EXAMINING TGF-beta INHIBITION VIA SMAD7 AND THE ROLE OF THE INHIBITORS OF DIFFERENTIATION (ID) IN METASTATIC MELANOMA

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By

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EXAMINING TGF-BETA INHIBITION VIA SMAD7 AND THE ROLE OF THE INHIBITORS OF DIFFERENTIATION (ID) IN METASTATIC MELANOMA

Kyle A. DiVito, M.S.

Thesis Advisor: Dean S. Rosenthal, PhD

ABSTRACT

TGF-β, Ids and tumorigenesis are interrelated. Smad7 (S7) has been shown to potently suppress melanomagenesis through downregulation of metastasis-related genes such as MMP2, MMP9, osteopontin and CXCR4. Here, we describe other contributing factors also play a role. Human skin grafts with fluorescently-tagged melanoma cells revealed Smad7-expressing cells positioned themselves proximal to the dermal-epidermal junction and failed to form tumors; control cells invaded and formed tumors within the dermis. Smad7 inhibited β-catenin T41/S45 phosphorylation, which is associated with degradation and induced a 4.5-fold increase in full-length N-cadherin. Cell adhesion assays confirmed a strong interaction between Smad7-expressing cells and primary dermal fibroblasts mediated via N-cadherin; while control cells were incapable of such interaction. Skin graft analysis indicated an N-cadherin-mediated homotypic interaction between Smad7-expressing cells and primary dermal fibroblasts; in contrast to control cells. Smad7 suppressed β-catenin degradation and promoted interaction with N-cadherin, stabilizing the association with neighboring dermal fibroblasts, thus mitigating invasion. We find Smad7 limits growth to a ‘radial growth phase’ melanoma, a significantly lessened state of disease.
To better define the role of Ids in melanomagenesis, we hypothesized expressing Id2, Id3 or Id4 along with Smad7 would revert 1205Lu/S7 cells back to the tumorigenic state. Subcutaneous injection of TGF-β-dependent 1205Lu cells co-expressing Id2, Id3, or Id4, with Smad7 bypassed the tumorigenic block mediated by Smad7, generating TGF-β-independent tumors and re-upregulated metastasis-related genes. Surprisingly, 1205Lu/S7 cells expressing Id4, but not Id2 or Id3, activated robust melanin production in amelanotic 1205Lu cells, confirmed by Fontana-Masson stain, de-novo expression of MART-1 and tyrosinase proteins. Mechanistic investigation revealed M-MITF phosphorylation and MART-1 promoter activation in 1205Lu and WM852 melanoma cells, suggesting broader implications for Id4 in melanoma. In human tumors melanin corresponded with Id4 localization. Additionally, pigment-laden CD163+ mouse histiocytes, with areas of extensive necrosis were found throughout S7/Id4 tumors only. Current chemotherapeutics for the treatment of melanoma are only marginally effective. Immunotherapy provides the most promise, yet innate immunity is often overlooked. This work better defines the role of the inhibitors of differentiation, particularly Id4, in advanced melanoma and may have implications for new pathways in developing immunotherapeutics in the treatment of melanoma.
This dissertation is dedicated to those whom have helped properly steer me along the, sometimes meandering, path of life. To Aunts, Uncles, Friends and Family especially my Mother, Father, sister and fiancée, Valerie, the words escape me to describe the roles you each have played in shaping the person whom I have become. I am forever indebted to you for your endearing advice, patience and love.

In the simplest terms possible...

Thank-you,

Kyle A. DiVito
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<tbody>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor- β</td>
</tr>
<tr>
<td>Smad</td>
<td>sons-of-mothers of decapentaplegic</td>
</tr>
<tr>
<td>R-Smad</td>
<td>receptor-associated Smad (e.g. Smad2/3)</td>
</tr>
<tr>
<td>I-Smad</td>
<td>inhibitory-Smad (e.g. Smad6 and 7)</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix loop helix</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>DsRed</td>
<td>red fluorescent protein</td>
</tr>
<tr>
<td>HLH</td>
<td>helix-loop-helix</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenic protein</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde dehydrogenase</td>
</tr>
<tr>
<td>Cdk</td>
<td>cyclin dependent kinase</td>
</tr>
<tr>
<td>CDKI</td>
<td>cyclin-dependent kinase inhibitor</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene-diamine-tetraacetic acid</td>
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<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>HFK</td>
<td>human foreskin keratinocytes</td>
</tr>
<tr>
<td>HFM</td>
<td>human foreskin melanocytes</td>
</tr>
<tr>
<td>Id</td>
<td>Inhibitor of Differentiation (or DNA binding)</td>
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<tr>
<td>MMP</td>
<td>matrix-metalloproteinase</td>
</tr>
<tr>
<td>ADAM</td>
<td>a disintegrin and metalloproteinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>CXCR4</td>
<td>chemokine receptor-4</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>Rb</td>
<td>retinoblastoma</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real-time-polymerase chain reaction</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>histiocyte</td>
<td>tissue macrophage</td>
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<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>Mab</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>Tet</td>
<td>tetracycline</td>
</tr>
<tr>
<td>Dox</td>
<td>doxycycline</td>
</tr>
<tr>
<td>MITF</td>
<td>microphthalmia-associated transcription factor</td>
</tr>
<tr>
<td>MART-1</td>
<td>melanoma antigen recognized by T-cells</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>1205Lu/Vc</td>
<td>1205Lu cells lacking Smad7 expression</td>
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<tr>
<td>1205Lu/S7</td>
<td>1205Lu cells expressing Smad7</td>
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<tr>
<td>S7/Id (2, 3 or 4)</td>
<td>1205Lu cells expressing Smad7 with Id2, Id3 or Id4</td>
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INTRODUCTION

