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A

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By

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Dedication

To the women in my life: My grandmothers, whose perseverance and defiant survival through wars and other hardships, served as a source of inspiration throughout my life. My mother, Varda Zigler, who was always there to give me strength, emotional support and the ability to thrive through out the challenges, knowing I would succeed.
To my father, Arie Zigler, who gave me the greatest gift – inspiring me with his passion for science and research. To both of my parents, for their continued and unwavering encouragement and tremendous support.

To my husband Tzur,

For all his love and patience, and for allowing me to fulfill my dream.

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MCAM/MUC18 REGULATES MELANOMA PROGRESSION BY MODULATING THE EXPRESSION OF ID-1

Publication No._____

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The acquisition of the metastatic melanoma phenotype is associated with increased expression of the melanoma cell adhesion molecule MCAM/MUC18 (CD146). However, the mechanism by which MUC18 contributes to melanoma metastasis remains unclear. Herein, we stably silenced MUC18 expression utilizing lentivirus-incorporated small hairpin RNA, in two metastatic melanoma cell lines, A375SM and C8161, and conducted cDNA microarray analysis. We identified and validated that the transcriptional regulator, Inhibitor of DNA Binding-1 (Id-1), previously shown to function as an oncogene in several malignancies, was downregulated by 5.6-fold following MUC18 silencing. Additionally, we found that MUC18 regulated Id-1 expression at the transcriptional level via ATF-3. Interestingly, ATF-3 was upregulated by 6.9 fold in our cDNA microarray analysis following MUC18 silencing. ChIP analysis showed increased binding of ATF-3 to the Id-1 promoter after MUC18 silencing, while mutation of the ATF-3 binding site on the Id-1 promoter increased Id-1 promoter activity in MUC18-silenced cells. These Data suggest that MUC18 silencing promotes inhibition of Id-1 expression by increasing ATF-3 expression and binding to the Id-1 promoter.

Rescue of MUC18 reverted the expression of Id-1 and ATF-3, thus validating that they are not off-target effects of MUC18. To further assess the role of Id-1 in melanoma invasion and metastasis, we overexpressed Id-1 in MUC18-silenced cells. Overexpression of Id-1 in MUC18-silenced cells resulted in increased cell invasion, as well as increased expression and activity of MMP-2. Our data further reveal that Id-1 regulates MMP-2 at the transcriptional level through Sp1 and Ets-1. This is the first report to demonstrate that MUC18 does not act exclusively in cell adherence, but is also involved in cell signaling that regulates the expression of genes, such as Id-1 and ATF-3, thus contributing to the metastatic melanoma phenotype.

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CHAPTER 1: Introduction and Background

Clinical Aspects of Melanoma

Malignant melanoma is an extremely aggressive disease with very few treatment options, thereby presenting a major medical challenge. In the past three decades, the incidence of melanoma has increased more than any other skin tumor [1, 2]. Currently, in North America, malignant melanoma is the fifth and seventh most common cancer in men and women, respectively [3]. It is expected that in 2010, 68,130 new cases of melanoma will be newly diagnosed with an estimated death of 8,700 patients [3]. To develop new treatment modalities for melanoma, it is of high importance to learn the molecular mechanisms which contribute to the metastatic melanoma phenotype.

Melanoma Development

Melanoma derives from melanocytes which originate from the multipotent cells of the neural crest. During embryonic development, melanocytes migrate and incorporate into the hair follicle and epidermis where they are distributed in the basal layer as single cells [4]. Melanoma development is influenced by a variety of genetic and epigenetic changes as well as environmental factors [5]. Clark et al. proposed a model for the development of melanoma by which it progresses as a multistep process, with distinct clinical and histopathological characteristics (Figure 1) [6]. In this five stage model, the first stage commences with the formation of common acquired nevi, with focal proliferation of melanocytes. Next, melanocytes develop into dysplastic nevi cells, displaying abnormal differentiation with architectural and cytological atypia [6].

The development of primary melanoma occurs at the third step of melanoma progression and is characterized by the radial growth phase (RGP) of cells [7]. At this step, tumors grow within the epidermis but do not have the capability to penetrate through the basement membrane [6]. It is only when the tumor progresses to the vertical growth phase (VGP) that cells gain the ability to grow vertically by invading through the basement membrane into the dermis [7]. The last stage of tumor progression is the formation of distant metastases [6]. The most common sites for melanoma metastasis are regional and distant lymph nodes, lung, brain, liver, central nervous system and bone. Another model for melanoma development suggests that melanoma arises de novo and not from dysplastic nevi [8], with activation of various cellular pathways which induce genetic and epigenetic changes, promoting the transformation of melanocytes to melanoma [9]. It is likely that both models of melanoma progression can occur in humans; however, the sequential progression model is most widely accepted.

Melanoma Staging and Treatment

Patient prognosis is largely dependent upon the stage of the disease. The most important factors for prognosis include the thickness and ulceration of the primary tumor, the presence of lymph node metastases, and sites of distant metastatic disease [10, 11]. The staging criteria for melanoma is comprised of both stage grouping and tumor-node-metastasis (TNM) classification of the disease. Clinical and pathological staging of melanoma classifies patients with stage I and II disease as those with no evidence of regional or distant metastasis [11, 12]. Stage III patients present with regional metastases to the regional lymph nodes or intralymphatic metastases. Stage IV

melanoma patients present with metastases at one or more distant sites [11, 12]. The group classification depends on the TNM status of disease. The TNM classification is based on the thickness of the primary tumor, the presence of ulceration and level of invasion (T category). Patients presenting with regional lymph node metastasis fall into the N category. The presence of distant metastatic melanoma and site of metastasis further categorize melanoma (M category) [11-13]. Both group and TNM classifications are important predictors of patient prognosis.

Early diagnosis of melanoma is crucial, since the five-year survival rate of patients presenting with early stage localized disease is 90%. However, patients presenting with wide spread metastases, or stage IV disease, have a much worst prognosis (10%-20%), with an overall median survival of 6 to 9 months [14, 15]. Treatment options are limited for patients with late stage melanoma, since metastatic melanoma is largely resistant to systemic treatment. Current treatment options for patients with metastatic disease include surgery chemotherapy and immunotherapy. Dacarbazine (DTIC) is currently the only FDA approved single agent chemotherapy for metastatic melanoma. DTIC is an alkylating agent that has been shown to have varying clinical effectiveness, with a patient response rate that ranges between 7.5%-20% and a median response duration of only 4 to 6 months [16-18]. Another alkylating agent currently used in clinical trials is Temozolomide (TMZ). Unfortunately, recent clinical trials have demonstrated that treatment with TMZ does not seem to have promising response rates as compared to treatment with DTIC [15]. Immunotherapy is also used as a treatment modality for patients with metastatic melanoma and includes the use of cytokines, such as high dose interleukin-2 (IL-2) and interferon-α (IFN−α). Studies with

either IL-2 or IFN−α alone, or in combination, have demonstrated approximately a 15% response rate, with median response duration of 6-9 months [19, 20]. Currently other treatment modalities for metastatic melanoma are being studied in clinical trials and demonstrate preliminary positive results [21]. These include biochemotherapy (a combination of chemotherapy and cytokine treatment, such as DTIC plus IFN-α), and targeted therapy such as RAF kinase inhibitors PLX4032, which showed promising results in phase I clinical trials, vide infra, and anti-Bcl-2 therapy [15]. As most of these treatment options offer very low response rates and do not improve patient prognosis, it is, therefore, important to identify the molecular mechanisms associated with melanoma progression. Defining these mechanisms will aid development of new therapeutic molecular targets.

Pathogenesis of Melanoma

Disruption of tissue homeostasis as well as the accumulation of genetic changes can result in development of melanoma. These alterations can be induced by both environmental factors and genetic susceptibility. The most studied environmental risk factor of melanoma development is ultraviolet (UV) exposure [5, 22-25]. Exposure to midrange UV radiation (UVR), ultraviolet B (UVB, 280–320 nm) and ultraviolet A (UVA, 320–400 nm), as well as the acquisition of multiple sunburns during childhood and adolescence, have been shown to increase the risk of melanoma development in adults, albiet the exact mechanisms of how UV induces melanoma are not clear [25]. Although melanocytes secrete melanin, which acts as a photoprotective filter, melanin cannot completely prevent UV-induced DNA damage [26]. Exposure to UV can cause

DNA mutations, such as pyrimidine dimers, DNA breaks, DNA-protein crosslinks and the formation of reactive oxygen species which promote cellular and DNA damage [26, 27]. Furthermore, UV can stimulate signaling pathways that ultimately result in increased cell proliferation and survival, by either inducing the secretion of growth factors or by promoting ligand-independent growth factor receptor activity [28]. Finally, UV can also act as an immunosupressant, by either inducing activation of suppressor T cells or by secretion of cytokines that inhibit the immune response, contributing to uncontrolled tumor growth [29, 30].

The transition from common acquired nevi to the vertical growth phase and the formation of metastasis does not arise solely due to environmental hazards, such as UV exposure, but also due to numerous germline and sporadic mutations, as well as molecular changes (Figure 1). A major risk factor for developing melanoma is familial history of melanoma [31]. About 10% of cutaneous melanoma arise from a familial setting [32]. Several germline mutations have been associated with the development of melanoma. In the mid 1990's two susceptibility genes were identified, cyclin dependent kinase 2a (CDKN2A) and cyclin dependent kinase 4 (CDK4), which are involved in cell cycle regulation. CDKN2A is a tumor suppressor gene, which codes for two distinct proteins, $p16^{INK4A}$ and $p14^{ARF}$, through alternative reading frames. Both $p16^{INK4A}$ and $p14^{ARF}$ function as tumor suppressors, as they regulate cell cycle progression through the G1/S checkpoint [33]. Germ line mutations in these genes can promote dysregulation of the cell cycle, thus contributing to melanoma development [34]. A small percentage of patients inherit the melanocortin 1 receptor (MC1R) gene which, is also involved in melanomagenesis. Germline mutation of the MC1R gene causes decreased production of

melanin. Additionally, MC1R mutation results in a reduced capacity to repair DNA, promoting the accumulation of UV–induced DNA damage [22].

Several cell signaling pathways that are involved in proliferation, senescence, apoptosis, invasion and angiogenesis are dysregulated during the progression of melanoma. These cellular activities are often the result of the aberrant activation of protein tyrosine kinases, as well as the abnormal expression of transcription factors and adhesion molecules. One of the most common pathways reported to play an important role in the progression of melanoma is the mitogen activated protein kinases (MAPK), extracellular signal-regulated kinase (ERK) pathway [35]. BRAF and N-RAS are frequently mutated during melanoma progression and lead to activation of RAS/RAF/MEK/ERK signaling pathway promoting cell proliferation, differentiation and survival [7, 36, 37]. Constitutive activation of BRAF, a serine/threonine kinase, results from a substitution mutation of Valine to Glutamic acid at codon 600 (V600E), located at the protein kinase domain. The V600E substitution is the most common type of BRAF mutation, shown to occur in up to 66% of all melanomas [38]. A novel BRAF inhibitor PLX4032, targeting V600E BRAF mutations, was recently developed. Preclinical experiments showed that PLX4032 can block the RAF/MEK/ERK pathway in BRAF mutant cells and cause regression in xenografts harboring the BRAF mutation [39]. Phase I clinical trial with PLX4032 demonstrated positive results as PLX4032 induced complete or partial tumor regression in 81% of patients who had melanoma with the V600E BRAF mutation. PLX4032, therefore, holds much promise for melanoma patients carrying the V600E BRAF mutation [21, 39].

The N-RAS mutation differs from the BRAF mutation, as constitutive activation of the N-RAS oncogene results from impaired hydrolysis of GTP due to a substitution mutation at codon 61 [40]. This mutation is particularly detrimental, as constitutively active N-RAS leads to aberrant activation of the RAS/RAF/MEK/ERK signaling pathway, promoting cell growth and survival.

c-Kit is a tyrosine kinase receptor and a member of the platelet-derived growth factor (PDGF) family of kinases and is also shown to be involved in the development of melanoma [23]. Activation of the c-Kit receptor by its ligand, stem cell factor (SCF), normally induces cellular apoptosis. Interestingly, its expression is lost during local tumor growth and invasion of melanoma, thus enabling melanoma cells a mechanism of escape from apoptosis [41].

Along with activation of receptor tyrosine kinases, other key molecular events have been shown to be associated with transition of melanoma from the RGP to the VGP including alterations in the expression of various transcription factors. For instance, loss of Activator Protein-2α (AP-2α) expression has been associated with the transition of melanoma from the RGP to the VGP. Additionally, loss of AP-2α strongly correlates with poor patient prognosis [42, 43]. AP-2α has been shown to play an important role in the transcriptional regulation of several key genes that have been associated with tumor progression and metastasis such as $p21^{Cip1/Waf1}$ [44], c-KIT[42], MCAM/MUC18 [42, 45], c-MYC [46], protease activated receptor 1 (PAR-1) [47], E-cadherin [48, 49] and vascular endothelial growth factor (VEGF) [50, 51]. Loss of AP-2α triggers uncontrolled expression of genes involved in critical cellular processes, such as cell

cycle progression, proliferation, adhesion, apoptosis and angiogenesis. Thus, the loss of AP-2α is a crucial event that contributes to the metastatic phenotype of melanoma.

Additional transcription factors that play an important role in melanoma progression are the cAMP-Responsive Element-Binding Protein/Activating Transcription Factor-1 (CREB/ATF-1) [52]. Overexpression and activation of both CREB and ATF-1 correlate with melanoma development [53]. These factors contribute to the progression of melanoma by promoting cell invasion through increased expression of a number of genes, including Matrix Metalloproteinase-2 (MMP-2) and MCAM/MUC18 [54]. CREB and ATF-1 can also contribute to melanoma progression by promoting survival in human melanoma cells [55].

Genomic amplification of the oncogenic transcription factor microphthalmia-associated transcription factor (MITF) has also been shown to promote melanoma development. MITF is involved in the regulation of melanocyte development and differentiation, as well as in maintaining melanocyte progenitor cell populations. During melanoma progression, genomic amplification of MITF promotes cell cycle progression and survival of melanoma cells [56].

Deregulation of two transcriptional repressors, SNAIL and SLUG, have been also implicated in melanoma progression. Both transcription factors have been shown to act as regulators of E-Cadherin expression, promoting the development of melanoma [57]. In melanoma cell lines, overexpression of SNAIL has been shown to negatively regulate E-Cadherin expression [58]. Furthermore, it has been recently demonstrated that using siRNA targeting SLUG resulted in enhanced melanoma cell susceptibility to drug exposure [59].

Another molecule that has been associated with the transition of melanoma cells from the RGP to the VGP is the thrombin receptor, PAR-1. PAR-1 is a G-protein coupled receptor belonging to the protease-activated receptor family that is activated by thrombin as well as other serine proteases [47]. PAR-1 has been shown to play an essential role in tumorigenesis and metastasis in several cancers, including breast cancer and melanoma [60, 61]. The role of PAR-1 in melanoma progression was further established by tissue microarray analysis which revealed that increased PAR-1 expression significantly correlates with melanoma progression [62]. Downstream signaling induced by PAR-1 suggests its importance in the progression of metastasis. PAR-1 activation results in upregulation of molecules involved in angiogenesis (Interleukin 8 (IL-8) and VEGF), invasion (MMP-2) and adhesion ($\alpha V\beta 5$, $\alpha V\beta 3$ and Connexin 43) [47, 61, 63]. Recently, Villares et al. demonstrated that systemic delivery of liposome (DOPC) incorporated PAR-1 siRNA decreased tumor growth and metastasis *in vivo* [61]. Moreover, the downstream targets of PAR-1, Connexin 43 and Maspin, have also been shown to be involved in the progression of melanoma [63].

The expression and secretion of angiogenic factors, growth factors, and cytokines also increases with melanoma progression, as they are secreted by both melanoma cells and cells in the microenvironment, acting in both an autocrine and paracrine manner. The recruitment of new vasculature, as well as the usage of existing vasculature, is essential for tumor growth beyond 1-mm in diameter and for tumor cells to metastasize [64, 65]. One of the molecules that plays an important role in melanoma progression is VEGF, also known as the vascular permeability factor. VEGF is a vascular endothelial cell growth factor, which regulates angiogenesis under both developmental and

physiological conditions in most organs [66-68]. During the progression of melanoma, the secretion of VEGF by melanoma cells increases [69, 70]. VEGF, in turn, stimulates endothelial cell proliferation, migration, tube formation, and survival, thus promoting angiogenesis, tumor growth, and metastasis, as seen in several animal models [71, 72], [73]. VEGF functions through two high affinity tyrosine kinase receptors on endothelial cells, Flt-1 and KDR/Flk-1, which results in the induction of p38, ERK and PI3K-AKT signaling pathways [74-76] [77-79]. Furthermore, following activation of these receptors by VEGF, the expression of matrix metalloproteinases (MMPs) and serine proteases is upregulated, which, in turn, promotes tumor growth and recruitment of tumor vasculature [80-83].

Basic fibroblast growth factor (bFGF) is another potent angiogenic factor known to regulate endothelial cell proliferation and act as a melanoma survival factor. bFGF can be produced by cells of the tumor microenvironment, such as keratinocytes and fibroblasts, and by transformed melanocytes themselves [84]. It has been demonstrated that release of matrix bound bFGF increases due to extracellular matrix degradation, leading to endothelial cell proliferation and vasculature formation in melanoma [71].

The angiogenic factor IL-8 is also upregulated during melanoma progression. It is secreted by melanoma cells as well as other cells in the microenvironment such as lymphocytes, monocytes, endothelial cells, fibroblasts and keratinocytes [85]. IL-8 is a CXC chemokine that activates two cell-surface G protein-coupled receptors (CXCR1 and CXCR2), inducing several downstream intracellular signaling [86]. It is a potent survival factor for melanoma cells, keratinocytes and vascular endothelial cells [87]. IL-8 plays an important role in angiogenesis and migration in melanoma [88, 89].

Specifically, our lab has previously demonstrated that the use of a neutralizing antibody targeting IL-8, ABX-IL8, inhibits angiogenesis, tumor growth and metastasis of human melanoma in mice [90]. Additionally, in melanoma patients, serum levels of IL-8 were significantly increased, compared to healthy controls, and these levels correlated with tumor progression and decreased survival [91].

Recruitment of vasculature to the tumor is dependent on secretion of MMPs. Secretion of MMPs is an important process facilitating tumor cell invasion, a crucial step in the progression of tumor metastasis. To detach from the primary tumor and to form metastases, cells degrade the basement membrane and remodel the extracellular matrix (ECM), by secreting high levels MMPs. Melanoma cells express several MMPs such as MMP-1, -2, -9, -13, and the membrane-type 1 matrix metalloproteinase (MT1-MMP) [92]. MMPs are a multigene family of zinc-dependent endopeptidases which are produced as prozymogens and are activated and released through cleavage of the N-terminal domain by proteins such as plasmin and MT1-MMP [93, 94]. The most widely studied MMPs in melanoma are MMP-2 (gelatinase A) and MMP-9 (Gelatinase B) which degrade type IV collagen found in the ECM. Increased expression and activation of both enzymes has been associated with increased melanoma cell invasiveness and metastasis [71]. Furthermore, only invasive melanoma cell lines express active MMP-2, while its expression was not observed in poorly invasive cell lines [95, 96]. It has also been demonstrated that adhesion and spreading of melanoma cells is modified by the active form of MMP-2, thus promoting melanoma cell migration and invasion [97].

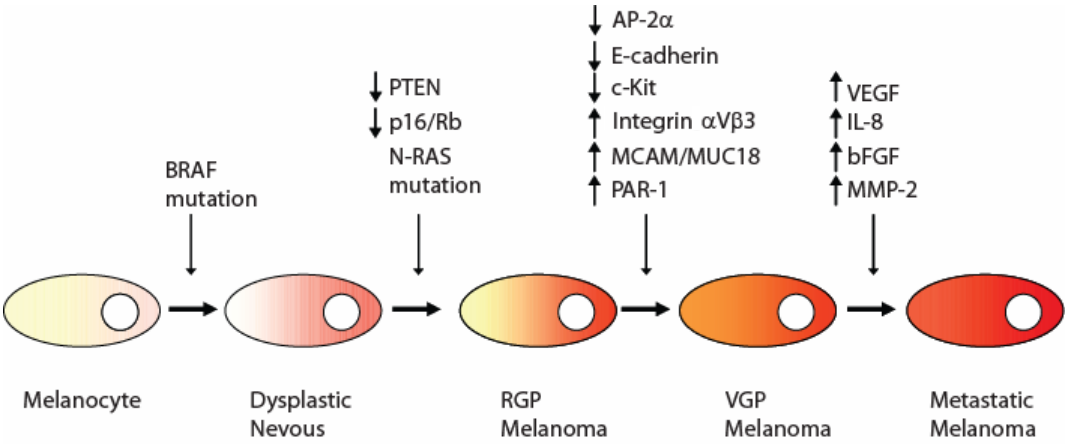


Figure 1. Molecular Events Contributing to the Development of Human Melanoma

Several key steps in the development of human melanoma are associated with the acquisition of mutations and molecular changes, including BRAF and N-RAS mutations, loss of PTEN and downregulation of p16, promoting the transition from normal melanocytes to dysplastic nevi cells. As melanoma progresses from RGP to VGP several other molecular events are acquired including changes in the expression of AP-2α, E-cadherin, c-Kit, integrin αvβ3, MCAM/MUC18 and PAR-1. The acquisition of metastatic capacity of melanoma cells at the final stage is associated with upregulation of various angiogenic and invasive factors including VEGF, IL-8, bFGF and MMP-2. (Adapted from [9, 70, 98, 99]).

Adhesion Molecules and Cancer

Cell adhesion molecules are cell-surface proteins that mediate either cell-to-cell or cell-to-ECM interactions. These molecules play a major role in a variety of physiological processes such as embryogenesis, organogenesis, inflammation, immunological function, wound healing, cell communication and cell growth. Furthermore, these molecules have a significant role in cancer progression as they are involved in tumor cell invasion, motility and aggregation [100]. The family of cell adhesion molecules include cadherins, immunoglobulins, integrins, and selectins (Figure 2) [101]. These proteins can mediate both homotypic and heterotypic adhesion, as well as homophilic and heterophilic interactions, and are known to be involved in different steps of the metastatic cascade [102]. These interactions can promote tumor cell survival in the vasculature as they allow cell clumping by mediating homotypic adhesion between tumor cells and heterotypic adhesion between tumor cells and platelets. Moreover, cell adhesion molecules are involved in extravasation and intravasation by mediating heterotypic adhesion between tumor cells and endothelial cells [103].

Cell adhesion molecules not only function in cell adherence, but they can also function as signaling molecules by mediating either inside-out signaling through their extracellular domain, or outside-in signaling through their cytoplasmic domain [104]. Changes in the expression of adhesion molecules on tumor cells modifies the interactions between tumor cells and their surrounding microenvironment , thus affecting their function and downstream signaling [104]. The importance of these changes and their contribution to cancer progression has been acknowledged in the early 20th century by Theodor Boveri [105].

