THE ROLE OF THE CELL ADHESION MOLECULES
N-CADHERIN, MCAM, AND BETA 3 INTEGRIN IN HUMAN MELANOMA

by

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Melanoma, which accounts for only 4% of all skin cancers, but 75% of skin cancer-related deaths, continues to rise at an alarming rate worldwide. When a melanoma is detected and resected at an early stage, the cure rate for patients is favorable. However, the response rate of patients with metastatic melanoma to chemotherapy is less than 15%, and biological therapies have limited efficacy. Therefore, identification of genes that can serve as therapeutic targets for advanced-stage melanoma is crucial. The cell adhesion molecules N-cadherin, MCAM, and $\beta_3$ integrin have been postulated to represent melanoma progression markers; yet, little is known regarding whether they may constitute valuable therapeutic targets for the disease. Furthermore, no studies conducted to date have examined the expression and function of these three molecules in concert in melanoma. The results of our whole-genome and tissue microarray profiling illustrate N-cadherin, MCAM, and $\beta_3$ integrin expression in the distinct stages of melanoma progression. We demonstrate that N-cadherin and $\beta_3$ integrin are melanoma progression markers, but MCAM is not. Furthermore, greater than 95% of metastatic melanomas analyzed in our study express at least one of the three adhesion molecules, and 50% express all three.

Our next objective was to determine whether inhibition of N-cadherin, MCAM, or $\beta_3$ integrin impairs melanoma cell proliferation, migration, and/or invasion. We hypothesized that due to redundancy in the functions of N-cadherin, MCAM, and $\beta_3$ integrin, simultaneous inhibition of all three molecules may elicit the most effective therapeutic response. We demonstrate that inhibiting expression of N-cadherin, MCAM, or $\beta_3$ integrin decreases melanoma cell proliferation. However, inhibiting their expression in parallel does not augment the anti-proliferative effect. In contrast, downregulation of N-cadherin, MCAM, and $\beta_3$ integrin in parallel inhibits melanoma cell migration and invasion to a significantly greater extent than targeting each gene alone. Our results indicate that of the three adhesion molecules, MCAM and $\beta_3$ integrin play the most pronounced role in migration and invasion, and therefore, in
combination, may represent the most promising therapeutic targets. The data presented in this dissertation provide the foundation for future clinical studies that target adhesion molecules in advanced-stage melanoma patients.
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ABBREVIATIONS

AS       Antisense
bFGF     Basic fibroblast growth factor
BSA      Bovine serum albumin
CAM      Chorioallantoic membrane
CDK      Cyclin-dependent kinase
CMV      Cytomegalovirus
Cy       Cyanine
DAPI     4’,6-diamidino-2-phenylindole
DMEM     Dulbecco’s modified eagle medium
DTIC     Dacarbazine
ECM      Extracellular matrix
FAK      Focal adhesion kinase
FBS      Fetal bovine serum
FGFR-1   Fibroblast growth factor receptor-1
HAV      Histine-Alanine-Valine
HGF      Hepatocyte growth factor
IFNα     Interferon alpha
Ig       Immunoglobulin
IS       In situ
kD       Kilodalton
LN       Lymph node (melanoma-infiltrated)
MAPK     Mitogen-activated protein kinase
MCAM     Melanoma cell adhesion molecule
MGP      Metastatic growth phase
MMP      Matrix metalloproteinase
OPN      Osteopontin
PBS      Phosphate-buffered saline
PCR      Polymerase chain reaction
PDGF     Platelet derived growth factor
RGP      Radial growth phase
RNAi     RNA interference
RSV      Rous sarcoma virus
siRNA    Small interfering RNA
TBS      Tris-buffered saline
TGF      Transforming growth factor
TMA      Tissue microarray
TYR      Tyrosinase
VEGF     Vascular endothelial growth factor
VGP      Vertical growth phase
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PREFACE

The work presented in this dissertation has been accepted for publication:

1.0 INTRODUCTION

In the last several decades, significant advances have been made in the field of cancer research and cancer therapeutics. This progress is attributed to several factors, including an improved understanding of the molecular basis of a given malignancy, efforts to emphasize better screening and early detection, and clinical implementation of gene targeting approaches for selective targeting of tumor cells. The monoclonal antibody Trastuzumab (Herceptin) is one of the classic examples of a targeted therapeutic approach, specific for the HER2 protein, and approved for advanced-stage HER2-positive breast cancers (as reviewed by Yeon and Pegram, 2005). Gleevec (Imatinib) is another example of a small molecule tyrosinase kinase inhibitor of the BCR-ABL fusion gene, for treatment of chronic myeloid leukemia (Ren, 2005). In the case of melanoma, however, the picture is different, because no molecular targeting therapies are yet approved, and chemotherapy, despite its lack of efficacy in improving overall survival, remains the standard of care for melanoma patients with distant metastases. For this reason, the development of novel and effective therapeutic approaches for advanced-stage melanoma patients is crucial.

1.1 MELANOMA

The three types of skin cancer are basal cell carcinoma, squamous cell carcinoma, and melanoma. In the United States, basal and squamous cell carcinomas occur in nearly one million cases per year. However, they rarely metastasize, and they have a high cure rate (ACS Cancer Facts and Figures 2006). Melanoma, on the other hand, is the most aggressive form of skin cancer, and accounts for 75% of all skin cancer-related deaths. The incidence and mortality are rising worldwide, and it is predicted that in 2006, melanoma will be diagnosed in approximately
62,000 people, with nearly 8,000 deaths (ACS Cancer Facts and Figures 2006). Despite a favorable cure rate when surgically removed at an early stage, the response rate of patients with metastatic disease to chemotherapy is less than 15%, and 5-year survival rates are between 5 and 15% (http://seer.cancer.gov). Furthermore, biologic therapies have only a marginal impact. Given this bleak picture, there is a great need to identify and characterize genes that play an important role in the advanced-stages of the disease and thus, may represent valuable and novel targets for therapy.

1.1.1 Progression Pathway

Melanocytes are the pigment-producing cells of the epidermis, which reside along the dermal-epidermal junction in the skin. Keratinocytes, which compose the majority of the cells in the epidermis, communicate with the melanocytes and help regulate melanocyte proliferation (Valyi-Nagy et al., 1993). The primary function of the melanocytes is to synthesize and transport melanin to the surrounding keratinocytes via melanosomes, which protects the keratinocytes from ultraviolet light (Hsu et al., 2002). Melanoma originates in melanocytes that have undergone malignant transformation. Risk factors for melanoma include repeated childhood or teenage sunburn, fair complexion, number of pigmented lesions, and a family history of the disease (for reviews, see Cummings et al., 1997; Houghton and Polsky, 2002; Tsao et al., 2004). In the majority of cases, melanoma arises in or in association with pigmented lesions, called nevi. Melanoma develops in distinct stages: atypical nevus > melanoma in situ > primary melanoma in the radial growth phase (RGP) > primary melanoma in the vertical growth phase (VGP) > metastatic growth phase melanoma (MGP). In RGP melanomas, melanoma cells spread horizontally within the confines of the epidermis, while in VGP melanomas, there is invasion of the melanoma cells into the dermal layer of the skin (Hsu et al., 2002). The transition from melanoma in situ to VGP melanoma, therefore, represents a key transition to the metastasis-competent stage of the disease.
1.1.2 **Genes and Pathways Implicated in Melanoma**

As in most other types of cancer, both genetic and environmental factors contribute to the development of melanoma. 90% of melanoma cases are of sporadic onset, and 10% of cases are of familial onset. Several studies have investigated chromosomal alterations in familial and sporadic melanomas. The most prominent germline mutations identified for familial melanoma to date are the tumor suppressor gene CDKN2A (which encodes INK4A/p16 and ARF), and the cyclin-dependent kinase 4 (CDK4) gene (Fountain et al., 1992; Loo et al., 2003; Soufir et al., 2004; Puig et al., 2005). The product of the CDKN2 gene is p16, which binds CDKs to inhibit their association with cyclin D, and blocks cell cycle progression at the G1 phase (Serrano et al., 1993). Mutations in these cell cycle regulators can mediate deregulation of cell growth during tumorigenesis. CDKN2/p16 has also been shown to be mutated in a small percentage of sporadic melanomas (Piccinin et al., 1997).

Polymorphisms of the melanocortin-1 receptor (MC1R), a seven-transmembrane G-protein-coupled receptor, whose ligand is melanocyte-stimulating hormone (MSH), have also been indirectly associated with melanoma predisposition (Chin, 2003). MC1R and MSH are critical components of the pigmentation pathway, and MC1R variants influence skin and hair coloring, with specific variants associated with the “red hair color (RHC)” phenotype (Flanagan et al., 2000). Because, as mentioned above, fair skin pigmentation is a significant risk factor for melanoma, it is hypothesized that polymorphisms in the MC1R gene may contribute to melanoma susceptibility. Interestingly, mutations in MC1R have been associated with reduced ability of melanocytes to protect against ultraviolet radiation (Scott et al., 2002).

A variety of different pathways have been implicated in melanoma development and progression. For example, melanoma cells, unlike melanocytes, produce growth factors that can function in an autocrine or paracrine manner, and therefore, lose their dependence upon external growth factors for survival. bFGF/FGFR-1 is the most prominently implicated growth factor signaling pathway in melanoma development and progression (Becker et al., 1989; Becker et al., 1992; Wang and Becker, 1997). Hepatocyte growth factor (HGF)/cMET (Otsuka et al., 1998) and transforming growth factor-β (TGF-β) (Hussein, 2005) have also been postulated to influence to melanoma development; however, these two pathways appear to be limited to a small fraction of melanomas.
The mitogen-activated protein kinase (MAPK) signaling pathway plays an integral role in the regulation of cell proliferation and survival. Somatic mutations resulting in constitutive activation of this RAS-RAF-ERK pathway, in particular, mutations in B-RAF, have been identified in a large percentage of melanoma cell lines and tissue specimens (for review, see Gray-Schopfer et al., 2005). One of several similar studies established that B-RAF is mutated in approximately 66% of malignant melanomas (Davies et al., 2002), usually within the kinase domain. A recent study has demonstrated an association between MC1R variants and increased frequency of mutations in B-RAF (Landi et al., 2006).

Mutations in the tumor suppressor PTEN, which negatively regulates the PI3K/AKT pathway and may result in increased activation of AKT, are hypothesized to reduce the sensitivity of melanoma cells to apoptosis (Stahl et al., 2004; Dai et al., 2005; Robertson, 2005). In addition, the transcription factors NF-κB (for review, see Amiri and Richmond, 2005), as well as signal transducers and activators of transcription (STATs) (Kirkwood et al., 1999), have emerged as regulators of gene expression in melanoma. From the implicated pathways, it is evident that deregulation of melanocyte/melanoma proliferation and survival are key events in melanoma pathogenesis. The ability to identify changes in these signaling pathways in patients with melanoma will facilitate the development of targeted therapeutic approaches for this disease.

1.1.3 Current Clinical Therapies

The current therapeutic options for metastatic melanoma are only marginally effective (for reviews, see Tsao et al., 2004; Atallah and Flaherty, 2005). The alkylating agent dacarbazine (DTIC) is the only chemotherapeutic reagent that is currently FDA-approved for melanoma and is considered the standard regimen for Stage IV melanoma. The objective response rate to DTIC is barely 10-20%, there is generally only a partial response, and overall survival is not improved significantly (Luikart et al., 1984; Chapman et al., 1999; Eggermont and Kirkwood, 2004). Likewise, other chemotherapeutic drugs, such as cisplatin and temozolomide, have shown only modest activity in clinical trials to date (Eigentler et al., 2003), and numerous clinical trials have failed to demonstrate that combining other chemotherapeutic drugs with DTIC yields any
consistent improvement in response rate compared with DTIC alone (Eggermont and Kirkwood, 2004).

In addition to standard chemotherapy regimens, cytokine therapy is another approach that is used for melanoma therapy. High dose interferon alpha 2b (IFNα2b), an FDA-approved cytokine therapy for melanoma, yields modest improvements in relapse-free and overall survival as an adjuvant therapy in stage IIb and stage III melanoma patients. However, toxicity associated with the drug is high (Kirkwood et al., 2002), and response rates for IFNα2b average around 10-15% (Atallah and Flaherty, 2005). IL-2 therapy generated an overall response of 16% in a trial composed of 270 patients with metastatic melanoma, but, as with IFNα2b, toxicity was high (Atkins et al., 1999). To date, no survival benefit has been demonstrated following combination of DTIC with IFNα2b and IL-2, compared with DTIC alone (Atkins et al., 2003; Keilholz et al., 2005).

As discussed above, somatic B-RAF mutations that result in increased kinase activity are detected in approximately 66% of malignant melanomas (Davies et al., 2002). The small molecule inhibitor, Nexavar (sorafenib, BAY 43-9006), inhibits the RAF kinase pathway, and, to some degree, the receptor tyrosine kinases VEGFR-2/3 and PDGFR-β (Wilhelm et al., 2004; Sridhar et al., 2005; Flaherty, 2006). Nexavar was one of the first small molecule inhibitors used for specific gene targeting in the treatment of advanced-stage melanoma. Early phase I/II trials showed activity of the drug in stage IV melanoma patients (Ahmad et al., 2004). Phase II and III trials are presently examining the efficacy of Nexavar in combination with temsirolimus, tipifarnib, or a combination of carboplatin and paclitaxel, in stage III/IV melanoma patients (www.clinicaltrials.gov).

In summary, it is clear from these clinical trial data that it is imperative that we develop novel therapeutic approaches for advanced-stage melanoma that are more specific, generate a more robust tumor response, and increase patient survival.
1.2 ADHESION MOLECULES

1.2.1 Invasion and Metastasis

In general, the cause of death from cancer is not attributed to the burden from the primary tumor, but to the metastasis of tumor cells to distant sites. In the case of melanoma, prognosis is directly related to the depth of invasion of the primary lesion at the time of diagnosis. During the process of metastasis, tumor cells detach from the primary tumor, migrate across the basement membrane, enter and spread throughout the circulatory system, and establish at distant sites. The metastatic tumor cell must therefore acquire the ability to migrate away from the primary site, to interact with different cell types encountered during invasion, and to proliferate and survive in the new tumor microenvironment (for review, see Chambers et al., 2002).

Two distinct hypotheses regarding the acquisition of metastatic potential have been described. The “seed and soil” hypothesis suggests that metastasis only occurs when the tumor cell (the seed) grows in the correct tumor microenvironment (the soil) (Fidler, 2001). In this model, interactions between tumor and host cells are crucial in mediating metastasis. On the other hand, Robert Weinberg and colleagues described a model whereby tumor progression and metastasis are “pre-programmed” at an early stage of tumor progression by genetic alterations, and that furthermore, the same oncogenes and tumor suppressors that mediate tumor initiation likely mediate subsequent tumor metastasis (Bernards and Weinberg, 2002). Whether one or a combination of both models represents the correct mechanism underlying tumor metastasis remains to be determined. Further understanding regarding the mechanism of the metastasis will be essential in the development of cancer therapeutics that can prevent the spread of tumors cells to distant sites.

1.2.2 Expression of Cell Adhesion Molecules in Melanoma

Cell adhesion molecules play a critical role in cancer metastasis by regulating tumor cell-cell and cell-extracellular matrix (ECM) adhesion. In addition, they mediate signal transduction following cell-cell and cell-ECM interactions. Tumor cells frequently display altered expression of cell adhesion molecules, which has been implicated in tumor invasion and metastasis in many cancer
types (Christofori, 2003), including melanoma (Johnson, 1999). Three cell adhesion molecules have been postulated to serve as progression-related factors in melanoma: N-cadherin, melanoma cell adhesion molecule (MCAM), and the β3 integrin subunit. These represent three distinct families of cell adhesion molecules: the cadherins, the immunoglobulin superfamily, and the integrins, respectively.

### 1.2.3 N-cadherin

The cadherins mediate calcium-dependent cell-cell adhesion, usually through homophilic binding at cell-cell junctions. Classical cadherins are composed of a single transmembrane domain, with the extracellular portion containing multiple calcium-binding domains. The cytoplasmic region interacts with catenins to provide a link to the actin cytoskeleton (Figure 1).

![Figure 1: N-cadherin Structure](image)

Classical cadherins are composed of a short cytoplasmic domain, a single transmembrane region, and multiple extracellular domains. The cytoplasmic domain interacts with catenins and links to the actin cytoskeleton. The extracellular domains contain Ca$^{2+}$-binding sites that stabilize the cadherin structure and facilitate interaction with cadherins on the surface of other cells. The His-Ala-Val (HAV) domain mediates specific cadherin-cadherin interactions.
Cadherins are essential for tissue development and are crucial for epithelial to mesenchymal transitions (Thiery, 2002). Homozygous deletion of E-cadherin (Larue et al., 1994) or N-cadherin (Radice et al., 1997) causes embryonic lethality in mice. The cadherins have emerged as important players in tumorigenesis, with changes in cadherin expression profiles frequently observed during tumor progression. Most prominently, a switch from E-cadherin to N-cadherin expression has been documented in numerous tumors of epithelial origin, including melanoma, breast, prostate, bladder, and squamous cell carcinoma (for review, see Cavallaro, 2004). The loss of E-cadherin expression can be genetic, through germline mutations (Guilford et al., 1998; Richards et al., 1999) or epigenetic, where downregulation at the transcriptional level is most common, through transcriptional repressors including SNAIL (Poser et al., 2001) and SLUG (Bolos et al., 2003), and/or promoter hypermethylation (Chen et al., 2003).