The incidence of cutaneous melanoma, the most common form of melanoma, has increased dramatically over the past 50 years. The NCI reports a dismal 10% of patients diagnosed with metastatic melanoma survive to 10 years (Horner MJ, 2008). Effective treatment modalities for this disease are exceptionally limited. Even in light of numerous clinical trials examining single agent or combinational chemotherapeutics, dacarbazine approved in 1975, remains the only Food and Drug Administration (FDA) approved therapy for metastatic melanoma and its 15% efficacy has not been shown to extend patient survival (Bhatia and Thompson, 2012; Bhatia et al., 2009; Divito et al., 2004; Falkson et al., 1998). Nonetheless, recent studies have employed T-cell based immunotherapy or small molecule inhibitors, such as imatinib (Gleevac®) with some success (Hodi et al., 2008). Additionally, clinical trials for the treatment of high-grade glioma targeting other signaling molecules such as TGF-β, whose overexpression is a hallmark of many cancers including melanoma, appear promising. If found efficacious TGF-β inhibitors would have broad implications for others diseases types where overexpression has been found to contribute to disease progression (Schlingensiepen et al., 2006).

The transforming growth factor-beta (TGF-β) super-family of proteins is involved in a myriad of cellular functions including development, cell migration and growth inhibition (Imoto et al., 2003). In normal cells and during early stage carcinogenesis, TGF-β exerts a potent growth inhibitory signal and thereby functions as a tumor suppressor. Yet, paradoxically, during advancing disease some cancers, including melanoma, have been found to secrete abnormally high levels of the cytokine to which the cell becomes desensitized (Krasagakis et al., 1995; Rodeck et al., 1999). In this case, the autocrine and paracrine effects of TGF-β result in a
contribution to, rather than protection from, advanced disease. In malignant melanoma, TGF-β overproduction correlates with increased tumor thickness and disease progression (Reed et al., 1994). In late stage disease, TGF-β overexpression is also associated with a significantly decreased survival time (Krasagakis et al., 1999; Reed et al., 1994; Van Belle et al., 1996).

TGF-β binds the extracellular domain of the constitutively active type-2 receptor (TβR2) resulting in recruitment and auto-phosphorylation of multiple residues along the GS domain of type-1 receptor (TβR1), thereby forming a heterotetrameric TGF-β receptor complex (TβR) (Shi et al., 2004; Shi and Massague, 2003). Following assembly, intracellular effector molecules, known as Smads, propagate the TGF-β signal (Massague et al., 2005). Upon ligand binding, cytoplasmic receptor-associated Smads (R-Smads), known as Smad2/3 are phosphorylated by TβR1 which then associate with the co-Smad, Smad4, to enter the nucleus and influence transcription. In normal cells, inhibitory-Smads (I-Smads) such as Smad7 limit the TGF-β signal either by competing with R-Smads for the receptor (TβR) or Smad4 binding (Imoto et al., 2003). Other inhibitory Smads, such as Smad6 function by limiting activation of the bone morphogenic protein (BMP) receptor family (Figure 1A). Inhibitory-Smads can also function to promote receptor degradation via recruitment of E3 ligases such as Smurf 1, 2 and WWP1 (Ebisawa et al., 2001; Komuro et al., 2004). Therefore, it is I-Smad activity itself that can determine the intensity and duration of the TGF-β signal.

However, recent evidence has shown that multiple Smad proteins are capable of engaging in signaling unrelated to the TGF-β superfamily, termed non-canonical signaling (Hoover and Kubalak, 2008). For example, posttranscriptional modification of microRNA-21 occurs via R-
**Figure 1A: Canonical TGF-β Signaling.** (A) TGF-β ligand exists as a homodimer which docks to the TGF-β receptor 2 (TβR2) also a homodimer, this induces auto-phosphorylation and heterotetrimerization with receptor 1 (TβR1). This recruits receptor-Smads (R-Smad), Smad2/3 which as phosphorylated and activated by the TGF-β receptor. Smad2/3 then interacts with the co-Smad, Smad4 which can enter the nuclear and influence gene transcription of hundreds of gene targets. The inhibitory-Smad, Smad7 blocks Smad2/3 activation by docking with the receptor or by docking with Smad2/3 and inhibiting Smad4 interaction. In normal cells, TGF-β inhibits growth by activating cyclin dependent kinase inhibitors (CDKI), such as p15, p21 and p27. TGF-β also restricts activation of Id expression in normal cells. The bone morphogenic protein (BMP) family is a member of the TGF-β superfamily and functions similarly. However, BMPs can activate Id synthesis, as opposed to TGF-β.