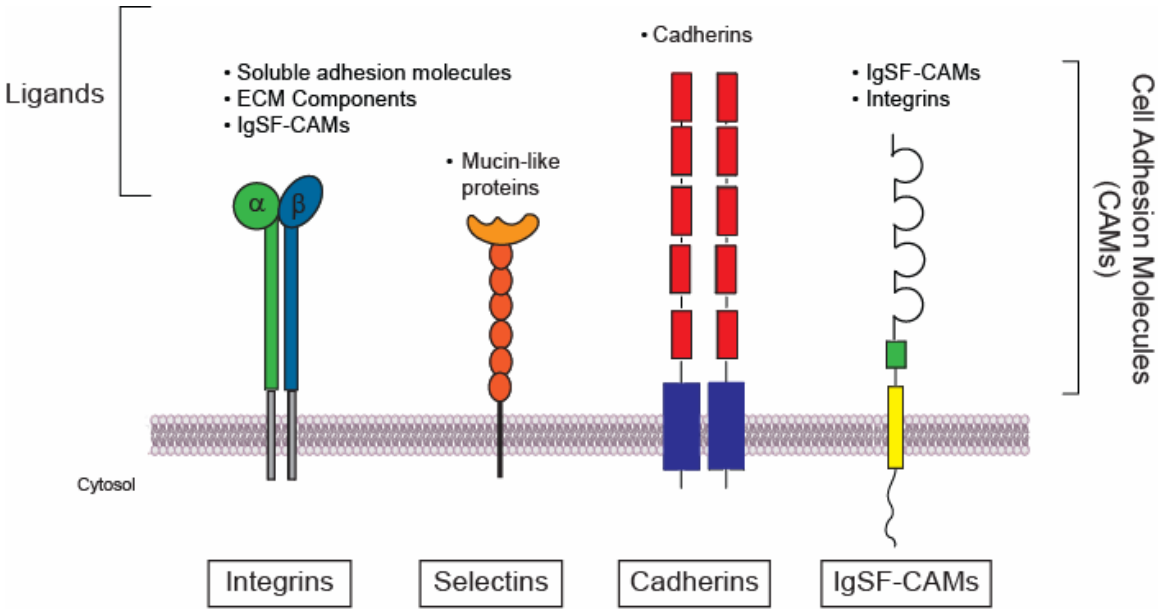


Figure 2. The Structure of Cell Adhesion Molecules (CAMs)

Each cell adhesion molecule contains several domains (cytoplasmic, transmembrane and extracellular domains) that can vary in number among members of each family and within each family. Integrins can mediate both homophilic and heterophilic interactions and bind several ligands including soluble adhesion molecules, such as fibrinogen; ECM components, such as fibronectin; and Ig Superfamily (IgSF) adhesion molecules such as N-CAM. Selectins bind highly glycosylated mucin-like proteins, such as P-selectin glycoprotein ligand-1 (PSGL-1). Cadherins mostly form homophilic interactions, thus binding identical cadherin subtypes. IgSF-CAMs can mediate homophilic as well as heterophilic interactions including interactions with other IgSF members and integrins. (Adapted from [106])

Adhesion Molecules and Melanoma

During the progression of melanoma, several changes in the expression of various adhesion molecules, including cadherins, integrins, and members of immunoglobulin superfamily, can occur.

Modulation of cadherin expression patterns have been associated with the progression of melanoma. The expression and function of E-cadherin, a member of the cadherin family of adhesion molecules, is lost during the progression of several malignant epithelial tumors, including melanoma [107]. E-cadherin is expressed mainly at the adherent junctions between melanocytes and keratinocytes and functions in two main capacities. It adheres these cells to one other and second, it plays a role in transducing downstream signaling by modulating β-catenin cellular localization, thus inhibiting the WNT signaling pathway [108]. Moreover, E-cadherin can inhibit ligand-dependent activation of EGFR by forming a complex with EGFR and consequently decreasing EGF binding affinity [109, 110].

The adhesion between keratinocytes and melanocytes has been shown to regulate normal melanocyte differentiation and proliferation [111]. Downregulation of E-cadherin expression is associated with metastatic progression of melanoma cell lines and with loss of regulation of proliferation by keratinocytes [112, 113]. Moreover, loss of E-cadherin expression is associated with upregulation of N-cadherin expression [114]. This switch is associated with changes in the interactions between melanoma cells and their microenvironment. Melanoma cells expressing N-cadherin can mediate homophilic adhesion with fibroblasts and vascular endothelial cells, which may be associated with increased migration and invasion of the melanoma cells [115]. Furthermore,

deregulation of E-cadherin signaling can occur not only due to its decreased expression but also due to mutations in β-catenin resulting in its inability to bind to the cytoplasmic domain of E-cadherin [116].

As melanoma progresses, the expression, function, and signaling of integrins is also distorted, further promoting tumor growth and metastasis. Integrins are composed of two transmembrane glycoprotein subunits, α and β, which form heterodimers and are involved in cell-matrix and cell-cell adhesion through the binding of different ligands which are dependent on the pairing of the α and β subunits [117]. In normal cells, integrins regulate embryonic development, cellular differentiation and anchorage-dependent proliferation. During neoplastic transformation, integrins promote invasion, anchorage independent growth and escape from apoptosis [118]. The acquired expression of β3 integrin in primary and metastatic melanoma is associated with the transition of melanoma from the RGP to the VGP. The expression of αVβ3 integrin during the VGP has been extensively studied and is mostly associated with angiogenesis and metastasis [119]. Seftor et al. demonstrated that in A375SM metastatic melanoma cells, association of αVβ3 integrin with its ligands, fibronectin and vitronectin, promotes activation of MMP-2 and increased cell invasiveness [120]. Furthermore, αVβ3 integrin promotes melanoma cell survival, due to its interaction with osteopontin, which is expressed in response to fibroblast growth factor receptor activation [121].

The expression of cell adhesion molecules from the immunoglobulin superfamily also increases in melanoma cells, as compared to melanocytes. These molecules include MCAM/MUC18, intracellular cell adhesion molecule 1 (I-CAM-1), vascular cell adhesion molecule 1 (VCAM-1), and L1 cell adhesion molecule (L1-CAM) [122]. The

adhesion molecules from the immunoglobulin superfamily can form homotypic and heterotypic adhesions, and they can also function as receptors or signaling molecules, similar to integrins [104]. For instance, the expression of I-CAM correlates with the vertical growth phase of melanoma and is associated with poor prognosis [123]. In fact, patients presenting with stage I disease and I-CAM positive tumors have decreased survival rates compared to patients with I-CAM negative tumors [124]. Furthermore, I-CAM expression is upregulated in response to TNF-α stimulation in melanoma cells and inhibition of I-CAM expression utilizing anti-sense oligonucliotides in C8161 metastatic melanoma cells, decreases their metastatic potential [125]. The mechanism of how I-CAM contributes to the melanoma progression has yet to be elucidated. However, it has been proposed that it may contribute to melanoma cell survival in the vasculature by promoting aggregation with leukocytes [126].

Melanoma Cell Adhesion Molecule MCAM/MUC18

MCAM/MUC18 was first identified as a human melanoma antigen in a study by Lehmann et al,. MUC18, also known as MCAM, CD146, Mel-CAM, A32 and S-Endo, is a 113-kDa type I transmembrane glycoprotein adhesion molecule and a member of the immunoglobulin superfamily [127]. It is rarely expressed on either normal epidermal melanocytes or moderate nevus cells *in situ,* but it is more abundantly expressed on primary and metastatic melanomas [128]. Moreover, increased expression of MUC18 has been shown to correlate with increased vertical thickness of primary tumors [127-129]. It was further established by tissue array analysis that the expression of MUC18 correlates with progression of cutaneous melanoma and decreased patient survival [127]. MUC18 is not present in biopsies from lesions smaller than 0.75 mm, although it is detected in more than 70% of biopsies from metastatic melanoma lesions [129].

Structure of MUC18

The extracellular domain of MUC18 contains five immunoglobulin–like domains which are connected through disulphide cross-links and have been shown to be highly glycosylated, suggesting that MUC18 may functionally interact with the ECM. MUC18 has a single transmembrane domain and a short cytoplasmic domain that consists of several potential protein kinase recognition motifs, suggesting its potential role in cell signaling (Figure 3) [45]. MUC18 is located on chromosome 11, spanning approximately 14 kb and consisting of 16 exons. It has sequence homology to other glycoprotein adhesion molecules, such as neural cell adhesion molecule (N-CAM) [127], deleted in colorectal cancer (DCC) [130], vascular cell adhesion molecule (V-

CAM) [131], the inter-cellular adhesion molecule (I-CAM) [132] and bursal epithelium and neurons [133] adhesion molecule [134].

The expression of MUC18 has been shown to correlate with the metastatic potential of melanoma cell lines *in vivo*. Luca et al., examined the expression of MUC18 in nine melanoma cell lines established from primary cutaneous tumors as well as lymph node and brain metastases. MUC18 mRNA and protein levels were low in cells with low metastatic potential such as SB2, DX3 and TXM40, whereas cells with high metastatic potential, A375SM, TXM-13 and A375, expressed high levels of MUC18 [135]. Another study examined the role of MUC18 in melanoma tumor growth and metastasis formation and demonstrated that ectopic expression of MUC18 in a non-metastatic primary cutaneous melanoma cell line, SB2, increased the tumorigenic and metastatic potential of cells *in vivo. In vitro,* expression of MUC18 increased MMP-2 collagenase activity, cell invasiveness, homotypic adhesion, attachment to human endothelial cells, and decreased cell adhesion to laminin, suggesting a functional role of MUC18 in promoting melanoma tumor progression [136].

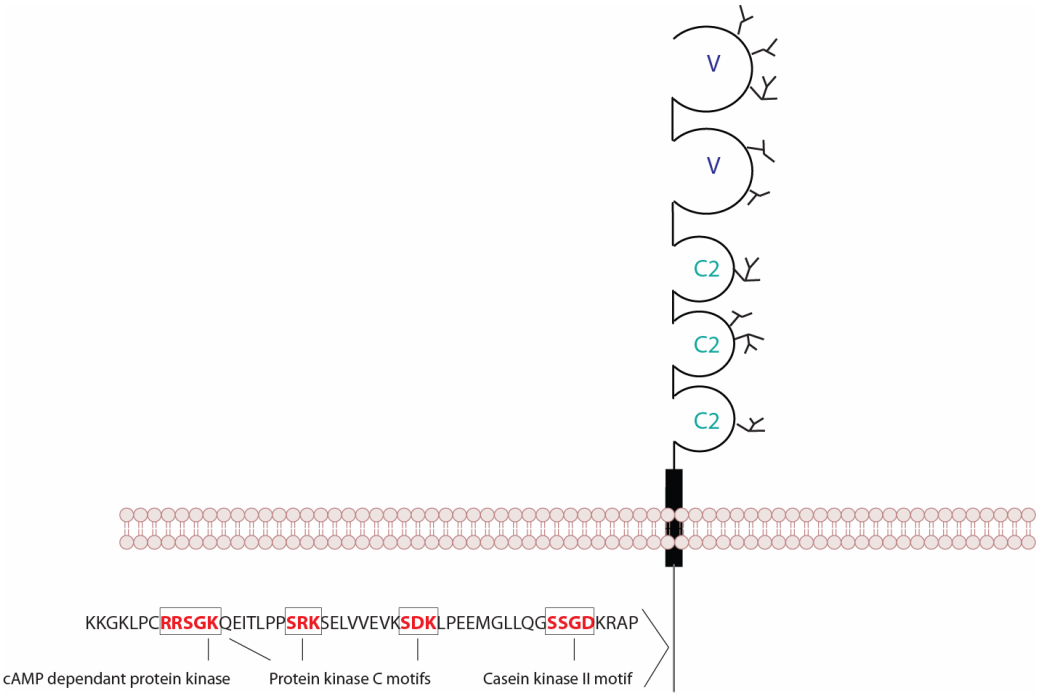


Figure 3. The Structure of MUC18

MUC18 is a 113-kDa transmembrane adhesion molecule that belongs to the Ig like family of adhesion molecules. The extracellular domain of MUC18 is comprised of a highly glycosylated (N-linked glycosylation) distinctive V-V-C2-C2-C2 immunoglobulin-like structure, (V-variable region, C-constant region), a single transmembrane domain and a short cytoplasmic domain. The cytoplasmic domain of MUC18 contains several potential protein kinase recognition motifs, including one similar to the cAMP dependent kinase recognition motif, a potential casein kinase II motif, and three potential protein kinase C recognition motifs that suggest the involvement of MUC18 in cell signaling [45]. Figure was adapted from Shih, I-M [137].

Regulation of MUC18 Expression

MUC18 expression in melanoma cells was shown to be modulated by adhesion to keratinocytes at early stages of disease progression. Adhesion between keratinocytes and melanocytes, nevus cells or melanoma cells from the RGP resulted in downregulation of MUC18 expression. Nevertheless, MUC18 expression was not altered upon adhesion between keratinocytes and cells from primary and metastatic melanoma, suggesting that late stage melanoma cells escape keratinocye regulation [138].

The transcriptional regulation of MUC18 expression was studied by Sers et al. Sequencing of the MUC18 promoter revealed that the promoter is GC rich, but does not contain the classical TATA or CAAT boxes. However, the binding sites for several transcription factors including c-MYB, Sp1, AP-1, AP-2, and CREB, were identified on the promoter [45]. Additional analysis of the MUC18 promoter demonstrated that several transcription factors, including AP-2α and CREB, can regulate MUC18 transcription. In fact, AP-2α overexpression in A375SM cells decreased MUC18 mRNA levels and promoter activity [139]. CREB has been found to positively regulate the expression of MUC18. Transfection of melanoma cells with a dominant negative CREB (kCREB) decreased MUC18 mRNA levels [140]. Importantly, a recent study from our laboratory demonstrated the regulation of MUC18 expression by a novel signaling pathway. Melnikova el al. established a connection between PAR-1, the platelet-activating factor receptor (PAFR), CREB and MUC18 [141]. Silencing the expression of PAR-1 decreased MUC18 expression at both the transcriptional and protein levels. This was shown to be mediated through decreased activation of CREB as well as decreased binding of both CREB and Sp1 to the promoter of MUC18. Furthermore,

downregulation of MUC18 expression was also observed after silencing PAFR, and overexpression of PAFR in PAR-1-silenced cells rescued the expression of MUC18. Taken together these data suggested that PAR-1 regulates the expression of MUC18 through PAFR, as well as the transcription factors CREB and Sp1. These data revealed, that MUC18 is modulated by two pro-inflammatory G-protein coupled receptors (PAR-1 and PAFR) that are overexpressed during melanoma progression. [141].

Role of MUC18 in Melanoma Progression

The function of MUC18 in cell adhesion was initially described by Shih et al. They demonstrated that melanoma cells express MUC18 at cell-cell adhesion sites and are able to adhere to a MUC18 antigen, A32, whereas non-melanoma cells failed to adhere to A32. Pre-incubation of melanoma cells with soluble MUC18 antigen abrogated this adhesion [142]. A different study demonstrated that SB-2 cells, non-metastatic melanoma cells that do not express MUC18, are able to adhere to nitrocellulose-immobilized MUC18 and form aggregates with human endothelial cells [143]. These studies suggest that MUC18 may be involved homotypic and heterotymic adhesions as well as heterophilic adhesion with an unidentified ligand. Due to the fact that MUC18 is expressed on endothelial cells, these interactions could potentially facilitate tumor cell invasion and metastasis.

Further studies of the function of MUC18 in adhesion and melanoma progression were performed by Satyamoorthy et al. Genetic suppressor elements (GSE) (antisense) targeting MUC18 expression in metastatic melanoma cell lines resulted in

downregulation of MUC18 expression in these cells. This led to decreased aggregation of melanoma cells and decreased tumorigenicity in immunodeficient mice [144].

The role of MUC18 in melanoma tumor growth, angiogenesis, invasion and metastasis was further studied utilizing a fully human anti-MCAM/MUC18 monoclonal Ab (ABX-MA1; produced by Abgenix Fremont, CA, USA). Treatment of nude mice with ABX-MA1 decreased tumor growth and metastasis formation in two metastatic melanoma cell lines, A375SM and WM2664, as compared to treatment with IgG control. *In vitro* inhibition of MUC18 by ABX-MA1 in A375SM and WM2664 disrupted the adhesive function of MUC18, decreasing both spheroid formation and attachment to human umbilical vein endothelial cells (HUVECs), further suggesting a role for MUC18 in tumor intravasation and extravasion. Because MUC18 is also expressed on HUVECs, treatment with ABX-MA1 hindered tube-like formation phenotype in an *in vitro* vessel formation assay. Additionally, the *in vivo* mean vessel density and the *in vitro* invasive capacity of both cell lines decreased significantly after treatment with ABX-MA1 [145]. Since MUC18 is expressed on both tumor cells and cells of the microenvironment, these results demonstrated a therapeutic potential of ABX-MA1 in human melanoma.

Given that the MUC18 expression correlates with melanoma development, targeting this protein may be beneficial for both prevention and treatment of patients. Our laboratory has developed a DNA-based vaccine targeting murine MUC18 and studied the effects on melanoma tumor growth and metastasis in nude mice [146]. Mice were injected with murine melanoma cells that express high levels of MUC18. Vaccination with the MUC18 vaccine induced both humoral and $CD8^+$ T-cell responses that significantly reduced both tumor growth and experimental lung metastasis formation

[146]. Though this study further confirms the importance of MUC18 in melanoma development, it raises the possibility that utilizing a DNA-based vaccine targeting MUC18 in patients, might cause an autoimmune response as well as destruction of other cells known to express MUC18. Thus, it is important to fully understand the signaling function of MUC18 potentially leading to the identification of new targets for melanoma treatment.

Expression of MUC18 in other Cell Lines and Neoplasms

MUC18 expression is detected as early as 7 weeks of human embryonic development. In normal adult tissues, MUC18 is expressed on a variety of cells including, smooth muscle cells, endothelial cells, Schwann cells, ganglion cells and myofibroblasts [147]. Expression of MUC18 on a spectrum of mesenchymal neoplasms was evaluated by Shih et al. Analysis of smooth muscle cell origin tumors demonstrated that 100% of leiomyoma and 91% of leimyosarcomas stained positive for MUC18. Additionally, MUC18 expression was detected in all examined neoplasms of vascular origin, including Kaposi's sarcoma, hemangioma, angiosarcoma, shwannoma, as well as neurofibromas, which, like melanoma, is derived from a neural precursor. This suggested that MUC18 might serve as a potential diagnostic marker for mesenchymal tumors of the smooth muscle cell, vascular or neural origin [148].

MUC18 expression is implicated in the metastatic behavior of various types of cancers. Wu et al. examined the expression of MUC18 in normal and prostate cancer cell lines and tissues. Analysis of clinical specimens demonstrated low expression of MUC18 in normal or benign hyperplastic epithelium (BPH), while high expression of

MUC18 was detected in high grade prostatic intraepithelial neoplasia [149], prostatic carcinoma tissue samples, and cells from lymph node metastasis [150]. It was also demonstrated that three of four prostate cancer cell lines (TSU-PR1, DU145, and PC-3) expressed high levels of MUC18 mRNA and protein. Interestingly, TSU-PR1, DU145, and PC-3, are metastatic cell lines, while the non-metastatic prostate cancer cell line, LNCaP, did not express MUC18 [150]. Ectopic expression of MUC18 in LNCaP cells increased metastatic potential of the cells *in vivo*. *In vitro,* MUC18 expression in LNCaP cells resulted in increased cell motility and invasiveness. This phenotype was significantly abrogated using anti-MUC18 antibodies [151].

A recent study examined the expression of MUC18 in malignant human breast cell carcinoma tissues and cell lines [152]. Tissue microarray analysis correlated MUC18 expression with high tumor grade, and triple-negative receptor status (Estrogen receptor/progesterone receptor/ERBB2) and associated MUC18 expression with poor overall survival [152]. Furthermore, flow cytometry and DNA microarray experiments suggest that MUC18 is highly expressed on human mammary cancer cell lines which have more mesenchymal characteristics, such as MDA-MB-231, MDA-MB-436 and BT-549 cells. Interestingly, cells that display more epithelial characteristics, or non-metastatic characteristics, express low levels of MUC18 [152].

MUC18 expression has also been detected on human bone sarcoma cells. A study by our laboratory has shown that osteosarcoma and Ewing's sarcoma cells, KRIB and TC-71, respectively, express high levels of MUC18. Inhibition of MUC18 *in vivo*, utilizing ABX-MA1, significantly decreased spontaneous metastasis formation of

osteosarcoma cells in nude mice [153], further demonstrating the role of MUC18 in metastasis formation.

Role of MUC18 in Mediating Downstram Signaling Events

The role of MUC18 in intracellular signaling has yet to be fully elucidated. The potential function of MUC18 in downstream signaling was proposed since the cytoplasmic domain of MUC18 contains several protein kinase recognition motifs [45]. Figure 4 illustrates previously described MUC18 downstream signaling pathways.

Outside-in signaling of MUC18 was studied in human endothelial cells and suggests a role for MUC18 in actin cytoskeleton reorganization [154, 155]. Cross linking of MUC18 with a stimulating antibody results in association of $p59^{fyn}$ with the cytoplasmic domain of MUC18. $p59^{fyn}$ is then phoshophorylated and, in-turn, phosphorylates $p125^{FAK}$. $p125^{FAK}$ consequently phosphorylates and associates with paxillin. [154]. A subsequent study demonstrated that activation of MUC18 via antibody engagement caused induction of Ca^{2+} through tyrosine phosphorylation of phospholipase C-γ by $p59^{Fyn}$, which then leads to phosphorylation and association with Pyk2 and $p130^{Cas}$ [155]. These results demonstrated the role of MUC18 as a signaling molecule in endothelial cells.

An additional signaling pathway downstream of MUC18 was identified by Meenhard Herlyn's laboratory, which established a connection between MUC18 and AKT [156]. Melanoma cells lines, as well as tumor samples, were shown to express higher levels of phosphorylated AKT, as compared to normal melanocytes. Overexpression of MUC18 in the non-metastatic melanoma cell line, SB2, increased

AKT activation and cell survival after drug treatment. These results indicate that MUC18 can regulate AKT activity, thereby promoting cell survival. [156].

MMP-2, which is known to contribute to the metastatic melanoma phenotype, has also been shown to be regulated by MUC18 [145]. Mills et al. have shown that both MMP-2 expression and activity can be modulated by MUC18. Blocking MUC18 with ABX-MA1 caused a decrease in MMP-2 expression *in vivo. In vitro*, ABX-MA1 decreased MMP-2 promoter and collagenase activity, ultimately resulting in decreased melanoma cell invasiveness through Matrigel [145]. Taken together, these studies suggest that MUC18 contributes to melanoma progression not solely as an adhesion molecule but also as plays a role in signal transduction.

Although these studies describe several signaling pathways downstream of MUC18, the role of MUC18 in regulation of gene expression has yet to be elucidatede. Furthermore, a gap in knowledge remains in the mechanism by which MUC18 promotes melanoma invasion and metastasis.