Cadherins also play a key role in the development and differentiation of human skin (Herlyn et al., 2000). The epidermis is composed primarily of keratinocytes and melanocytes, at a ratio of approximately 5-10:1. Melanocyte proliferation is carefully regulated by signaling from the keratinocytes. Keratinocytes and melanocytes both express E-cadherin, which has been shown to mediate interactions and communication between the two cell types (Valyi-Nagy et al., 1993). Notably, however, expression of E-cadherin is significantly reduced or absent in most human melanoma cell lines, while N-cadherin, which is not expressed by melanocytes in normal skin, is highly expressed (Hsu et al., 1996). A general pattern toward decreased expression of E-cadherin and increased expression of N-cadherin in MGP melanoma tissue specimens has been suggested (Danen et al., 1996; Hsu et al., 1996; Sanders et al., 1999), although the expression patterns in these reports were variable in the small number of tissue samples assayed.

Based on these findings, a loss of E-cadherin expression and/or de novo N-cadherin expression in the melanocytes may impair their interactions with keratinocytes. A loss of communication between the two cell types may thereby promote melanocyte proliferation and migration. This hypothesis is supported by experiments in several cell types. First, re-expression of E-cadherin in an E-cadherin negative melanoma cell line inhibits invasion in skin reconstruct models, and reduces growth in soft agar and tumor formation in nude mice (Hsu et al., 2000). Second, in the E-cadherin-expressing MCF7 breast cancer cell line, exogenous expression of N-cadherin increases cell migration, invasion, and adhesion to endothelial cells in vitro, and increases metastasis in vivo (Hazan et al., 2000). Finally, transfection of a squamous cell
carcinoma cell line with antisense N-cadherin results in a change from scattered fibroblastic to normal epithelial morphology, and a re-induction of E-cadherin expression (Islam et al., 1996).

Because N-cadherin is expressed by fibroblasts and vascular endothelial cells, it is possible that N-cadherin expression may promote the interaction of melanoma cells with these cell types during invasion and metastasis. Transendothelial migration assays indicate that N-cadherin is expressed in regions of heterotypic contact between melanoma and endothelial cells during the transmigration (Sandig et al., 1997). It has also been reported that N-cadherin can mediate adhesion between melanoma cells and dermal fibroblasts/vascular endothelial cells in vitro. Furthermore, an N-cadherin blocking antibody inhibits the migration of melanoma cells across a monolayer of fibroblasts (Li et al., 2001).

N-cadherin expression has been linked to several signal transduction pathways. An N-cadherin antibody inhibits the phosphorylation of Akt/PKB in melanoma cells (Li et al., 2001). Likewise, N-cadherin expression in prostate cancer cells has been implicated in upregulation of Bcl-2 and activation of PI3 kinase/Akt (Tran et al., 2002). Furthermore, in breast cancer cells, a direct association between N-cadherin and fibroblast growth factor receptor-1 (FGFR-1) occurs, resulting in a decrease in ligand-induced receptor downregulation and sustained MAPK/ERK activation (Suyama et al., 2002). From these studies, it is clear that N-cadherin mediates tumor cell-cell interactions, as well as signal transduction pathways involved in cell proliferation and migration, and represents an intriguing molecule for further analysis in melanoma.

1.2.4 MCAM

Cell adhesion molecules of the immunoglobulin (Ig) superfamily mediate cation-independent cell-cell adhesion, which can be either homophilic or heterophilic (Johnson, 1991). MCAM is a 113 kD integral membrane protein, with an extracellular domain composed of repeated Ig-like folds, stabilized by disulfide bonds (Figure 2). The cytoplasmic domain contains several putative protein kinase recognition sites, which may mediate signal transduction (Sers et al., 1993).
Figure 2: MCAM Structure
MCAM is composed of a short cytoplasmic domain, a single transmembrane domain, and an extracellular domain containing five immunoglobulin-like units (V-V-C2-C2-C2). MCAM interacts with an as yet unknown receptor.

MCAM was first identified on MGP melanoma cells (Lehmann et al., 1987), but is also expressed in cell types including endothelial, smooth muscle, activated T lymphocytes, and hair follicles (Lehmann et al., 1987; Shih et al., 1997). MCAM expression has also been documented in prostate carcinoma (Wu et al., 2001), choriocarcinoma, angiosarcoma, Kaposi’s sarcoma, and leiomyosarcoma (Shih et al., 1998). MCAM is not expressed by melanocytes in normal skin; however, when melanocytes are propagated in vitro, they begin to express MCAM (Shih et al., 1994). MCAM interacts heterophilically with an as yet unidentified ligand that is typically co-expressed with MCAM on melanoma cells (Johnson, 1997; Shih et al., 1997). In human tissues, an early study reported that greater than 70% of melanoma metastases express MCAM and that its expression correlates with increasing tumor thickness (Holzmann et al., 1987). Furthermore, it has been suggested that loss of expression of the transcription factor AP-2 may mediate MCAM upregulation in advanced-stage melanoma (Jean et al., 1998).

Several studies have implicated MCAM in melanoma invasion in vitro and metastasis in vivo. Transfection of an MCAM-negative melanoma cell line with an MCAM cDNA was reported to increase invasion, attachment to endothelial cells, subcutaneous tumor formation, and lung metastases formation in nude mice (Xie et al., 1997; Schlagbauer-Wadl et al., 1999). MCAM-positive cells also displayed reduced adhesion to laminin (Xie et al., 1997). Another study reported that inhibiting MCAM expression in melanoma cells may lead to loss of gap-junction communication, as evidenced by reduced invasion in a 3D skin reconstruct model.
(Satyamoorthy et al., 2001). Furthermore, an MCAM antibody decreases melanoma cell invasion, tumor vascularization, and MMP-2 expression, and reduces formation of lung metastasis in nude mice (Mills et al., 2002).

MCAM expression is thought to play a role in several signal transduction pathways. A reciprocal relationship between MCAM expression and Akt phosphorylation in melanoma cells has been suggested. Blocking Akt activation results in decreased MCAM expression, while overexpression of MCAM, likewise, increases phosphorylation of Akt and Bad (Li et al., 2003). In addition, engagement of MCAM in endothelial cells leads to tyrosine phosphorylation of focal adhesion kinase (p125\textsuperscript{FAK}/FAK) (Anfosso et al., 1998). Overall, these results demonstrate that MCAM, like N-cadherin, plays a role in both cell-cell interactions and signal transduction in melanoma cells.

1.2.5 \( \beta_3 \text{ integrin} \)

Integrins are heterodimeric cell surface receptors, which mediate divalent cation-dependent cell-matrix interactions. Integrin complexes are composed of an alpha and a beta subunit, which are non-covalently associated. Different combinations of alpha and beta subunits convey ligand specificity. Most integrin subunits are composed of a large extracellular domain, a single transmembrane region, and a short cytoplasmic domain (Figure 3). The cytoplasmic tail of the beta subunit binds to intracellular proteins, including \( \alpha \)-actinin and filamin, to provide a physical linkage between the ECM and the cytoskeleton. The alpha and beta cytoplasmic tails also associate with a variety of adaptor proteins, including Shc, Grb2, paxillin, and FAK, which provides a mechanism for intracellular signaling (Berman et al., 2003).
Integrins are composed of an α and β subunit, which are non-covalently associated. The extracellular regions of the integrin subunits bind to ECM ligands, with ligand specificity determined by the particular combination of α and β subunits. The cytoplasmic tail of the β subunit binds to intracellular proteins that anchor to the cytoskeleton, thus providing a physical linkage between the ECM and the cytoskeleton. The α and β cytoplasmic tails also associate with a variety of adaptor proteins, which can mediate intracellular signaling.

αvβ3 integrin is one of the most “promiscuous” integrin receptors, recognizing ligands including vitronectin, fibrinogen, von Willebrand factor, fibronectin, laminin, thrombospondin, and osteopontin (Cheresh, 1991). αvβ3 is expressed by vascular endothelial cells, smooth muscle cells, osteoclasts, macrophages, and some lymphocytes (B-cells, T-cells, and NK cells) (Nip and Brodt, 1995). β3 integrin is not expressed by melanocytes in normal skin. However, in melanoma, expression of the β3 integrin subunit is associated with the transition from RGP to VGP melanoma (Albelda et al., 1990). The αv subunit, on the other hand, is expressed throughout all stages of melanoma progression.

Exogenous expression of β3 integrin in RGP melanoma cells increases invasion in a skin reconstruct model and increases tumorigenicity in SCID mice (Hsu et al., 1998). αvβ3 has also been implicated in transendothelial migration of melanoma cells in culture, with strong expression in regions of heterotypic contact between melanoma and endothelial cells (Voura et al., 2001). β3 integrin expression has been linked with the activation of focal adhesion kinase (FAK) (Li et al., 2001), the production of mature MMP-2 and MMP-9 (Brooks et al., 1996; Felding-Habermann et al., 2002; Rolli et al., 2003), and the activation of Akt (Zheng et al.,
In melanomas, expression of $\alpha_v\beta_3$ integrin is associated with increased tumor thickness, reduced disease-free and overall survival (Hieken et al., 1996), and elevated risk of melanoma recurrence (Natali et al., 1997).

The suggestion that $\alpha_v\beta_3$ integrin plays a crucial role in angiogenesis was based initially on studies using a function-blocking $\alpha_v\beta_3$ integrin antibody to inhibit the growth of human tumor fragments implanted onto chicken chorioallantoic membrane (CAM) (Brooks et al., 1994; Brooks et al., 1994). These studies generated substantial enthusiasm for $\alpha_v\beta_3$ integrin as a putative therapeutic target for inhibiting angiogenesis in diseases including cancer, diabetic retinopathy, and rheumatoid arthritis. However, studies with $\beta_3$ integrin knockout mice have challenged the hypothesis that $\beta_3$ integrin functions exclusively as a pro-angiogenic molecule. $\beta_3$-deficient mice are viable and fertile, with defects in platelet aggregation and clot retraction, and prone to prolonged bleeding (Hodivala-Dilke et al., 1999). Unexpectedly, however, mice deficient in $\beta_3$ integrin or both $\beta_3/\beta_5$ integrin display enhanced tumor growth and angiogenesis compared with controls (Reynolds et al., 2002; Taverna et al., 2004). This experimental outcome generated uncertainty with respect to the role of $\beta_3$ integrin in angiogenesis and tumorigenesis. Future studies will be necessary, to further elucidate the mechanism of $\beta_3$ integrin in tumorigenesis and determine its value as a therapeutic target.

### 1.2.6 Clinical Trials

Ongoing clinical trials address the potential of adhesion molecules as therapeutic targets for cancer. An N-cadherin cyclic peptide inhibitor, Exherin™ (Adherex Technologies, Inc.) is designed to target N-cadherin interactions and is thought to block the interaction between endothelial cells and/or tumor cells (Williams et al., 2000). Exherin contains the His-Ala-Val (HAV) sequence, which is located in the extracellular region of N-cadherin near the N-terminus and mediates cadherin cell-cell adhesion. In a phase I trial comprised of patients with N-cadherin-positive tumors of various cancer types, Exherin was well tolerated, and modest anti-tumor activity was observed in two patients (Jonker et al., 2004). An ongoing phase II trial further investigates the efficacy of this drug (www.clinicaltrials.gov). In the case of MCAM, the
humanized antibody described by Mills et al, 2002 (ABX-MA1), developed by Abgenix, and had entered phase I clinical trials for melanoma. However no follow-up information is available.

In addition to the targeting agents described above, inhibitors of integrin subunits are the focus of several clinical trials. Cilengitide, an Arg-Gly-Asp (RGD) peptide that blocks both $\alpha_v \beta_3$ and $\alpha_v \beta_5$ integrin, is currently in phase I/II trials for cancers including head and neck, glioblastoma, non-small-cell lung, and pancreatic (Smith, 2003). However, therapeutic efficacy has not been demonstrated to date. Additional integrin antagonists are in the early stages of clinical investigation, including CNTO 95 ($\alpha_\text{v}$), Volociximab ($\alpha_5 \beta_1$), and ATN-161 ($\alpha_5 \beta_1, \alpha_v \beta_3$) (Tucker, 2006). In the case of melanoma, Phase I/II trials are investigating Abegrin/Vitaxin/MEDI-522, a humanized monoclonal antibody that blocks $\alpha_v \beta_3$ integrin (Gutheil et al., 2000; Posey et al., 2001; Shreyaskumar et al., 2001). Abegrin is thought to function as an anti-angiogenic inhibitor, through inhibition of tumor blood vessel growth. An ongoing phase II study is examining the efficacy of Abegrin in stage IV melanoma patients. Results obtained to date indicate that although patients with MGP melanoma treated with this antibody have no objective tumor response, they live longer compared to historic controls (Hersey et al., 2005). The results of these ongoing studies will help indicate whether blocking the function of integrins represents a viable approach for cancer therapy.

1.3 RATIONALE AND HYPOTHESIS

Data in the literature suggest that N-cadherin, MCAM, and $\beta_3$ integrin may contribute to melanoma progression. Yet, little is known as to whether they may constitute therapeutic targets for the disease, and, equally importantly, no previous studies have examined the expression or function of the three adhesion molecules in concert. In this dissertation, we will illustrate the expression of N-cadherin, MCAM, and $\beta_3$ integrin mRNA and protein throughout the distinct stages of melanoma progression using microarray analyses, and, in addition, evaluate whether MGP melanomas co-express all three adhesion molecules. This critical question will help to establish whether all three adhesion molecules are essential for melanoma metastasis. Next, we will test our hypothesis that individual inhibition of N-cadherin, MCAM, or $\beta_3$ integrin in melanoma cells may decrease proliferation, migration, and/or invasion. However, it is important
to note that research to date has indicated substantial redundancy in the signaling pathways for N-cadherin, MCAM, and β3 integrin, including bFGF/FGFR-1, FAK, MMP’s, MAPK/ERK, Akt/PKB, and others (Xie et al., 1997; Anfosso et al., 2001; Hood and Cheresh, 2002; Derycke and Bracke, 2004). As such, it is possible that upon inhibition of one of these molecules, the other two may readily compensate for any anti-tumorigenic impact. Therefore, this dissertation will also evaluate our hypothesis that simultaneous inhibition of multiple adhesion molecules may be required for the most effective inhibition of melanoma cell proliferation, migration, and/or invasion. Given the lack of effective therapeutic strategies for advanced-stage melanoma, our data will provide valuable evidence as to whether these cell adhesion molecules, alone or in concert, represent useful and effective therapeutic targets for advanced-stage melanoma.
2.0 MATERIALS AND METHODS

2.1 CELL CULTURE

The human metastatic (MGP) melanoma cell lines, WM1158, WM852, and WM983B, were cultured in 3 parts DMEM, 1 part L-15 (Mediatech, Herndon, VA), and 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT) and 100 µg/mL penicillin-streptomycin. Cells were maintained at 37°C and 5% CO₂.

2.2 RT-PCR

Total RNA was isolated from WM1158, WM852, and WM983B cells using TRIzol reagent (Invitrogen, Carlsbad, CA). RT-PCR was performed to amplify N-cadherin, MCAM, and β₃ integrin cDNA (1.9, 1.8, and 2.3 kb fragments, respectively). Primer sequences were as follows: N-cadherin (Forward: 5’-AGACTGGATTTCTCTGAAG-3’, Reverse: 5’GGGAACCTT CATAGATACC-3’); MCAM (Forward: 5’-AGCACACGCCCCTTCTGAAG-3’, Reverse: 5’-ATTTCTCTCCCCTGCTCTC-3’); β₃ integrin (Forward: 5’AACATCT GTACCACGCGA3’, Reverse: 5’CGTGTATTTTGTGAAGGT3’).

2.3 WHOLE-GENOME MICROARRAY ANALYSIS

Total RNA was extracted from tissue specimens - two normal skin, two benign nevi, two atypical nevi, two melanomas in situ, two primary melanomas (VGP), two metastatic melanomas...
(MGP), and three melanoma-infiltrated lymph nodes, as well as from WM1158 cells, with the 
RNeasy Lipid Midi Extraction Kit (Qiagen, Valencia, CA). RNAs were subsequently processed 
using the RNeasy Mini Kit RNA clean-up protocol (Qiagen). Human neonatal melanocyte 
(HEMn) and keratinocyte (HEKn) RNA were obtained from Cell Applications, Inc. Samples 
were analyzed on an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA) to verify 
RNA integrity. RNAs were transcribed into cRNAs, biotinylated, and hybridized to Human 
Genome U133 Plus 2.0 gene chip microarrays (Affymetrix, Santa Clara, CA) (Smith et al., 
2005). Normalization of intensity values was performed using GeneSpring 7.1 bioinformatics 
software (Silicon Genetics, Agilent Technologies).