Smad-dependent interaction (Davis et al., 2008). Furthermore, Smad7’s traditional role as the predominant mechanism by which the TβR complex is inhibited and degraded has recently been expanded. The non-canonical functions of Smad7 include direct interaction with several signaling proteins such as the signal activator of transcription (STAT) as well as protein inhibitor of activated signal transduction; PIAS (Imoto et al., 2003). Furthermore, Smad7 appears to
directly influence overall β-catenin stability and, in turn, TGF-β-dependent apoptosis and cell adhesion (Figure 1B) (Edlund et al., 2005). Recently, Tang et al. showed, in breast cancer cells, that Smad7 directly binds β-catenin, effectively sequestering it, resulting in a loss of both GSK3β-directed β-catenin phosphorylation and subsequent ubiquitin-mediated degradation (Tang et al., 2008). As a result β-catenin/ E-cadherin complexes were stabilized, resulting in stabilized adherens junctions. In light of these findings, exploration of the Smad proteins and their diverse roles outside traditional TGF-β signaling, especially cell adhesion, have become of particular interest.

Cell adhesion proteins such as catenins/ cadherins are important players during cellular transformation and progression to metastasis. Evidence supporting cadherin switching during carcinogenesis is extensive (Cavallaro and Christofori, 2001; Cavallaro et al., 2002; Hazan et al., 2000; Herlyn et al., 2000; Islam et al., 1996; Herlyn et al., 2000; Islam et al., 1996; Maeda et al., 2005; Schmitt et al., 2007). Under this model, epithelial cells normally expressing epithelial-cadherin (E-cadherin) remain polarized and ‘locked’ in position via neighboring cell-cell interactions. During transformation, cadherin switching proposes E-cadherin is lost, through yet undetermined mechanisms, at which point epithelial cells express alternative cadherins such as neural-cadherin (N-cadherin). For example, normal melanocytes reside at the dermal-epidermal junction interspersed throughout the basal layer of the epithelium. Melanocytes retain this position through E-cadherin adherens junctions established with neighboring keratinocytes, while underlying dermal fibroblasts predominantly express N-cadherin. During initial transformation, melanocytes can downregulate the E-cadherin gene resulting in a subsequent loss of keratinocyte anchorage and can begin to alternatively
Figure 1B: Non-canonical TGF-β signaling. (B) Smad7 docks with the cell adhesion protein, β-catenin inhibiting its degradation. Once stabilized, β-catenin can accumulate in the cytoplasm and bind to other cell adhesion proteins, such as E- and N-cadherin, thus promoting cell-cell interactions and limiting invasion potential.

express N-cadherin (Hsu et al., 2000; Hsu et al., 1996). This cadherin switch along with upregulated proteases, such as MMP, allow transformed melanocytes to break their keratinocyte contacts, digest the basement membrane and enter the dermis via newly upregulated N-cadherin (Herlyn et al., 2000). However, evidence has suggested that an additional event such as the loss of N-cadherin is required to potentiate migration; however, to date this has not been explored in melanoma (Maretzky et al., 2005; Mochizuki and Okada, 2007; Nakagawa and Takeichi, 1998; Van Hoorde et al., 1999). In this model, N-cadherin is either downregulated or cleaved, in the ectodomain, by proteases such as the disintegrin and metalloproteinase-10 (ADAM-10) resulting
in cell migration and invasion. N-cadherin can then later be upregulated at distant sites such as to engage endothelial cells and enter the vasculature (Kohutek et al., 2009; Reiss et al., 2005).

We have recently shown stable over-expression of Smad7 in 1205Lu metastatic melanoma cells inhibits tumor formation when subcutaneously injected into nude mice and bone metastasis following intracardiac injection (Javelaud et al., 2005; Javelaud et al., 2007). These changes were accompanied by significant reduction in secretion of metastasis-related genes including matrix metalloproteinases (MMP)-2 and -9, and reduced expression of interleukin-11, CXCR4 and osteopontin (Javelaud et al., 2007).