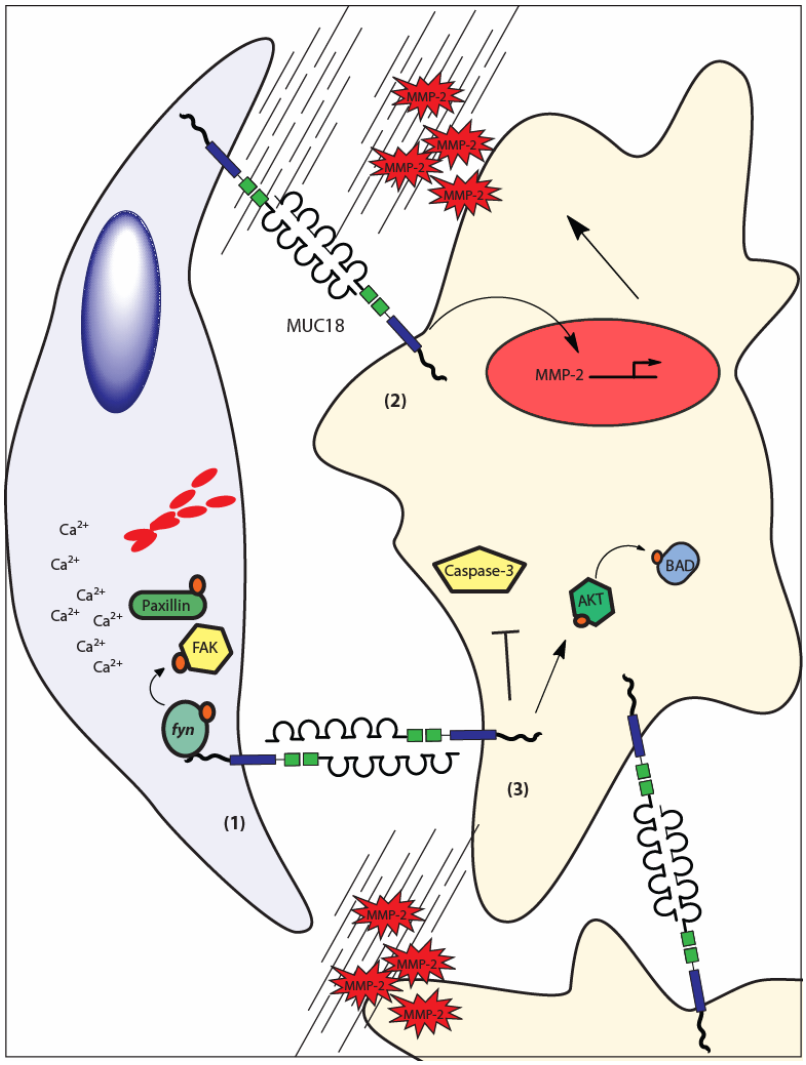


Figure 4. The Downstream Signaling Events Induced by MUC18 Following Homotypic and Heterotypic Interactions

(1) Activation of MUC18 in endothelial cells results in association of $p59^{fyn}$ with the cytoplasmic domain of MUC18. $p59^{fyn}$ is then phoshophorylated and, in-turn, phosphorylates $p125^{FAK}$. $p125^{FAK}$ consequently phosphorylates and associates with paxillin. Activation of MUC18 also promotes increase of cytosolic Ca^{2+} [154, 155]. **(2)** In melanoma cells, MUC18 transcriptionally regulates MMP-2 expression and promotes its collagenase activity, indicating a role for MUC18 in melanoma cell invasion [136,

145]. **(3)** MUC18 promotes AKT activation, leading to the phosphorylation and inactivation of Bad. MUC18 also inhibits Caspase-3 activation, demonstrating that MUC18 can promote melanoma cell survival [156].

Specific Aims

The acquisition of the metastatic melanoma phenotype of melanoma is associated with increased expression of the melanoma cell adhesion molecule MCAM/MUC18. Herein, we aim to establish the molecular mechanism by which MUC18 regulates melanoma cell invasion to promote melanoma progression. Our study will further elucidate the function by which MUC18 acts not only to adhere cells together but also as a signaling molecule, identify novel downstream potential targets, characterize their regulation by MUC18 and their contribution to melanoma progression.

Therefore, we hypothesize that MUC18 expression promotes differential expression of genes which contribute to the acquisition of the metastatic melanoma phenotype.

To test this hypothesis we developed the following three specific aims

1. **To determine the effect of MUC18 on the tumorigenic and metastatic potential of melanoma cell lines by silencing MUC18 expression utilizing lentiviral-based small hairpin RNA (shRNA)**
2. **To identify potential downstream target genes regulated by MUC18 and determine the mechanisms of their regulation**
3. **To determine the contribution of the genes regulated by MUC18 to the malignant phenotype of melanoma**

CHAPTER 2: Material and Methods

Cell Lines

The human metastatic melanoma cell line A375SM was established from pooled lung metastases produced by A375-P cells injected i.v. into nude mice [157] . The SB-2 cell line was isolated from a primary cutaneous lesion and was a gift of Dr. B. Giovanella (St. Joseph's Hospital Cancer Center, Research Laboratory, Houston, TX). The A375SM and SB-2 cell lines were maintained in cell culture as monolayers in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, HEPES buffer, and penicillin-streptomycin. The amelanotic human metastatic melanoma cell line C8161 [158] was maintained in Dulbecco's modified Eagle's medium-F12 (DMEM-F12) supplemented with 5% fetal bovine serum, sodium pyruvate, nonessential amino acids, and penicillin-streptomycin. The 293FT cells (Invitrogen) used to produce the lentiviral shRNA were maintained as previously described [159]. All cell lines were incubated at 37°C with 5% CO_2.

Protein Extraction

Total protein extracts were obtained from 80% confluent cells plated on a 100-mm culture dishes or 6 well plates. Cells were washed once with PBS and were incubated for 30 minutes on ice with either 300 µl (for 100-mm culture dish) or 100 µl (for 6 well plate) of Triton X Lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA and 1% Triton X-100) supplemented with 1% Protease Inhibitor Cocktail containing six protease inhibitors for the inhibition of aspartic, cysteine, serine, and

aminopeptidases (Merck KGaA, Darmstadt, Germany). Proteins were then collected and cenrifugated at 4°C at 14, 000 G for 10 minutes. Supernatant was transferred to a new tube and protein concentration was determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA).

Nuclear extract were extracted from cells cultured in 6 well plate at 80% confluence. A commercial nuclear extraction kit (Panomics, Inc., Fremont, CA) was used to extract nuclear proteins. Briefly, cells were exposed to a hypotonic buffer allowing cells to swell. Next, the cells were scraped and the cytoplasmic fraction was removed. The nuclear proteins were then released from the nuclei by incubation in a high-salt buffer. Samples were centrifuged at 4°C at 14,000 x *g* for 5 minutes and supernatants were collected. Protein concentration was determined using Bradford assay (Bio-Rad Laboratories, Hercules, CA).

Western Blot Analysis

Melanoma cell lines were seeded at 1×10^6 in 100-mm culture dishs in 10 ml of media. Protein concentrations were determined by using the Bradford protein assay (Bio-Rad). 20 μg of protein was resolved using a 10% SDS-PAGE gel in a BioRad Mini Protean III gel aparatus (BioRad, Hercules, CA) and transferred to a 0.45-μm nitrocellulose membrane (Millipore, Bedford, MA). The membrane was then blocked with 5% milk in Tween 20 Tris-HCL buffered saline for 1 h. Subsequently, blots were incubated overnight at at 4 °C with primary antibodies at the following dillution of 1:1000 for anti-ATF-3 or anti-Id-1, (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-MMP-2 (Cell Signaling Technology) and 1:10000 for anti-Ets-1 (Santa Cruz

Biotechnology, Santa Cruz, CA). Proteins were detected by enhanced chemiluminescence utilizing ECL detection system according to the manufacturer's instructions (GE Healthcare). For detecting ATF-3 and Ets-1, nuclear extracts were prepared and 20 μg of protein was analyzed as described above. Densitometry was carried out to determine the intensities of bands of interest in the Western blots, using ImageJ software (NIH).

Chromatin Immunoprecipitation Assay (ChIP)

ChIP Assay was performed utilizing ChIP-IT Express Kit (Active Motif Carlsbad, CA) according to the manufacturer's protocol. 1.5×10^7 cells were plated in 15 cm culture dishes. The following day ells were fixed with 37% Formaldehyde containing medium. The plates were then scraped, cell suspensions centrifuged for 10 minutes at 2500 rpm at 4 °C, and supernatants were discarded. Pelleted cells were then lysed and incubated on ice for 30 minuets. Next, enzymatic shearing was performed and sheared chromatin containing DNA fragments between 200-bp and 1000-bp was isolated. Protein-DNA complexes were immunoprecipitated with magnetic beads usng the following antibodies: anti-ATF-3 antibody (sc-188, Santa Cruz, Santa Cruz, CA), Anti-Sp1 antibody (sc-14027X, Santa Cruz, Santa Cruz, CA), anti-Ets-1 antibody (sc-55581X, Santa Cruz, Santa Cruz, CA), or anti-AP-2α Antibody (sc-184X, Santa Cruz, Santa Cruz, CA). The complexes were then separated from the magnetic beads, and protein-DNA complexes were reverse-crosslinked at 65°C for 2.5 hours. Next, the proteins were digested for one hour at 37°C with of Proteinase K and DNA was analyzed by PCR. PCR was carried out using either a Taq PCR Master Mix Kit (Qiagen,

Valencia, CA) in a 30µl reaction mixture or with REDAccuTaq LA DNA Polymerase (Sigma, St Louis, MO) in a 25µl reaction mixture.

The following primer sequences were used to amplify the binding sites of the analyzed transcription factors:

Sp1/AP-2 binding site: forward 5'-GGTCGTGCACTGAGGGTGGACGTAG-3' and reverse 5'-GTTTAAAGCCCCAGATGCGCAGCCTC-3', spanning a region of 273 bp from -218 to +55.

AP-1/Ets-1- forward 5'-CATTGTCAATGTTCCCTAAAACATTC-3' and reverse 5'-CTCCCTCTCTCAGGAAAGACAGTTG-3' spanning a region of 185 bp from -1355 to -1145.

ATF-3- forward 5'-GCGCCAGCCTGACAGTCCGTCCGGG-3' and reverse 5'-CTTCTCAAAGACCTCAGAGCAGGG-3' spanning a region of 181-bp from -1052 to -871

Matrigel Invasion Assay

Invasion assay was carried out using BioCoat Matrigel invasion chambers (BD Biosciences Pharmingen, San Diego, CA). Briefly, $2.5x10^4$ cell diluted in 500 µl of serum-free DMEM-F12 or MEM were placed in triplicates in the upper chamber of the Matrigel plate. The lower chamber contained medium supplemented with 20% FBS. Matrigel plates were incubated for 24 h at 37 °C. Hema3 stain set (Fisher Scientific) was utilized to stain cells that migrated to the lower surface of the Matrigel filter. Stained cells were then counted under the microscope.

Lentivirus shRNA Targeting MUC18

A MUC18 targeting shRNA sequence 5'-TGATATCGCTGCTGAGTGA-3' and a nontargeting (NT) shRNA sequence 5'-TTCTCCGAACGTGTCACGT-3' (with no homology to any known human genes) were designed with a hairpin and sticky ends (Cla1 and Mlu1). Using the Cla1 and Mlu1 restriction enzymes the oligos were digested and cloned into a lentiviral gene transfer vector, pLVTHM. The annealed vector was then transformed into competent E.coli bacteria (XL-10-Gold Ultracompetent Cells, Stratagene, Cedar Creek, TX) and plated on Luria broth [160]-Agar plates over night. Several bacterial colonies were then isolated and grown in LB overnight. DNA was isolated from the bacterial culture using a Maxi Plasmid DNA purification kit (Qiagen, Santa Clarite, CA), and then sequenced to test for proper insertion and length of the inserts. The pLVTHM vector containing the MUC18 shRNA sequence, a plasmid coding for the virion packaging system (MD2G), along with a plasmid coding for an envelope (PAX2), were transfected into 293T (human embryonic kidney cell) to generate lentivirus particles. 72 hours later the supernatant containing the lentiviral particles was collected and filtered to remove debris. The lentivirus system was developed as described previously [61, 161] and kindly provided by Didier Trono (Ecole Polytechnique Fe´de´rale de Lausanne). To silence MUC18, A375SM and C8161 cell lines were plated at 70% confluence in 6-well plates and were transduced with the virus overnight 37 °C. Subsequently, the virus containing medium was removed and replaced with normal growth medium, cells were sorted for expression of green fluorescent protein using Coulter Epics XL-MCL Flow Cytometer (Beckman Coulter, Fullerton, CA).

In vitro Proliferation Assay

Ninety-six well plates containing 1500 cells/well from MUC18 shRNA-transduced A375SM or C8161 cell lines or NT shRNA-transduced A375SM or C8161 cell lines were cultured for 4 days in normal growth medium. Cell growth was analyzed by MTT assay, which determines relative cell numbers based on the conversion of MTT to formazan in viable cells. 40 µg/ml MTT (Sigma St. Louis, MO) was added to each well. After a 2 hour incubation period at 37°C, growth medium and MTT were removed and cells were reconstituted in 100µl of dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO). Viable cells were then quantified by measuring the absorbance at 570nm using a BIO-TEK uQuant plate reader (BIO-TEK, Winooski, VT). This procedure was repeated after a 4 day incubation to determine the proliferation rate between NT and MUC18 shRNA-transduced cell.

Non-Targetable MUC18 Expression Vector

Five silent point mutations were introduced in to a MUC18 pCDNA 3.1 plasmid vector, targeting shRNA sequence. A non-targetable MUC18 expression vector containing the mutations was obtained by QuikChange II XL site-directed mutagenesis kit (Staratagene) according to manufacturer's procedures. The following primers were used to create silent mutations:

Forward- 5'

CAGGGCCTGGACTTGGACACCATGAT**T**TC**C**CT**C**CT**C**AG**C**GAACCACAGGAA

CTACTGGTG-3',

Reverse-

5’CACCAGTAGTTCCTGTGGTTCGCTGAGGAGGGAAATCATGGTGTCCAAGTC CAGGCCCTG -3’.

PCR was performed using *PfuUltra* high-fidelity (HF) DNA polymerase, generating a mutated plasmid containing staggered nicks. Following PCR *Dpn* I endonuclease was used to digest the parental DNA template. The amplified sequence was then transformed into XL-10-Gold ultracompetent cells (Stratagene, Cedar Creek, TX). Bacteria cells were plated on LB-Agar plates over night. Several bacterial colonies were then isolated and grown in LB overnight. The DNA was isolated from the bacterial culture using a Maxi Plasmid DNA purification kit (Qiagen, Santa Clarite, CA), and was sequenced to test for proper insertion and length of the MUC18 mutations. MUC18 cDNA containing the mutations was then ligated into the pLVX-DsRed-Monomer-C1 vector (Clontech, Mountain View, CA) replacing the red protein coding sequence of DsRed. The pLVX-DsRed-Monomer-C1 vector containing the mutated MUC18 sequence, a plasmid coding for the virion packaging system (MD2G), along with a plasmid coding for an envelope (PAX2), were transfected into 293T (human embryonic kidney cell) to generate lentivirus particles. 72 hours later the supernatant containing the lentiviral particles was collected and filtered to remove debris and a final rescue lentiviral vector was obtained as described above. To stably rescue MUC18 expression in MUC18-silenced cells, A375SM and C8161 MUC18-shRNA or NT-shRNA were plated in 6-well plates and transduced with the virus containing either the non-targetable mutated MUC18 expression vector or empty vector control. After 48 h, the cells were replated and

selected with growth medium containing 500 μg/ml puromycin. MUC18 expression was confirmed by Western blot.

Luciferase Assay

2.5×10^4 cells/well of A375SM, C8161 and SB-2 were plated in a 24-well plate. 0.8 μg of luciferase reporter plasmids containing either the Id-1 promoter, Id-1 mutant promoter, MMP-2 promoter or a promoterless luciferase PGL3-basic vector (which was used as negative control) were diluted in serum free medium. Diluted reporter plasmid DNA were combined with 2 μl of Lipofectamine 2000 diluted in serum free medium to form DNA-Lipofectamine complexes. For each transfection, 2.5 ng of cytomegalovirus-driven *Renilla* luciferase reporter construct (pRL-CMV, Promega, Madison, WI) was included as internal control for detection of transfection efficiency. DNA-Lipofectamine complexes were incubated for 20 minutes at room temperature. Cells were washed with serum free medium and the DNA-Lipofectamine complexes were added to the cells and incubated at 37°C. At 4 hours following transfection, medium was changed to complete growth medium containing serum. Cells were harvested 48 h after transfection. Luciferase activity was assayed utilizing a Dual-Luciferase reporter assay system ((Promega, Madison, WI) according to the manufacturer's instructions. The luciferase luminescence (relative light intensity) was measured with a LUMIstar microplate reader (BMG Labtech Durham, NC). The ratio of firefly luciferase activity to cytomegalovirus-driven *Renilla* luciferase activity was used to normalize any differences in transfection efficiency among samples.

Zymography

A375SM and C8161 cells were plated in a 6-well plate at a concentration of 5×10^5 cells/well. After 24 h, the medium in each well was replaced with 500 μl of serum-free medium and incubated overnight at 37 °C. Following incubation, the medium was collected and the volume was adjusted according to cell number. Fifty μl of samples including positive control (10% FBS containing media) and negative control (serum-free medium) were loaded on a substrateimpregnated gel (10% polyacrylamide [wt/vol], 0.1% SDS, 0.1% gelatin) to determine MMP-2 activity. The gel was then washed twice for 30 min in 2.5% Triton X-100. Subsequently, the gel was placed in incubating buffer (50 mM Tris-Cl, pH 7.5, 200 mM NaCl, 5mM $CaCl_2$) at 37°C for 16 h to optimize metalloproteinase activity. The gel was then stained for 1 h in 40% methanol/10% acetic acid/0.5% (wt/vol) Coomassie brilliant blue G-250. At the end of the incubation the gel was destained utilizing destaining buffer (45% methanol/10% acetic/45%H_2O). Proteinase activity was indicated by the presence of clear bands.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were deparaffinized by sequential washing with xylene, graded ethanol, and PBS. Pepsin was utilized for antigen retrieval for 10 min at room temperature. Subsequently, sections were washed with PBS and blocked with endogenous peroxide containing 3% hydrogen peroxidase inhibitor in PBS for 12 min. Nonspecific proteins were blocked in 5% horse serum and 1% goat serum for 20 min. Slides were incubated with anti-MMP-2 (1:400; Chemicon, Temecula, CA) or anti-Id-1(polyclonal, 1:50; Santa Cruz, Santa Cruz, CA) overnight at 4 °C and then

with a peroxidase-labeled anti-rabbit antibody (1:500; Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. Signal was detected by staining with 3,3′-diaminobenzidine (DAB; Phoenix Biotechnologies, San Antonio, TX) substrate for 6 min and then counterstaining with Gill's hematoxylin No. 3 (Sigma, St. Louis, MO) for 20 s. For MUC18 staining, 1× Divi deckloaker (BioCare Medical) was utilized for antigen retrival. Sections were then washed with PBS and blocked with endogenous peroxide containing 3% hydrogen peroxidase inhibitor in PBS for 12 min. Fragment blocking was used overnight at 4 °C with Affini fregment blocking anti mouse antibody (1:10; Jackson ImmunoResearch, West Grove, PA) to block nonspecific binding on tissue sections. Slides were then blocked in 5% horse serum and 1% goat serum for 5 min and were incubated overnight at 4 °C with anti-MUC18 (1:50; BD Biosciences Pharmingen San Diego, CA). A peroxidase-labeled anti-mouse IgG_1 antibody (1:400; BD Biosciences Pharmingen San Diego, CA) was used for 1 h at room temperature. Signal was detected as mentioned above. Slides were imaged using a Nikon Microphot-FXA microscope at 10X magnification. Pictures were taken using a Leica DFC 320 R2.

For CD31 staining, frozen sections were fixed for 5 minutes in each of the following solutions acetone, acetone:chloroform (1:1) and acetone. Slides were then washed with PBS three times and blocked with protein block 4% Fish gelatin in PBS for 20 minutes at room temperature. Slides were incubated at 4 °C over night with primary antibody (rat anti-mouse CD31, PharMingen Inc. Cat. #01951A). Alexa 494 [37] was incubated for 1 hour at room temperature. Slides were then washed 3 times for 3 minutes in PBS.

TUNEL staining was performed utilizing a TUNEL assay kit (Promega, Promega, Madison, WI). Slides were fixed in 4% Paraformaldehyde for 10 minutes at room

temperature followed by two 5 minute washes with PBS. Slides were then incubate in 0.2% Triton X-100 in PBS for 15 minutes and washed twice with PBS. Subsequently, slides were incubate with DNase 10 minutes at 37ºC and washed four times with PBS. Equalibrium buffer was then used on slides for 10 minutes room temperature. Incubation buffer containing 5ul Nucleotide Mix and 1 ul of TDT enzyme, was then applied on slides. Slides were then incubated at 4 °C over night. The following day, slides were washed with SSC buffer twice for 15 minutes followed by washes with PBS to remove unincorporated Fl-dUTP. Hoechst (Molecular Probes, Carlsbad CA) diluted 1:10,000, was used to counterstain slides and is in PBS.

Plasmid Construction

The promoter of Id-1 gene (2.2kb) cloned into pGL3 was kindly donated by Dr. Rhoda M Alani [162]. A promoter region of 1.4 kb (-1370+40) was amplified by PCR utilizing the following primer sequences: forward 5'-GGGTACCCGAAATTAATAATGGTC-3' and reverse 5'-GAAGATCTGAATGGGCAAAGCGAAAAAAATGAGG-3'. The amplified fragment was digested with KpnI and BglII and ligated into a promoterless luciferase pGL3-basic vector (Promega, Madison, WI). Site-directed mutagenesis of the ATF-3 site, replacing TGACGTCA to TGTGCAGCA, was performed using the QuikChange II XL site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) according to manufacturer's instructions. The promoter of region of MMP-2 from -1766 to +55 was amplified from A375SM genomic DNA utilizing PCR primers:

forward 5'- GGGGTACCTTTAAAACTGACTCTGG-3' and

reverse 5'- GAAGATCTAAAGCCCCAGATGCGC-3'. The amplified fragment was digested with KpnI and BglII and was cloned into a promoterless luciferase pGL3-basic vector (Promega, Madison, WI).

To generate an Id-1 expression vector, the Id-1 sequence was amplified from an Id-1-pLXSN expression vector kindly provided by Dr. Pierre-Yves Desprez [163] using the following primer sequences:

Forward- 5'-CCGCTCGAGCGGATGAAAGTCGCCAGTGGCAGCACCG-3' and reverse- 5'-CGGGATCCCGCTAGTGGTCGGATCTGGATCTCACCTC-3'. The amplified fragment was digested with Xho1 and BamH1 and ligated into the pLVX-DsRed-Monomer-C1 vector (Clontech) replacing the red protein coding sequence of DsRed.

To generate the ATF-3 expression vector, a pCDNA 3.1 expression vector containing ATF-3 with an N-terminal flag tag sequence was kindly provided by Dr. Douglas Boyd [164]. The coding sequence of ATF-3 with an N-terminal flag tag was digested with Hind IV and HpH1 enzymes and filled in with large (Klenow) fragment. The product was then ligated into the pLVX-DsRed-Monomer-C1 vector (Clontech, Mountain View, CA) replacing the red protein coding sequence of DsRed .The final lentiviral particles, for all plasmids, were obtained as described above.

