 1993). Several other studies also link α4 expression to melanoma progression (Danen et al., 1994; Edward, 1995; Okahara et al., 1994; Rebhun et al., 2010). In addition, enhanced endothelial transmigration and adhesion to mesothelium due to α4-VCAM-1 interaction supports a role for α4 in ovarian cancer (Slack-Davis et al., 2009).

Besides metastasis, α4 plays a role in chemoresistance. A recent study in ovarian cancer found that an α4 ‘inhibitor’ sensitizes cells to carboplatin treatment (Scalici et al., 2014). This combinatorial treatment increased cell death and reduced doubling time, although no alterations in cell cycle progression were detected. In addition, apoptosis did not appear to be the cause of cell death. Thus, other forms of cell death such as necrosis, ER stress or aberrant autophagy may be involved.

Integrin α4 has also been linked to chemoresistance in leukemia and lymphoma. In leukemia, α4-mediated adhesion to bone marrow mesenchymal stromal cells was associated with increased nuclear factor kappa B (NFκB) activation and increased chemoresistance (Jacamo et al., 2014). A similar mechanism has been linked to chemoresistance in lymphoma (Weekes et al., 2001). Taken together, these studies indicate that the role of integrin α4 in metastasis and chemoresistance is a general phenomenon that applies to a variety of cancer models.
5.7 Integrin α4 as a therapeutic target in cancer

The consistent role of integrin α4 in enhanced migratory and metastatic phenotypes in multiple cancers warrants further investigation of α4 as a therapeutic target. There are a variety of α4 antagonists in development including, antibodies and small molecules (Millard et al., 2011). Currently, natalizumab, a humanized monoclonal antibody, is the only FDA-approved α4 antagonist. Natalizumab has shown efficacy in patients with multiple sclerosis and Crohn’s disease, which are chronic inflammatory conditions (Dalton et al., 2004; Havrdova et al., 2009; Targan et al., 2007). This antibody functions by blocking the physical interactions of T cells with α4’s endothelial ligand, VCAM-1. This blockage prevents leukocyte extravasation resulting in decreased inflammation. Notably, natalizumab functions similarly in *in vitro* cancer cell studies, blocking adhesion of C1300 NB cells to VCAM-1 and CS1 FN (data not shown).

Natalizumab treatment is well tolerated by most patients. However, its use is limited due to the rare occurrence (2.71 in 1,000 patients) of progressive multifocal leukoencephalopathy (PML), a severe side effect of the treatment (Benkert et al., 2012). In addition, a recent study showed that some patients experience more aggressive disease upon cessation of treatment (Benkert et al., 2012). Though natalizumab blocks the functional interaction of T cells and endothelial cells, it also stimulates signaling that leads to an increase in T cells in the peripheral blood. It is unclear what effect, if any, natalizumab might have on cancer progression. Therefore, caution is warranted before simply applying natalizumab to oncological applications. In a pilot study examining the effect of natalizumab on α4-mediated NB metastasis, I
found that treatment with natalizumab tended to increase liver metastases (Fig. 5.2).

My data are consistent with those obtained in a very recent study in ovarian cancer, where treatment of platinum resistant ovarian cancer cell lines with natalizumab alone increased tumor burden compared to the IgG control (Scalici et al., 2014). Interestingly, natalizumab did increase response to carboplatin. This is consistent with observations that increased proliferative signals may render cells more sensitive to chemotherapeutics (Chabner, 2006). It is not clear whether the use of an \( \alpha_4 \) antagonist that inhibits both interaction and signaling (such as P1H4) would be preferable to the use of an \( \alpha_4 \) ‘blocker’ that stimulates signaling (natalizumab) but depends upon combinatorial treatment for long-term success. VCAM-1 blockade might also serve as an alternative to \( \alpha_4 \) inhibition, though VCAM-1 is only one of several \( \alpha_4 \) ligands.

5.8 Integrin \( \alpha_4 \) as a prognostic marker in NB

In addition to its role as a potential therapeutic target in cancer, integrin \( \alpha_4 \) may serve as a prognostic indicator in NB. Amplification of the MYCN oncogene is the strongest predictor of prognosis for NB patients. However, MYCN is only amplified in 25% of neuroblastoma (Ishola and Chung, 2007; Kelleher, 2013). For patients lacking amplification, there is no standard molecular marker used to gauge prognosis. These patients are classified as low- or intermediate-risk based on tumor histology, age, and the degree of tumor excision possible (Brodeur et al., 1993).

Previous studies have identified members of the inhibitor of apoptosis (IAP) family, survivin and livin as prognostic indicators correlated with poor prognosis. However, these and other indicators were often observed in conjunction with MYCN.
amplification (Islam et al., 2000). This work identifies high integrin α4 expression as an indicator of poor prognosis in non-MYCN amplified patients (See Fig. 3.1D). Though tumor cell α4 does enhance NB malignancy, high α4 expression, whether on tumor cells or infiltrating lymphocytes is associated with worse prognosis. Thus, α4 may serve as a tool to better determine treatment regimens for this group of patients.
5.9 Figures and tables

Figure 5.1: An integrated model: Integrin α4 in neuroblastoma metastasis

a) Integrin α4 promotes the later stages of neuroblastoma metastasis. The presence of α4 has no impact on initial cell lodging but promotes cell adhesion and can enhance extravasation through blood vessels that express its ligand, VCAM-1. The α4 cytoplasmic domain is important for metastatic outgrowth as cells with truncated α4 (Δcyto-GFP) have significantly less metastasis. b) Full-length α4 can bind α4 ligand, is expected to have functional integrin trafficking and can promote downstream signaling. c) Integrin α4 Δcyto-GFP cells retain adhesive (and migratory) abilities. However, truncation of the cytoplasmic domain impairs downstream signaling and may affect integrin trafficking. The c-terminal GFP fusion may permit inhibitory proteins to access the β1 tail and affect signaling. d) Complete truncation of the α4 cytoplasmic domain (Δcyto) results in impaired adhesion (and migration).
$1 \times 10^6$ C1300 α4-gfp neuroblastoma cells were injected into the tail vein of A/J mice. Following tumor initiation (7 days), mice were injected (tail vein) with 170 ug of Natalizumab (Nat.) or IgG. Mice were sacrificed after two additional weeks and examined for metastases. (A) The number of macroscopic metastatic liver lesions (n=4). Values represent the mean ± standard error of the mean. (B,C) Adapted from Scalici JM, et al. Nude mice were injected intraperitoneally (IP) with SKOV3ip1Luc (luciferase) cells (n=10). Following tumor initiation (7 days), mice were treated IP with 200 ug of Natalizumab (black) or IgG control (gray) antibody twice weekly for four weeks. (B) Quantification of light emission by luciferase expressing cells in the abdominal cavity after 4 weeks of treatment. Values represent means ± standard deviation. (C) Number of visible tumors in the abdominal cavity. In cases where mice had too many tumors to count, mice were assigned a value of 100.
5.10 References