In this, the first chapter of my thesis, we sought to further understand the mechanism by which Smad7 expression in metastatic melanoma inhibits tumorigenesis within the context of skin development. Prior subcutaneous injection models are not well suited for understanding the complex mechanisms involved in skin formation and fail to address questions pertaining to the effects of Smad7 upon cellular interactions. A more diagnostic model using an in vivo human skin grafting system, coupled with fluorescently tagged melanoma cells allows precise assessment of the effects of Smad7 on human melanoma cell proliferation, localization and potential cell-cell interactions. We present data here indicating that Smad7 is capable of directing 1205Lu positioning within human skin grafts, a mechanism previously unreported in response to Smad7. Interestingly, we find that Smad7 blocks invasion in vivo, with cells residing in close proximity to the dermal-epidermal junction or in some cases within the epidermis interacting with keratinocytes much like normal melanocytes. In contrast, vector control (1205Lu/Vc) cells readily invaded into the lower dermis. We propose these differences are the result of stabilized cell adhesion proteins β-catenin and, consequently, N-cadherin resulting in abrogated tumor
formation. To our knowledge this is the first report defining *in vivo* modification to β-catenin and N-cadherin in response to Smad7 expression.

The second chapter of this thesis seeks to better define the role of the Inhibitors of Differentiation (Id) in melanoma with particular respect to Id2, 3, or 4 co-expression along with Smad7 and then asse any alteration to cell growth, proliferation or tumorigenesis using the 1205Lu metastatic melanoma cell line as a model. The incidence of cutaneous melanoma has increased more rapidly than most cancers over the past 50 years. One of the most alarming increases is observed in young women aged 15-39 and is attributable to increased exposure to UV (Purdue et al., 2008). Indeed men are also at risk for advanced melanoma as men are 55% more likely to die from the disease versus age-matched women (Gamba et al., 2013).

Dacarbazine is a common treatment modality for metastatic melanoma but its limited efficacy has been the impetus for the development of newly approved therapeutics such as ipilimumab (anti-CTLA-4), vemurafenib (BRAF V600E inhibitor), and TGF-β inhibitors currently underway in clinical trials, all of which offer a more targeted therapy as well as the promise of improved overall survival (Bhatia and Thompson, 2012; Sosman et al., 2012).

Different TGF-β family members are involved in diverse cellular functions (Imoto et al., 2003). In normal cells, TGF-β induces phosphorylation of Smads 2/3, resulting in association with Smad4, and in turn inhibits Id synthesis (Kang et al., 2003; Ruzinova and Benezra, 2003). It has been suggested that Id de-regulation is a contributing factor to cancer initiation and progression.

In malignant melanoma, TGF-β overproduction correlates with increased tumor thickness and disease progression (Reed et al., 1994). In late-stage disease, TGF-β is also associated with a
significantly decreased survival time (Javelaud et al., 2008; Reed et al., 1994). The switch from TGFβ growth inhibition to growth promotion has been linked to Ids, which are suppressed by TGFβ in normal cells, although not in all malignant melanomas.

Ids are a small family of helix-loop-helix (HLH) factors, which lack a basic domain and the ability to associate directly with DNA. Inhibitors of differentiation, also referred to as inhibitors of DNA binding were first identified in 1990 by Robert Benezra (Benezra et al., 1990). The Id genes contain a helix-loop-helix domain, a structure common in many transcription factors. Helix-loop-helix regulatory proteins have diverse roles by controlling cell growth and terminal differentiation in many cell types. The HLH motif in particular mediates dimerization among other HLH proteins. Normally, adjacent to HLH domains are a stretch of basic amino acid residues that mediate direct DNA interaction at regions termed E-boxes (Christy, 2001). Id proteins retain the HLH dimerization characteristic, yet lack the basic domain refraining them from direct DNA interaction. Ids play an important cellular function by binding and thereby sequestering E-proteins, widely expressed bHLH transcription factors. This sequestration interferes with E-protein/ DNA interaction and negatively regulates gene transcription; therefore Ids operate in a dominant negative manner. The inhibitor of differentiation gene family comprises four known members (Id1-Id4) each with distinct, yet somewhat overlapping roles. From an evolutionary standpoint the Id gene family may have emerged as mutant members of the HLH family of transcription factors where, instead of being selected against, they evolved into the negative regulatory occupation they have today (Rosenzweig et al., 1995).

The four known Id genes, Id1-4, each reside on separate chromosomes and are therefore not the products of splice variants; though, to date, one splice variant termed Id3-Long has been
described in smooth muscle cells (Matsumura et al., 2001). Id proteins readily bind class A transcription factors of which E2A, E12 and E47 are common members. Class A E-proteins heterodimerize with class B molecules such as myogenic regulatory factors (MRFs) like myoD, myogenin and Myf-5 involved in terminal differentiation. Together, class A and B factors induce gene transcription including binding to gene promoters involved in cell differentiation (Figure 1C). Since E-proteins are ubiquitously expressed, they are consequently imperative for many cellular functions. Some of these functions include cellular differentiation, therefore E-proteins provide Ids with an enormous regulatory task that indirectly influences gene expression through a wide repertoire of protein-protein interactions.