Animals, Tumor Growth and Metastasis

Female athymic BALB/c nude mice were purchased from the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD) and housed in specific pathogen-free conditions. All studies were approved and supervised

by The University of Texas M.D. Anderson Cancer Center Institutional Animal Care and Use Committee (IACUC). Subcutaneous tumors were produced as described previously [159]. Briefly, 5×10^5 A375SM cells or 2.5×10^5 C8161 cells suspended in 0.1 ml of Hanks' buffered salt solution were injected into the right flank of each mouse (*n*=8/group). Tumor growth was measured three times weekly in two dimensions with a caliper and calculated as $axb^2/2$ cm^3 (*a*, long diameter; *b*, short diameter). Mice were sacrificed in a CO_2 chamber, either 35 days after injection or when the tumor reached 1.5 cm^3 in volume (according to guidelines of the IACUC, which specifies that when the largest dimension of a subcutaneously injected tumor reached 1.5 cm^3, mice should be considered moribund and then sacrificed). Tumors were harvested and processed for hematoxylin and eosin staining and for immunohistochemical staining. To determine metastatic potential 5×10^5 A375SM cells and 3.75×10^5 C8161 cells suspended in 0.1 ml of Hanks' buffered salt solution were injected into the tail vein of nude mice (*n*=7/group). Mice were sacrificed 35 days after injections, lungs harvested and fixed in Bouin's solution, and the number of macroscopic surface tumors nodules was counted.

cDNA Microarray

Total RNA was isolated from NT A375SM cells and MUC18-silenced A375SM cells utilizing the Clontech Advantage RT-for-PCR kit according to the manufacturer's instructions. A human genome U133 Plus 2.0 array (Affymetrix) was used for the microarray analysis. The microarrays were produced in the microarray core facility of Codon Bioscience (Houston, TX). Affymetrix software was utilized to analyze the results as described previously [165].

Statistical Analyses

Student's *t* test was used to evaluate the statistical significance of differences the *in vitro* data. Statistical analysis of the results of the animal studies was performed using the Mann-Whitney *U* test. Values for tumor growth are given as a mean volume ± S.E.M., and p values < 0.05 were considered statistically significant.

CHAPTER 3: Specific Aim 1

Determine the Effect of MUC18 on Tumor Growth and the Metastatic Potential of Melanoma Cell Lines by Silencing MUC18 Expression Utilizing Lentiviral-Based shRNA.

Introduction

The melanoma cell adhesion molecule, MUC18, was first identified as a melanoma antigen whose expression has been shown to correlate with poor prognosis and progression of cutaneous melanoma [127]. Our lab has previously demonstrated that MUC18 expression in melanoma cell lines is associated with their ability to form subcutaneous (s.c.) tumors and metastases *in vivo* [135]. Direct evidence of the role of MUC18 in the progression and metastasis of melanoma was established by overexpressing MUC18 in the non-metastatic melanoma cell line, SB-2, which resulted in increased tumor growth and metastatic potential in nude mice [136]. Furthermore, inhibition of MUC18 activity utilizing a fully human antibody, ABX-MA1, decreased tumor growth and reduced the formation of experimental lung metastasis in nude mice after challenging them with the metastatic melanoma cell lines, A375SM and WM2664. *In vitro*, blocking MUC18 in melanoma cell lines with ABX-MA1 decreased both MMP-2 promoter activity, as well as collagenase activity, and subsequently resulted in decreased cell invasion through Matrigel [145]. To further establish the role of MUC18 in melanoma progression and metastasis, we silenced MUC18 expression in two metastatic melanoma cell lines, A375SM and C8161. Utilizing these metastatic

melanoma cells that lack MUC18 expression will provide a unique tool in which we can identify novel downstream gene targets that are regulated by MUC18. Identification of new MUC18-regulated genes will shed light on the molecular signaling pathways which contribute to the metastatic melanoma phenotype.

Results

MUC18 silencing in the Metastatic Melanoma Cell Lines A375SM and C8161

To further establish the role of MUC18 in promoting tumor growth and metastasis of human melanoma cells, we stably silenced MUC18 expression in two human melanoma cell lines, A375SM and C8161. We utilized a lentiviral based shRNA vector containing a green fluorescence protein (GFP) sequence to clone either a MUC18 shRNA sequence or a control non-targeting (NT) shRNA sequence. Importantly, the NT shRNA sequence used had no homology to any known human gene. Lentivirus containing the shRNA sequences were produced utilizing the method developed by Didier Trono [161]. Transduced cell populations were sorted by Fluorescent Activated Cell Sorting (FACS) on the basis of GFP expression. Reduction of MUC18 expression was then verified by western blot analysis. Indeed, MUC18 protein levels decreased by 90% and 80% in MUC18-silenced A375SM and C8161 cells, respectively, as compared to the non-targeting (NT) shRNA transduced cells (Figure 5).

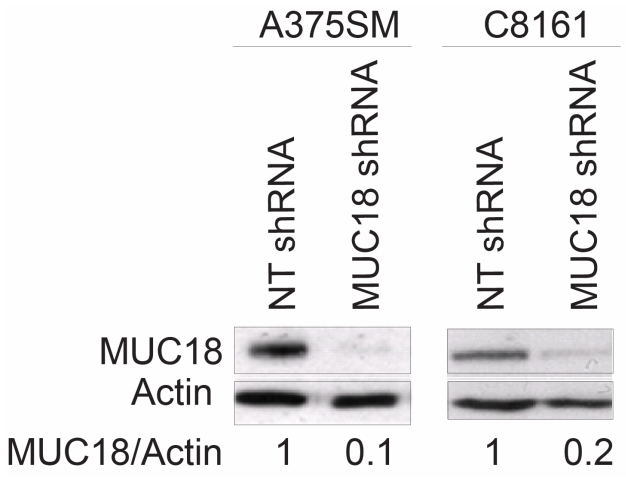


Figure 5. MUC18 Expression is Downregulated in Metastatic Melanoma Cell Lines Transduced with Lentivirus Containing MUC18 shRNA

Western blot analysis of MUC18 expression in A375SM and C8161, after stable transduction with lentivirus containing either MUC18 shRNA or a Non-Targeting (NT) control vector, shows decreased expression of MUC18 by 90% and 80% after MUC18 silencing in A375SM and C8161, respectively.

Effect of MUC18 Silencing on Melanoma Tumor Growth and Experimental Lung Metastasis Formation

To determine the effect of MUC18 silencing on melanoma tumor growth, we injected the two MUC18-silenced melanoma cell lines, A375SM and C8161 s.c. ($0.5x10^6$ A375SM cells and $0.25x10^6$ C8161 cells) into the flanks of nude mice (n=8/group). Tumor growth was measured twice a week for 35 days. MUC18 silencing resulted in a significant decrease in tumor growth in both cell lines, as compared to mice injected with the NT shRNA-transduced cells; mean tumor volume at day 35 was 621.4 mm^3 in mice injected with NT A375SM cells, whereas in mice injected with MUC18-silenced A375SM cells it was 233.6 mm^3. (*$p<0.05$) (Figure 6A). Similar results were observed with NT shRNA and MUC18-silenced C8161 cells; mean tumor volume was 662.3 mm^3 versus 221.9 mm^3 (*$p<0.001$) (Figure 6B).

To assess whether the differences observed in tumor growth between MUC18 silenced cells and NT-transduced cells were due to changes in cell proliferation rates, we evaluated *in vitro* cell proliferation (Figure 7). Although the MTT assay detected no significant changes in *in vitro* cell proliferation, we examined the *in vivo* expression of the proliferation marker, Ki67, in tumor samples derived from MUC18-silenced and the NT-transduced A375SM cells (Figure 8A). Interestingly, we observed a decrease in Ki67 immunohistochemical staining in the tumor samples from the MUC18-silenced A375SM cells, compared to the samples from NT-transduced cells. These data suggested that the changes in tumor growth between the MUC18 silenced cells and NT-transduced cells were, at least in part, due to differences in their *in vivo* cell proliferation rates. To next determine whether apoptosis contributed to the differences observed in tumor

growth, we evaluated the effect of MUC18 silencing on apoptosis and performed TUNEL staining on tumors from both MUC18 silenced and NT-transduced cells (Figure 8B). Increased apoptosis of tumor cells was observed in samples obtained from MUC18-silenced cells. Taken together, these data indicate that *in vivo* MUC18-silenced cells proliferate slower and have increased apoptosis resulting in significantly smaller tumors produced by MUC18-silenced cells, compared to NT-transduced cells.

Because of their smaller tumor size, decreased proliferation and elevated level of apoptosis, we hypothesized that tumors formed from MUC18-silenced cells lacked sufficient supply of nutrients and oxygen. We, therefore, evaluated the effect of MUC18 silencing on the formation of tumor vasculature by staining of endothelial cells (Figure 8C). Immunohistochemical analysis demonstrated a significant decrease in vessel size, as well as number, in tumor samples obtained from MUC18-silenced cells. Collectively, these results suggest that the reduction of tumor growth was due to a number of factors including decreased tumor cell proliferation, increased apoptosis, and decreased vessel size and number as a result of MUC18 silencing in metastatic melanoma cells.

To confirm the validity of the immunohistochemical staining patterns, we evaluated MUC18 expression *in vivo*. Immunohistochemical staining of the s.c. tumors verified that MUC18 expression decreased in tumors derived from MUC18-silenced A375SM (Figure1D) as compared to tumors from NT-transduced cells and that the decreased expression of MUC18 was sustained until the end of the experiment at day 35 (Figure 1D).

Next, to determine the effect of MUC18 silencing on melanoma metastasis, MUC18-silenced A375SM and C8161 cell lines were injected i.v. into nude mice (n=7

mice/group). Six weeks after injections, experimental lung metastasis formation was assessed. Lung metastases were significantly decreased in mice injected with MUC18-silenced A375SM and C8161 cells as compared to NT-transduced cells (median= 78 vs 279 for A375SM, median= 133 vs 290 for C8161) (*p<0.05) (Figure 9).

Taken together, these data corroborate previous results demonstrating the role of MUC18 in promoting melanoma tumor growth and lung metastasis formation *in vivo* [135, 136, 145]

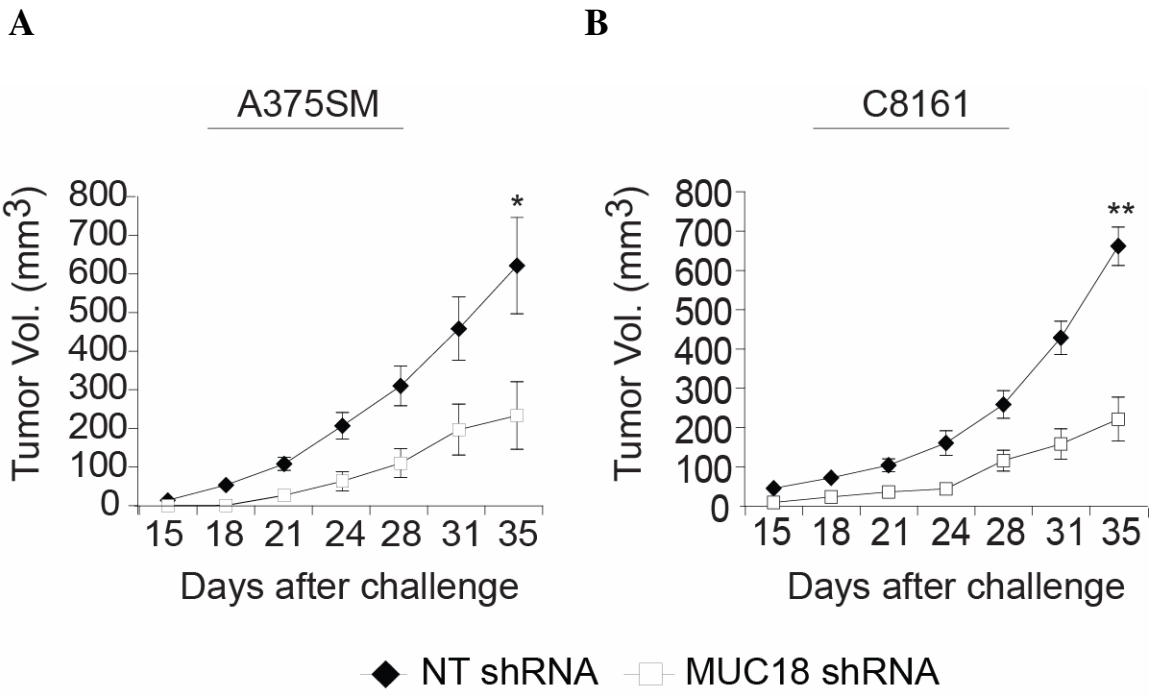


Figure 6. The Effect of MUC18 Silencing on Subcutaneous Tumor Growth

Silencing of MUC18 in two melanoma cell lines, A375SM and C8161, significantly decreased subcutaneous tumor growth in nude mice. Tumors were measured twice weekly for 35 days. **(A)** A375SM cells expressing either MUC18 shRNA or NT shRNA ($0.5x10^6$/ animal) were injected subcutaneously into the flank of nude mice (n=8/group). Mean tumor volumes at day 35 were 621.4 mm^3 in mice injected with NT A375SM cells, whereas it was 233.6 mm^3 in mice injected with MUC18-silenced A375SM cells (*p<0.05). **(B)** $0.25x10^6$ C8161 cells expressing MUC18 shRNA or NT shRNA were injected subcutaneously into the flank of nude mice (n=8/group). At day 35 mean tumor volumes were 662.3 mm^3 in tumors expressing NT shRNA and 221.9 mm^3 in tumors expressing MUC18 shRNA (**p<0.001).

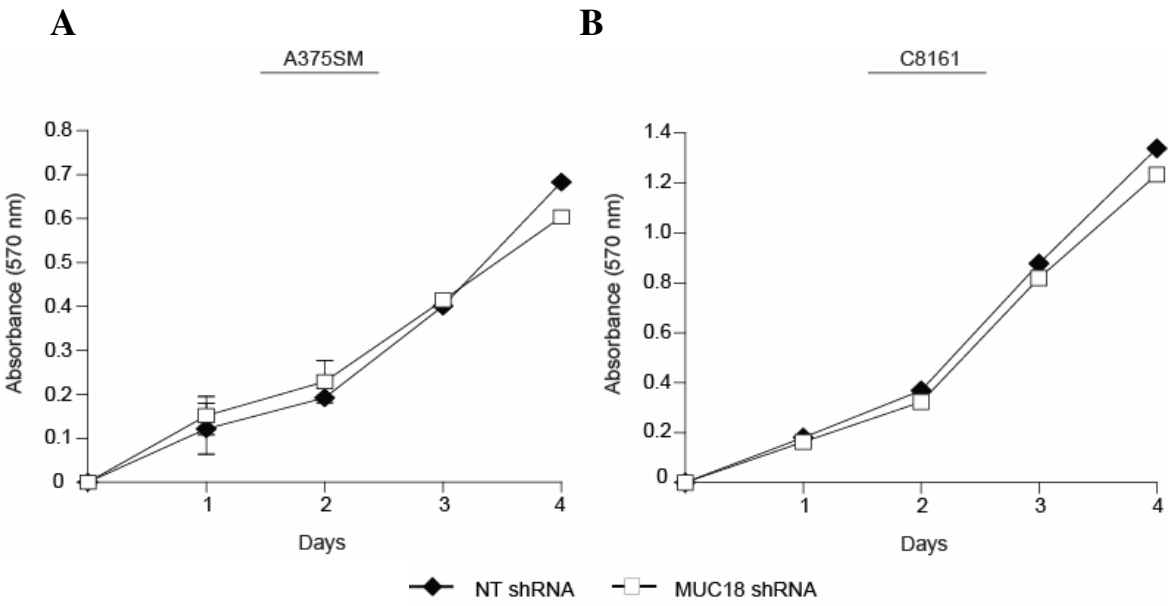


Figure 7. The Effect of MUC18 Silencing on Melanoma Cell Growth *In vitro*

MTT assay demonstrates that silencing of MUC18 in two melanoma cell lines, **(A)** A375SM and **(B)** C8161, does not affect cell doubling time *in vitro*. Cells were grown for 4 days in a 96 well plate, and cell growth was determined daily.

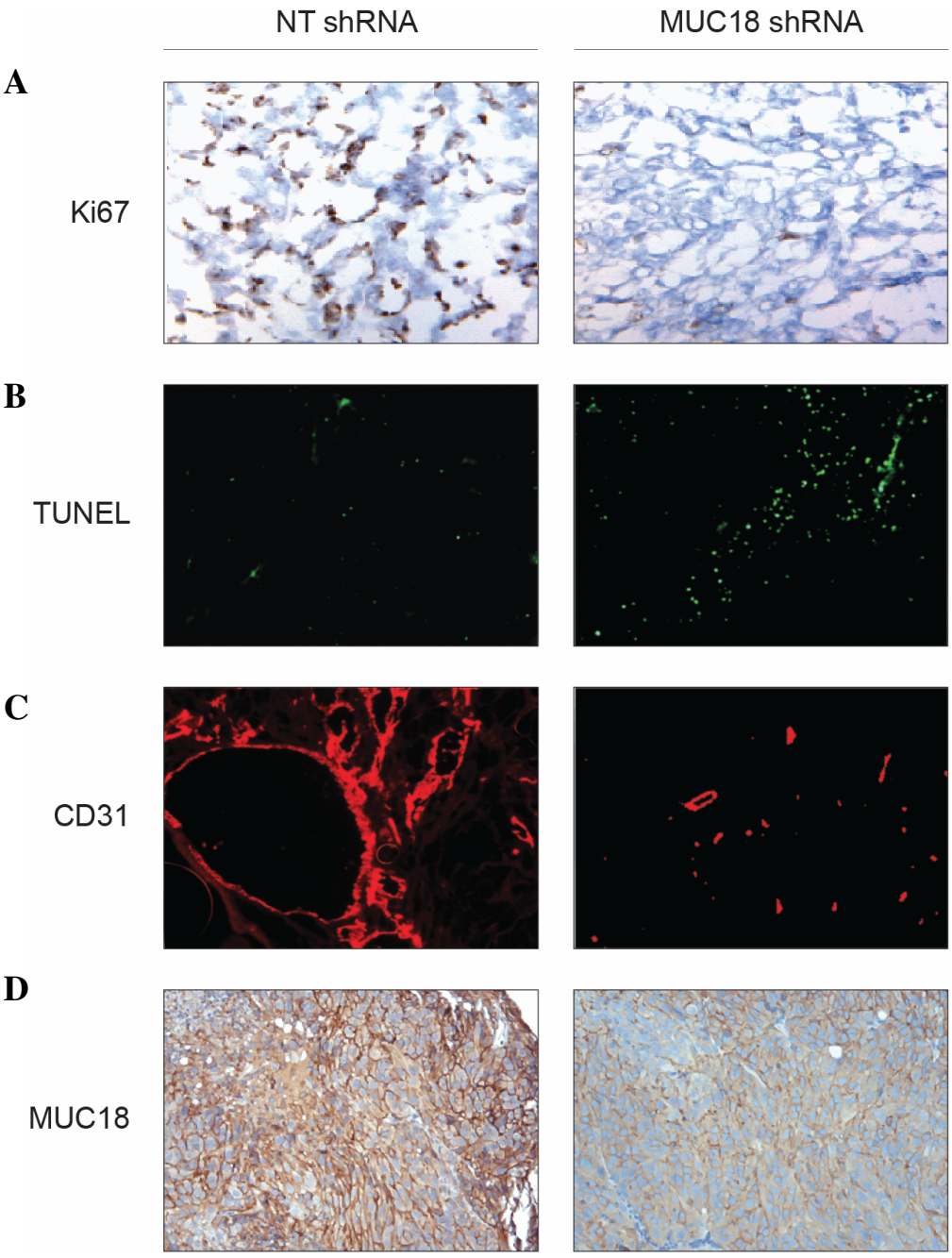


Figure 8

Figure 8. Immunohistochemical Analysis of the Effects of MUC18 Silencing on Tumor Growth

Representative images of immunohistochemical staining of subcutaneous tumors 35 days post-injections with either MUC18-shRNA or NT-shRNA-transduced A375SM. **(A)** Evaluation of tumor cell proliferation by Ki67 staining demonstrates decreased cell proliferation in tumors obtained from MUC18-silenced cells. **(B)** TUNEL staining illustrates increased apoptosis in MUC18-silenced tumors, as compared to tumors obtained from NT-transduced cells. **(C)** CD31 staining demonstrates decreased vessel size and number in tumors from MUC18-silenced cells. **(D)** Staining for MUC18 expression shows that MUC18 expression was downregulated in tumors obtained from MUC18-silenced cells. All images are shown at 10X magnification and are representative sections from the respective experimental groups.

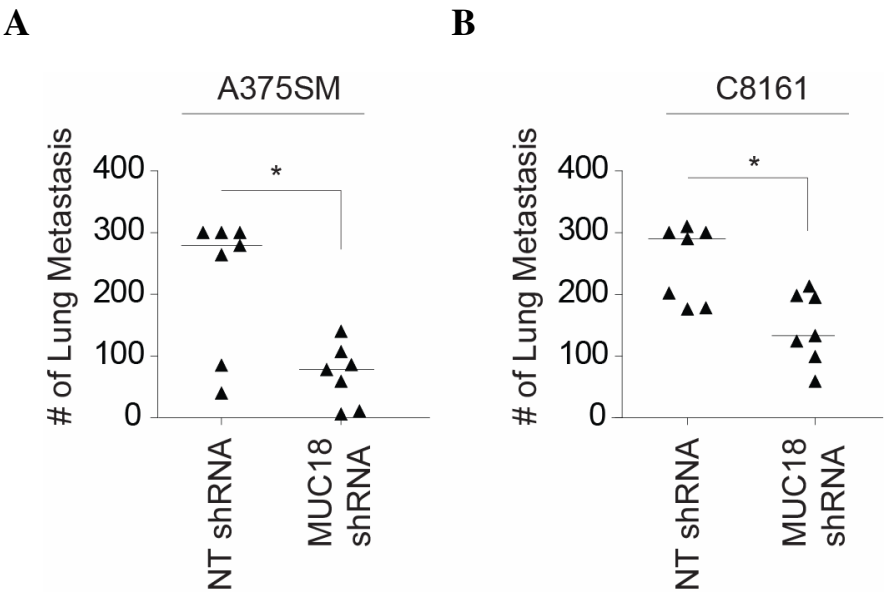


Figure 9. Effect of MUC18 Silencing on Experimental Lung Metastasis Formation

MUC18-silenced and NT-transduced A375SM **(A)** and C8161 **(B)** cells were injected into the tail vein of nude mice (n = 7 animals/cell line/group). After 6 weeks, mice were sacrificed, lungs were removed and number of lung colonies was counted in each lung. MUC18 silencing results in a significant decrease in experimental lung metastasis formation in **(A)** A375SM (median: NT-shRNA, 279; MUC18-shRNA, 78) and **(B)** C8161 (median: NT-shRNA, 290; MUC18-shRNA, 133) melanoma cell lines. *p<0.05 for both cell lines.

MUC18 Silencing Decreases MMP-2 Expression *In vivo* Along with MMP-2 Activity and Cell Invasion *In vitro*

We have previously demonstrated that MUC18 contributes to the metastatic phenotype of melanoma by increasing the melanoma cell invasion through regulation of MMP-2 expression and activity [136, 145]. MUC18 blockade using ABX-MA1 decreased MMP-2 expression *in vivo* [145]. To confirm that MUC18 silencing also results in decreased expression of MMP-2 *in vivo*, immunohistochemical analysis was performed. We found that the expression of MMP-2 was decreased in tumor samples obtained from MUC18-silenced cells (Figure 10). These data support our previous work demonstrating that MUC18 inhibition *in vivo* results in diminished levels of MMP-2.