2.4 TISSUE MICROARRAY ANALYSIS

Nevus, VGP, MGP, and melanoma-infiltrated lymph node TMAs were constructed by the 
National Cancer Institute - Center for Cancer Research (NCI-CCR) Tissue Array Research 
Program (TARP). TMA slides were deparaffinized and rehydrated, following by antigen 
unmasking using Target Retrieval buffer, pH 6 (Dako, Glostrup, Denmark) in a decloaking 
chamber. Endogenous peroxidases were then quenched using 3% hydrogen peroxide. Standard 
immunohistochemistry was performed using the VECTASTAIN elite ABC kit (Vector 
Laboratories, Burlingame, CA). Tissue cores were probed with antibody to N-cadherin (sc-7939, 
rabbit polyclonal, 1:50), MCAM (sc-18942, goat polyclonal, 1:50), or \( \beta_3 \) integrin (sc-13579, 
mouse monoclonal, 1:100) (Santa Cruz Biotechnology, Santa Cruz, CA). As a negative control, 
each array was also probed with antibody to PERP (ab-5986, rabbit polyclonal, 1:250, ABCAM, 
Cambridge, MA). Peroxidase substrate was NovaRED (Vector Laboratories). Slides were 
counterstained with hematoxylin, dehydrated, and mounted in non-aqueous mounting media.

TMAs were scored using a scale of 0 (no signal), 1 (weakly positive), or 2 (strongly 
positive). The total number of tissue cores scored for N-cadherin expression were nevi (38), VGP 
melanomas (43), MGP melanomas (60), melanoma-infiltrated lymph nodes LN (30); for MCAM 
expression: nevi (43), VGP melanomas (35), MGP melanomas (66), melanoma-infiltrated lymph 
nodes (35); and for \( \beta_3 \) integrin expression: nevi (63), VGP melanomas (36), MGP melanomas 
(68), melanoma-infiltrated lymph nodes (38).
2.5 RNA INTERFERENCE

N-cadherin individual siGENOME duplex (5’-GAAACTTGCTGACATGTAT-3’), and MCAM siRNA duplex (5’-AAGATCCACAGCGAGTCTCTGA-3’) were purchased from Dharmacon (Lafayette, CO). Pre-designed β3 integrin silencer siRNA (5’-GGAGAATCTGCTGAAGGAT-3’), and a scrambled siRNA negative control were purchased from Ambion (Austin, TX). Melanoma cells were transfected with 100 nM siRNA mixed with Lipofectamine 2000 (Invitrogen), as per the manufacturer’s recommendations. For combined treatment with multiple siRNAs, a mixture of 100 nM of each siRNA was used. 48 or 72 hours post-transfection, cell lysates were subjected to immunoblot analysis.

N-cadherin, MCAM, and β3 integrin siRNAs were labeled with Cy3, by way of a Silencer siRNA Labeling Kit (Ambion), and transfected into WM1158 cells. The cells were imaged in the Cy3 channel.

2.6 ANTISENSE VECTOR CONSTRUCTS

Total RNA was extracted from WM1158 cells using TRIzol reagent (Invitrogen). RT-PCR was performed to amplify partial cDNA transcripts of N-cadherin (2.0 kb), MCAM (1.1 kb), and β3 integrin (1.3 kb). The primer sequences included the restriction sites XhoI or BamHI for sub-cloning (underlined): N-cadherin (Forward: 5’GCCATGGGATCCATGTGGCGGATAGCGGGA3’, Reverse: 5’GCTGTACCTCGAGGGGAACCTTCATAGATACC3’) - NCBI sequence X54315 nucleotide 102-2121; MCAM (Forward: 5’GCCAGTGGATCCAGCATGGGGCTTCCCAGG3’, Reverse: 5’GCTGTACCTCGAGTGCTCACCAGGTAGGT3’) - NCBI sequence M28882 nucleotide 5-1108; β3 integrin (Forward: 5’GCCAGTGGATCCACTCGAACATCTGTACCACCGA3’, Reverse: 5’GCTGTACCTCGAGGGTCCCAATCCATTGTT3’ - NCBI sequence NM_000212 nucleotide 101-1439. Each of the cDNA sequences were subcloned, in their antisense orientation, into the multiple cloning site of the cytomegalovirus (CMV) promoter-driven pcDNA3.1 Hygro (-) mammalian expression vector (Invitrogen). Sequencing analysis was performed to verify correct insertion and orientation of the cDNAs.
N-cadherin, MCAM, and β3 integrin antisense plasmids, as well as empty pcDNA 3.1 plasmid, were transfected into WM983B cells using Lipofectamine 2000 (Invitrogen), as per the manufacturer’s recommendations. Stable transfectants were selected in the presence of 150 µg/mL hygromycin B (Invitrogen). Individual hygromycin-resistant colonies were isolated and screened for reduction of respective protein levels compared with controls, by immunoblot analysis.

2.7 IMMUNOBLOTTING

Total cell lysates were prepared using Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA) supplemented with 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich, St. Louis, MO), as per manufacturer’s instructions. Lysates were separated on 8% SDS-PAGE gels and transferred to nitrocellulose (Biorad, Hercules, CA) or PVDF (Millipore, Billerica, MA) membrane. Following blocking with 5% dry milk/TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween), the membranes were probed with antibodies to N-cadherin, MCAM, β3 integrin, E-cadherin (Santa Cruz Biotechnology); pAKT (Ser473) (Cell Signaling Technology); αv integrin (BD Biosciences, San Diego, CA); or actin (ABCAM), all diluted in TBST. Membranes were washed three times with TBST for 5 minutes each, and then probed with secondary antibody. After three washes for 5 minutes each with TBST and a 5-minute wash with TBS (without Tween), signals were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore). ImageJ densitometry software was used for quantification of levels of protein expression.

2.8 IMMUNOHISTOCHEMISTRY

Snap-frozen tissue specimens were embedded in O.C.T. (Tissue-Tek, Sakura Finetech U.S.A., Inc., Torrance, CA), and 5 µm sections were prepared and fixed in ice-cold acetone. Standard immunohistochemistry was performed using the R.T.U VECTASTAIN elite ABC kit (Universal)
The tissue sections were probed with antibody to N-cadherin (sc-7939, rabbit polyclonal), MCAM (sc-18942, goat polyclonal), or β3 integrin (sc-6626, goat polyclonal) (Santa Cruz Biotechnology), at a dilution of 1:20, or osteopontin (rabbit polyclonal, LabVision, Freemont, CA), at a dilution of 1:50. These incubations were performed in a humidified incubator, at 37°C. The peroxidase substrate was AEC (Vector Laboratories).

2.9 IMMUNOFLORESCENCE AND PHASE-CONTRAST IMAGING

Melanoma cells were plated on coverslips in 24-well culture dishes. Cells were fixed in ice-cold acetone, rehydrated in PBS, and blocked with 2.5% normal goat serum (Vector Laboratories). Cells were probed with primary antibody, as described in Section 2.8, and then probed with FluoroLink Cy5-conjugated secondary antibody at a 1:1000 dilution (Amersham Biosciences, Piscataway, NJ). Fluorescent DAPI (Molecular Probes, Invitrogen) was used as a nuclear counterstain. Incubations were performed at 37°C in a humidified incubator. Fluorescent signal was visualized using a Nikon TE2000 microscope, with Photometrics CoolSNAP HQ charge-coupled device (CCD) camera (Roper Scientific, Tuscon AZ), 40X oil immersion objective, and DAPI or Cy5 excitation/emission filters. Images were acquired using MetaMorph® imaging software (Universal Imaging, Sunnyvale, CA).

Phase-contrast images of antisense-transfected or control vector only-transfected MGP melanoma cells were captured, using a 10X objective, with phase-contrast light settings.

2.10 APOPTOSIS

Apoptosis was assessed in cultured melanoma cells using the TMR Red In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN), as per the manufacturer’s instructions. For positive control, cells were pre-treated with 500 U/mL DNase I, for 10 minutes at room temperature. For negative control, cells were incubated in the absence of terminal transferase.
enzyme. A 40X oil immersion objective and Cy3 excitation/emission filters were utilized to acquire images of cells having undergone apoptosis.

2.11 MELANOMA CELL PROLIFERATION

Melanoma cells were seeded, in triplicate, into 24-well plates, at a density of 7x10^4 cells per well. Following siRNA transfection, adherent cells were detached with trypsin/ethylenediaminetetraacetic acid (EDTA), and counted using a hemacytometer. Replicate counts were averaged for each sample.

Proliferation rates of the pcDNA N-cadherin, MCAM, and β3 integrin antisense-transfected cells and control pcDNA vector-transfected cells were determined via the MTT colorimetric assay. 1x10^4 cells were plated in quadruplicate in 96 well dishes. At the indicated times, the media was replaced with phenol red-free media, and 10 µl of 12 mM MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to each well, and samples were incubated at 37°C for 4 hours. Thereupon, 100 µl of 10% SDS in 0.01 M HCl was added to each well, and cells were incubated overnight at 37°C. Samples were read at an absorbance of 570 nm.

2.12 MELANOMA CELL MIGRATION AND INVASION

For determination of melanoma cell migration and invasion, at 48 hours post siRNA transfection, cells were trypsinized and resuspended in serum-free media. 4x10^4 cells were plated per well, in triplicate, into transwell migration chambers, containing an 8 µm pore-size filter with (for analysis of invasion) or without (for analysis of cell migration) a Matrigel coating (BD Biosciences). Heat-inactivated 5% fetal bovine serum (FBS), mixed with Dulbecco's Modified Eagle's Medium (DMEM), served as chemoattractant in the lower wells. Twenty-two hours later, cells that had migrated to the lower side of the filter(s) were fixed, stained, and counted in seven randomly selected 40x fields/filter.
2.13 IN VIVO GROWTH OF ANTISENSE-TRANSFECTED CELLS

N-cadherin, MCAM, or $\beta_3$ integrin antisense-transfected cells, pcDNA vector only-transfected cells, or non-transfected control cells were isolated and washed three times in PBS. 5x10^6 cells, in 100 µl PBS, were injected subcutaneously, into the lower right dorsal region of mice (nu/nu BALB/c, Tac:Cr:(Ncr)-Foxn1^nu, Taconic, Hudson, NY). Tumor size was measured three times a week, and tumor volume was calculated using the formula: $\frac{(\text{Length} \times \text{Width}^2)}{2}$. Mice were sacrificed by CO$_2$ inhalation, and tumors were resected and snap-frozen in an ethanol-dry ice bath. 5 µm sections were prepared from O.C.T-embedded tumors, and immunohistochemistry was performed as described in Section 2.8.

2.14 VECTOR CONSTRUCTS FOR IN VIVO TARGETING

For in vivo gene-targeting experiments, two additional plasmid vectors were generated. pREP7-RSV, an episomal expression vector, contains the Rous sarcoma virus (RSV) promoter (Invitrogen). pREP7-TYR, contains a 430 bp cDNA fragment of the tyrosinase promoter (TYR), which replaces the RSV promoter. The tyrosinase promoter mediates tissue-specific expression of the vector in cells of melanocytic origin (Bentley et al., 1994). Antisense (AS) and sense-oriented vector constructs for N-cadherin, MCAM, and $\beta_3$ integrin were generated using the sequences and methods described in Section 2.6. For sense-oriented plasmids, cDNA sequences were amplified using the primers described in Section 2.6, containing the appropriate restriction sites for subcloning in their sense orientation.

2.15 INTRATUMORAL PLASMID INJECTIONS

5x10^6 cells (WM983B) were injected subcutaneously, as described in Section 2.13. Tumor volume was monitored, and when the tumors reached a perpendicular diameter of approximately 5x5 mm, intratumoral plasmid injections were initiated, and continued on a twice-weekly basis.
To determine the most optimal in vivo delivery strategy, several different gene delivery reagents were tested. In the case of Lipofectamine 2000 (Invitrogen), 50-100 µg of plasmid DNA was diluted in either PBS or TE pH 8.0 (10mM Tris-Cl, pH 8.0, 1 mM EDTA). 3-5 µg of Lipofectamine 2000 was added to the diluted DNA, and incubated for 25 minutes at room temperature prior to injection. For Mirus TransIT In Vivo Gene Delivery (Mirus Bio Corporation), 290 mM glucose, 20 µg of plasmid DNA (1 µg/µL), and 20 µL of TransIT reagent were mixed, vortexed, and incubated for 5 minutes at room temperature prior to injection. For the In vivo jetPEI reagent (Bridge Biosciences), 10 µg of plasmid DNA was diluted into 5% glucose to a volume of 50 µL, while 2 µL of jetPEI was diluted into 48 µL of 5% glucose. The jetPEI mixture was added to the diluted DNA, and incubated for 15 minutes at room temperature prior to injection. For Gene/NovaFECTOR reagent (Venn Nova, Inc), 25 µg of plasmid DNA was diluted in 50 µL of water, and 25 µL of Gene/NovaFECTOR was diluted into 25 µL of water, to give a final ratio of 1:1 µg of DNA:liposome. The DNA was added to the Gene/NovaFECTOR mixture, and incubated for 20 minutes at room temperature prior to injection. For injection of “naked” plasmid DNA, 50 µg of pcDNA-LacZ control plasmid was diluted in PBS and injected. At the completion of the experiments, tumors were extracted and snap-frozen.

2.16 DNA EXTRACTION FROM MELANOMA XENOGRAFTS

Total DNA was extracted from tumors using the DNeasy Tissue kit (Qiagen). PCR was performed with primers specific for each vector. The forward primers were designed to anneal to the promoter region of each vector - RSV promoter: 5’ GCTCGATACAATAACGCC 3’, TYR promoter: 5’ CACTGTAGTAGTAGCTGG 3’, and CMV promoter: 5’ TAATACGACTCACTATAGGG 3’. The reverse primers were specific for the 3’ end of each antisense or sense-oriented cDNA insert. As a positive control, the sense or antisense cDNA was amplified from purified sense or antisense plasmid construct via PCR.
2.17 PROTEIN ISOLATION FROM MELANOMA XENOGRAFTS

75-100 mg of frozen tumor tissue was minced and placed into 250 uL of Cell Lysis Buffer (Cell Signaling Technology), supplemented with 1 mM PMSF, and processed as described in Section 2.7.

2.18 BETA-GALACTOSIDASE ASSAY

Tumors were injected with pcDNA3.1-CMV plasmid containing the LacZ reporter gene, using several gene delivery reagents. 48 hours later, the mice were sacrificed and their tumors snap-frozen. 5 µm sections were prepared and processed as per the manufacturer’s instructions for the β-Gal Staining Kit (Invitrogen).

2.19 TARGETING ALPHA V BETA 3 INTEGRIN THROUGH A FUNCTION-BLOCKING ANTIBODY (ABEGRIN)

1x10^5 cells were plated in 24-well culture dishes. Twenty-four hours later, 50 µg/mL (in the case of WM1158) or 75 µg/mL (in the case of WM983B) of Abegrin antibody (from Medimmune Inc., Gaithersburg, MD) diluted in PBS was added to the culture medium. At the indicated times, cells were detached with trypsin/EDTA, and counted using a hemacytometer. Replicate counts were averaged for each sample.

WM983B cells were injected into nude mice to generate melanoma xenografts, as described in Section 2.13. When the tumors reached approximately 5x5 mm in perpendicular direction, 100 µg of Abegrin diluted in 75 µL PBS was injected directly into the tumors. The injections were repeated three times a week. At the completion of the experiment, tumors were snap-frozen as described in Section 2.13.
WHOLE-GENOME AND TISSUE MICROARRAY ANALYSIS DEMONSTRATE THAT N-CADHERIN AND BETA 3 INTEGRIN ARE MELANOMA PROGRESSION MARKERS, WHEREAS MCAM IS NOT

3.1 INTRODUCTION

Previous research has documented that N-cadherin, MCAM, and β3 integrin are not expressed in melanocytes of normal skin, but they are expressed at high levels in a substantial number of melanoma cell lines and tissues (Holzmann et al., 1987; Albelda et al., 1990; Hsu et al., 1996). However, the expression of the three adhesion molecules has never been examined in concert, and furthermore, this is the first study to examine the expression of these molecules through microarray analyses. Our whole-genome and tissue microarray analyses illustrate both mRNA and protein expression for N-cadherin, MCAM, and β3 integrin, during the distinct stages of melanoma progression. This systematic evaluation of their expression in tissues facilitates the determination of the specific stage of progression whereby the expression of each molecule is substantially increased. Second, the TMAs made it possible to examine, for the first time, the co-expression of N-cadherin, MCAM, and β3 integrin within the same clinical specimens, thus answering the question, do individual metastatic melanomas express more than one of these adhesion molecules, or is one sufficient?