TGF-β is capable of regulating literally hundreds of genes; some of those gene targets include the Ids (Ruzinova and Benezra, 2003). In epithelial cells it has been described that TGF-β inhibits Ids through activation of receptor-associated Smad3. In this case, TGF-β stimulation results in activation Smad3 and, subsequently, activation of the transcriptional repressor ATF3, which in turns results in ATF/ CREB binding and inhibition of the Id1, 2 and 3 promoters (Goumans et al., 2002). However, other members of the TGF-β superfamily, such as the BMPs, oppose the TGF-β inhibitory response and can induce Id transcription. Here, activation of the BMP receptor stimulates Smad1/ 5 phosphorylation which motivates the binding of Smad4 to the Smad responsive element (SRE) on the Id1 promoter(Goumans et al., 2002; Valdimarsdottir et al., 2002), this binding induces activation of the Id1 promoter. Other than a specific methylation pattern correlating with advanced breast cancer, little work has been done describing the functional role of Id4 in melanoma. In fact no peer-reviewed literature describing the function of either Id3 or Id4 in melanoma have been described, leaving these particular Ids as interesting
targets of future investigation, some of which is reported here. In summary, Id genes can be directly induced by BMP activation or indirectly inhibited through TGF-β activation.

Ids are believed to function primarily by sequestration of other factors, including certain bHLH, ETS, and Rb proteins, thereby acting as dominant-negative transcription factors. In this way, Ids regulate a myriad of cellular functions including cell cycle progression and proliferation, migration, angiogenesis, and invasion, while simultaneously stalling differentiation (Benezra et al., 2001; Fong et al., 2004). Id expression is upregulated in many types of cancer, including those of the breast, pancreas, ovaries, and head and neck; implicating Ids as cooperating oncogenes (Ruzinova and Benezra, 2003). Id1 and Id2 show some intriguing associations with melanoma, while the roles of Id3 and Id4 have not been well examined,

![Diagram](image)

**Figure 1C: Ids Operate via Sequestration.** (C) Left, bHLH proteins are inhibited from binding DNA as Id proteins sequester necessary E-proteins. Right, in the absence of Ids bHLH/ E-proteins can associate with DNA.

begging the question of the potential role of Ids in this disease. Limited data focusing on Ids in the skin has shown that ectopic expression of Id1, and to a lesser degree, Id2 and Id3, delays senescence in primary human keratinocytes (Alani et al., 1999; Nickoloff et al., 2000) as well as
melanocytes (Cummings et al., 2008), via inhibition of p16\(^{\text{Ink4A}}\), a common target of deletions, inactivating mutations or methylation in familial and sporadic melanoma (Bartkova et al., 1996; Hussussian et al., 1994; Kamb et al., 1994). As with most cancers, Id1 is generally expressed at high levels in melanoma and is associated with decreased survival (Straume and Akslen, 2005), although it does not appear to play a role in silencing tumor suppressor genes in melanoma, as in other cancers (Cummings et al., 2008).

In some melanomas, unlike primary cells, Id2 is not downregulated by TGF-β, a mechanism proposed to explain loss of melanoma growth inhibition by TGF-β (Schlegel et al., 2009). Id2, but not Id1 or Id3, was found to interact physically and genetically with hypophosphorylated retinoblastoma (Rb) family members, important in cell cycle progression and melanomagenesis (Iavarone et al., 1994; Lasorella et al., 2000). Consensus on the correlation between Id3 and Id4 expression and prognosis in human cancer has been mixed. Id3 is upregulated in some cancers including prostate (Sharma et al., 2012), ovarian (Shepherd et al., 2008), and NSCLC (Castanon et al., 2013), but, conversely, carries potentially inactivating mutations in 36/53 cases of Burkitt’s Lymphoma (Richter et al., 2012). In breast and prostate cancer, as well as leukemia, the Id4 gene has been shown to be silenced by hypermethylation, suggesting its role as a putative tumor-suppressor. However, Id4 silencing correlates with improved clinical outcome in glioblastoma (Carey et al., 2009; Martini et al., 2012; Yu et al., 2005) suggesting that Id3 and Id4 can function either as cooperating oncogenes, or tumor suppressors, depending on the type of cancer. The role of Id genes in melanoma are, as yet, not fully realized, especially for Id3 and Id4.
Melanoma originates from melanocytes, responsible for pigmentation of skin and hair. Over 125 genes have been implicated in four intricate stages of pigment production. Stage I of melanin synthesis begins with the pre-melanosome, composed mainly of an amorphous protein matrix of full-length gp100/Pmel17 and tyrosinase-related protein-1 (TRP-1). Stage II is marked by expression of melanoma antigen recognized by T-cells (MART-1/Mel-A/MLANA) and cleaved gp100, all present within a melanosome with a fibrillar matrix. MART-1 directly associates with gp100 and is vital for its function and, therefore, the initial processes of pigmentation (Hoashi et al., 2005). By stage III, both gp100 and MART-1 are reduced, and expression of tyrosinase (TYR) is upregulated as melanin begins to fill the melanosome. Stage IV is marked primarily by TYR and dopachrome tautomerase (DCT) expression. The rate-limiting step in melanin production is the TYR-mediated hydroxylation of tyrosine to 3,4-dihydroxyphenlyalanine (DOPA) and subsequent oxidation to dopaquinone, which is ultimately converted to pheo- or eu-melanin via one of three pathways (Hearing, 2011; Hearing and Jimenez, 1989; Hoashi et al., 2005; Scherer and Kumar, 2010). Furthermore, much work exploring pigmentation in melanoma has centered on the microphthalmia-associated transcription factor (MITF), a bHLH protein important in proliferation and survival of melanocytes; MITF is amplified in 10-15% of melanomas. MITF plays a dual role as a key regulator of both melanomagenesis and pigment-associated gene expression, most notably through E-box binding of the TYR, MART-1 and TRP1+2 promoters (Du et al., 2003; Goding, 2000). Further upstream, TGF-β has also been implicated in pigmentation. Immortalized mouse melanocytes treated with TGF-β1 downregulated the MITF promoter, which resulted in loss of TYR and TRP1 proteins, ultimately blocking melanin synthesis (Kim et al., 2004). The roles of Ids in pigmentation have not
previously been investigated. Yet, have only been tangentially explored in osteoclast precursors, where Id1, Id2 and Id3 are direct binding partners for MITF, which prevent it from binding and inducing the osteoclast-associated receptor (OSCAR), and osteoclast differentiation (Lee et al., 2006). The effects of Id4 and MITF on differentiation or the roles of Id/MITF interaction in pigmentation and melanomagenesis have yet to be elucidated.