To confirm that MUC18 silencing in A375SM and C8161 cell lines also results in decreased *in vitro* MMP-2 activity, we performed a zymography assay. Serum containing medium was used as a positive control, while serum free medium was used as a negative control for MMP-2 activity. As shown in Figure 11, decreased gelatin degradation of the gelatin impregnated gel, indicating less MMP-2 activity, was obtained in both MUC18-silenced A375SM and C8161 cells as compared to NT-transduced cells.

To assess whether decreased MMP-2 activity following MUC18 silencing contributes to decreased *in vitro* invasiveness of melanoma cells, a Matrigel invasion assay was used. $2.5x10^4$ MUC18-silenced A375SM and C8161 cells were placed in the upper chamber of a Matrigel coated Boyden chamber, incubated for 24 h, and the number of invaded cells were then evaluated. Our results demonstrate a greater than 2-fold significant decrease in the *in vitro* invasive capability of MUC18-silenced A375SM and C8161 cells, as compared to NT-transduced cells *(*$p<0.05$)* (Figure 12).

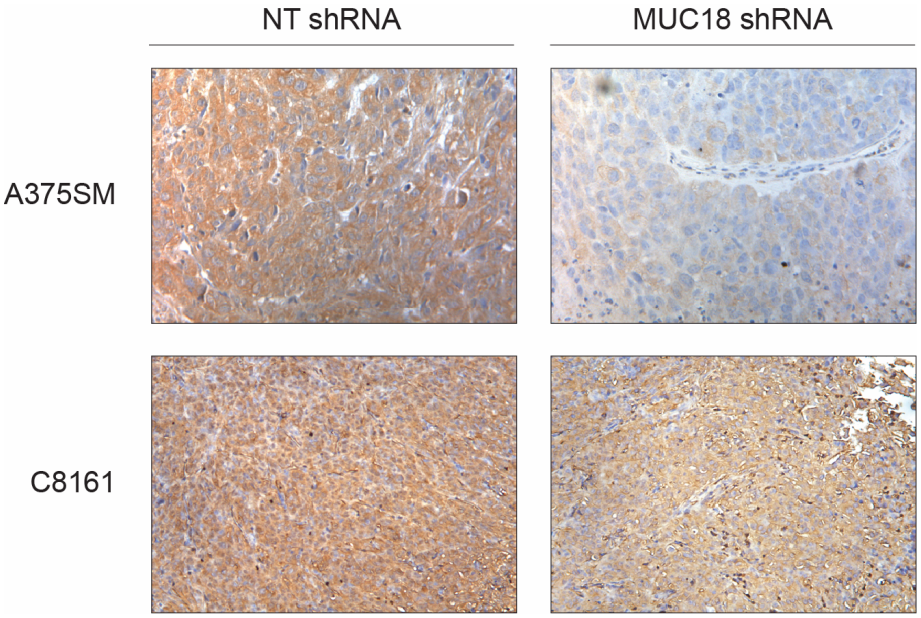


Figure 10. Decreased MMP-2 Expression *In vivo* Following MUC18 Silencing

Immunohistochemical staining of subcutaneous tumors demonstrates decreased MMP-2 expression in tumors obtained from MUC18-silenced cells, 35 days after cell injections.

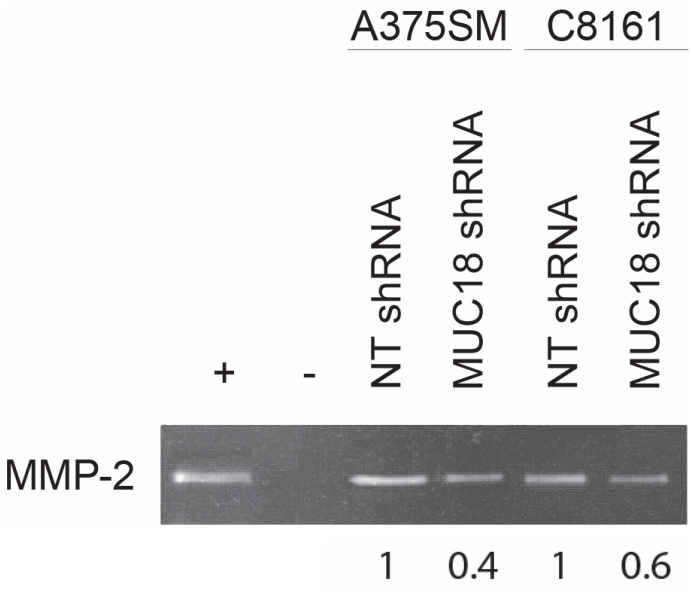


Figure 11. *In vitro* Effect of MUC18 Silencing on MMP-2 Activity

Zymography assay demonsrates the proteolytic activity of MMP-2, following MUC18 silencing. Samples were loaded on a gelatin impregnated SDS gel. Denscytometry demonstrates decrease of 60% and 40% in MMP-2 activity in both MUC18-silenced A37SM and C8161, as compared to NT-transduced cells.

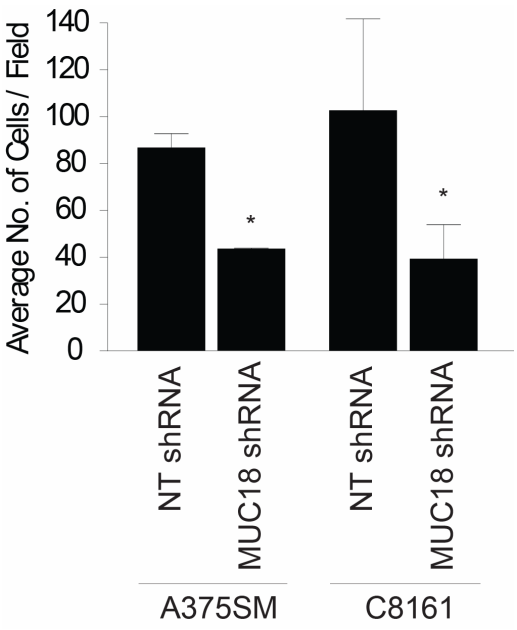


Figure 12. Effect of MUC18 Silencing on the Invasive Capacity of Metastatic Melanoma Cell Lines

A Matrigel invasion assay demonstrates a significant decrease in invasion following MUC18 silencing in both A375SM and C8161. Each group was plated in Matrigel invasion chambers, in triplicates, and in serum free medium. Data represents number of invaded cells and is expressed as mean ± SEM. *p<0.05.

Discussion

The correlation between MUC18 and melanoma progression was previously described by both Lehamann et al. and Holzmann et al. Their work demonstrated that MUC18 is highly expressed in advanced and metastatic melanoma and that this expression is associated with tumor thickness, while weaker expression is detected in benign nevi and thin tumor lessions [127-129]. Our laboratory further demonstrated that the expression of MUC18 directly correlates with the potential of melanoma cells to form metastases *in vivo* [135]. Although the expression of MUC18 correlated with melanoma progression, the role of MUC18 in the metastatic process was not established.

In an effort to elucidate the contribution of MUC18 to melanoma progression, MUC18 was overexpressed in a MUC18 negative, non-metastatic melanoma cell line, SB2. Overexpression of MUC18 in these cells resulted in increased tumor growth and experimental lung metastasis formation in nude mice. Furthermore, *in vitro,* ectopic expression of MUC18 resulted in increased homotypic and heterotypic adhesion between melanoma cells and endothelial cells, decreased attachment to laminin and increased MMP-2 activity, resulting in increased cell invasion [136]. This study revealed a critical role for MUC18 in melanoma progression, suggesting that by promoting melanoma cell intravasation, extravasation and intravascular survival, MUC18 is involved in metastasis formation.

Because MUC18 is overexpressed on metastatic melanoma lesions as compared to benign nevi, it is possible that MUC18 can serve as a target for melanoma therapy. Our lab previously developed a fully human antibody against MUC18 (ABX-MA1, in collaboration with Abgenix Fremont, CA, USA) and demonstrated that treatment of

nude mice with the antibodies after i.v. or s.c. inoculation of melanoma cells, significantly decreased tumor growth and metastasis formation in nude mice, compared to treatment with control IgG. These data further describe the important role of MUC18 in promoting the metastatic process in melanoma cell adhesion, invasion and angiogenesis. Moreover, these results suggest that ABX-MA1 can be used to target melanoma metastasis, as immunotherapy either alone or in combination with chemotherapy.

In this aim, we further established the central role of MUC18 in the progression of melanoma. We demonstrate that stable silencing of MUC18 in two metastatic melanoma cell lines significantly decreases both tumor growth and experimental lung metastasis formation in nude mice, corroborating our previous results. Immunohistochemical analysis revealed that the diminished tumor size in MUC18-silenced cells is a consequence of decreased cell proliferation and increased apoptosis, due, at least in part, to insufficient tumor vasculature. We believe that the discrepancy between the effect of MUC18 silencing on cell proliferation *in vitro* and *in vivo* is likely due to interactions between tumor cells and the microenvironment. *In vivo*, melanoma cell proliferation and angiogenesis can be mediated by secretion of various cytokines and growth factors from fibroblasts, immune cells, and other cells in the tumor microenvironment.

Although it has been demonstrated that MUC18 contributes to melanoma progression by increasing homotypic interactions between melanoma cells and heterotypic interactions between melanoma cells and the extracellular matrix and vascular endothelial cells, it was proposed that MUC18 can also promote melanoma

progression by mediating downstream signaling events. Indeed, corroborating previous data, we validated that MUC18 silencing results in downregulation of MMP-2 expression *in vivo*, and MMP-2 activity *in vitro*. During tumor progression, increased expression of MMP-2 often stimulates cell signaling pathways which promote angiogenesis and contribute to tumor invasion and metastasis [166, 167]. Thus, we speculate that the reduction in tumor size and angiogenesis following MUC18 silencing is partially due to decreased MMP-2 expression and activity.

These data suggest that MUC18 may promote melanoma progression by functioning both in regulation of downstream signaling events and interacting with cells in the microenvironment. The data obtained from this set of experiments enabled us to continue and study the role of MUC18 as a signaling molecule in melanoma progression.

CHAPTER 4: Specific Aim 2

Identify Potential Downstream Target Genes Regulated by MUC18 and Determine the Mechanisms of their Regulation

Introduction

The role of MUC18 in melanoma intracellular signaling has not been fully elucidated. Very few studies have examined the involvement of MUC18 in cell signaling pathways; however, since the cytoplasmic domain of MUC18 contains several protein kinase recognition motifs, its potential involvement in intracellular signaling was proposed [45]. Specifically, outside-in signaling of MUC18 was studied in human endothelial cells and revealed that MUC18 functions in actin cytoskeleton reorganization, intercellular interaction, maintenance of cell shape, and control of cell migration [154]. Its role in cytoskeleton rearrangement was further described. Activation of MUC18 resulted in its association with the protein tyrosine kinase $p59^{Fyn}$ leading to phosphorylation of the cytoskeleton associated proteins $p125^{FAK}$ and Paxillin [154]. Additionally, MUC18 has been implicated in cell survival signaling pathways. A reciprocal regulation loop between MUC18 and AKT was described, but these signaling functions of MUC18 were not further characterized [156]. To further elucidate the mechanism by which MUC18 acts as a signaling molecule in melanoma, we conducted a cDNA microarray analysis utilizing a MUC18-silenced and control melanoma cell line to potentially identify novel target genes regulated by MUC18 (see Table-1). We identified a number of genes as downstream targets of MUC18, but focused our studies

on the protein, Id-1, because of its involvement in melanoma progression [162, 168-170].

Id-1 is part of the Id protein family, which is comprised of 4 members (Id-1-4). The Id proteins are known as "Inhibitors of differentiation" or "Inhibitors of DNA binding" and belong to the helix-loop-helix (HLH) family of transcription factors (but do not act as transcription factors). These proteins lack the basic DNA binding domain and thus function as dominant-negative regulators of basic HLH (bHLH) transcription factors by physically binding and hetero-dimerizing with the HLH domain to prevent their binding to the DNA [171-173]. Specifically, transcriptional inhibition by Id proteins is mediated either by their binding to bHLH proteins that are ubiquitously expressed or by their binding to tissue restricted bHLH transcription factors such as the myogenic marker, MyoD [174, 175].

Id proteins, including Id-1, have been shown to be involved in regulation of several developmental and cellular processes [176]. As "inhibitors of differentiation", Id proteins have been shown to be down-regulated during cell differentiation. For example, Id-1 overexpression in embryonic and postnatal cerebral cortex inhibited both early and late stages of neuronal differentiation [177]. The critical role of Id-1 in regulation of embryogenesis was also shown, as knock out mice for Id-1 and Id-3 (Id-$1^{-/-}$ Id-$3^{-/-}$) were embryonically lethal. In fact, early embryos display a variety of phenotypic anomalies, including abnormal neurogenesis, vascular malformations and lack of blood vessel sprouting and branching into neuroectoderm [178]. In addition to cell differentiation, Id-1 plays an important role in regulation of cell growth. For instance, Id-1 was shown to down-regulate the expression of the CDK inhibitors $p21^{Cip1/Waf1}$ and

$p16^{INK4a}$, promoting cell cycle progression [168, 179, 180]. Moreover, premature senescence due to upregulation of $p16^{INK4a}$ expression was demonstrated in Id-1 deficient mouse embryo fibroblasts [168-170].

Because Id-1 plays an important role in regulating cellular processes such as differentiation and cell cycle progression, it is not surprising that aberrant expression of Id-1 results in cellular transformation. It was suggested that Id proteins, particularly Id-1, function as oncogenes in several malignancies [162]. Upregulation of Id-1 expression was demonstrated in various primary human cancers such as breast [181], prostate cancers [182], endometrial [183], ovarian [184] gastric [185, 186], and melanoma [170].

In melanoma, increased Id-1 expression was shown to correlate with melanoma progression. A tissue microarray study of 119 melanoma cases demonstrated that Id-1 expression significantly associates with increased tumor thickness and decreased patient survival. In addition, it was shown that in patients harboring the BRAF mutation, Id-1 expression was significantly increased, suggesting that Id-1 is a downstream target of the RAS signaling pathway [187]. Moreover, analysis of tissue samples from various stages of melanoma demonstrated that increased Id-1 expression at the RGP is associated with decreased expression of the CDK inhibitor $p16^{INK4a}$ [170]. Increased Id-1 expression in melanoma was also demonstrated by Byungwoo et al. Analysis of Id-1 expression in human melanoma cell lines from various stages of melanoma progression revealed that Id-1 expression is increased at the transcriptional level during the RGP and the metastatic phase of melanoma. Interestingly, no gene amplification or epigenetic changes were detected in these cells [162]. Despite the evidence establishing elevated levels of Id-1 in melanoma, the mechanism of its regulation in melanoma has yet to be

elucidated. Herein, we propose that MUC18 promotes melanoma progression by modulating the expression of downstream genes.

Results

Id-1 is a Downstream Target of MUC18 in Melanoma

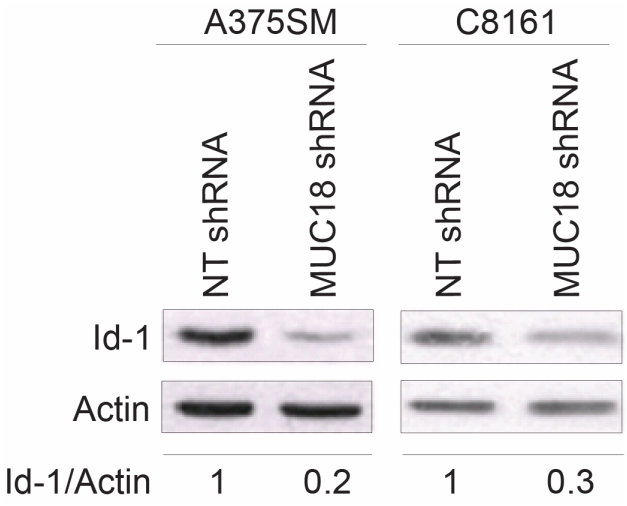
To determine the mechanism by which MUC18 contributes to melanoma tumor growth and metastasis, we performed a cDNA microarray analysis, comparing the gene expression pattern of NT-transduced cells and MUC18-silenced cells. Our analysis revealed a large number of genes differentially expressed following MUC18 silencing (partial list in Table-1). We decided to focus our studies on the differential expression of transcriptional regulators and found that Id-1 was down-regulated by 5.6-fold after MUC18 silencing. Western blot validated the cDNA microarray results, showing that Id-1 was downregulated by 80% in MUC18-silenced A375SM cells and by 70% in MUC18-silenced C8161 cells, compared to NT-transduced cells (Figure 13A). Moreover, immunohistochemical analysis of s.c tumor samples after 35 days confirmed that Id-1 expression was diminished in tumors formed from MUC18-silenced cells (Figure 13B). Taken together, these data demonstrate that in the absence of MUC18, Id-1 expression is attenuated, establishing the link between MUC18 and Id-1 in our experimental system.

DECREASED EXPRESSION		INCREASED EXPRESSION	
FOLD	GENE NAME	FOLD	GENE NAME
3.0	CYCLIN A	2.1	CANNABINOID RECEPTOR 2 (MACROPHAGE) (CNR2)
3.0	HIGHLY EXPRESSED IN CANCER (HEC)	2.6	GROWTH ARREST DNA-DAMAGE-INDUCIBLE 34 (GADD34)
3.0	T-CELL ORIGINATED PROTEIN KINASE (TOPK)	2.8	TUMOR REJECTION ANTIGEN (GP96)
3.0	FORKHEAD BOX M1 (FOXM1)	4	DELETED IN ORAL CANCER-RELATED 1 (DOC-1R)
3.2	HYALURONAN-MEDIATED MOTILITY RECEPTOR (HMMR)	4.7	TISSUE FACTOR PATHWAY INHIBITOR 2
3.2	SERINETHREONINE KINASE 18	5.3	IL-24 (SUPPRESSION OF TUMORIGENICITY 16)
3.5	TRANSFERRIN RECEPTOR (P90, CD71) (TFRC),	5.3	SEL-1 (SUPPRESSOR OF LIN-12, C.ELEGANS)-LIKE
4	TRANSFORMING GROWTH FACTOR, ALPHA (TGFα)	6.5	ARGININOSUCCINATE SYNTHETASE (ASS)
5.6	INHIBITOR OF DNA BINDING 1 (ID-1)	6.9	ACTIVATING TRANSCRIPTION FACTOR 3 (ATF-3)
7.0	CYCLIN F	13	DNAJ (HSP40) HOMOLOG, SUBFAMILY B, MEMBER 9

Table-1. Partial list of differentially expressed genes following cDNA microarray analysis, comparing MUC18-silenced and NT-transduced A375SM cells

The expression of two transcriptional regulators, Inhibitor of DNA binding 1 (Id-1) and Activating transcription Factor 3 (ATF-3), was modulated following MUC18 silencing in A375SM cells; The expression of Id-1 was decreased by 5.6 fold while the expression of its transcriptional inhibitor, ATF-3, was increased by 6.9 fold.

A


A375SM
C8161
NT shRNA
MUC18 shRNA
NT shRNA
MUC18 shRNA
Id-1
Actin
Id-1/Actin
1
0.2
1
0.3

B

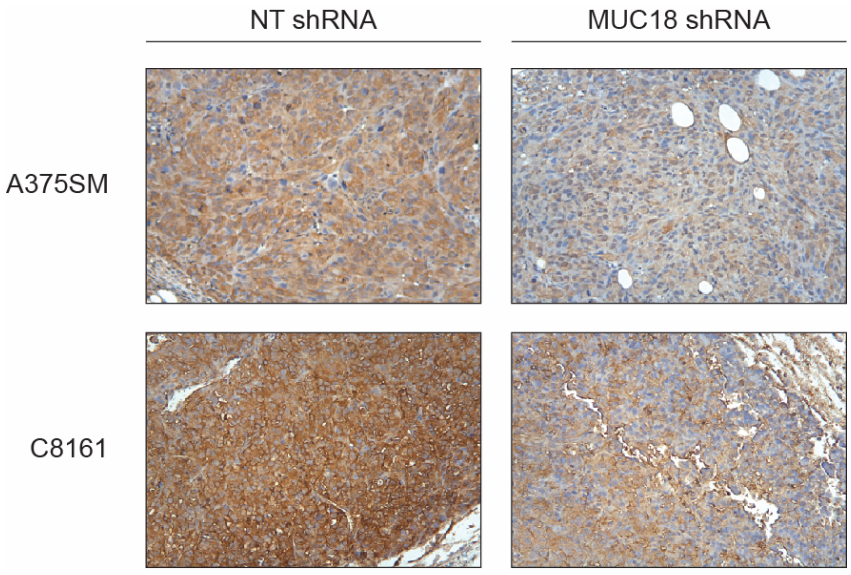

NT shRNA
MUC18 shRNA
A375SM
C8161

Figure 13

Figure 13. Expression of Id-1 Following MUC18 Silencing in the Metastatic Melanoma Cell lines, A375SM and C8161

(A) Id-1 was identified in a cDNA microarray as a downstream target of MUC18. Western blot analysis of Id-1, expression after MUC18 silencing in A375SM and C8161, shows decreased Id-1 expression by 80% and 70% respectively, as compared to NT-transduced cells. **(B)** Immunohistochemical staining for Id-1 illustrates downregulation of Id-1 expression in MUC18-silenced tumors 35 days after injection with MUC18-silenced or NT-shRNA-transduced A375SM or C8161 cells. Images are shown at 10X magnification.

MUC18 Regulates Id-1 Expression at the Transcriptional Level

To further analyze the mechanism of Id-1 regulation by MUC18, we cloned the Id-1 promoter (a promoter region of 1.4 kb from -1370 kb to +40 kb from transcription initiation site) in front of a luciferase reporter gene. We then evaluated Id-1 promoter activity in both A375SM and C8161 MUC18-silenced cells and found that Id-1 promoter activity decreased by more than 2.5-fold in MUC18-silenced A375SM cells (*p<0.01) and by more than 2-fold in MUC18-silenced C8161 (**p<0.001), as compared to NT-transduced cells (Figure 14). These experiments revealed, for the first time, that MUC18 regulates Id-1 expression at the transcriptional level.