In line with the goals described above, tissue specimens ranging from normal human skin all the way to melanoma-infiltrated lymph nodes were subjected to whole-genome expression profiling. N-cadherin, MCAM, and β3 integrin mRNA expression will be presented. Next, in order to examine the protein expression and localization of the three adhesion molecules, four separate tissue microarrays, composed of either nevi, VGP melanomas, subcutaneous and visceral MGP melanomas, or melanoma-infiltrated lymph nodes (LN), were probed with antibody specific for N-cadherin, MCAM, or β3 integrin. Together, the microarray data expand
upon gaps in the literature regarding the expression of N-cadherin, MCAM, and $\beta_3$ integrin in the distinct stages of melanoma progression.

3.2 RESULTS

3.2.1 Whole-genome microarrays

For the whole-genome expression profiling, biotinylated cRNAs were generated from tissue specimens representing normal skin, benign and atypical nevi, and early and advanced-stage melanomas, as well as cultured human keratinocytes, melanocytes, and human MGP melanoma cells, and hybridized to Affymetrix Human Genome U133 Plus 2.0 Gene Chip Arrays (Smith et al., 2005). Normalized signal intensity values for N-cadherin, MCAM, and $\beta_3$ integrin expression are displayed in Figure 4. Each bar represents one tissue specimen. The microarray data reveal an increase in N-cadherin mRNA expression concordant with the transition from melanoma in situ to VGP melanoma. $\beta_3$ integrin expression in the samples was lower than anticipated, increasing only slightly in the VGP and MGP melanomas, but highly expressed in one lymph node metastasis. In contrast to N-cadherin and $\beta_3$ integrin, MCAM is expressed in benign and atypical nevi, as well as in melanomas in situ. MCAM mRNA levels are further increased in VGP and MGP melanomas, and in melanoma-infiltrated lymph nodes.

The whole-genome arrays indicate that all three adhesion molecules are expressed in short-term cultures of melanocytes, as previously described, although melanocytes of normal skin do not express these molecules to a significant degree (Albelda et al., 1990; Shih et al., 1994). This observation was expected, given that in the absence of keratinocyte-mediate growth control, it is well-established in the literature that cultured melanocytes begin to express melanoma antigens, and that cultured melanocytes do not accurately represent the phenotype of melanocytes of normal skin (Herlyn et al., 1987). All three cell adhesion molecules are likewise expressed in the MGP melanoma cell line WM1158.

Because we only subjected one MGP melanoma cell line to whole-genome microarray analysis, we performed RT-PCR to determine the level of N-cadherin, MCAM, and $\beta_3$ integrin
mRNA in additional cell lines. The expression of N-cadherin, MCAM, and $\beta_3$ integrin mRNA in three MGP melanoma cell lines is illustrated in Figure 5. In the WM983B cells, the $\beta_3$ integrin primers also amplify a smaller fragment of approximately 600 bp, which may represent a splice product.
Figure 4: N-cadherin, MCAM, and \( \beta_3 \) integrin mRNA expression in cells and tissues subjected to whole-genome expression profiling
The samples represent short-term cultures of human keratinocytes (Ker) and neonatal melanocytes (Mel), and tissue specimens including normal skin (NS1; NS2), benign nevi (BN1; BN2), atypical nevi (AN1; AN2), melanomas in situ (in situ1; in situ2), VGP melanomas (VGP1; VGP2), MGP melanomas (MGP1; MGP2), melanoma-infiltrated lymph nodes (LN1; LN2; LN3), and cultured MGP melanoma cells (WM1158).

Figure 5: N-cadherin, MCAM, and \( \beta_3 \) integrin expression in MGP melanoma cells as determined by RT-PCR
Total RNA was extracted from WM1158, WM852, and WM983B cells, and N-cadherin, MCAM, or \( \beta_3 \) integrin cDNA was amplified by RT-PCR. The sizes of the RT-PCR-amplified fragments are: N-cadherin 1.9 kb, MCAM 1.8 kb, \( \beta_3 \) integrin 2.3 kb.
3.2.2 Tissue microarrays

To independently validate the whole-genome microarray data, we used tissue microarrays (TMAs), consisting of four individual TMAs composed of benign and atypical nevi, VGP melanomas, subcutaneous and visceral MGP melanomas, or melanoma-infiltrated lymph nodes (LN). Each TMA was probed with antibody specific for human N-cadherin, MCAM, or $\beta_3$ integrin.

The percentage of tissue cores from each TMA exhibiting expression of each protein, or lack thereof, is illustrated in Table 1. We found that expression of N-cadherin and $\beta_3$ integrin increases from nevi $\rightarrow$ VGP $\rightarrow$ MGP melanoma. N-cadherin is detected in 39.5% (nevi), 60.5% (VGP), and 65.0% (MGP) of cores, while $\beta_3$ integrin is detected in 0% (nevi), 30.6% (VGP), and 77.9% (MGP) of cores. In contrast, MCAM is expressed in a significant percentage of both VGP and MGP melanomas (85.7 and 77.3%), but is also expressed in 81.4% of nevi. Therefore, the TMA, like the whole-genome profiling data (Smith et al., 2005), do not support earlier suggestions that MCAM is a melanoma progression marker. In Table 2, the data are expressed as the average score (0-2) for each adhesion molecule, in each TMA. These data clearly confirm the results described in Table 1.

It should be noted that the LN tissue cores did not yield strong/overall good quality signal for any of the molecules examined in the context of the TMA project. We did not observe this technical problem with the nevus, VGP, or MGP TMAs. Therefore, this observation is currently being addressed through the generation of a new LN TMA.
Table 1: N-cadherin, MCAM, and $\beta_3$ integrin expression in melanoma progression pathway TMAs
TMA slides were stained with N-cadherin, MCAM, or $\beta_3$ integrin antibody. Antibody-probed tissue cores were scored using a scale of 0 (no signal), 1 (weakly positive), or 2 (strongly positive). The results are displayed as the percentage of tissue cores that were scored 0, 1, 2, or $\geq 1$.

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<tr>
<th></th>
<th>Nevi</th>
<th>VGP</th>
<th>MGP</th>
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<th>MGP</th>
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<td><strong>MCAM</strong></td>
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<th>MGP</th>
<th>LN</th>
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<td><strong>$\beta_3$ integrin</strong></td>
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<td>% scored 1 or 2</td>
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<td>69.4</td>
<td>22.1</td>
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Table 2: Average scores for N-cadherin, MCAM, and $\beta_3$ integrin expression in each TMA
The data are expressed as the average score (0-2) for N-cadherin, MCAM, and $\beta_3$ integrin expression, on each TMA

<table>
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<tr>
<th>Average Score (0-2)</th>
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<th>MGP</th>
<th>LN</th>
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<td><strong>N-cadherin</strong></td>
<td>0.42</td>
<td>0.70</td>
<td>0.75</td>
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<td><strong>MCAM</strong></td>
<td>1.21</td>
<td>1.11</td>
<td>1.33</td>
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<td><strong>$\beta_3$ integrin</strong></td>
<td>0</td>
<td>0.42</td>
<td>1.24</td>
<td>0.45</td>
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</table>
Selected images of N-cadherin-probed TMA cores are shown in Figure 6. Overall, N-cadherin expression was somewhat weak across the TMA slides, and we believe this may have been a result of the antibody dilution. However, it is clear that N-cadherin expression is weak or absent in nevi, and high in some, but not all, VGP and MGP melanomas.

**Figure 6: Expression of N-cadherin in melanoma progression pathway TMAs**
Tissue cores, representing nevi, VGP melanomas, MGP melanomas, and melanoma-infiltrated lymph nodes (LN), were probed with antibody to N-cadherin.
Selected images of MCAM-probed TMA cores are shown in Figure 7. They represent the same set of tissue cores depicted in Figure 6. MCAM, unlike N-cadherin and β3 integrin, is expressed in most nevi. In addition to its expression in nevocytes, which are clusters of melanocytes, MCAM was also expressed in certain cells of the dermis, including endothelial cells and hair follicles (data not shown). In the majority of VGP and MGP melanoma cores, MCAM is expressed at high levels.

**MCAM**

![Image of MCAM-expressed tissue cores](image)

**Figure 7: Expression of MCAM in melanoma progression pathway TMAs**
Tissue cores, representing nevi, VGP melanomas, MGP melanomas, and melanoma-infiltrated lymph nodes (LN), were probed with antibody to MCAM.
Selected images of β3 integrin-probed TMA cores are illustrated in Figure 8. The same corresponding tissue cores are illustrated as in Figure 6 and 7, except for the core in the upper right-hand corner, which was missing from the β3 integrin TMA. β3 integrin is not expressed in any of the nevi, but is first detected in VGP melanomas, and is highly expressed in the MGP melanomas. In several of the VGP and MGP melanomas, β3 integrin is also detected in blood vessels (Figure 9).

Figure 8: Expression of β3 integrin in melanoma progression pathway TMAs
Tissue cores, representing nevi, VGP melanomas, MGP melanomas, and melanoma-infiltrated lymph nodes (LN), were probed with antibody to β3 integrin.
Figure 9: β3 integrin expression in melanoma-infiltrating blood vessels

As a “negative” control, we probed each TMA with antibody to PERP (p53 apoptosis Effector Related to PMP-22 (Attardi et al., 2000), a molecule that was first identified as a tumor suppressor of melanoma (Hildebrandt et al., 2001), and which we have confirmed as being downregulated in advanced-stage melanomas (Smith et al., 2005). The absence of staining in two selected PERP-probed TMA cores, compared with the same cores probed for N-cadherin, MCAM, or β3 integrin, is illustrated in Figure 10. The results shown are representative of all PERP-stained TMA cores.

Figure 10: Selected tissue cores probed with antibody to PERP, serving as a “negative” control for the TMA staining
Given the finding that every one of the three cell adhesion molecules is expressed in some VGP and MGP melanomas, we next determined to what extent subcutaneous and visceral MGP melanoma lesions from individual patients express one, two, or all three proteins. As summarized in Table 3, 97% of the 59 tumors analyzed expressed at least one, 80% two, and 48% all three. These data suggest that simultaneous expression of all three adhesion molecules may not be required for melanoma metastasis. However, the fact that 97% of tumors express at least one molecule points to a critical role of the three adhesion molecules in the process of metastasis formation, whereby expression of at least one of these three molecules is essential.
Table 3: Co-expression of N-cadherin, MCAM, and/or \( \beta_3 \) integrin in MGP melanomas
Fifty-nine MGP melanoma tissue cores, composed of subcutaneous and visceral metastases. Cores were scored on a scale of 0 (no stain), 1 (weakly positive), or 2 (highly positive).

<table>
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<th>Level of Expression</th>
<th>Tumor</th>
<th>Level of Expression</th>
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<td></td>
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<td>1</td>
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Another question we asked with respect to the TMA data was whether expression of N-cadherin, MCAM, and/or \( \beta_3 \) integrin correlates with formation of metastases at a particular organ site. We organized the available data for the subcutaneous and visceral MGP melanoma TMA into four categories of organ-site of metastasis: soft tissue metastases (10), lung metastases (6),
subcutaneous metastases (27), and gastrointestinal metastases (12). Expression of N-cadherin, MCAM, and β3 integrin (scored 0, 1, or 2) in each organ site is illustrated in Figure 11. Although the overall number of tissue samples per group is relatively low, the data provide some insights correlating expression of each adhesion molecule with the site of metastasis formation. Soft tissue metastases show the most prominent expression of β3 integrin, while lung metastases exhibit high-level expression of both MCAM and β3 integrin in a significant percentage of tissue cores. In subcutaneous metastases, MCAM is the most highly expressed adhesion molecule, although N-cadherin and β3 integrin are also present in a majority of tissue specimens. Interestingly, 100% of the gastrointestinal metastases analyzed express β3 integrin to some degree. Furthermore, of the four organ-site categories, the gastrointestinal metastases were the most likely to co-express all three adhesion molecules (8 out of 12 cores examined - 66%).

Figure 11: Expression of N-cadherin, MCAM, and β3 integrin in melanomas metastasized to different organ sites

The data from the MGP melanoma TMA were evaluated according to metastasis to four distinct organ sites: soft tissue (10), lung (6), subcutaneous (27), or gastrointestinal (12). They are expressed as the percentage of cores in each group expressing N-cadherin, MCAM, or β3 integrin: 0 (no stain), 1 (weakly positive), 2 (highly positive)
3.3 DISCUSSION

Our whole-genome expression profiling data, combined with the results of our TMA analysis, demonstrate that N-cadherin and β3 integrin are melanoma progression markers, whereas MCAM, despite its strong expression in advanced-stage melanomas, is not. The latter finding corroborates previous data, obtained in the context of a small-scale immunohistochemical analyses of benign and atypical nevi (Wang et al., 1996). Furthermore, we demonstrate that 80% of subcutaneous and visceral MGP melanoma tissue cores express at least two of the respective three adhesion molecules, and approximately 50% of them reveal expression of all three (Table 1). On the one hand, this suggests that all three cell adhesion molecules need not be expressed at the same time in a given MGP melanoma to govern metastasis. On the other hand, given that one of the three adhesion molecules is nearly always present in the MGP melanomas, we postulate that every one of these molecules plays a role in metastasis.

These observations are important in the context of melanoma treatment. If all three adhesion molecules are essential for metastasis, then blocking one should, in theory, suffice to inhibit metastasis. However, the TMA data suggest that, in fact, melanomas can metastasize without expressing all three molecules. The fact that each adhesion molecule is not essential suggests that it may be necessary to simultaneously target more than one adhesion molecule for the most effective treatment. This strategy may be of particular importance in patients who co-express the molecules – in these patients, upon blocking one adhesion molecule, another may compensate for its function, rendering the therapy ineffective. Furthermore, and equally important, the whole-genome and TMA data demonstrate that expression profiles vary in melanomas from patient-to-patient, which should be taken into consideration when determining the most effective therapeutic strategy for individual patients. Finally, our data raise the important question, are advanced-stage melanomas that express all three molecules more aggressive with respect to metastasis formation at distant sites than the ones expressing only one or two molecules? This important aspect will be further addressed in our overall discussion.
4.0 INHIBITION OF N-CADHERIN, MCAM, OR BETA 3 INTEGRIN REDUCES PROLIFERATION OF HUMAN MGP MELANOMA CELLS

4.1 INTRODUCTION

Following the whole-genome and TMA analysis, our next objective was to investigate the functional role of N-cadherin, MCAM, and β3 integrin in human MGP melanoma cells. To obtain information regarding the biological function(s) of each of the three molecules in advanced-stage melanomas, the first question we asked was, do these genes play a role in melanoma cell proliferation? Previous literature has suggested an association of these adhesion molecules with signaling pathways and genes that regulate proliferation and survival, such as bFGF/FGFR-1 (Suyama et al., 2002; Sahni and Francis, 2004) and Akt (Zheng et al., 2000; Li et al., 2001; Tran et al., 2002; Li et al., 2003). However, studies examining the effect upon proliferation following inhibition of the endogenous molecules in melanoma cells are lacking. We hypothesized that inhibiting N-cadherin, MCAM, or β3 integrin expression may decrease melanoma cell proliferation. In addition, we tested whether blocking expression of all three molecules in concert would result in a greater decrease in proliferation compared with their individual inhibition. We used two experimental approaches – RNA interference (RNAi) and antisense targeting - to analyze the effect on melanoma cell proliferation following inhibition of N-cadherin, MCAM, and/or β3 integrin.
4.2 RESULTS

4.2.1 RNA interference

RNA interference (RNAi) is a conserved biological mechanism, whereby fragments of double-stranded RNA are processed into 20-25 nucleotide fragments by an enzyme called Dicer. Subsequently, the RNA fragments assemble into an RNA-induced signaling complex (RISC), which targets the complementary mRNA for cleavage and degradation. Small interfering RNAs (siRNAs) are short double-stranded RNAs, usually 19-22 nucleotides in length, which can be synthesized for inhibition of a specific gene in vitro. We will also demonstrate that multiple siRNAs can be utilized in order to simultaneously downregulate the expression of more than one gene.

4.2.1.1 Downregulation of N-cadherin, MCAM, and/or $\beta_3$ integrin expression in MGP melanoma cells by siRNA(s)

siRNAs were designed to target human N-cadherin, MCAM, or $\beta_3$ integrin. Two MGP melanoma cell lines (WM1158 and WM852) were transfected with N-cadherin, MCAM, or $\beta_3$ integrin-specific siRNA. In order to examine the transfection efficiency, each siRNA was labeled with the Cy3 fluorochrome, and subsequently transfected into WM1158 cells. The inset in Figure 12 depicts Cy3-labeled siRNA taken up by the cells (pseudocolored yellow), with fluorescent DAPI nuclear counterstain (pseudocolored blue). The majority of the cells contained multiple copies of siRNA. Immunofluorescence staining indicated that the levels of N-cadherin, MCAM, and $\beta_3$ integrin are reduced following transfection with each respective siRNA (Figure 12). Immunoblot analysis further demonstrated that N-cadherin, MCAM, and $\beta_3$ integrin protein levels are decreased in both WM1158 and WM852 cells, 48 hours after treatment with each respective siRNA (+), compared with Lipofectamine 2000 only-transfected control cells (-) (Figure 13).
Figure 12: siRNA-mediated inhibition of N-cadherin, MCAM, or β3 integrin expression
Fluorescence images of WM1158 melanoma cells not transfected (-), or transfected (+) with N-cadherin, MCAM, or β3 integrin siRNA, 72 hours post-transfection. Cells were probed with antibody to N-cadherin (pseudocolored red), MCAM (pseudocolored green), or β3 integrin (pseudocolored orange), followed by Cy5-labeled secondary antibody. Nuclei are counterstained with fluorescent DAPI (pseudocolored blue). Insets illustrate cells transfected with Cy3-labeled siRNA (pseudocolored yellow) to N-cadherin, MCAM, or β3 integrin.
Figure 13: siRNA-mediated inhibition of N-cadherin, MCAM, or β3 integrin expression

Immunoblot analysis of MGP melanoma cell lines, WM1158 and WM852, in the absence (-) or presence of 100 nM siRNA (+) targeting N-cadherin, MCAM, or β3 integrin, 48 hours following siRNA transfection.