Smad7 expression in 1205Lu metastatic melanoma cells results in a tumorigenic block through both TGF-β-dependent and -independent mechanisms. Using multiple approaches including subcutaneous injection, intra-cardiac injection and human skin grafts, we have found that Smad7 not only blocked melanoma formation but also mitigated metastasis in highly aggressive, TGF-β-dependent 1205Lu cells. Mechanisms for these observations include down-regulation of metastasis-related genes such as MMP2, MMP9, osteopontin and CXCR4, as well as stabilization of cell adhesion related proteins β-catenin and N-cadherin (DiVito et al., 2010; Javelaud et al., 2011; Javelaud et al., 2005; Javelaud et al., 2007).

The second Aim of my thesis seeks to evaluate the role of Ids in melanomagenesis and pigmentation in the presence of a functioning or blocked TGF-β pathway, via Smad7. Here, we show that Id2, Id3 or Id4 are each able to overcome the potent tumorigenic block imposed by Smad7. This lends confirmation that Ids2-4 alone can promote tumorigenesis independently, to varying degrees depending upon the Id, following TGF-β inhibition (Norton and Atherton, 1998; Wilson et al., 2001). We also observe, somewhat unexpectedly that Id4, interacts with MITF and derepresses MART-1 and TYR, leading to robust melanin synthesis and histiocyte infiltration in melanoma xenografts, a unique finding establishing a role for Id4 in melanomagenesis, as well as
pigmentation and subsequent immune response. This work seeks to assess a deeper investigation into Id function with respect to TGF-β in melanoma.
**Materials and Methods**

**Chapter I**

**Cell Culture and Retroviral Transductions:** 1205Lu metastatic melanoma cells expressing Smad7-FLAG (1205Lu-Smad7) or vector (pcDNA3-FLAG) clones (1205Lu-Vc) were fully characterized and cultured as previously described (Javelaud et al., 2005). Primary human keratinocytes and dermal fibroblasts were cultured in KSFM (Invitrogen Carlsbad, CA) and DMEM plus 10% FBS, respectively. Primary human melanocytes were cultured in Media 254CF plus supplements and 0.08mM CaCl₂ (Gibco Portland, OR). 1205Lu cells and human keratinocytes were transduced with pLHCX-DsRed and pLHCX-GFP, respectively using the φNX retroviral system (Clontech Mountainview, CA) as previously described (Trabosh et al., 2009).