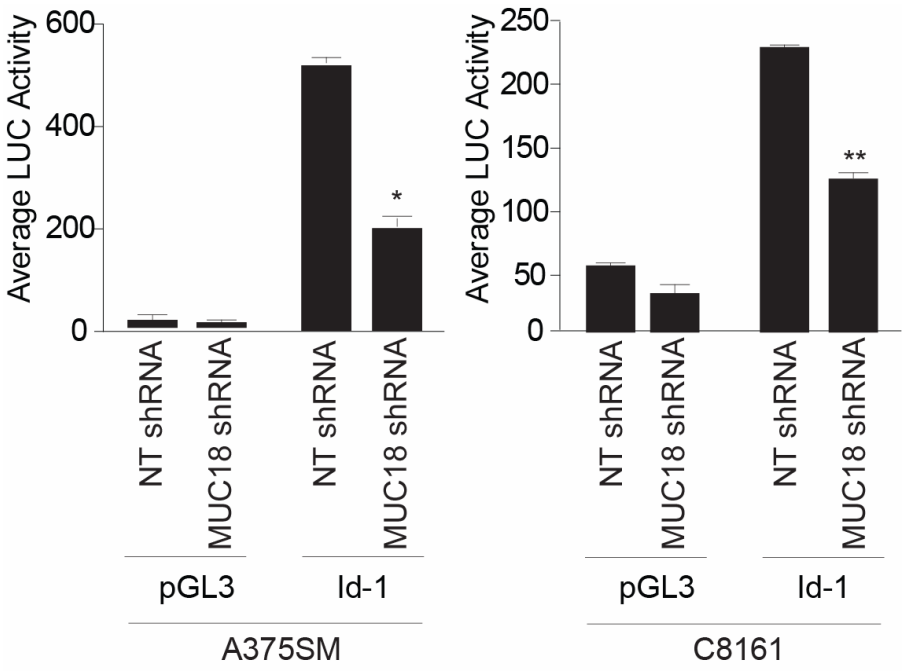


Figure 14. Transcriptional Regulation of Id-1 Expression by MUC18

The promoter of the Id-1 gene (1.4 kb, from -1370 to +40 from transcription start site) was cloned in front of a lucifearase expression vector (pGL3-basic vector) and was transiently transfected into both A375SM and C8161 expressing either MUC18 shRNA or NT shRNA. Relative Id-1 promoter-driven luciferase activity was compared to the luciferase activity of cells transfected with a promoterless luciferase pGL3-basic vector. Dual-Luciferase reporter assay demonstrates that MUC18 silencing significantly decreases Id-1 promoter-driven luciferase activity in both A375SM and C8161cells. *p<0.01, **p<0.001.

ATF-3 Represses Id-1 Promoter Activity in MUC18-Silenced Cells

Having established that MUC18 transcriptionally regulates Id-1 expression, we then aimed to determine the mechanism of regulation of Id-1 by MUC18. The Id-1 promoter was analyzed for potential transcription factor binding sites. We found putative binding sites of several transcription factors including Early Growth Factor-1 (EGR-1), Sp1, Nuclear Factor-1 (NF-1), CCAAT enhancer-binding protein (C/EBP) and Activating Transcription Factor/CREB (ATF/CREB), all of which were described previously [173, 188-190]. Interestingly, ATF-3, which is part of the ATF/CREB transcription factor family, was upregulated by 6.9-fold after MUC18 silencing in our cDNA microarray analysis (Table-1). The role of ATF-3 in regulation of Id-1 expression was also previously described in epithelial cells. ATF-3 has been shown to act as a repressor of Id-1 expression through increased binding to the Id-1 promoter [188, 191]. To confirm that MUC18 negatively regulates ATF-3 expression, Western blot analysis was performed. These data corroborated the cDNA microarray results and demonstrated that, as compared to NT-transduced cells, ATF-3 protein expression was up regulated by more than 9-fold in both MUC18-silenced A375SM and C8161 respectively (Figure 15), linking MUC18 and ATF-3 .

To determine whether MUC18 transcriptionally regulates Id-1 expression via differential binding of ATF-3 to the Id-1 promoter, Chromatin immunoprecipitation (ChIP) was performed. These experiments revealed increased binding of ATF-3 to the Id-1 promoter in both MUC18-silenced A375SM and C8161 cells (Figure 16). Taken together, these data suggest that in the absence of MUC18, the expression and binding of ATF-3, is enhanced, leading to decreased expression of Id-1.

To further delineate whether ATF-3 acts as a repressor of Id-1 expression in melanoma, we exogenously expressed ATF-3 along with the Id-1 promoter in MUC18-silenced and NT-transduced A375SM cells and measured the Id-1 promoter-driven luciferase activity (Figure 17A). Overexpression of ATF-3 resulted in a significant decrease of Id-1 promoter activity in both MUC18-silenced and NT-transduced A375SM cells (*p<0.05) (Figure 17B). To confirm that ATF-3 acts as a transcriptional inhibitor of Id-1, we mutated the Id-1 promoter using site-directed mutagenesis at the ATF-3 binding site located at -1016 Kb replacing TGACGTCA to TGTGCAGCA, as described previously (Figure 17A) [188]. Mutation of the Id-1 promoter at the ATF-3 binding site significantly increased Id-1 promoter activity (*p<0.05), further validating that ATF-3 acts as a transcriptional inhibitor of Id-1 (Figure 17B).

To determine that ATF-3-mediated transcriptional inhibition of Id-1 results in a functional reduction in Id-1 protein expression, we cloned the ATF-3 cDNA into a lentiviral expression vector, and transduced parental A375SM and C8161 cells, which do not endogenously express ATF-3. Overexpression of ATF-3 in both melanoma cell lines resulted in decreased expression of endogenous Id-1 protein by 90% and 70%, respectively (Figure 18). Collectively, MUC18 silencing resulted in upregulation of ATF-3 expression and increased binding of ATF-3 to the Id-1 promoter, resulting in diminished expression of Id-1 protein. Accordingly, we conclude that ATF-3 acts to repress Id-1 transcription and protein expression in melanoma cells in the absence of MUC18.

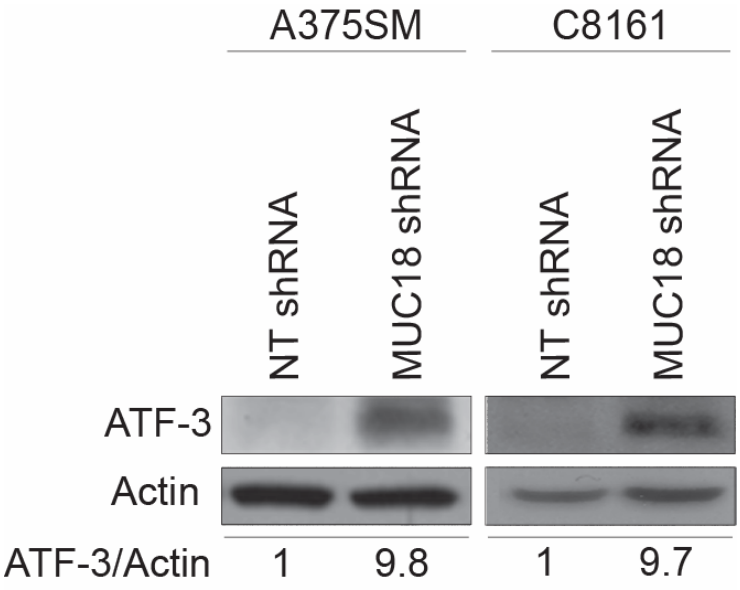


Figure 15. Expression of ATF-3 Following MUC18 Silencing in the Metastatic Melanoma Cell Lines, A375SM and C8161

ATF-3 was identified in a cDNA microarray as a downstream target of MUC18. Western blot analysis of ATF-3 expression in MUC18-silenced A375SM and C8161 cells confirms increased expression of ATF-3 by 9.8-fold and 9.7-fold in A375SM and C8161, respectively, as compared to NT-transduced cells.

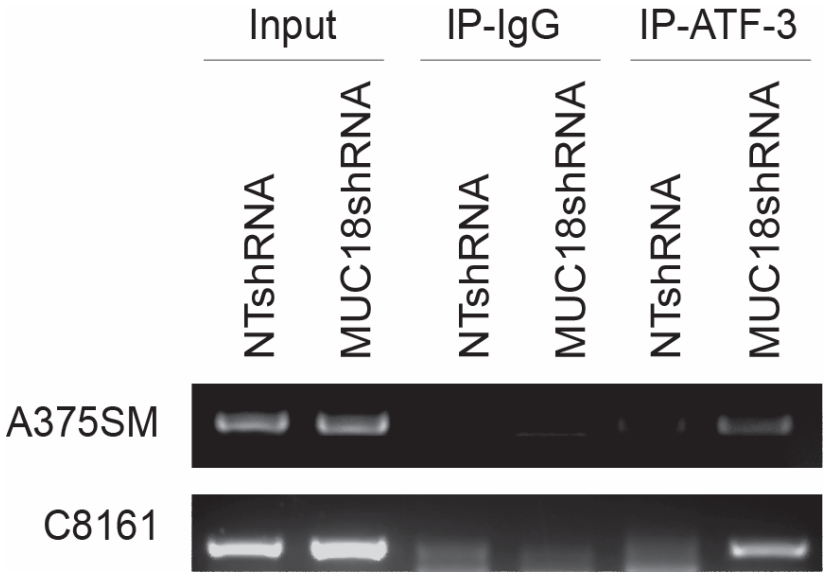


Figure 16. Chromatin Inmmunoprecipitation Assay to Identify Differential Binding of ATF3 to the Id-1 Promoter in MUC18-Silenced A375SM and C8161 Cells

The region of the ATF-3 binding site on the Id-1 promoter was amplified by PCR following chromatin immunoprecipitation of ATF-3 bound DNA. Increased binding of ATF-3 to the Id-1 promoter is demonstrated in both MUC18-silenced cell lines as compared to NT-trasduced cell lines (A375SM and C8161). IgG antibodies were used as negative controls and input DNA was used to ensure an equal amount of chromatin used in each assay.

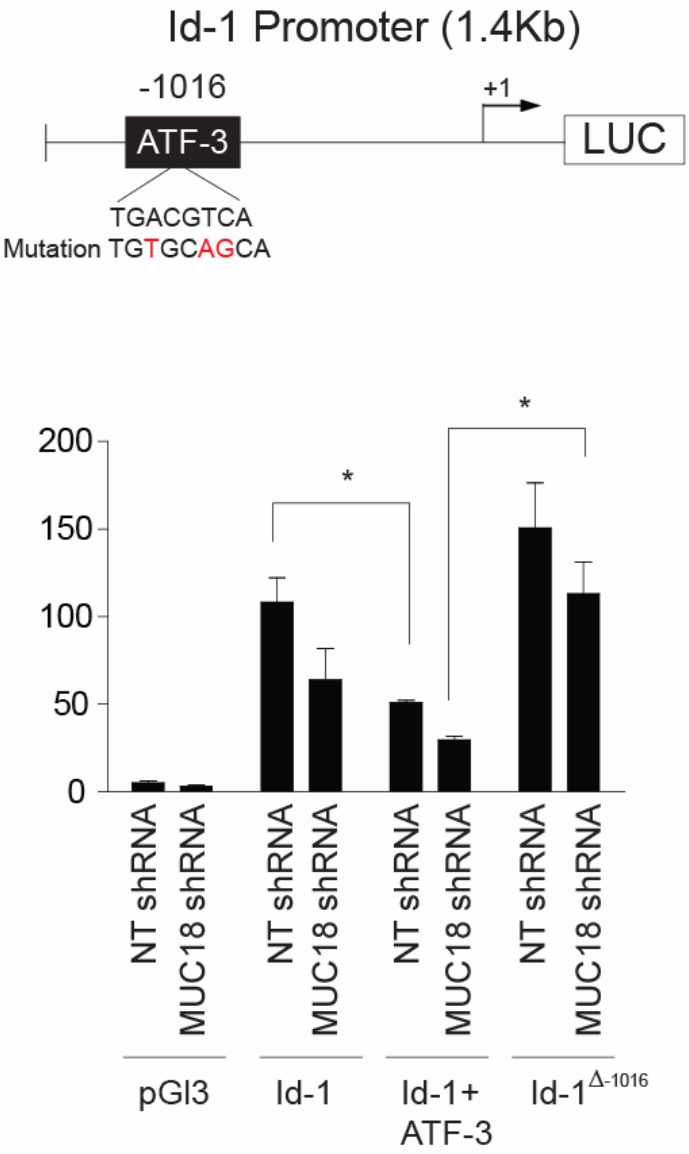


Figure 17. MUC18 Regulates Id-1 Promoter Activity Through ATF-3

(A) Schematic illustration of the Id-1 promoter shows an ATF-3 binding site located -1016 bp from the transcription start site. Mutation of the ATF-3 binding site is represented in red. **(B)** A375SM cells stably expressing either MUC18 or NT shRNA were transiently transfected with the Id-1 promoter-driven luciferase construct and with

either the ATF-3 expression plasmid or an empty vector control (pcDNA3.1). Transduced cells were also transfected with a mutated Id-1 promoter-driven luciferase construct Luciferase-driven Id-1 promoter activity in NT-transduced cells significantly decreased after transient transfection of cells with an ATF-3 expression vector. Point mutation at the ATF-3 binding site on the Id-1 promoter (-1016) significantly increased luciferase-driven Id-1 promoter activity in MUC18-silenced cells, as compared to promoter activity in MUC18-silenced cells following transient transfection with an ATF-3 expression vector. *$p<0.05$.

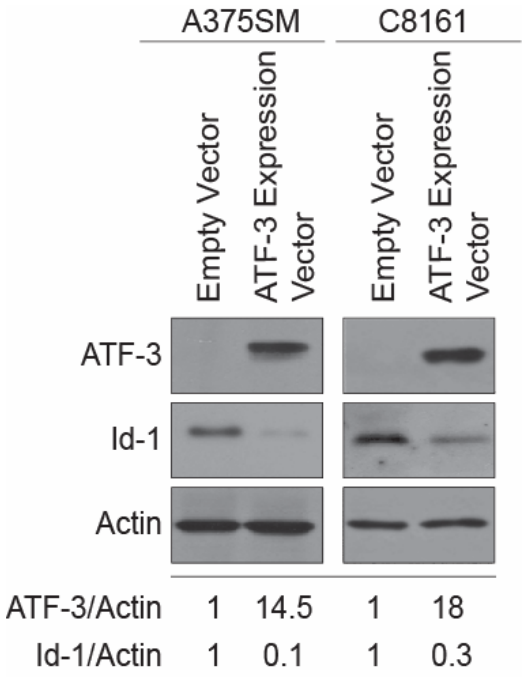


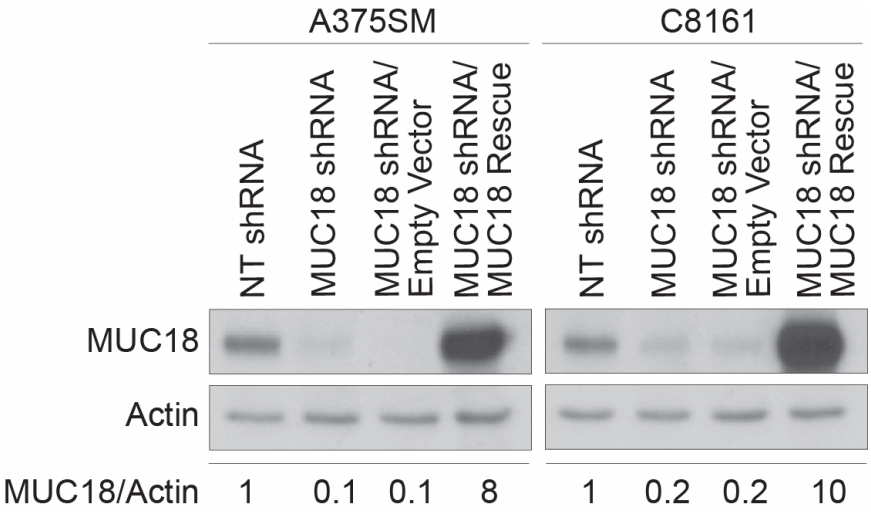
Figure 18. ATF-3 Overexpression Inhibits Endogenous Expression of Id-1 in the Metastatic Melanoma Cell Lines, A375SM and C8161

Western blot analysis depicting decreased expression of endogenous Id-1 protein by 90% and 70% in A75SM and C8161, respectively, after stable overexpression of ATF-3 in the low ATF-3-expressing metastatic melanoma cell lines, A375SM and C8161, as compared to control cells expressing an empty vector.

Rescue of MUC18 in MUC18-Silenced Cells Reverts the Expression of Id-1 and ATF-3

To ascertain that the differential expression of Id-1 and ATF-3 were not off target effects of MUC18, rescue of MUC18 expression in MUC18-silenced cells was performed. A non-targetable MUC18 expression vector was generated by creating five silent point mutations in the MUC18 cDNA at the sequence which is targeted for silencing of MUC18 by the MUC18 shRNA. MUC18-silenced A375SM and C8161 cells were stably transduced with lentiviruses containing the puromycin resistant, non-targetable-MUC18 expression vector or the puromycin resistant control vector. Western blot analysis demonstrated that MUC18 expression was successfully rescued in both MUC18-silenced A375SM and C8161 cells as compared to control vector-transduced cells (Figure 19A). Since MUC18 silencing resulted in decreased Id-1 expression and increased ATF-3 expression, we hypothesized that these expression patterns would be reverted following re-expression of MUC18 in MUC18-silenced cells. Indeed, rescue of MUC18 resulted in increased Id-1 expression and decreased ATF-3 expression (Figure 19B), thereby demonstrating that both Id-1 and ATF-3 were specifically regulated by MUC18.

A


A375SM
C8161
NT shRNA
MUC18 shRNA
MUC18 shRNA/ Empty Vector
MUC18 shRNA/ MUC18 Rescue
NT shRNA
MUC18 shRNA
MUC18 shRNA/ Empty Vector
MUC18 shRNA/ MUC18 Rescue
MUC18
Actin
MUC18/Actin 1 0.1 0.1 8 1 0.2 0.2 10

B

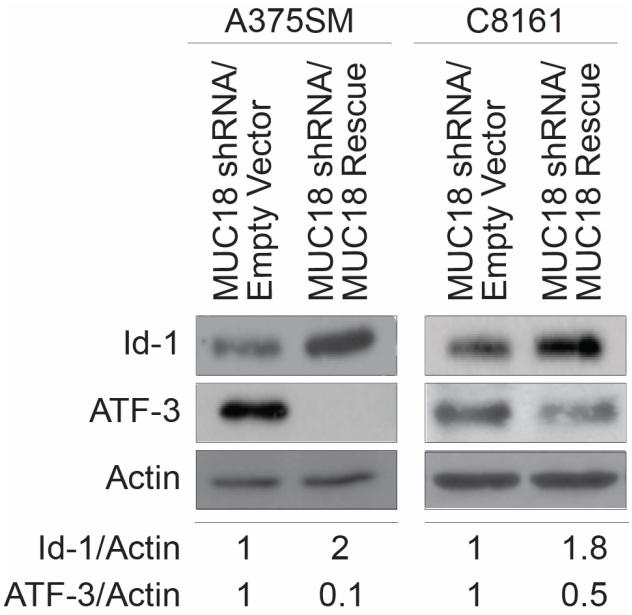

A375SM
C8161
MUC18 shRNA/ Empty Vector
MUC18 shRNA/ MUC18 Rescue
MUC18 shRNA/ Empty Vector
MUC18 shRNA/ MUC18 Rescue
Id-1
ATF-3
Actin
Id-1/Actin 1 2 1 1.8
ATF-3/Actin 1 0.1 1 0.5

Figure 19

Figure 19. Rescue of MUC18 Expression in MUC18-Silenced Cells Reverts the expression of Id-1 and ATF-3

(A) MUC18-silenced A375SM and C8161 cells were stably transduced with either a non-targetable MUC18 expression vector or an empty vector control. Western blot analysis demonstrates that MUC18 expression is restored in MUC18-silenced A375SM and C8161 cells transduced with the rescue vector, compared to MUC18 silenced cells transduced with an empty vector. **(B)** Rescue of MUC18 expression in MUC18-silenced cells results in increased Id-1 expression by 2-fold in A375SM and by 1.8-fold in C18161 cells. The expression of ATF-3 decreased by 10-fold in A375SM and by 2-fold in C8161, compared to cells transduced with an empty vector control.

Discussion

It has been well established that increased expression of the adhesion molecule, MUC18, correlates with disease progression and metastasis in melanoma [127, 135, 136, 145]. Outside-in signaling mediated by other types of cell adhesion molecules such as integrins, cadherins as well as adhesion molecules from the immunoglobulin-like family, have been shown to contribute to metastatic progression by promoting cell survival, migration, invasion and angiogenesis [105, 148]. For instance, clustering of I-CAM in endothelial cells induces an intracellular signaling cascade which leads to activation of the small GTPase, RhoA, and increased free, cytosolic Ca^{2+}. RhoA-mediated release of cytosolic Ca^{2+} leads to cytoskeleton reorganization and can influence cell migration [192-194]. Moreover, it has been demonstrated that N-CAM regulates the adhesion between tumor cells and the extra cellular matrix by promoting the activation of the fibroblast growth factor receptor (FGFR) resulting in β1 integrin-mediated cell adhesion, and ultimately preventing tumor cell dissemination [195].

Thus, it is possible that upregulation of MUC18 in melanoma can contribute to acquisition of the metastatic melanoma phenotype not only by modulating cell adhesion but also by mediating cell signaling [45]. Several potential protein kinase recognition domains, including protein kinase A (PKA), protein kinase C (PKC) and Casein Kinase 2(CK2), reside in the cytoplasmic domain of MUC18, suggesting its putative role in initiating downstream cell signaling events [45, 196]. Much effort has been made to identify downstream signaling events initiated by MUC18, although the MUC18 ligand has yet to be identified. Anfosso et al. were the first to demonstrate that antibody cross linking of MUC18 in endothelial cells resulted in focal adhesion assembly, cytoskeleton

reorganization and cell migration. Activation of MUC18 induced recruitment of the protein tyrosine kinase, $p59^{Fyn}$, to its cytoplasmic domain and resulted in tyrosine phosphorylation of both $p125^{FAK}$ and Paxillin [154]. A subsequent study further established that upon antibody activation of MUC18, Ca^{2+} influx was increased, in a $p59^{Fyn}$ dependent manner, resulting in phosphorylation of phospholipase C-γ (PLC-γ), proline-rich tyrosine kinase 2 (Pyk2) and $p130^{cas}$ and the formation of a complex between PyK2 and $p130^{cas}$. This study demonstrated the role of MUC18 in regulating cytoskeleton reorganization through outside-in signaling mediated by non-receptor tyrosine kinases [155]. Another study has revealed that MUC18 is involved in cell survival signaling and identified a reciprocal regulation between MUC18 and AKT. That is, overexpression of MUC18 resulted in activation of AKT. Likewise, constitutive activation of AKT resulted in elevated levels of MUC18 protein expression. This regulatory loop ultimately resulted in increased cell survival in stress conditions via inhibition of the pro-apoptotic protein BAD [156].

Despite the fact that the cytoplasmic tail of MUC18 contains several protein kinase recognition motifs as well as a tyrosine residue (Tyr^{641}), no phosphorylation has been described [45, 127]. This might suggest that in melanoma MUC18 might act either in a similar fashion to N-CAM, and interact in cis with adjacent molecules, such as integrins, to induce downstream signaling or by regulating secondary messengers, such as Ca^{2+}.