In order to address our hypothesis that simultaneous inhibition of all three adhesion molecules may have a more pronounced effect upon melanoma cell proliferation, we wanted to establish a method for inhibiting expression of all three genes at the same time. Once we had demonstrated successful downregulation of N-cadherin, MCAM, and β3 integrin using siRNA to each molecule, the next step was to attempt to simultaneously inhibit N-cadherin, MCAM, and β3 integrin expression using a combination of all three siRNAs. Figure 14 illustrates that the levels of each respective protein are decreased following combined targeting of all three adhesion molecules (+), compared with Lipofectamine 2000 only (-).
4.2.1.2 Inhibition of N-cadherin, MCAM, and/or β3 integrin expression through siRNA slows melanoma cell proliferation, but does not arrest it

Having shown that siRNAs are an effective tool to downregulate expression of the three adhesion molecules, we next determined the impact on melanoma cell proliferation following siRNA-mediated inhibition of N-cadherin, MCAM, and/or β3 integrin. Melanoma cells were plated in triplicate and counted at 24, 48, and 72 hours after siRNA transfection. At the first two time points, we did not observe noticeable differences in the rate of proliferation of the siRNA-transfected cells compared to controls (with the exception of β3 integrin siRNA-treated cells at 48 hours). However, at 72 hours following siRNA-transfection, proliferation was decreased by approximately 50% in the cells that received N-cadherin, MCAM, and/or β3 integrin siRNA (Figure 15). Interestingly, this reduction in proliferation was not augmented by inhibiting, in parallel, the expression of all three genes.
Figure 15: Proliferation of melanoma cells is decreased when expression of N-cadherin, MCAM, and/or β3 integrin is inhibited through siRNA
WM1158 cells were transfected with 100 nM of N-cadherin, MCAM, or β3 integrin siRNA, or a combination of 100 nM of each siRNA. At 24, 48, and 72 hours post-transfection, cells were counted using a hemacytometer. The data are expressed as the average of triplicate counts. * = p < 0.05, Student’s t-test

Because of the noted decrease in proliferation, we next asked whether there is a link between inhibition of proliferation and onset of apoptosis in the MGP melanoma cells. The cell morphology following siRNA treatment did not provide an indication that apoptosis was occurring (data not shown). However, to obtain a more definitive answer to this question, we analyzed the siRNA-treated cells for apoptosis, using the terminal transferase dUTP nick end labeling (TUNEL) method, which is designed to recognize DNA strand breaks through labeling of free 3’OH groups. TUNEL staining was performed 72 hours following siRNA transfection, the same timepoint at which proliferation was significantly decreased. Consistent with our observations of cell morphology, cells treated with N-cadherin, MCAM, or β3 integrin siRNA, or a combination of all three siRNAs, did not display increased apoptosis compared with non-transfected or Lipofectamine 2000 only-treated cells (Figure 16).
Figure 16: Absence of apoptosis following inhibition of N-cadherin, MCAM, and/or β3 integrin
WM1158 or WM983B cells were transfected with 100 nM of N-cadherin, MCAM, or β3 integrin siRNA, or with a combination of 100 nM each siRNA (combined siRNAs). Controls were not transfected, transfected with Lipofectamine alone, or transfected with GFP siRNA. TUNEL assay was performed 72 hours following siRNA transfection. Positive control cells were pre-treated with DNase I. Green pseudocolor indicates the presence of DNA strand breaks. The cells were not counterstained.
4.2.2 Antisense targeting

The inhibition of gene expression via siRNA is transient. In contrast, vectors containing antisense cDNA sequences can mediate stable downregulation of expression, when transfected cells are grown in the presence of a selective antibiotic. Thus, we utilized this technique to further investigate the functional consequence of stably downregulating expression of N-cadherin, MCAM, or β3 integrin in an MGP melanoma cell line.

4.2.2.1 Reduced expression of N-cadherin, MCAM, and β3 integrin in antisense-transfected cells

In parallel with the siRNA targeting approach, we generated pcDNA3.1 expression vectors containing antisense-oriented N-cadherin, MCAM, or β3 integrin cDNA, driven by a CMV promoter. Each plasmid vector was transfected into WM983B MGP melanoma cells, as described in the Materials and Methods section. Transfectants were selected in the presence of hygromycin, the antibiotic marker contained in the pcDNA3.1 vector, and screened for protein expression. Illustrated in Figure 17 are three cell lines that stably express N-cadherin, MCAM, or β3 integrin antisense plasmid and show reduced levels of each respective protein. Therefore, both siRNA and antisense plasmid vectors successfully downregulated expression of the adhesion molecules in MGP melanoma cells.

Figure 17: N-cadherin, MCAM, and β3 integrin antisense-transfected cells
WM983B cells were transfected with pcDNA vector only, or with pcDNA vector containing antisense-oriented cDNA for N-cadherin, MCAM, or β3 integrin. Hygromycin-resistant colonies were propagated. Whole-cell lysates were collected and analyzed by immunoblot analysis for expression of N-cadherin, MCAM, or β3 integrin. Actin serves as a loading control.
4.2.2.2 N-cadherin and β3 integrin antisense-transfected cells display altered morphology

Phase-contrast images were acquired of the antisense-transfected N-cadherin, MCAM, and β3 integrin cell lines and depicted in Figure 18. In the N-cadherin antisense transfectants, about 50% of the cells exhibit a more rounded, epithelial morphology, compared with the fibroblastic morphology of control non-transfected or vector only-transfected cells. This fact is intriguing, considering that E-cadherin is generally expressed in epithelial cell types, while N-cadherin expression is associated with mesenchymal cells. The MCAM antisense cells, on the other hand, show no substantial alterations in morphology compared with controls. The β3 integrin antisense cells, however, tend to grow more in clusters or groups of cells, rather than in a uniform sheet across the surface of the culture dish. This suggests that there is an impact upon adhesion of the melanoma cells to the matrix following downregulation of β3 integrin expression.

In the case of the N-cadherin, MCAM, and/or β3 integrin siRNA-transfected cells, no significant changes in morphology were observed up to 72 hours following transfection (data not shown). However, it is important to note that the transient inhibition of expression following siRNA transfection may yield a less pronounced effect upon morphology compared with a stable downregulation of expression by means of an antisense plasmid.
4.2.2.3 Proliferation of MCAM and $\beta_3$ integrin antisense-transfected cells is impaired, but not arrested

We next determined the rate of proliferation of the N-cadherin, MCAM, and $\beta_3$ integrin antisense transfectants in comparison to non-transfected or pcDNA vector only-transfected cells. The cells were plated in 96-well culture dishes, and the MTT colorimetric assay was performed at the indicated timepoints. Similar to the MCAM and $\beta_3$ integrin siRNA transfected cells, the MCAM and $\beta_3$ integrin antisense transfectants demonstrated a 30-40% lower growth rate compared to the controls (Figure 19). However, proliferation of the N-cadherin antisense transfectants did not differ from the controls.

The different effect upon proliferation following inhibition of N-cadherin through siRNA compared with antisense targeting could be a result of several factors. First, siRNA and antisense-mediated inhibition may elicit somewhat different cellular responses, due to their different mechanism of inhibition. Furthermore, the siRNA inhibition is transient, whereas the
stable antisense-mediated downregulation is maintained long-term. Second, the two MGP melanoma cell lines utilized for the experiment (WM1158 versus WM983B) may respond differently to the inhibition of N-cadherin. In the future, it would be valuable to generate WM1158 cells that stably express antisense N-cadherin, MCAM, or $\beta_3$ integrin vector. Overall, however, the trend toward decreased proliferation following inhibition of the three cell adhesion molecules is similar with both experimental approaches.

**Figure 19: Proliferation of melanoma cells is decreased when expression of MCAM or $\beta_3$ integrin is downregulated via pcDNA antisense plasmid**

The MTT assay was performed at timepoints following plating of each cell lines in 96-well culture dishes ($^* = p < 0.05$, Student’s t-test).

### 4.3 DISCUSSION

To address the question of whether N-cadherin, MCAM, and/or $\beta_3$ integrin play a role in melanoma cell proliferation, RNAi and antisense targeting were used to downregulate expression of each gene in MGP melanoma cells. Furthermore, we simultaneously inhibited expression of
all three adhesion molecules using a combination of all three siRNAs. Whereas morphological differences were not observed following transfection with siRNAs, the N-cadherin and β3 integrin stable antisense transfectants display altered morphology, suggesting changes in the adhesion properties of these cells.

siRNA-mediated inhibition of N-cadherin, MCAM, or β3 integrin led to a 50% decrease in melanoma cell proliferation. Simultaneous inhibition of N-cadherin, MCAM, and β3 integrin did not augment this effect. In the case of the MCAM and β3 integrin antisense transfectants, proliferation was decreased by approximately 30-40%, but proliferation of N-cadherin antisense cells was not significantly altered. Again, it is likely that this discrepancy is due in part to the different methodological approach – in particular, the fact that siRNA-mediated inhibition is transient, while the antisense approach we utilized is maintained long-term.

Although a decrease in proliferation was observed as indicated, the fact that it was possible to propagate colonies stably expressing N-cadherin, MCAM, or β3 integrin antisense plasmid was a preliminary indication that proliferation was not completely arrested. Neither cell morphology nor TUNEL staining of the siRNA-transfected cells compared to the two controls provided evidence that the decrease in proliferation was accompanied by onset of melanoma cell apoptosis. Therefore, the data support the hypothesis we put forward that inhibition of N-cadherin, MCAM, and/or β3 integrin partially impairs MGP melanoma cell proliferation. However, when compared to the results of targeting basic fibroblast growth factor (bFGF; FGF2) and/or FGFR-1, which both in vitro and in vivo, lead to complete arrest of melanoma growth (Wang and Becker, 1997), the data presented here do not make an overly strong argument for N-cadherin, MCAM, and/or β3 integrin being absolutely essential for melanoma cell proliferation.

The residual growth of the MGP melanoma cells is likely maintained through alternative signaling pathways. In epithelial cells (MDCK), for example, although the cells undergo anoikis following disruption of cell-ECM interactions, overexpression of bcl-2 can rescue the cells from apoptosis, as can transformation with the v-src oncogene, or treatment with scatter factor/HGF (Frisch and Francis, 1994). In melanoma cells, bFGF (Becker et al., 1992) and pAkt (Dai et al., 2005), to name a few, have been implicated in melanoma progression, and may mediate, to some degree, cell proliferation and anti-apoptotic signaling in the absence of N-cadherin, MCAM, and/or β3 integrin expression.
Overall, the results demonstrate that N-cadherin, MCAM, and $\beta_3$ integrin partially mediate melanoma cell proliferation, but that blocking their expression does not fully arrest proliferation, and does not drive the cells into apoptosis. Although N-cadherin, MCAM, and $\beta_3$ integrin play a role in melanoma cell proliferation, they may not represent the primary mediators of proliferation.
5.0 INHIBITION OF N-CADHERIN, MCAM, AND/OR BETA 3 INTEGRIN EXPRESSION DOES NOT CAUSE DEREGLULATION OF RELATED MOLECULES

5.1 INTRODUCTION

In light of the observed effect upon proliferation following inhibition of N-cadherin, MCAM and/or β3 integrin, we next addressed the questions; what signaling pathways are altered in these MGP melanoma cells? Is the expression of other adhesion molecules impacted? Three major pathways were investigated following inhibition of N-cadherin, MCAM, and/or β3 integrin: the putative reciprocal relationship between N-cadherin and E-cadherin, the expression of the αv integrin subunit, and the Akt signaling pathway.

In the case of E-cadherin, there is a well-established switch from E to N-cadherin expression in numerous cancers of epithelial origin (for review, see Cavallaro, 2004), including melanoma (Hsu et al., 1996). As described in the introduction, re-expression of E-cadherin in a melanoma cell line results in decreased invasion and tumor formation (Hsu et al., 2000). However, to date, it is unknown whether E-cadherin expression can be restored in melanoma cells following inhibition of N-cadherin expression. Antisense-mediated downregulation of N-cadherin in a squamous cell carcinoma cell line suggested that in some cell types, E-cadherin expression might be restored via this approach (Islam et al., 1996). This question is intriguing, given that E-cadherin-mediated interactions between melanocytes and keratinocytes help to keep melanocyte proliferation in check (Valyi-Nagy et al., 1993). Re-expression of E-cadherin in melanoma cells might therefore result in decreased melanoma cell proliferation.

The αv integrin subunit, which associates with the β3 integrin subunit, represents another relevant molecule for investigation. Expression of the αvβ3 integrin complex increases concordant with melanoma progression (Natali et al., 1997); yet, expression of the αv integrin subunit remains unchanged (Albelda et al., 1990). The αv integrin subunit can also associate with
other β subunits, including β₁ (Bodary and McLean, 1990), and β₅ (Smith et al., 1990). We examined whether αv integrin expression is altered following downregulation of β₃ integrin.

Akt is known to promote survival/proliferation in many human cancers, including melanoma (for review, see Vivanco and Sawyers, 2002). There have been several reports in the literature linking the expression of N-cadherin, MCAM, and β₃ integrin with Akt signaling. For example, in two melanoma cell lines, an N-cadherin function-blocking antibody was shown to inhibit the phosphorylation (activation) of Akt (Li et al., 2001). Also, transfection of additional copies of MCAM into a melanoma cell line lead to phosphorylation of Akt, while expression of a constitutively active Akt also increases MCAM expression (Li et al., 2003). This study generated some uncertainty as to whether Akt acts as an upstream regulator or a downstream “effector” of MCAM in melanoma cells, or whether a reciprocal relationship exists. A link between the engagement of αvβ₃ integrin through vitronectin and/or osteopontin and the activation of Akt has been demonstrated in prostate carcinoma cells (Zheng et al., 2000). Most of these studies utilized antibodies that block protein function, and none addressed the consequence of inhibiting of N-cadherin, MCAM, or β₃ integrin mRNA/protein expression in melanoma cells. We examined the phosphorylation of Akt at Serine 473 following inhibition of these molecules by way of siRNA or stable antisense inhibition. Activation of Akt requires phosphorylation at two sites (Threonine308 and Serine473), with phosphorylation at Serine473 required for complete activation. The goal of our approach was to determine whether the observed decrease in proliferation following inhibition of N-cadherin, MCAM, and/or β₃ integrin was accompanied by changes in Akt activation.

Finally, the hypothesis that inhibition of one adhesion molecule might lead to a compensatory change in expression of the other adhesion molecules was addressed, by examining whether individual inhibition of N-cadherin, MCAM, or β₃ integrin leads to changes in expression of the remaining two molecules. Because N-cadherin, MCAM, and β₃ integrin are reported to perform overlapping functions in the melanoma cells, an increase in the expression of another adhesion molecule might indicate that it is compensating for the function of the downregulated molecule. In the development of future therapeutic approaches, this issue will be crucial, because a drug designed to block expression of one adhesion molecule may not generate a robust tumor response if another adhesion molecule is capable of compensating for its loss of function.
5.2 RESULTS

5.2.1 Expression of related molecules following inhibition of N-cadherin, MCAM, and/or β₃ integrin through siRNA

Because E-cadherin expression is decreased concurrent with melanoma progression, while N-cadherin expression is upregulated (Tang et al., 1994; Danen et al., 1996; Hsu et al., 1996), we investigated whether blocking N-cadherin would result in re-expression of E-cadherin in two MGP melanoma cell lines that no longer express E-cadherin. Upon inhibition of N-cadherin by RNAi, E-cadherin expression is not restored (Figure 20) (MCF-7 cells were used as a positive control for efficacy of the E-cadherin antibody). This result is in contrast to a previous study examining a squamous cell carcinoma cell line, whereby antisense-mediated downregulation of N-cadherin did, in fact, lead to restored expression of E-cadherin (Islam et al., 1996). Next, we demonstrated that following inhibition of β₃ integrin, expression of the αᵥ integrin subunit, which associates non-covalently with the β₃ subunit, was unchanged (Figure 22). This result suggests that αᵥ integrin is likely associating with an alternative beta subunit in the absence of β₃ integrin.