**Adhesion Assay:** Assays were performed as previously reported (Maretzky et al., 2005). Briefly, 5x10⁴ primary human foreskin fibroblasts (HFF) or primary human foreskin keratinocytes (HFK) were seeded onto 96-well plates and grown to confluence. 1.2x10⁶ 1205Lu-Vc or 1205Lu-Smad7 melanoma cells were re-suspended in 1x PBS/ 0.1%BSA and labeled with 2.5µM calcein AM (Invitrogen Carlsbad, CA) for 30 min, 37°C. Cells were washed in 1xPBS to remove excess dye. 1205Lu-Vc and 1205Lu-Smad7 cells were pre-incubated with either N-cadherin (GC4) antibody (50µg/ mL), IgG control (50µg/ mL) each 2 hrs; EGTA (5mM) for 20 min; or left untreated. The cells were added to confluent monolayers at 5x10⁴ cells/ well in growth media and incubated for 20 min, 37°C. Plates were read at 480/530nm excitation/ emission. Plates were subsequently
washed 3x in 1xPBS and fluorescence was measured. Mean calcein-AM fluorescence is a direct measure of cell number (Maretzky et al., 2005). Remaining fluorescence was expressed as a percentage compared to initial reading; done according to previously established protocol. Representative bar graphs reflect the percentage of adherent cells. All experiments were reproduced three independent times. Results are the means ± SDs of three biological replicates of a representative experiment.

**Immunoblotting/ Immunoprecipitation:** Protein analyses were performed according to standardized protocols. Briefly, cells were collected at confluency and lysed in RIPA buffer containing protease inhibitors under confluent conditions. For immunoprecipitation, 250µg of total protein was incubated with either: 0.5µg/ mL β-catenin antibody or 40µl of anti-FLAG affinity gel (Sigma; St. Louis, MO) and precipitated overnight. Protein A/G agarose beads (Santa Cruz, CA) were used to pellet the β-catenin immunoprecipitate. For western blot 20µg total protein was resolved on 8% SDS-PAGE, transferred to nitrocellulose membrane and blocked. Corresponding primary antibodies were incubated in 5% non-fat milk overnight and then washed 3x in PBS-Tween. Secondary donkey anti-mouse-HRP or sheep anti-rabbit-HRP was used at 1:8000 dilution. After washing, ECL (Thermo Rockford, IL) was used to detect proteins. Smad7 (P20) and β-catenin (E5) antibodies were purchased from Santa Cruz (Santa Cruz, CA). Mouse monoclonal antibodies to N-cadherin recognizing the C-terminal domain (clone 32) and N-terminal domain (GC4) were purchased from BD Biosciences (San Jose, CA) and Sigma-Aldrich (St. Louis, MO), respectively. E-cadherin (24E10) was purchased from Cell Signaling (Danvers, MA). Phospho-Smad3 was a generous gift from Dr. Edward Leof (Mayo Clinic Rochester, MN).
GAPDH antibody used as loading control (Ambion Austin, TX). All experiments were reproduced three independent times. Results are the means ± SDs of three biological replicates of a representative experiment.

**siRNA Transfection:** 120nM of Smad7 siRNA (SantaCruz Biotechnology Santa Cruz, CA) was transfected into 1205Lu cells using Lipofectamine 2000; cells were re-plated and incubated for up to 72hrs. Cells were collected in RIPA buffer, separated by 8% SDS-PAGE, transferred and probed using anti-β-catenin, anti-N-cadherin and anti-Smad7.

**Immunofluorescence/ Confocal/ In-Vivo Animal Imaging:** 1205Lu cells were seeded into 8-well chamber slides at 20x10^3 cells/ well. Cells were fixed in 4% paraformaldehyde, permeabilized, blocked in SuperBlock (Thermo Rockford, IL) and stained overnight with primary antibody. Goat anti-mouse or rabbit AlexaFluor® 488, 594, or 647 (Invitrogen Carlsbad, CA) secondary antibodies were used at 1:500 for 1hr. DAPI (1µg/ mL) was used to identify nuclei. For apoptosis experimentation, cultured cells or grafted tissue sections were stained overnight with antibody recognizing cleaved caspase-3 (Asp175) (Cell Signaling Danvers, MA). Five high power fields, representing >500 cells, were then captured and used for quantitation. Figures show representative images taken from three independent experiments. Confocal images were obtained using Olympus Fluoview-FV300 laser scanning confocal microscope. *In vivo* imaging was performed using Maestro II® Imaging (CRi Woburn, MA). Briefly, animals were anesthetized using 2% isoflurane and analyzed for GFP and DsRed
fluorescence. Animals were allowed to recover and imaged every other day for one month. All experiments were reproduced three independent times.

**Skin Grafts/ Frozen Sectioning:** Primary HFFs and HFKs cultures were derived from neonatal foreskin obtained, with permission, from Georgetown University Hospital (Washington, D.C.) and processed according to previously established protocol (Rosenthal et al., 1995). Skin grafts were prepared by making oval incisions onto the dorsal region of nude mice into which silicon domes were secured under the skin, against the muscle fascia. HFF, HFK and 1205Lu cells were then seeded into the domes at 8x10^6, 5x10^6 and 1x10^6 cells respectively, per animal. Four animals were grafted per condition. Cells were allowed to grow for 1 week; domes were removed and then allowed to grow for the remainder of the experiment. Animals were euthanized and human skin reproductions were immediately surgically excised. Tissue was fixed in 4% paraformaldehyde for 1-2 hrs to preserve fluorescence and processed according to established protocol (Ueno and Weissman, 2006). Grafts were then sectioned at 5µm using a cryostat and placed onto SuperFrost glass slides (Fisher Scientific Pittsburg, PA).