To further describe the role of MUC18 as a signaling molecule and mediator of outside in signaling regulating gene expression, we conducted a cDNA microarry analysis comparing gene expression patterns between MUC18-silenced cells and NT-

transduced cells. Since MUC18 has been shown to contribute to melanoma cell invasion and MMP-2 expression we decided to focus our studies on the differential expression of two transcriptional regulators found in our microarray analysis, Id-1 and ATF-3, which were previously shown to regulate cell invasion and MMP-2 expression. Id-1 is a regulator of differentiation and DNA binding, that functions by regulating gene transcription as a result of its binding and inhibition of bHLH proteins [171]. In recent years, the role of Id-1 in tumorigenesis has been extensively studied in several different malignances and has been shown to correlate with poor prognosis and decreased survival in a variety of different cancers including breast, ovarian, endometrial and melanoma [183, 187, 197, 198]. Furthermore, Id-1 has been shown to play a role in promoting angiogenesis, invasion and inhibition of cellular senescence [178, 199-202]. While increased Id-1 expression correlates with melanoma progression and Id-1 was shown to be transcriptionally regulated during the melanoma progression, the precise mechanism of its transcriptional regulation in melanoma has yet to be elucidated. Hence, we decided to study whether Id-1 expression in melanoma is regulated by MUC18. Our cDNA microarry analysis revealed a 5.6-fold decrease in Id-1 expression MUC18 following MUC18 silencing. We confirmed that Id-1 expression is indeed decreased in MUC18-silenced cells, as well as in tumor samples obtained from mice injected with MUC18-silenced cells, thereby establishing a clear link between MUC18 and Id-1 in melanoma. Since cDNA analysis is based on the differential expression of mRNA in the cell, we decided to study whether MUC18 regulates Id-1 at the trancriptional level. Utilizing a lucifearase-driven Id-1 promoter, we demonstrated regulation of Id-1 by MUC18 at the transcriptional level. Several transcription factors binding sites including

Sp1, EGR1, NF-1 and ATF/CREB are located on the Id-1 promoter and have been previously shown to be involved in the transcriptional regulation of Id-1 [173, 188, 190]. Loss of the repressor complex formed between NF-1, the retinoblastoma protein [203], and histone deacetylase 1 (HDAC1) has been shown to increase Id-1 transcription in breast cancer cells [173]. Additionally, previous studies have linked ATF-3 to transcriptional inhibition of Id-1 [188, 191]. Because we identified ATF-3 as a MUC18 downstream target in our cDNA microarray, we pursued its potential involvement in Id-1 regulation.

ATF-3 is a stress-inducible protein which is part of the ATF/CREB family of transcription factors induced by the p38 signaling pathway [204]. It has been shown to act in a promoter-dependent manner, either as a repressor or an activator of transcription [205]. Therefore, the role of ATF-3 in cancer development has been suggested to be dichotomous [206]. While the oncogenic role of ATF-3 has been implicated in cancers such as prostate [207] and breast [206], it has also been shown to function as a tumor suppressor in colorectal cancer [208] and ovarian cancer cells [209]. A recent study has described ATF-3 as having a tumor suppressive role in melanoma by negatively regulating IL-6 expression [210]. Furthermore, Kang et al. have demonstrated that, in epithelial cells, ATF-3 binds to the Id-1 promoter and represses its transcription due to a stress response of the cells [188]. Activation of ATF3 was further shown to inhibit Id-1 expression in a malignant mesothelioma orthotopic mouse model [211]. Our finding that MUC18 silencing increased the expression of ATF-3 further supports the tumor-suppressive role of ATF-3 in melanoma.

These results raised the possibility that MUC18 negatively regulates ATF-3 expression, and promotes expression of Id-1. In this regard, promoter and ChIP analyses illustrated that, indeed, in MUC18-silenced cells upregulation of ATF-3 results in increased binding of ATF-3 to the Id-1 promoter and subsequent repression of Id-1 transcription. Additionally, stable overexpression of ATF-3 in two metastatic melanoma cell lines, A375SM and C8161, decreased endogenous Id-1 expression, further characterizing the role of ATF-3 in repression of Id-1 expression. Although this work only describes ATF-3-mediated Id-1 transciptional regulation, it is likely that many transcription factors work in concert to regulate Id-1. It is well understood that the transcriptional regulation of Id-1 is not mediated by ATF-3 alone, but rather by well orchestrated differential binding of several transcriptional regulators and co-factors. These interactions are currently being explored.

Since ATF-3 is a stress inducible transcription factor which is upregulated by the p38 MAPK signaling pathway [205], it is possible that MUC18 inhibits the p38 MAPK signaling pathway and subsequently ATF-3, thus promoting Id-1 expression. Similarly, Harper et al. have shown that suspension or wounding of leading keratinocytes, decreases their adhesion to laminin 5. This resulted in increased activation of p38, which leads to increased phosphorylation of ATF-2, a trans-activator of ATF-3, and consequently increased ATF-3 expression [212]. Furthermore, Kang et al. have demonstrated that Id-1 expression decreases following upregulation of ATF-3 at the transcriptional level [188]. These data further suggest that upon MUC18 silencing, melanoma cell adhesion also decreases, resulting in phosphorylation of both p38 and ATF-2 leading to inhibition of Id-1 by ATF-3. An additional mechanism for the

regulation of Id-1 expression might also exist. CREB shares the same binding site of ATF-3 on the Id-1 promoter, indicating that they may bind in a competitive manner. It can be further speculated that the increase in MUC18 expression which promotes Ca^{2+} influx and CREB activation, can also increase CREB binding to the Id-1 promoter, preventing ATF-3 from binding, and ultimately promoting Id-1 expression. Further studies are needed to determine the balance of transcription factor binding to the Id-1 promoter.

Finally, we confirm the link between MUC18, Id-1 and ATF-3, by rescuing MUC18 expression in MUC18-silenced cells. The expression patterns of both Id-1 and ATF-3 reverted after rescue of MUC18 expression in both A375SM and C8161, confirming that the modulation of Id-1 and ATF-3 was not an off-target effect of MUC18 silencing.

In conclusion, we demonstrate that MUC18 regulates Id-1 expression through inhibition of ATF-3. Our current studies support the notion that MUC18 does not simply function to promote cell adhesion, but rather is involved in cell signaling events which affect the expression of various genes, including Id-1 and ATF-3. We thus propose a novel signaling function of MUC18 by which it acts to promote melanoma progression through regulation of gene expression.

CHAPTER 5: Specific Aim 3

Determine the Contribution of the Genes Regulated by MUC18 to the Malignant Phenotype of Melanoma

Introduction

The results from this dissertation, thus far, demonstrate that MUC18 plays a key role in regulating the expression of genes, such as Id-1 and ATF-3, which contribute to the progression and metastasis of melanoma. A critical step in the metastatic process is the ability of cells to invade through the basement membrane, intravasate and extravasate through the vasculature to form distant metastases. MMPs, such as MMP-2 and MMP-9, have been shown to play pivotal role in tumor invasion, migration, and angiogenesis, as they degrade the basement membrane and ECM [213, 214]. Increased expression of MMP-2 has been shown to be important for melanoma progression [96]. We have previously established the role of MUC18 in melanoma cell invasion and demonstrated that MUC18 regulates MMP-2 expression and activity. Overexpression of MUC18 in a non-metastatic melanoma cell line, SB-2, resulted in elevated levels of MMP-2 activity and increased invasiveness through Matrigel-coated filters [136]. Moreover, inhibition of MUC18 by ABX-MA1 in melanoma cell lines inhibited MMP-2 transcription, activity, and cell invasion [145]. However the mechanism of how MUC18 regulates MMP-2 expression and activity has yet to be described.

Interestingly, the MUC18 downstream target, Id-1, has also been implicated in promoting tumor cell invasion and MMP-2 expression. In breast and endometrial

carcinomas, among other cancers, Id-1 was shown to promote cell invasion [183, 215]. Interestingly, it was demonstrated that Id-1 promotes tumor cell invasion by increasing MMP-2 expression and activity. In fact, it was observed that B6RV2 lymphoma cells tumors from Id-$1^{+/-}$ Id-$3^{-/-}$ knockout mice displayed lower levels of MMP-2 expression [178]. In Id-$1^{-/-}$ knockout mouse embryo fibroblasts, MMP-2 activity was significantly diminshed [199]. On the other hand, Id-1 overexpression in a prostate cancer cell line resulted in enhanced cell invasion as well as MMP-2 activity [216]. Based on these data, we hypothesized that MUC18 promotes melanoma cell invasion through Id-1-mediated regulation of MMP-2 expression and activity.

Results

Increased Invasive Capacity of MUC18-Silenced Cells Following Id-1 Overexpression

Because Id-1 is a downstream target of MUC18, we rescued its expression in MUC18 silenced-cells to examine its role in melanoma progression. Id-1 cDNA was cloned into a lentiviral expression vector and lentivirus containing the Id-1 cDNA and an empty vector control were produced. MUC18-silenced A375SM and C8161 cells were transduced and then selected for puromycin resistance. Western blot analyses confirmed the Id-1 overexpression in MUC18-silenced A375SM and C8161 cells resulted in 2.5- and 3.3-fold increases, respectively, in Id-1 protein levels, compared to control cells (Figure 20).

Recent studies in several tumor models, including breast [163, 215] and bladder [217] cancers have demonstrated that Id-1 participates in tumor cell invasiveness. Having established that MUC18 expression promotes melanoma cell invasion and upregulation of MMP-2 expression and activity (Figures 10, 11) [135, 145], we assessed whether MUC18 contributes to the invasive phenotype of melanoma cells through regulation of Id-1 expression. A Matrigel invasion assay was used to evaluate the invasive capacity of MUC18-silenced cells overexpressing Id-1, compared to that of MUC18-silenced cells expressing an empty vector control. As seen in Figure 20A and B, cell invasiveness was promoted following Id-1 overexpression in MUC18-silenced A375SM cells by 5-fold (Figure 21A) and by nearly 2-fold in MUC18-silenced C8161 cells (Figure 21B) (**$p<0.001$, *$p<0.01$, respectively). Because Id-1 rescued the

invasive activity of MUC18-silenced cells, these data suggest that MUC18 mediates melanoma cell invasion through Id-1.

To further examine the mechanism by which Id-1 contributes to increased invasiveness of MUC18-silenced cells, we analyzed the effect of Id-1 overexpression on MMP-2 expression and activity. Id-1 overexpression in MUC18-silenced A375SM and C8161 resulted in increased MMP-2 expression by 3.4-fold and by 1.5-fold, respectively, compared to MUC18-silenced cells expressing empty vector control (Figure 22A). Furthermore, zymography assay demonstrated that Id-1 overexpression in MUC18-silenced cells increased the MMP-2 collagenase activity in MUC18-silenced cells, as compared to control cells (Figure 22B). These data describe a novel mechanism by which Id-1 acts as a signaling intermediate between MUC18 and MMP-2 .

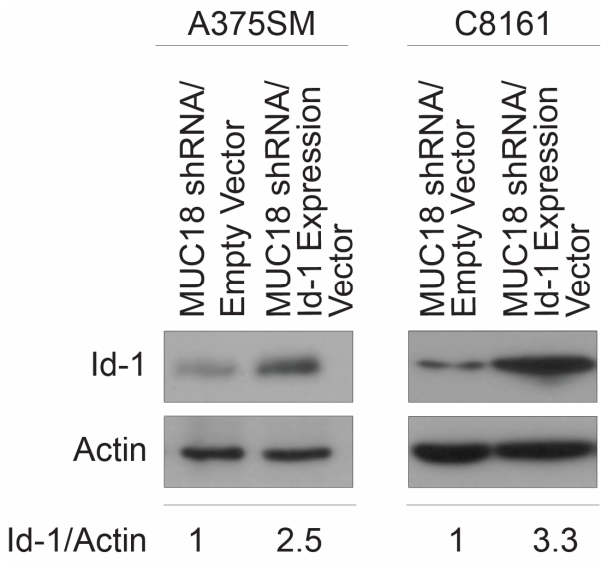


Figure 20. Rescue of Id-1 Expression in MUC18-Silenced Cells

Id-1 expression is restored by stable transduction of an Id-1 expression vector in MUC18-silenced A375SM and C8161 cells. Id-1 expression increased in MUC18-silenced A375SM and C8161 transduced with Id-1 expression vector by 2.5-fold and by 3.3-fold, respectively, as compared to control cells transduced with empty vector.

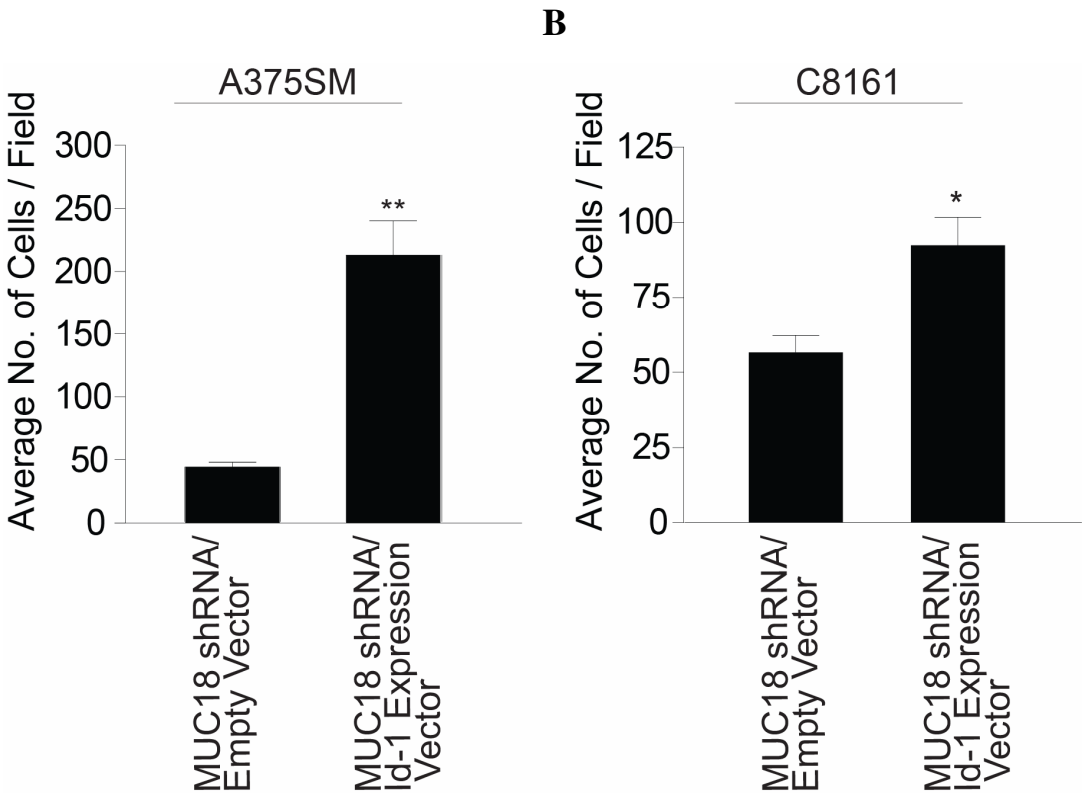


Figure 21. Rescue of Id-1 Expression in MUC18-Silenced A375SM and C8161 Cells Increases the Invasive Phenotype of the Cells

Matrigel invasion assay demonstrating **(A)** overexpression of Id-1 in MUC18-silenced A375SM significantly increases cell invasiveness. ***p*<0.001. **(B)** Overexpression of Id-1 in MUC18-silenced C8161cell significantly increases their invasive capacity. **p*<0.01. Data are expressed as mean ± SEM.

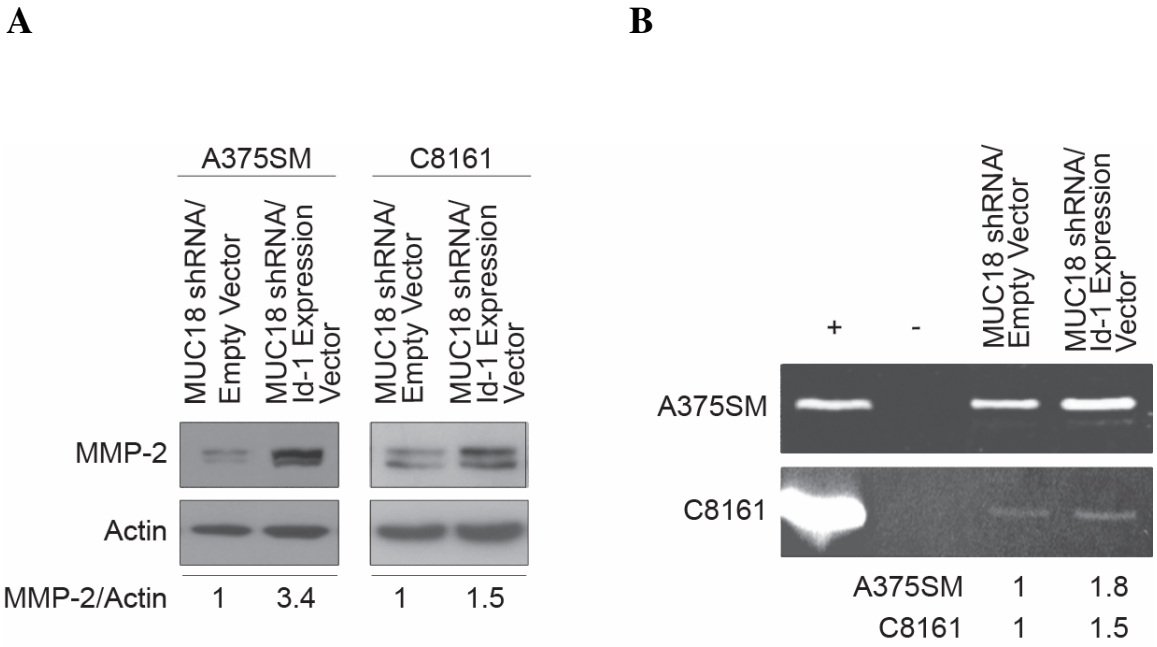


Figure 22. Rescue of Id-1 Expression in MUC18-Silenced A375SM and C8161 Cells Increases MMP-2 Expression and Activity

(A) Western blot analysis demonstrates increased expression of MMP-2 after rescuing Id-1 expression in MUC18-silenced A375SM and C8161 cells. MMP-2 expression increases by 3.4-fold in MUC18-silenced A375SM cells overexpressing Id-1 and by 1.5-fold in MUC18-silenced C8161 cells overexpressing Id-1, as compared to empty vector control expressing cells **(B)** Zymography assay demonstrates the proteolytic activity of MMP-2. Samples were loaded on a gelatin impregnated SDS gel. As seen by increased band intensity, MMP-2 activity increases following overexpression of Id-1 in both MUC18 silenced A375SM and C8161 cells, compared to control cells.

Id-1 Contributes to the Invasive Capacity of SB-2 Cells and Promotes Expression of MMP-2

Since melanoma cell invasiveness, and MMP-2 expression and activity were rescued in MUC18-silenced cells overexpressing Id-1, we sought to further delineate the role of Id-1 in regulating MMP-2 expression and melanoma cell invasion by utilizing non-metastatic melanoma cells, SB-2, which do not express MUC18 and express low levels of Id-1. Id-1 was stably expressed in SB-2 cells via lentiviral-mediated Id-1 overexpression. Following puromycin selection, expression of Id-1 was analyzed. Western blots confirmed that Id-1 was overexpressed in SB-2 cells with a 7-fold increase in expression, compared to control cells (Figure 23A).

To further assess the function of Id-1 in melanoma cell invasion, we examined the invasive capacity of SB-2 cells overexpressing Id-1 using a Matrigel invasion assay. Id-1 overexpression in SB-2 cells resulted in a greater than 3-fold increase in cell invasion, as compared to control SB-2 cells (Figure 23B). This increase in invasiveness correlated with elevated expression of MMP-2. Western blot analysis showed that Id-1 overexpression in SB-2 cells resulted in 2-fold increase in MMP-2 protein expression, as compared to control cells (Figure 24). These data further establishes the regulation of MMP-2 by Id-1.

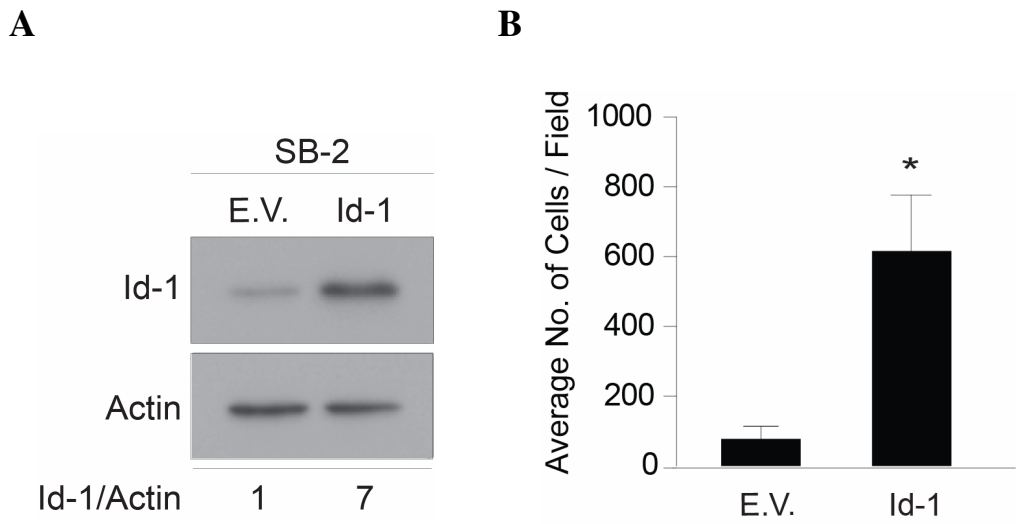


Figure 23. Overexpression of Id-1 in the Non-Metastatic Melanoma Cell Line, SB-2 Increases their Invasive Capacity

(A) Western blot analysis demonstrates increased Id-1 expression by 7-fold, following stable transduction of Id-1 expression vector into SB-2 cells, compared to cells transduced with empty vector (E.V). **(B)** A Matrigel invasion assay demonstrates a significant increase in SB-2 cell invasion capacity after overexpression of Id-1, as compared to SB-2 cells expressing empty vector (E.V). *p<0.01. Each group was plated in Matrigel invasion chambers, in triplicates, and in serum free medium. Data represents the average number of invaded cells and is expressed as mean ± SEM.

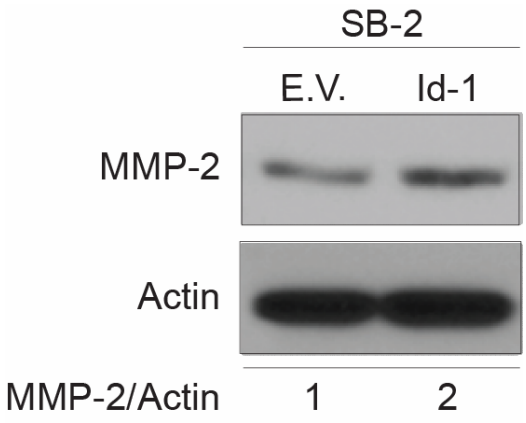


Figure 24. Id-1 Expression in the Non-Metastatic Melanoma Cell Line, SB-2, Increases MMP-2 Expression

Western blot analysis shows that overexpression of Id-1 in SB-2 cells results in increased MMP-2 expression by 2-fold as compared to control cells (E.V.).

Id-1 Transcriptionally Regulates MMP-2 Expression by Inducing Differential Expression and Binding of Sp1 and Ets-1 to the MMP-2 Promoter

It has been previously demonstrated by others that in prostate cancer cells, lymphoma cells and mouse embryo fibroblasts as well as by our studies in melanoma cell lines (Figure 22 and 24) that Id-1 expression correlates with increased MMP-2 expression [178]. However, how Id-1 participates in the regulation of MMP-2 expression has not been fully elucidated. To ascertain the mechanism by which Id-1 regulates MMP-2, the MMP-2 promoter (1.8 kb) was cloned in front of a luciferase reporter gene and MMP-2 promoter activity was evaluated in SB-2 cells overexpressing Id-1. MMP-2 promoter-driven luciferase activity was increased by more than 2.5-fold in SB-2 cells overexpressing Id-1, as compared to control SB-2 cells (*p<0.01) (Figure 25). Because the SB2 cells utilized in these experiments lack endogenous MUC18 expression, these results clearly establish a link between Id-1, melanoma cell invasiveness and MMP-2 expression.