Due to the observed decrease in proliferation following siRNA transfection, as well as previous literature linking N-cadherin, MCAM, and β₃ integrin with Akt signaling (Zheng et al., 2000; Li et al., 2001; Li et al., 2003), we investigated whether Akt phosphorylation was altered in siRNA-treated melanoma cells. In melanoma cells transfected with Lipofectamine 2000 only, immunoblotting shows that Akt is phosphorylated at serine 473. However, following siRNA treatment, there is no change in the level of Ser473-phosphorylated Akt protein at 48 (data not shown) or 72 hours post-transfection (Figure 23), despite the decrease in proliferation previously indicated at 72 hours. Therefore, the observed decrease in proliferation is not a consequence of inhibition of Akt phosphorylation.

Finally, we determined whether siRNA-mediated inhibition of N-cadherin, MCAM, or β₃ integrin would increase or decrease the protein levels of one another. Western blotting demonstrated that upon inhibition of N-cadherin, MCAM, or β₃ integrin, there is no substantial change in the protein levels of the other two molecules (Figures 20-22).
Figure 20: siRNA-induced inhibition of N-cadherin expression does not lead to changes in levels of MCAM, β3 integrin, or E-cadherin protein
WM1158 or WM852 cells were transfected with 100 nM of N-cadherin siRNA (+), or treated with Lipofectamine only (-). Cell lysates were prepared at 48 hours post-transfection, and expression of MCAM, β3 integrin, E-cadherin, and actin was analyzed by immunoblots.

![Diagram showing protein expression levels](image)

Figure 21: siRNA-induced inhibition of MCAM expression does not lead to changes in levels of N-cadherin or β3 integrin protein
WM1158 or WM852 cells were transfected with 100 nM of MCAM siRNA (+), or treated with Lipofectamine only (-). Cell lysates were prepared at 48 hours post-transfection, and expression of N-cadherin, β3 integrin, and actin was analyzed by immunoblots.

![Diagram showing protein expression levels](image)
Figure 22: siRNA-induced inhibition of β3 integrin expression does not lead to changes in levels of N-cadherin, MCAM, or the αv integrin subunit

WM1158 or WM852 cells were transfected with 100 nM of β3 integrin siRNA (+), or treated with Lipofectamine only (-). Cell lysates were prepared at 48 hours post-transfection, and expression of N-cadherin, MCAM, αv integrin, and actin was analyzed by immunoblots.

Figure 23: Inhibition of N-cadherin, MCAM, and/or β3 integrin expression does not alter levels of phosphorylated Akt, as documented at 72 hours post siRNA transfection

WM1158 cells were transfected with 100 nM of N-cadherin, MCAM, or β3 integrin siRNA, or with a combination of 100 nM of each siRNA (combined siRNAs). 72 hours following siRNA transfection, cell lysates were prepared, and expression of pAkt (Ser473) was determined by immunoblot analysis. Actin serves as a loading control.
5.2.2 Expression of related molecules following antisense-mediated inhibition of N-cadherin, MCAM, and/or β₃ integrin

Because siRNA exerts only a transitory effect upon inhibition of gene expression, we deemed it important to also assess expression patterns in the stable antisense-transfected cells. Therefore, the WM983B N-cadherin, MCAM, and β₃ integrin antisense transfectants were analyzed for the same pathways described following siRNA-mediated inhibition. E-cadherin expression was not restored in the transfected cells following stable downregulation of N-cadherin. Nor were levels of the αᵥ integrin protein substantially altered in cells following stable downregulation of β₃ integrin. Furthermore, levels of phosphorylated Akt protein were found to be unchanged following stable downregulation of N-cadherin, MCAM, or β₃ integrin in these melanoma cells (Figure 24). These results were consistent with the results obtained following the inhibition of N-cadherin, MCAM, or β₃ integrin through siRNA, in two different melanoma cell lines.

Next, we investigated whether stable antisense-mediated downregulation of N-cadherin, MCAM, or β₃ integrin expression deregulates the protein levels of one another. In the MCAM antisense-transfected MGP melanoma cells, N-cadherin expression was increased by approximately 100%, or 2-fold, while the level of β₃ integrin was reduced by approximately 75% (ImageJ Densitometry). In addition, in the β₃ integrin antisense-transfected cells, levels of N-cadherin were decreased by 30% (Figure 24). This result demonstrates that following stable downregulation of N-cadherin, MCAM, or β₃ integrin, either increases or decreases in the protein levels of the other two adhesion molecules can occur.
5.3 DISCUSSION

To assess the potential impact of inhibiting expression of N-cadherin, MCAM, or β3 integrin on genes/pathways potentially linked with these three molecules, we first asked the question; does E-cadherin become re-expressed as a result of N-cadherin downregulation? The answer was a clear no. E-cadherin expression is not restored; neither in the two MGP melanoma cell lines transfected with N-cadherin siRNA (Figure 20), nor in the N-cadherin antisense transfectants (Figure 24). Therefore, our findings clearly demonstrate that in melanoma cells, a direct reciprocal relationship between expression of E-cadherin and N-cadherin does not exist. In other words, unlike in squamous carcinoma cells (Islam et al., 1996), it is not possible restore E-cadherin expression by blocking expression of N-cadherin alone. This observation is significant.
because studies of melanocyte-keratinocyte interactions have suggested that E-cadherin plays a key role in regulating melanocyte proliferation (Valyi-Nagy et al., 1993). As such, the continued absence of E-cadherin expression in melanoma cells may contribute to the promotion of melanoma cell proliferation.

The $\alpha_v$ integrin subunit has been shown to associate with $\beta_3$, $\beta_5$, or $\beta_1$ (Bodary and McLean, 1990; Smith et al., 1990; Cheresh, 1991). Neither the $\beta_3$ integrin siRNA-transfected MGP melanoma cells (Figure 22) nor the $\beta_3$ integrin antisense transfectants (Figure 24) exhibited a change in level of expression of the $\alpha_v$ integrin subunit. One possible explanation for this observation is that $\alpha_v$ integrin associates with $\beta_1$ or $\beta_5$ following downregulation of $\beta_3$ integrin; however, we have not tested this hypothesis to date.

As described above, several studies have suggested a link between N-cadherin, MCAM, and $\beta_3$ integrin, and Akt activation in normal and malignant cells (Zheng et al., 2000; Li et al., 2001; Tran et al., 2002; Li et al., 2003). Akt signaling, which is highly active in many tumors, can mediate proliferation, as well as anti-apoptotic signaling (reviewed by Vivanco and Sawyers, 2002). Serine 473-phosphorylated Akt is present in control MGP melanoma cells, but protein levels are unchanged following inhibition of N-cadherin, MCAM, and/or $\beta_3$ integrin through siRNA (Figure 23). Levels of Serine 473-phosphorylated Akt are also unchanged in the antisense-transfected melanoma cells (Figure 24). These results show that following inhibition of N-cadherin, MCAM, and/or $\beta_3$ integrin expression, we do not observe an inhibition of Akt phosphorylation/signaling. This observation may explain, in part, the lack of complete arrest of proliferation following inhibition of these molecules, as well as the lack of onset of melanoma cell apoptosis. Overall, our studies suggest that in the case of MGP melanoma cells, Akt does not represent a direct downstream target or effector of N-cadherin, MCAM, or $\beta_3$ integrin. However, our results do not preclude the possibility that Akt might act as an upstream regulator of N-cadherin, MCAM, and/or $\beta_3$ integrin, which we did not investigate.

Finally, the potential for compensatory up/down-regulation of N-cadherin, MCAM, or $\beta_3$ integrin following individual targeting was examined. Upregulation of N-cadherin, MCAM, or $\beta_3$ integrin protein following inhibition of either alone was not detected in siRNA-treated cells (Figure 20-22). On the other hand, in the antisense-transfected melanoma cell lines, where inhibition of expression was stable and prolonged, MCAM antisense cells displayed increased levels of N-cadherin and decreased $\beta_3$ integrin, while $\beta_3$ integrin antisense cells had moderately
decreased levels of N-cadherin (Figure 24). The increased levels of N-cadherin in the MCAM antisense-transfected cells may indicate that the cells upregulate N-cadherin in order to compensate for the absence of MCAM. However, it is evident that expression of another molecule may either increase or decrease following inhibition of an individual adhesion molecule. This result suggests that there is some degree of interplay between expression of the three adhesion molecules, and it will be important to consider these mechanisms when developing therapeutic approaches and predicting how patient populations may respond to a given drug.

Overall, it is clear from these studies that upregulation of E-cadherin or $\alpha_v$ integrin does not occur following inhibition of N-cadherin or $\beta_3$ integrin, respectively, and that Akt phosphorylation is not perturbed following inhibition of one or all three of the adhesion molecules examined. Our results also provide a preliminary indication that, in some cases, the inhibition of expression of N-cadherin, MCAM, or $\beta_3$ integrin may result in a change in the protein levels of one another.
6.0 SIMULTANEOUS INHIBITION OF N-CADHERIN, MCAM, AND BETA 3 INTEGRIN IMPAIRS MELANOMA CELL MIGRATION AND INVASION

6.1 INTRODUCTION

Migration and invasion are crucial features of cancer metastasis, and cell adhesion molecules are essential for these processes. Not only do they mediate interactions between the tumor cells, but they also govern important interactions with cells of the tumor’s microenvironment. Changes in the profile of adhesion molecules expressed by tumor cells can enable them to survive, migrate, and invade in foreign environments.

Previous data in the literature demonstrated a pivotal role for N-cadherin, MCAM, and β3 integrin in migration and invasion. However, knowledge regarding the impact on migration/invasion following inhibition of N-cadherin, MCAM, and/or β3 integrin in MGP melanomas is lacking. Moreover, our TMA analysis demonstrated that 97% of subcutaneous and visceral melanoma metastases express at least one of these three adhesion molecules, in direct contrast to their normal melanocyte counterparts, which do not express N-cadherin, MCAM, or β3 integrin (Albelda et al., 1990; Shih et al., 1994; Hsu et al., 1996). The TMA data, therefore, strongly suggest that N-cadherin, MCAM, and/or β3 integrin expression might play a role in migration and/or invasion, two key properties in the acquisition of metastatic potential.

We hypothesized that inhibition of N-cadherin, MCAM, or β3 integrin may decrease melanoma cell migration/invasion, and further predicted that simultaneous inhibition of all three molecules may elicit an even greater response. Thus, we conducted a series of in vitro studies to evaluate whether N-cadherin, MCAM, and/or β3 integrin mediate melanoma cell migration and/or invasion.
6.2 RESULTS

6.2.1 Migration is decreased in melanoma cells following transfection with either MCAM siRNA, or with a combination of N-cadherin, MCAM, and β3 integrin siRNAs

To analyze cell migration, melanoma cells were transfected with N-cadherin, MCAM, or β3 integrin siRNA, or a combination of all three siRNAs. 48 hours post-transfection, cells were seeded into transwell migration chambers and allowed to migrate for 22 hours, with 5% FBS serving as a chemoattractant. Migration of cells treated with MCAM siRNA or a combination of N-cadherin MCAM, and β3 integrin siRNA was reduced by 30 and 50%, respectively, in comparison to cells transfected with only Lipofectamine 2000 or with a scrambled siRNA control (Figure 25). Individual inhibition of N-cadherin or β3 integrin expression, on the other hand, did not result in a significant change in cell migration.

![Melanoma Cell Migration](image)

**Figure 25:** Transfection of MGP melanoma cells with MCAM siRNA, or a combination of N-cadherin, MCAM, and β3 integrin siRNAs leads to a decrease in migration

WM1158 cells were transfected with Lipofectamine 2000 alone (control), 100 nM of a scrambled siRNA, 100 nM of siRNA specific for N-cadherin, MCAM, or β3 integrin, or a combination of 100 nM each of N-cadherin, MCAM, and β3 integrin siRNA. 48 hrs following siRNA-transfection, cells were plated in transwell migration chambers. Cells that migrated to the lower membrane surface were fixed, stained, and counted. Data are expressed as the average number of cells per 40X field that migrated to the lower surface of the filter. (* = p < 0.05, Student’s t-test)
6.2.2 Invasion is decreased in melanoma cells following transfection with MCAM siRNA, β3 integrin siRNA, or a combination of N-cadherin, MCAM, and β3 integrin siRNAs

In addition to examining the impact of inhibiting N-cadherin, MCAM, and/or β3 integrin upon melanoma cell migration, we sought to examine whether N-cadherin, MCAM, and/or β3 integrin play a role in melanoma cell invasion. For analysis of invasion, the cells were plated into transwell chambers containing Matrigel-coated filters. Downregulation of MCAM, β3 integrin, or all three genes at once, through siRNA, reduced invasion by 30, 34, and 50%, respectively (Figure 26). In contrast, melanoma cells did not demonstrate a major change in their ability to invade when transfected with N-cadherin siRNA alone.

![Figure 26: Transfection of MGP melanoma cells with MCAM siRNA, β3 integrin siRNA, or a combination of N-cadherin, MCAM, and β3 integrin siRNAs results in decreased invasion](image)

WM1158 cells received Lipofectamine 2000 alone (control), 100 nM of a scrambled siRNA, 100 nM of siRNA specific for N-cadherin, MCAM, or β3 integrin, or a combination of 100 nM each of N-cadherin, MCAM, and β3 integrin siRNA. 48 hrs following siRNA-transfection, cells were plated in transwell migration chambers containing a Matrigel-coated filter. Cells that invaded to the lower membrane surface were fixed, stained, and counted. Data are expressed as the average number of cells per 40X field that invaded through to the lower surface of the filter. (* = p < 0.05, Student’s t-test)
The results of both the migration and invasion assays confirm that simultaneous inhibition of N-cadherin, MCAM, and β3 integrin leads to a substantially greater decrease in cell migration and invasion compared to targeting the molecules individually. Inhibition of N-cadherin, in fact, did not inhibit migration or invasion to any significant degree. On the other hand, blocking the expression of MCAM or β3 integrin did have a substantial impact on these two interlinked biological processes, which suggests that these two adhesion molecules play a role in melanoma cell migration and invasion.

It is important to note, however, that although inhibition of N-cadherin did not impair migration/invasion, we cannot rule out the possibility that N-cadherin plays some role in these processes. For instance, inhibiting expression of N-cadherin in the WM1158 cells, which also express MCAM and β3 integrin, does not lead to a decrease in migration or invasion. In these cells, however, the other two adhesion molecules may fully compensate for the loss of N-cadherin function. On the other hand, it is possible that inhibiting N-cadherin in a cell line that does not also express MCAM and β3 integrin may lead to inhibition of cell migration and/or invasion. It would therefore be useful, if feasible, to identify a melanoma cell line that expresses N-cadherin, but not MCAM or β3 integrin, and determine whether migration and invasion are impaired when N-cadherin expression is inhibited in the absence of these adhesion molecules with redundant functions.

Furthermore, although the MGP melanoma cell lines utilized in our experiments co-express N-cadherin, MCAM, and β3 integrin, the TMA data clearly demonstrate that only about 50% of metastatic melanoma patients express all three. Thus, in the clinic, the efficacy of individual or combined targeting of adhesion molecules may differ based on whether an individual patient’s melanoma expresses only one or two adhesion molecules versus all three. Therefore, before initiating a therapeutic regimen, it would be important to evaluate which cell adhesion molecules a particular melanoma expresses, to help determine which patients are most likely to respond to a given treatment.

Taken as a whole, our results support the hypothesis that therapeutic strategies that target a combination of adhesion molecules may induce a more pronounced impairment of melanoma cell migration and invasion compared with targeting only a single molecule.
7.0 INHIBITION OF N-CADHERIN, MCAM, OR BETA 3 INTEGRIN IN VIVO

7.1 INTRODUCTION

In addition to the *in vitro* studies described above, a second aim of this dissertation was to determine the impact of inhibiting the expression of N-cadherin, MCAM, and/or $\beta_3$ integrin in melanoma xenografts, by examining the potential for tumor regression and/or decreased metastasis formation. These *in vivo* studies have the potential to provide additional preliminary evidence regarding whether inhibition of cell adhesion molecules represents a viable therapeutic approach for melanoma. We used two approaches to investigate this question: analysis of the growth patterns of the N-cadherin, MCAM, or $\beta_3$ integrin stable antisense-transfected cells *in vivo*, and direct intratumoral injection of antisense plasmids targeting individual adhesion molecules in established melanoma xenografts. In previous studies, our lab demonstrated successful inhibition of bFGF/FGFR-1 expression in xenografts via intratumoral injection of antisense plasmid (Li et al., 1996; Wang and Becker, 1997; Valesky et al., 2002). This chapter will discuss the approaches and challenges associated with our goal, as well as alternative methods that may be used in future investigations.
7.2 RESULTS

7.2.1 Tumor-forming ability of N-cadherin, MCAM, and β3 integrin antisense transfectants

In the previous chapters, I described the generation of MGP melanoma cells transfected with antisense-oriented N-cadherin, MCAM, or β3 integrin plasmid. Our next objective was to determine whether the antisense transfectants would form tumors in nude mice, and if so, whether the tumors would grow at a reduced rate compared to control cells. In order to assess the growth and tumor-forming ability of these antisense-transfected cells in an \textit{in vivo} setting, we subcutaneously injected $5 \times 10^6$ non-transfected, vector only-transfected, or antisense-transfected cells into each nude mouse. Two mice were injected per cell line. Tumor size was measured until the tumors reached a maximum of 2 cm in perpendicular diameter. Tumor volume for each experimental group is illustrated in Figure 27. The growth of N-cadherin, MCAM, or β3 integrin antisense-transfected cells was substantially reduced (between 2 to 8 fold) compared with non-transfected control cells, beginning as early as day 21 following injection. However, the growth of two vector-transfected cell lines was equally impaired. This experiment was repeated twice, with the same result. Therefore, conclusive results regarding the \textit{in vivo} growth potential of the antisense-transfected cells could not be obtained.

One possible explanation for the technical difficulties encountered with this approach is the impact of growing the cell lines in the selective antibiotic hygromycin prior to injection. It is possible that cells grown in hygromycin have impaired ability to establish as a tumor \textit{in vivo}. We do not believe that the nonspecific inhibition of \textit{in vivo} growth is a direct consequence of the pcDNA vector itself, because we determined that melanoma cells transfected with a different vector control (pREP7-RSV, Invitrogen, for episomal expression) also exhibited impaired growth \textit{in vivo} (data not shown).
Figure 27: Growth of antisense-transfected cells in nude mice
N-cadherin, MCAM, or β3 integrin antisense-transfected WM98B melanoma cells, pcDNA vector only-transfected cells, or non-transfected cells were injected subcutaneously into nude mice (5x10^6 cells per mouse). Tumor size was measured three times per week, and tumor volume was calculated using the formula: [(Length x Width^2)/2].

7.2.2 Direct intratumoral targeting of N-cadherin, MCAM, or β3 integrin in established human melanoma xenografts

While in the previous section we asked whether melanoma cells transfected with antisense-oriented N-cadherin, MCAM, or β3 integrin would exhibit reduced tumor formation and/or tumor growth, our next goal was to determine whether we could inhibit expression of the three adhesion molecules in established melanoma xenografts. The latter method is therapeutically more relevant compared with the in vivo analysis of the antisense transfectants. As mentioned above, our lab previously demonstrated successful inhibition of bFGF/FGFR-1 expression in xenografts, through intratumoral injection of antisense plasmid, using DcChol liposomes as the delivery reagent (Li et al., 1996). However, prepared DcChol liposomes are no longer available.
Therefore, it was necessary to identify a new source of lipid particles for intratumoral delivery of the plasmid constructs.

We utilized several approaches to develop an experimental protocol whereby antisense plasmids could be efficiently expressed \textit{in vivo} and yield subsequent downregulation of expression of the targeted molecule in a melanoma xenograft. First, pREP7-TYR vectors (driven by the tyrosinase promoter, which is expressed in cells of melanocytic origin), containing sense or antisense-oriented $\beta_3$ integrin cDNA, were injected intratumorally into melanoma xenografts, using Lipofectamine 2000 as the delivery reagent. However, we did not observe significant differences in the growth of xenografts injected with antisense or sense $\beta_3$ integrin plasmid, compared with controls, which included no injection, Lipofectamine 2000 only, or empty pREP7-TYR vector (Figure 28).

![Growth of Melanoma Xenografts](image)

**Figure 28:** Melanoma xenografts injected intratumorally with sense or antisense-oriented $\beta_3$ integrin pREP7-TYR plasmid do not display altered growth

Three mice per vector construct were injected intratumorally with Lipofectamine 2000, pREP7-TYR vector, sense-oriented $\beta_3$ integrin pREP7-TYR vector, or antisense-oriented $\beta_3$ integrin pREP7-TYR vector. Two mice received no injection. 100 $\mu$g of plasmid DNA was used for the first injection, with 50 $\mu$g for each injection thereafter. 3 $\mu$g of Lipofectamine 2000 was complexed with the plasmid DNA for each injection. The arrow indicates the first day of plasmid intratumoral injections.
Next, to determine the expression of $\beta_3$ integrin following injection of sense or antisense-oriented $\beta_3$ integrin plasmid, we probed the tumors for $\beta_3$ integrin through standard immunohistochemistry. Selected tumors are shown in Figure 29. Overall, $\beta_3$ integrin levels are unchanged in sense or antisense-injected tumors, compared with controls. However, $\beta_3$ integrin expression is slightly decreased in a small part of the antisense-injected tumor (lower left-hand corner of image).

![Figure 29: Expression of $\beta_3$ integrin is not significantly altered in tumors injected with sense or antisense-oriented $\beta_3$ integrin pREP7-TYR plasmids](image)

Snap-frozen tumors were embedded in O.C.T., sectioned, and stained by standard immunohistochemistry for expression of $\beta_3$ integrin protein.

Given our finding that $\beta_3$ integrin expression was not significantly decreased in the tumors injected with antisense $\beta_3$ integrin plasmid vector, we extracted DNA from the tumors and performed PCR to determine the presence or absence of each plasmid. The sense-oriented $\beta_3$ integrin plasmid is present in the tumors injected with this plasmid, but as expected, is absent in tumors receiving either no injection, vector only, or antisense $\beta_3$ integrin plasmid (Figure 30, left panel). Purified $\beta_3$ integrin sense plasmid served as a positive control. Likewise, $\beta_3$ integrin antisense plasmid is present only in the tumors that were injected with this plasmid. Purified $\beta_3$ integrin antisense plasmid served as a positive control (Figure 30, right panel). These results indicate that the plasmids were delivered to the tumor cells. However, the immunostaining data
suggest that the antisense transcript may not be expressed at sufficiently high levels to produce downregulation of the endogenous $\beta_3$ integrin transcript.

Figure 30: $\beta_3$ integrin sense and antisense pREP7-TYR plasmid DNA is present in tumors injected intratumorally with each respective vector.
DNA was extracted from snap-frozen tumors, and PCR was performed to amplify the sense or antisense $\beta_3$ integrin plasmid vector (1.3 kb fragment).

The previous experiment was repeated, using 5 $\mu$g of Lipofectamine 2000 (rather than 3 $\mu$g) per injection, complexed with $\beta_3$ integrin sense or antisense plasmid vector. In addition, one mouse each was injected intratumorally with N-cadherin or MCAM antisense-oriented pREP7-TYR plasmid. The growth of melanoma xenografts following injection of $\beta_3$ integrin sense or antisense pREP7-TYR plasmid is illustrated in Figure 31.
Figure 31: Melanoma xenografts injected intratumorally with β3 integrin sense or antisense-oriented pREP7-TYR plasmid do not display significant alterations in growth.

Three mice per vector construct were injected intratumorally with Lipofectamine 2000 alone, empty pREP7-TYR vector, sense-oriented β3 integrin pREP7-TYR vector, or antisense-oriented β3 integrin pREP7-TYR vector. Two mice received no injection. 100 µg of plasmid DNA was used for the first injection, with 50 µg for each injection thereafter. 5 µg of Lipofectamine 2000 was complexed with the plasmid DNA for each injection. The arrow indicates the first day of intratumoral plasmid injections.

For a second time, the growth of the xenografts was not significantly altered following injection of β3 integrin sense or antisense plasmid. Immunoblot analysis illustrates that in tumors injected with β3 integrin antisense plasmid, levels of β3 integrin protein were only slightly decreased compared with controls. In the mice injected with N-cadherin or MCAM antisense plasmid, levels of each respective protein were unchanged (Figure 32).
Figure 32: N-cadherin, MCAM, and β3 integrin protein levels are not significantly decreased in tumors following intratumoral injection with antisense pREP7-TYR plasmids targeting each adhesion molecule.
Protein was isolated from snap-frozen tumors and analyzed by immunoblotting for expression of N-cadherin, MCAM, or β3 integrin.

The PCR data indicated that the plasmids were delivered to the cells; yet, effective downregulation of expression of the targeted molecules was not achieved. We hypothesized that the tissue-specific tyrosinase promoter may not generate high enough copy numbers of the antisense transcript to mediate downregulation of the targeted proteins in vivo. In order to address our hypothesis, we generated new N-cadherin, MCAM, and β3 integrin antisense vectors using the pREP7-RSV plasmid, as well as the pcDNA3.1-CMV plasmid, for expression in all cell types. Tumors were injected intratumorally with N-cadherin, MCAM, or β3 integrin antisense-oriented pREP7-RSV or pcDNA-CMV plasmid. It is important to note that only one tumor per plasmid was injected for this preliminary experiment, and as such, the growth results are likely not significant. However, the tumors injected with antisense-oriented N-cadherin, MCAM, or β3 integrin pREP7-RSV plasmid grew slightly slower than the control tumor, receiving Lipofectamine 2000 alone (Figure 33). The tumors injected with antisense-oriented N-
cadherin or β3 integrin pcDNA-CMV plasmids grew at approximately the same rate as control tumors (Figure 34). Although the single tumor injected with antisense-oriented MCAM pcDNA-CMV plasmid grew slowly compared with controls, it is important to note that this tumor was smaller from the beginning of the experiment (Figure 34).

![Growth of Melanoma Xenografts](image)

**Figure 33:** Tumor volume of melanoma xenografts following intratumoral injection of N-cadherin, MCAM, or β3 integrin antisense-oriented pREP7-RSV plasmids
One tumor per vector construct was injected with plasmid DNA complexed with 5 µg of Lipofectamine 2000. 100 µg of plasmid DNA was used for the first injection, with 50 µg for each injection thereafter. The arrow indicates the first day of intratumoral injections.
Figure 34: Tumor volume of melanoma xenografts following intratumoral injection of N-cadherin, MCAM, or β3 integrin antisense-oriented pcDNA-CMV plasmids

One tumor per vector construct was injected with plasmid DNA complexed with 5 µg of Lipofectamine 2000. 100 µg of plasmid DNA was used for the first injection, with 50 µg for each injection thereafter. The arrow indicates the first day of intratumoral injections.

PCR analysis of DNA extracted from these tumors showed that, in contrast to previous experiments, the injected plasmid DNA was detected in only some of the respective tumors (Figure 35). Of the six antisense plasmid vectors that were intratumorally injected, only antisense MCAM pcDNA-CMV and antisense β3 integrin pREP7-RSV were detected in the respective tumors. The control reactions, designed to amplify each purified plasmid, were all positive as expected. These results suggest that delivery of the plasmid DNA into the tumor cells was only achieved in a fraction of the tumors.
Figure 35: PCR analysis of plasmid DNA in intratumorally-injected melanoma xenografts

DNA was isolated from the snap-frozen tumors, and PCR was performed to amplify each injected plasmid (antisense-oriented N-cadherin (2.0 kb), MCAM (1.1 kb), or β3 integrin (1.3 kb), in pREP7-RSV or pcDNA-CMV plasmid).

Because delivery of the plasmid DNA into the tumor cells was not persistent and uniform, we obtained several additional transfection delivery reagents, including Mirus TransIT In Vivo delivery reagent, In vivo jetPEI, and GeneFECTOR/NovaFECTOR. A control pcDNA vector containing the LacZ reporter gene was complexed with each transfection reagent, and intratumorally injected into the melanoma xenografts. In addition, one mouse was injected with naked pcDNA LacZ plasmid. Each mouse received two injections of the pcDNA LacZ plasmid, two days apart. Two days after the second injection, the tumors were extracted, snap-frozen, and assayed for expression of β-galactosidase. However, we were unable to detect expression of β-galactosidase, using any of the delivery methods, as evidenced by a lack of tumor cells stained blue (images not shown).

In parallel, mice were also injected with Mirus TransIT or jetPEI reagent, complexed with N-cadherin, MCAM, or β3 integrin antisense pcDNA-CMV plasmid, so that the protein levels could be examined in these tumors. The tumor injected with antisense β3 integrin plasmid with MIRUS delivery reagent grew faster compared with the other tumors, which all grew at approximately the same rate (Figure 36). Again, it is important to note, that only one mouse was utilized per group for this experiment. Immunoblot analyses illustrated that in the case of N-cadherin and MCAM, levels of the targeted protein were still unchanged. In the case of β3 integrin, protein levels were also unchanged, although an unexplained shift in band size was
observed for $\beta_3$ integrin antisense pcDNA-CMV plasmid injected with jetPEI reagent (Figure 37).

**Figure 36: Tumor volume of melanoma xenografts intratumorally injected with antisense N-cadherin, MCAM, or $\beta_3$ integrin pcDNA-CMV plasmid, using jetPEI or MIRUS delivery reagent**

One tumor per vector construct was injected with antisense N-cadherin, MCAM, or $\beta_3$ integrin pcDNA-CMV plasmid complexed with jetPEI or MIRUS delivery reagent, as described in Section A.2.2. Control tumors were injected with pcDNA LacZ plasmid. The arrow indicates the start of intratumoral injections.
7.3 DISCUSSION

In the context of the in vivo studies, we encountered a variety of challenges. Conclusive differences between the growth of control versus antisense-transfected cells in nude mice could not be observed. This result may have been due to the fact that prior to injection, the cells were cultured in the antibiotic hygromycin, which may have impaired the ability of the cells to establish tumors in vivo. In addition, none of the intratumoral plasmid delivery techniques produced a significant downregulation of expression of the targeted molecules. Based on the PCR results, we found that delivery of the plasmid DNA into the tumor cells was inconsistent from experiment-to-experiment, although it was achieved in some cases. We were unable to detect a significant decrease in expression of N-cadherin, MCAM, or β3 integrin protein following intratumoral injection of any of the antisense plasmids. β-galactosidase expression was not detected following intratumoral injection of pcDNA-CMV plasmid containing the LacZ gene, using various delivery reagents. This suggests that the antisense transcripts, at least in the case of pcDNA-CMV, are not efficiently expressed within the tumor cells. Overall, it is likely that a combination of poor delivery of the plasmid into the tumors cells and insufficient
expression/copy number of the antisense transcripts contributed to our lack of success at inhibiting N-cadherin, MCAM, or β3 integrin in MGP melanoma xenografts.

There are several alternative approaches that have been described in the literature and might be used in future experiments. Several groups have recently demonstrated that intratumoral injection of siRNA can effectively downregulate expression of specific genes (Leng and Mixson, 2005; Ogushi et al., 2005). Intratumoral injection of siRNAs has the advantage of directly paralleling the in vitro siRNA methods utilized to inhibit N-cadherin, MCAM, and β3 integrin in this dissertation. If effective, one might also readily achieve simultaneous inhibition of N-cadherin, MCAM, and β3 integrin expression in vivo. In fact, simultaneous inhibition of two genes through intratumoral injection of siRNA has been recently reported (Ducker et al., 2005). Similarly, one might utilize antisense oligonucleotides to inhibit expression of the adhesion molecules in vivo, which would present many of the same benefits described for siRNAs.

In the future, it will be important to examine the individual and simultaneous inhibition of N-cadherin, MCAM, and β3 integrin in an in vivo setting, where the tumor microenvironment may play a substantial role in tumor growth and metastasis. Once an effective method is achieved for inhibition of N-cadherin, MCAM, and/or β3 integrin in an in vivo setting, these additional questions can be addressed.
Over the last decade, blocking the function of specific molecules by way of antibodies has become one of several promising approaches for cancer therapeutics. The monoclonal antibody, Herceptin, which blocks HER2 protein, is an example of a targeted therapy for HER2-positive breast cancers (as reviewed by Yeon and Pegram, 2005). Similarly, in the case of integrins, several antibodies that target specific integrin α or β subunits, or αβ complexes, are being investigated for their therapeutic efficacy.

The first antibody targeting the αvβ3 integrin complex was developed about a decade ago. This function-blocking mouse monoclonal antibody, called LM609, has specificity for human αvβ3 integrin (Cheresh and Spiro, 1987). Studies showed that when human tumor fragments were implanted onto the chicken chorioallantoic membrane (CAM), LM609 impaired angiogenesis and resulted in tumor regression (Brooks et al., 1994). The M21-L human melanoma cells used in this study did not express αvβ3 integrin, which indicated that the tumor regression was due to inhibition of angiogenesis of the tumor neovasculature, and not an inhibition of αvβ3 integrin on the melanoma cells. In subsequent experiments, LM609 was also shown to decrease the transendothelial migration of melanoma cells in culture (Voura et al., 2001). Studies were then expanded to an in vivo setting, whereby it was shown that LM609 treatment decreased the formation of metastases of human M21 melanoma cells, and increased animal survival (Felding-Habermann et al., 2002).

Based on these promising data, a humanized LM609 antibody, Abegrin® (Vitaxin, MEDI-522), was generated and tested in clinical trials (Wu et al., 1998). A Phase I trial, comprised of patients with various types of late stage cancer, demonstrated that Abegrin was well-tolerated. In addition, one patient had a partial response, and seven patients showed stable
disease (Gutheil et al., 2000). However, not all of the results were as promising. In another Phase I trial of metastatic melanoma patients, Abegrin caused little toxicity, but no anti-tumor responses were reported (Posey et al., 2001), while in a Phase I trial comprised of patients with advanced leiomyosarcoma, Abegrin was well-tolerated, but, again, no tumor response was observed (Shreyaskumar et al., 2001). On the other hand, preliminary results from an ongoing Phase II trial designed to test the efficacy of the Abegrin antibody in stage IV melanoma patients demonstrated that although patients treated with Abegrin had no objective tumor response, they lived longer compared to historic controls (Hersey et al., 2005).