MMP-2 transcription has been previously shown to depend on the recruitment and coordination of several transcription factors. Figure 26A illustrates a schematic of the MMP-2 promoter, depicting potential transcription factor binding sites [218]. To determine whether Id-1 regulates MMP-2 transcription through differential binding of transcription factors, we performed a ChIP assay. Specific primers were designed to amplify the potential transcription binding site of p53, Ets-1, CREB, Sp1 and AP-2α. ChIP assays demonstrated that Id-1 overexpression in SB-2 cells resulted in increased recruitment and binding of both Sp1 and Ets-1 (located between positions -70 and -91 and 1255 and -1248 respectevly) to the MMP-2 promoter, as compared to control cells

expressing an empty vector (Figure 26B). The role of Ets-1 and Sp1 in promoting MMP-2 expression was previously studied; in pancreatic cancer cells, it was established that MMP-2 expression is dependent on Ets-1 binding to the MMP-2 promoter [219]. Additionally, Sp1 was shown to play an important role in promoting MMP-2 transcription in astroglioma cells [218]. Although we examined the binding of other transcription factors, including AP-2α, CREB and p53, to the MMP-2 promoter, their recruitment was unaffected by Id-1 overexpression (Figure 26B and data not shown).

To determine whether the enhanced binding of Ets-1 and Sp1 to the MMP-2 promoter is due to their elevated expression following Id-1 overexpression, western blots were performed. Id-1 overexpression in SB-2 cells resulted in increased expression of both Ets-1 and Sp1 by 1.7-fold as compared to control cells (Figure 27A, B). These results indicate that Id-1 positively regulates MMP-2 transcription by modulating both the expression and binding of Ets-1 and Sp1 transcription factors to the Id-1 promoter.

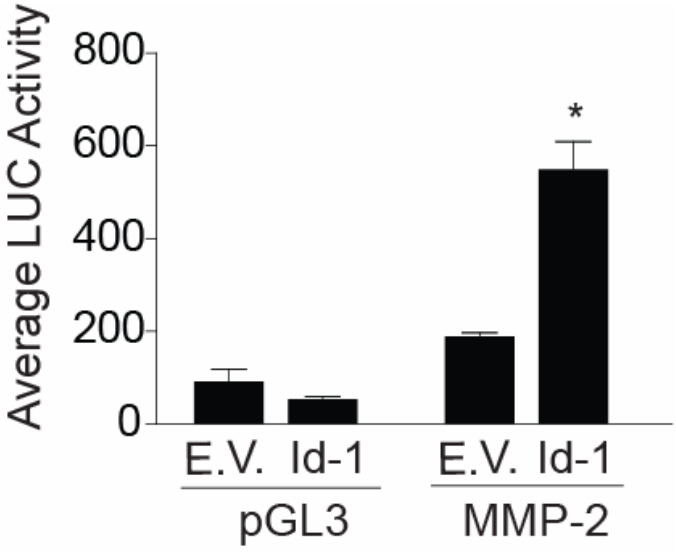


Figure 25. Transcriptional Regulation of MMP-2 by Id-1

SB-2 cells stably expressing Id-1 were transiently transfected with either a MMP-2 promoter-driven luciferase vector (MMP-2-pGL3) or a promoterless luciferase vector (pGL3). MMP-2 promoter-driven luciferase activity increased by more than 2.5-fold in SB-2 cells stably overexpressing Id-1 as compared to control cells (E.V.). *p<0.01. Lysates were analyzed in triplicate for luciferase activity and expressed as mean ± SEM.

A

B

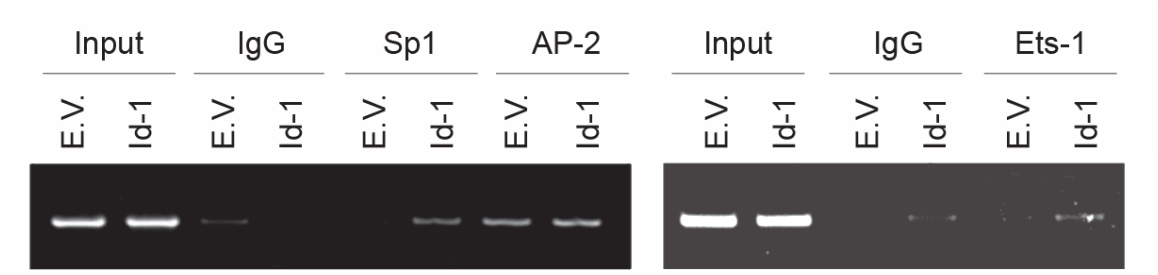


Figure 26. Regulation of MMP-2 Transcription by Id-1 Through Differential Binding of Regulatory Factors

(A) Schematic representation of transcription factor binding sites on the MMP-2 promoter. **(B)** Chromatin Immunoprecipitation analyses were performed utilizing the Sp1, AP-2 and Ets-1 antibodies, followed by PCR amplification of their binding sites on the MMP-2 promoter. Stable expression of Id-1 in SB-2 cells increases Sp1 and Ets-1 binding to the MMP-2 promoter as compared to SB-2 control cells (E.V.). No differences were observed in AP-2 binding of to the MMP-2 promoter following Id-1 expression. IgG antibodies were used as negative controls and input DNA was used to ensure an equal amount of chromatin used in each assay.

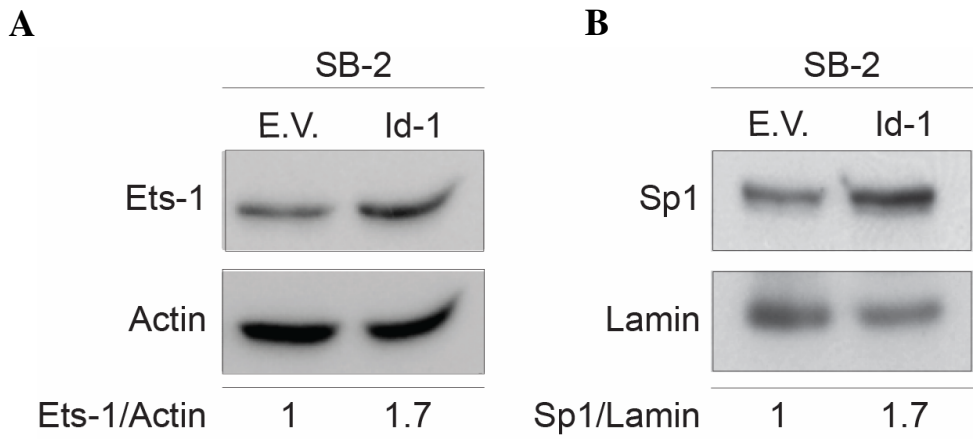


Figure 27. Id-1 Regulates the Expression of Ets-1 and Sp-1

Western blot analysis depicting increased protein expression of **(A)** Ets-1 and **(B)** Sp1 by 1.7-fold, following overexpression of Id-1 in SB-2 cells, as compared to cells expressing empty vector (E.V.).

Discussion

In this aim, we elucidated the role of Id-1 in melanoma progression as a downstream target of MUC18. Id-1 was shown to promote tumorigenesis in several cancers by acting as a regulator of various cellular processes, including growth and survival [220, 221], cellular senescence [168, 169, 200, 222], angiogenesis [178, 199, 223, 224] and invasion [183, 201, 215, 225]. Because we have discovered that MUC18 regulates Id-1 and showed that MUC18 promotes melanoma cell invasion (Figure 11 and [136, 145]), we were particularly interested in studying the function of Id-1 in melanoma cell invasion. First, to establish whether Id-1 participates in melanoma cell invasion, we overexpressed Id-1 in MUC18 silenced cells. The invasive capacity of MUC18-silenced cells (A375SM and C8161) was significantly increased in cells that overexpress Id-1, implicating that Id-1 is a signaling mediator involved in MUC18 induced melanoma cell invasion.

This study, as well as previous work from our laboratory, established that MUC18 regulates both MMP-2 expression and activity (Figure 10, 11) [136, 145]. Numerous connections between Id-1 expression and the expression of MMPs have been previously suggested. Lyden et al. have shown that in $Id1^{+/-}$ $Id3^{-/-}$ mice, MMP-2 expression was decreased as compared to wild-type mice. Id-1 was also shown to play a role in regulating MMP expression in various breast cancer models. In fact, constitutive expression of Id-1 in mammary epithelial cells increased the expression of a novel 120 kDa MMP-like protein [201, 225]. Moreover, siRNA-mediated inhibition of Id-1 expression in metastatic breast cancer cells resulted in a significant decrease of MT1-MMP protein expression [215]. In the non-aggressive prostate cancer cells, LNCaP,

ectopic overexpression of Id-1 resulted in increased MMP-2 activity and cell invasion [216]. Experiments utilizing mouse embryo fibroblasts that lacked Id-1 expression revealed that MMP-2 gelatinase activity was decreased as compared to wild type cells [199]. Additionally, cDNA microarray analysis of $Id1^{-/-}$ endothelial cells identified a reduction in MMP-2 expression, compared to their wild type counterparts [226]. In light of these findings, we raised the question of whether Id-1 mediates the diminished MMP-2 expression in MUC18-silenced cells. To that end, we examined MMP-2 expression and activity in MUC18-silenced cells overexpressing Id-1. We found that overexpression of Id-1 in MUC18-silenced A375SM and C8161 increased MMP-2 expression and activity, as compared to MUC18-silenced cells expressing empty vector control. Our studies, therefore, implicate Id-1 as a positive regulator of MMP-2, downstream of MUC18. The increased levels of MMP-2 expression and activity may account for the increased cell invasiveness through Matrigel in MUC18-silenced cells overexpressing Id-1.

It should be noted that Id-1 was shown to upregulate the expression of other MMPs, that can, in turn, promote cell invasion. For instance, in breast cancer sells, Fong et al. have demonstrated that siRNA mediated Id-1 inhibition resulted in decreased MT1-MMP expression [215]. MT1-MMP itself promotes ECM degradation, but can also act as an activator of MMP-2 by proteolysis of the pro-MMP-2 enzyme. Furthermore, MMP-2 activity can also be regulated by the tissue inhibitor of metalloproteinase 2 (TIMP-2) [227]. It is possible that Id-1 acts as an inhibitor of TIMP-2 expression. Id-1-mediated MMP-2 expression could therefore be influenced by the potential interaction of Id-1 with TIMP-2. Id-1 may inhibit TIMP-2, thereby promoting increased MMP-2

proteolysis by MT1-MMP. Id-1 likely modulates several related pathways which ultimately lead to enhanced melanoma cell invasion.

We initially observed that MMP-2 expression is decreased both *in vitro* and *in vivo* following MUC18 silencing in metastatic melanoma cell lines. This reduction could possibly result from a synergistic effect of the attenuation of Id-1 expression and increased ATF-3 expression. Yan et al. demonstrated that ATF-3 acts to repress MMP-2 expression and activity via p53 [228]. Thus, silencing of ATF-3 in MUC18-silenced cells overexpressing Id-1 could potentially elevate MMP-2 expression even further in these cells.

Because Id-1 functions to regulate gene expression, we hypothesized that Id-1 regulates MMP-2 expression at the transcriptional level. To that end we stably overexpressed Id-1 in a non-metastatic melanoma cell line, SB-2, which lacks or expresses MUC18 and Id-1 at very low levels. Like Id-1 overexpressing MUC18-silenced cells, SB-2 cells that overexpress Id-1 exhibited enhanced cell invasion. Moreover, MMP-2 expression also increased following Id-1 expression in SB-2 cells. This set of experiments demonstrated that overexpression of Id-1 results in a functional protein, modulating both the invasive behavior of melanoma cells and MMP-2 expression. These data confirm that, in melanoma cells, Id-1 alone can promote the switch from non-invasive to invasive behavior.

Although previous studies determined that Id-1 expression correlates with MMP-2 expression, the mechanism by which Id-1 regulates MMP-2 expression had yet to be elucidated. To establish that Id-1 functions as a regulator of MMP-2 gene expression, we cloned the MMP-2 promoter in front of a lucifearase reporter gene. Our results

demonstrate that MMP-2 promoter activity significantly increased following overexpression of Id-1 in SB-2 cells. Thus, these results demonstrate that Id-1 regulates MMP-2 at the transcriptional level.

Id-1 can regulate gene expression by binding and sequestering ubiquitously expressed bHLH E-proteins. This binding inhibits E-proteins from binding either to the DNA alone or to tissue specific bHLH transcription factors, thereby inhibiting their transcriptional activity [229]. As such, we propose that Id-1 can promote MMP-2 transcription via two pathways, either directly by sequestering a bHLH E-protein that functions as an inhibitor of MMP-2 expression or indirectly by promoting the expression of a downstream gene which then functions to promote MMP-2 expression. We thus hypothesized that Id-1 regulates MMP-2 expression via differential binding of transcription factors to the MMP-2 promoter. To that end, we examined the differential binding of transcription factors to the MMP-2 promoter following Id-1 overexpression in SB-2 cells. Several transcription factor binding sites, including those of Ets-1 and Sp1, are located on the MMP-2 promoter, and we discovered that the DNA binding and the expression of both Ets-1 and Sp1 to the MMP-2 promoter increased following Id-1 overexpression. These results are in accord with previous evidence demonstrating the role of these transcription factors in promoting MMP-2 expression. The PI3K-AKT signaling pathway has been shown to increase expression of MMP-2 via increased Sp1 expression and binding to the MMP-2 promoter [218, 230]. Additionally, the role of Sp1 in regulation of MMP-2 transcription and expression was described in two additional studies. The first demonstrated that genetic variation of the MMP-2 gene at the Sp1 binding site resulted in decreased Sp1 binding and subsequent MMP-2 promoter activity

[231]. A second study utilized deletion mutations of the Sp1 binding site to demonstrate decreased MMP-2 promoter activity [218]. These studies further substantiate the role of Sp1 in regulation of MMP-2 transcription. A number of studies also support the role of Ets-1 in transcriptional activation of ECM-degrading enzymes, including serine proteases (uPA) and matrix metalloproteinases [232]. Indeed, overexpression of Ets-1 in squamous cell carcinoma induced the promoter activation and subsequent expression of MMP-2 [233], while silencing of Ets-1 in pancreatic cancer cells abrogated cell invasiveness as well as MMP-2 expression [219]. Transcriptional activation of genes is dependent on binding of several transcription factors which function in a cooperative manner. Increased affinity of Ets proteins for their DNA binding domain has been shown to occur following their interactions with various transcription factors, including Sp1 [234, 235]. It would be interesting to determine whether Ets-1 and Sp1 work alone or in conjunction to promote MMP-2 expression in a similar fashion.

MMP-2 can be transactivated by transcription factors other than Sp1 and Ets-1 including CREB [236], Sp3, AP-2α [218] and p53 [237]. In our study we did not observe differential binding or expression of these transcription factors following Id-1 overexpression in SB-2 cells, which suggests that they do not play a role in MMP-2 expression in our system. Although we demonstrated that Id-1 positively regulated the expression and binding of Ets-1 to the MMP-2 promoter, it should be noted that in other non-melanoma systems, Id-1 has been shown to inhibit Ets-1 transcriptional activity [168]. It is possible that, in our system, increased expression of Ets-1 promotes its interaction with other transcription factors or co-activators (i.e. c-Jun, c-fos and

CBP/p300). Rather than being inhibited by Id-1, it competes with Id-1 for promoter binding and leads to transactivation of MMP-2.

Positive regulation of transcription of various other genes by Id-1 has also been shown. It was recently demonstrated that Id-1 expression increased c-Myc expression and its subsequent binding to the E-box region on the promoter of Bmi-1, resulting in increased Bmi-1 expression [238]. This study is in agreement with our findings, suggesting that Id-1 can act to promote gene expression by increasing the expression and binding of upstream transcription factors.

In this aim we conclude that Id-1 promotes melanoma cell invasion by affecting differential expression and binding of transcription factors to the MMP-2 promoter, resulting in increased MMP-2 expression.

Summary

Herein, our study further establishes the cardinal role of MUC18 in promoting the metastatic melanoma phenotype. Our investigation revealed novel findings which can be summarized as follows:

1. Stable silencing of MUC18, by means of lentiviral based shRNA delivery, in two metastatic melanoma cell lines, A375SM and C8161, significantly decreased both melanoma tumor growth and experimental lung metastases *in vivo*.
2. MUC18 inversely regulated the expression of two transcriptional regulators: Id-1 and ATF-3.
3. *In vivo*, MUC18 silencing decreased the expression of both Id-1 and MMP-2.
4. MUC18 upregulates Id-1 expression at transcriptional level via inhibition of ATF-3 expression.
5. Rescue of MUC18 in MUC18-silenced cells restored the protein expression of Id-1 and ATF-3, confirming that this expression pattern is not an off-target effect of MUC18 silencing.
6. MUC18 regulated MMP-2 expression and activity as well as melanoma cell invasion through Id-1.
7. Id-1 transcriptionally regulated MMP-2 expression through increased expression and differential binding of Ets-1 and Sp1.

Taken together, our study describes a previously unrecognized, signaling role for MUC18 and identifies its ability to modulate the expression of genes which are involved in melanoma metastasis. We discovered a novel pathway by which MUC18 regulates the expression of both Id-1 and ATF-3. MUC18–regulated Id-1, in turn, promotes MMP-2

expression and activity, contributing to cell invasion and ultimately to the metastatic phenotype of melanoma. Figure 28 depicts the model we described in this work.

MUC18 was initially identified as a melanoma cell adhesion molecule, and although it was shown to correlate with the acquisition of the metastatic phenotype and poor patient prognosis, its involvement in promoting melanoma progression mainly focused on its adhesive functions. Indeed, several lines of evidence support the role of MUC18 in facilitating melanoma progression through increasing cell adhesion. MUC18 has been shown to promote melanoma cell adhesion to endothelial cells and to the basement membrane by forming both homotypic and heterotypic adhesions. Thus, it was suggested that MUC18 participates in the metastatic cascade by promoting melanoma cell intravasation and extravasation, mediated by melanoma–endothelial cell interactions, as well as intravascular survival, mediated by melanoma-melanoma cell interactions. Due to its cytoplasmic protein kinase recognition motifs, early studies of MUC18 suggested that it might be involved in intracellular signaling; however, the role of MUC18 in outside-in signaling had not been fully elucidated. While our investigation demonstrated that MUC18 was involved in the regulation downstream gene expression in melanoma, it would be interesting to characterize the involvement of the cytoplasmic domain in downstream signaling and determine the precise intracellular signaling pathways induced by MUC18. Another missing link needed to establish the mechanism by which MUC18 promotes melanoma is the indentification of the unknown ligand of MUC18. Identifying this ligand is critical to the understanding of outside-in signaling of MUC18. More significantly, the ligand of MUC18 can be a potential target for melanoma therapy.

As MUC18 is expressed in melanoma and other malignancies, including Kaposi's sarcomas, angiosarcomas and leiomyosarcomas, our findings, together with future studies, will broaden understanding of the molecular changes associated with these diseases and further emphasize the need to target MUC18 as a modality for melanoma treatment.

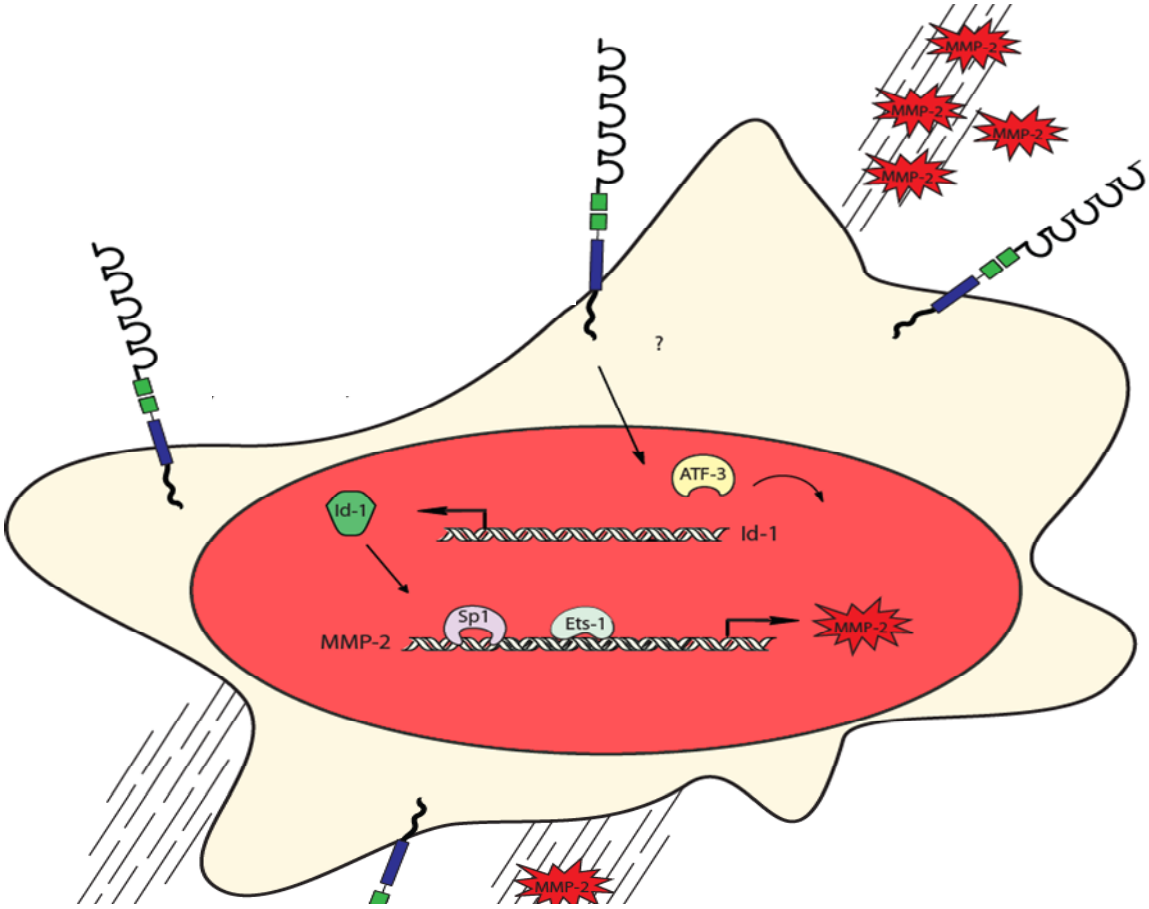


Figure 28. Proposed mechanism by which MUC18 contributes to melanoma invasion

Increase of MUC18 expression in metastatic melanoma cell promotes downstream signaling (possibly through inhibition of the p38 signaling pathway) which resultes in decreased ATF-3 expression and binding to the Id-1 promoter. This consequently releases the inhibition of Id-1 transcription by ATF-3 calumniating an increase of Id-1 transcription and expression. Upregulation of Id-1 increases the expression and binding of Sp1 and Ets-1 to the MMP-2 promoter, causing upregulation of MMP-2 transcription and expression, and, thus resulting in increased MMP-2 activity and melanoma cell invasion.

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Vita

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