In light of these ongoing trials, we examined the proliferation of MGP melanoma cells treated with Abegrin, both in vitro and in vivo, with the goal of addressing the hypothesis that Abegrin may impair melanoma cell proliferation. This set of experimental assays enabled us to make a preliminary assessment of the consequence of inhibiting $\alpha_v\beta_3$ integrin in melanoma cells in vivo, as well as to compare the results of the siRNA/antisense-mediated inhibition of $\beta_3$ integrin in vitro to an antibody-mediated approach.

### 8.1 RESULTS

**8.1.1 Proliferation of human MGP melanoma cells is not impaired by a function-blocking $\alpha_v\beta_3$ integrin antibody, Abegrin**

We first examined the effect of the $\alpha_v\beta_3$ integrin function-blocking antibody, Abegrin, on the proliferation of the MGP melanoma cell lines WM1158 and WM983B. Cells were counted at 24, 48, and 72 hours following addition of Abegrin antibody to the cell cultures. Proliferation of both MGP melanoma cell lines is unchanged following treatment with the antibody (Figure 38 and 39). This is in contrast to the effect following inhibition of $\beta_3$ integrin through siRNA or antisense targeting in the same melanoma cell lines, whereby proliferation was decreased by between 30 and 50%. 

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Figure 38: Abegrin does not decrease the proliferation of WM1158 cells
WM1158 cells were seeded into 24-well plates, and 24 hours later, 50 µg/mL of Abegrin antibody diluted in PBS was added to the culture medium. At the indicated times following Abegrin treatment, the cells were detached with trypsin and counted. The data are expressed as the average of triplicate counts.

Figure 39: Abegrin does not decrease the proliferation of WM983B cells
WM983B cells were seeded into 24-well plates, and 24 hours later, 75 µg/mL of Abegrin antibody diluted in PBS was added to the culture medium. At the indicated times following Abegrin treatment, the cells were detached with trypsin and counted. The data are expressed as the average of triplicate counts.
8.1.2 Growth of established human melanoma xenografts is not impaired by intratumoral injection of Abegrin

Following the in vitro proliferation analysis, our second goal was to examine the impact of blocking αvβ3 integrin function in vivo. WM983B MGP melanoma xenografts were generated, and when the tumors reached approximately 5x5 mm, Abegrin was injected intratumorally, three times weekly, for the duration of the experiment. Similar to the in vitro analyses, the Abegrin antibody does not significantly alter the growth of melanoma xenografts (Figure 40).

**Figure 40: Abegrin does not slow the growth of established human melanoma xenografts**

Melanoma xenografts were generated via subcutaneous injection of WM983B cells. When the tumors reached approximately 5x5 mm, 100 µg of Abegrin antibody, diluted in PBS, was injected intratumorally three times per week, until the tumors reached the maximum permissible size at day 38. Control mice were injected with PBS alone. Three mice were included per group.
8.1.3 Expression of β₃ integrin and osteopontin in human melanoma xenografts injected with Abegrin

In order to assess whether Abegrin had an impact on the expression of β₃ integrin, the tumors were extracted, snap-frozen, and sectioned for standard immunohistochemistry. β₃ integrin expression is unchanged following Abegrin injection, except for a slight decrease in Tumor 2 (selected tumor sections are shown in Figure 41). Immunoblot analysis also showed that levels of β₃ integrin protein are unchanged an Abegrin-treated tumor (data not shown).

![β₃ integrin expression](image)

**Figure 41: β₃ integrin expression is unchanged in tumors injected with Abegrin.**

Tumors were snap-frozen, sectioned, and probed with β₃ integrin antibody, through standard immunohistochemistry. Selected images of tumor sections are shown.

As part of the whole-genome microarray analysis, we recently identified osteopontin (OPN, SPP1) as the gene exhibiting the most profound change in expression correlating with the transition from melanoma **in situ** to VGP melanoma (Smith et al., 2005). OPN serves as an extracellular matrix ligand to αᵥβ₃ integrin, and has been associated with melanoma progression, proliferation, and invasion (Philip et al., 2001; Zhou et al., 2005). We hypothesized that
expression of OPN may be altered following the inhibition of $\alpha_\text{v}\beta_3$ integrin signaling. We examined the expression of OPN in the xenografts injected with Abegrin, or with PBS alone. However, we did not observe major differences in the expression of OPN in three tumors injected with Abegrin (Figure 42).

![Osteopontin](image)

Figure 42: OPN expression in tumors injected with Abegrin
Tumors were snap-frozen, sectioned, and probed with OPN antibody. Selected images of tumor sections are shown.

### 8.2 DISCUSSION

Targeting $\alpha_\text{v}\beta_3$ integrin via a function-blocking antibody does not decrease the proliferation of MGP melanoma cells, \textit{in vitro} or \textit{in vivo}. In contrast, siRNA or antisense-mediated inhibition of $\beta_3$ integrin expression reduces the proliferation of the MGP melanoma cells by 30-50% as determined \textit{in vitro}. There are several possible explanations for this discrepancy. First, Abegrin targets and binds to the $\alpha_\text{v}\beta_3$ integrin complex, whereas siRNA or antisense-mediated inhibition of $\beta_3$ integrin expression impacts all integrin complexes that contain $\beta_3$ integrins. This may have
the subsequent effect of leaving the $\alpha_v$ subunit available to interact with other $\beta$ subunits. As such, the biological consequences of these two different targeting approaches, though similar in concept, may differ with respect to their impact on the melanoma cells. Second, downregulation of an adhesion molecule at the level of mRNA/protein expression may elicit a more potent effect compared to a function-blocking antibody approach, wherein the expression of the targeted protein could remain unchanged.

It is worthwhile to consider our findings when examining the results of ongoing clinical trials of Abegrin in advanced-stage melanoma patients. Results from a Phase II clinical trial suggest that patients receiving Abegrin have improved survival compared with historic controls, but no objective tumor response (Hersey et al., 2005). In a previous study, the non-humanized antibody, LM609, impaired the growth of $\alpha_v\beta_3$-negative melanoma fragments implanted on the chicken CAM (Brooks et al., 1994) – indicating that the observed tumor regression was due to the anti-angiogenic effect of LM609 on the blood vessels, rather than the direct binding of the antibody to the melanoma cells. In light of these results, one hypothesis is that inhibition of $\alpha_v\beta_3$ integrin in advanced-stage melanoma patients blocks angiogenesis, primarily by targeting $\alpha_v\beta_3$ integrin on the tumor-interspersing blood vessels, rather than on the melanoma cells themselves. In our in vivo study, the humanized Abegrin antibody should not be expected to block $\alpha_v\beta_3$ integrin on the mouse blood vessels – therefore, our approach examined the impact of targeting $\alpha_v\beta_3$ integrin on the melanoma cells only. As we observed, Abegrin does not substantially impact the growth of the human melanoma xenografts, perhaps due to the fact that mouse blood vessel growth/angiogenesis is not impaired in this setting. Taken together, our results are in agreement with the proposed hypothesis that Abegrin’s primary mechanism might be the inhibition of angiogenesis, rather than a direct impact of the antibody on the $\alpha_v\beta_3$ integrin expressed by the melanoma cells.
9.0 DISCUSSION

9.1 SUMMARY

The focus of this dissertation was to elucidate the role of the cell adhesion molecules N-cadherin, MCAM, and $\beta_3$ integrin in melanoma progression, proliferation, migration, and invasion, with the ultimate goal of determining whether any of these genes may serve as valid and effective therapeutic targets for the disease. This study represents the first time that all three adhesion molecules have been studied in concert. We addressed the hypothesis that combined targeting of all three adhesion molecules might generate a more robust anti-tumor response compared with inhibition of each individual gene.

Previous reports in the literature documented that N-cadherin, MCAM, and $\beta_3$ integrin are expressed at high levels in a significant number of human metastatic melanoma cell lines and tissues. However, knowledge regarding the expression level of each molecule during the individual stages of melanoma progression was incomplete. To help fill this gap, we used whole-genome and TMA analyses to generate a comprehensive picture of N-cadherin, MCAM, and $\beta_3$ integrin mRNA and protein expression, throughout the distinct stages of melanoma progression. We also documented, for the first time, the co-expression of one, two, or all three adhesion molecules in subcutaneous and visceral melanoma metastases. We demonstrate that although N-cadherin and $\beta_3$ integrin are melanoma progression markers, whereby expression increases from early to late stages of the disease, MCAM is not. MCAM is expressed in many benign and atypical nevi, and is also expressed at high-levels in metastatic melanomas. This suggests that MCAM may also function in the early stages of melanoma development.

In addition, the TMA analysis revealed that only about 50% of the subcutaneous and visceral MGP melanoma tissues examined co-expressed N-cadherin, MCAM, and $\beta_3$ integrin, but 97% expressed at least one of the three. This indicates that all three adhesion molecules need
not be expressed in the same melanoma for metastasis formation to occur. N-cadherin, MCAM, and β3 integrin may each be capable of mediating melanoma invasion and metastasis, and, as a result, inhibition of greater than one molecule may be required for substantial anti-tumor effects.

We next investigated the consequence of inhibiting expression of N-cadherin, MCAM, or β3 integrin on the proliferation, migration, and invasion of MGP melanoma cells. First, by using the same cell lines and assays for inhibiting expression of each adhesion molecule, we were able to analyze the relative importance of N-cadherin, MCAM, and β3 integrin expression compared to one another. In contrast, previous studies of individual adhesion molecules were performed using a variety of different cell lines and experimental approaches, thus making direct comparisons challenging. Furthermore, the inhibition of endogenous gene expression more closely resembles a viable clinical therapy, compared with previous studies that, in most cases, involved overexpression of the adhesion molecules. Second, we demonstrate, for the first time, the result of simultaneously inhibiting expression of N-cadherin, MCAM, and β3 integrin in MGP melanoma cells.

We show that blocking expression of N-cadherin, MCAM, or β3 integrin leads to decreased melanoma cell proliferation, but that simultaneous targeting of all three adhesion molecules does not indicate synergistic effects. Proliferation is decreased by about 50%, but is not completely blocked, suggesting that proliferation is maintained in the melanoma cells via a different signaling pathway(s). On the other hand, simultaneous inhibition of N-cadherin, MCAM, and β3 integrin results in a decrease in migration and invasion that is more substantial than that following individual inhibition of the three adhesion molecules. Therefore, the migration/invasion data support the hypothesis that simultaneous inhibition of multiple cell adhesion molecules generates a more robust anti-tumorigenic effect on the melanoma cells.

As a final point, we show that a fully humanized α,β3 integrin antibody, currently assessed in clinical trials, does not impair melanoma cell proliferation - neither in vitro nor in vivo. This result is in contrast to the inhibition of β3 integrin subunit expression via siRNA or antisense plasmids in the same melanoma cell lines, whereby proliferation was decreased. This finding suggests that an antisense or siRNA targeting approach, whereby the mRNA/protein is downregulated, may elicit a different biological response compared with a function-blocking antibody approach, where the antibody binds directly to the integrin target. Moreover, it is likely
that targeting an individual integrin subunit can generate a different cellular response than targeting a specific integrin alpha-beta complex.

9.2 FUTURE DIRECTIONS

Several avenues for future investigation can be based on the results presented in this dissertation. First, it will be essential to extend the work to determine the impact of inhibiting expression of N-cadherin, MCAM, and/or β3 integrin in mouse models. This is crucial, because in vitro experiments do not address the interaction of tumors cells with the tumor microenvironment - an event that is likely impacted by the cell-cell and cell-matrix adhesion molecules expressed both by tumor cells and by cells in the surrounding tumor microenvironment. In Chapter 7, I described our attempts to accomplish this goal; however, to date, we have been unable to achieve measurable downregulation of the adhesion molecules in melanoma xenografts. In the future, alternative approaches should be explored. Possible strategies include the use of siRNAs, shRNA vectors, or antisense oligonucleotides. These pre-clinical animal studies will help further illuminate whether targeting a combination of cell adhesion molecules is likely to represent a useful and effective therapeutic strategy.

Future studies should also further investigate the signaling mechanisms of N-cadherin, MCAM, and β3 integrin in metastatic melanoma cells. For example, it would be valuable to examine the expression of additional integrin subunits, such as β1 and β5, which can also associate with αv integrin, following the inhibition of β3 integrin. This would help to determine with which β subunit αv integrin associates following the downregulation of β3 integrin, and to investigate whether this alternative integrin complex has the ability to mediate cell proliferation, migration, and/or invasion.

In addition, a physical association between FGFR-1 and N-cadherin has been demonstrated in breast cancer cells (Suyama et al., 2002), which results in persistent activation of MAPK/ERK and enhanced cell invasion. However, this phenomenon has not been investigated in melanoma cells to date. Given that bFGF/FGFR-1 are known to play a key role in melanoma proliferation (Becker et al., 1992; Wang and Becker, 1997; Valesky et al., 2002), it would be worthwhile to investigate whether a direct association between N-cadherin and FGFR-
1 occurs in melanoma cells, and whether a loss of such association may be linked to the impairment of melanoma cell proliferation following inhibition of N-cadherin expression.

Finally, we showed that inhibition of MCAM or $\beta_3$ integrin had a more pronounced effect upon melanoma cell migration and invasion compared with inhibition of N-cadherin alone. For this reason, inhibition of the specific combination of MCAM and $\beta_3$ integrin should be explored, to determine whether this combination is equally effective compared with inhibition of all three adhesion molecules.

### 9.2.1 Clinical Applications

The data from this dissertation also point to avenues for future investigation in a clinical setting. First, it would be worthwhile to explore whether there is a correlation between co-expression of N-cadherin, MCAM, and/or $\beta_3$ integrin and survival of patients with metastatic melanoma. We show that approximately 50% of MGP melanomas express all three adhesion molecules, and that the three adhesion molecules play a role in proliferation, migration, and invasion *in vitro*. This leads to the hypothesis that patients whose melanomas co-express all three molecules may have poorer prognosis/survival compared with patients whose melanomas express only one or two of the three adhesion molecules. The TMA technique could be used to address this question in a large number of patients, in the context of a retrospective clinical trial of patients whose progression-free and overall survival were documented.

Translating our findings into the clinical setting has additional implications. First, treatment of patients with advanced-stage melanoma with, for example, an antibody or peptide that blocks only one of these molecules may not be as efficacious as blocking a combination of adhesion molecules, such as MCAM and $\beta_3$ integrin. This may be of particular importance in the subset of patients whose melanomas express all three adhesion molecules. Therefore, sequential or simultaneous treatment with antagonists to multiple adhesion molecules may yield a noticeably greater benefit regarding progression-free and overall survival than targeting a single one of these adhesion molecules. Second, if indeed in the clinical setting it turns out that targeting cell adhesion molecules impairs the formation of additional metastases and/or causes tumor regression, it may be desirable to establish their most effective combination, as N-cadherin, MCAM, and $\beta_3$ integrin likely do not represent the only therapeutic targets.
In recent years, combined treatment modalities have generated significant attention in clinical trials. In this dissertation, I suggest that inhibiting a combination of cell adhesion molecules may generate a more robust therapeutic response compared with targeting a single one of them. To take this approach even further, one might also combine inhibitors of cell adhesion molecules with cytotoxic agents, such as dacarbazine and cisplatin, which interfere with DNA replication and cause cell death. It is likely that in the case of melanoma, it may be necessary to combine an inhibitor of a cell adhesion molecule(s) with a cytotoxic drug, to elicit the most effective tumor response. To make a preliminary assessment of this hypothesis, siRNA or antisense-transfected melanoma cells could be treated with various chemotherapeutic reagents, such as such as dacarbazine, cisplatin, or paclitaxel, and the relative effect upon proliferation, migration, and/or invasion examined. If promising, this approach could be extended to in vivo models.

In summary, the proposed experiments will further our understanding of the biological function(s) of N-cadherin, MCAM, and $\beta_3$ integrin in metastatic melanoma, and help determine whether simultaneous targeting of multiple adhesion molecules is an approach that should be taken to the clinic.

9.3 CONCLUDING REMARKS

The development of novel therapies for treatment of advanced-stage melanoma is essential, and the identification of genes that may serve as viable targets will facilitate this goal. We have characterized the expression of N-cadherin, MCAM, and $\beta_3$ integrin throughout the stages of melanoma progression, and shown that simultaneous downregulation of the expression of all three adhesion molecules represents a valuable approach for inhibition of melanoma cell proliferation, migration, and invasion. Our findings contribute to a better understanding of the function of N-cadherin, MCAM, and $\beta_3$ integrin in metastatic melanoma, and provide the foundation for future work targeting the cell adhesion molecules N-cadherin, MCAM, and $\beta_3$ integrin in a clinical setting.


directed against the human alpha v beta 3 (avb3) integrin, +/- dacarbazine (DTIC) in patients with metastatic melanoma (MM). ASCO Annual Meeting.


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