**Statistical Analyses:** Bar graphs and statistical analysis were performed using SigmaStat and SigmaPlot (Systat San Jose, CA). Error bars represent ±SD. Student’s t-test was used to calculate p-values. P-values of less than 0.05 were considered statistically significant.
**CHAPTERS II & III**

**Cell culture, retroviral transductions, and expression vector clones**

1205Lu, 1205Lu/Smad7, WM852, Sk-28 and Mel-501 were kindly provided by Drs. A. Mauviel (Institut Curie; Orsay, France) and T. Guise (Indiana University). Cells have been cultured and characterized as previously described (DiVito et al., 2010; Javelaud et al., 2007). Pigmented MNT1 melanoma cells were kindly provided by Dr. V. Hearing (NIH; Bethesda, MD) and maintained as previously described (Kushimoto et al., 2001). Primary human foreskin keratinocytes (HFK) and human foreskin melanocytes (HFM) were derived from neonatal foreskin, obtained from Georgetown University Hospital (Washington, DC) and processed according to previously established protocols (Rosenthal et al., 1995). Briefly, HFKs and HFMps were cultured in KSFM and Media 254, respectively, with appropriate supplements (Invitrogen; Carlsbad, CA), plus 1% gentamicin; melanocytes were additionally supplemented with 0.16 mM CaCl$_2$. 1205Lu cells stably and constitutively expressing Smad7 (1205Lu/S7) or vector only (1205Lu/Vc) were transduced with pLHCX-DsRed retroviral vector (Clontech; Mountainview, CA) using the ΦNX delivery system as previously described (Trabosh et al., 2009). Id2, Id3, and Id4 were separately cloned into the pcDNA4/TO Tet-on expression vector (Invitrogen). 1205Lu cells were transfected first with pcDNA6/TR, selected in 10 µg/ml blasticidin, transfected with pcDNA4/TO-Id constructs, and further selected with 1000 µg/ml Zeocin™ (Invitrogen) for >2 weeks. Highly expressing Tet-inducible individual clones were isolated, confirmed by immunoblot analysis and used for subsequent studies.
Immunoblot analysis, immunoprecipitation, and antibodies

Protein analyses were performed according to standard protocols. Briefly, cells were collected and lysed in RIPA buffer containing protease and phosphatase inhibitors. For immunoprecipitation, 500 μg (unless otherwise stated) of total protein was incubated with 40 μl of anti-FLAG affinity gel (Sigma) and precipitated overnight. FLAG beads were pelleted and washed 3x in PBS according to manufacturer’s protocol. For immunoblot analysis, 40 μg (unless otherwise stated) total protein was resolved on 12% SDS-PAGE, transferred to nitrocellulose membrane and blocked in 5% non-fat milk. Corresponding primary antibodies were incubated in 5% non-fat milk overnight and then washed 3x in PBS-Tween. Secondary donkey anti-mouse-HRP or sheep anti-rabbit-HRP was used at 1:8000 dilution. After washing, ECL (Thermo, Rockford, IL, USA) was used to detect proteins. Smad7 (N19), Id2 (C20), Id3 (C20) Id4 (L20), tyrosinase (C19) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal (21D1418) and rabbit polyclonal antibodies to MITF were purchased from Abcam (Cambridge, MA). Phospho-Smad3 was a generous gift from Dr. Edward Leof (Mayo Clinic; Rochester, MN, USA). GAPDH was used as a loading control using antibody from Millipore (Billerica; MA). All experiments were reproduced three independent times. Results shown are the mean ± SDs of three biologic replicates of a representative experiment.

In vivo subcutaneous injection

Subcutaneous injections were performed using athymic NCr-nu/nu mice (Harlan Laboratories; Indianapolis, IN). Animals were anesthetized using 2% isoflurane. 1x10^6 cells of each type were then injected into hind flanks using a 20 gauge syringe. At least 4 animals were used for each
condition. Animals were allowed to recover from anesthesia and monitored for tumor growth for 60 days via Maestro II™ live-animal fluorescence imaging (Caliper Life Sciences; Hopkinton, MA). Animals were then euthanized, and tumor tissue was harvested, fixed in either 10% neutral-buffered formalin and sectioned using a microtome, or in 4% paraformaldehyde-fixed for 4-6 h to preserve DsRed fluorescence and sectioned using a cryostat according to established protocol (Ueno and Weissman, 2006). Sections (5 µm) were then placed onto SuperFrost glass slides (Fisher Scientific Pittsburg, PA) for further analysis.

**Histochemistry and immunofluorescence**

To detect melanin, formalin-fixed paraffin sections were freshly cut and stained using Fontana-Masson stain kit (Fisher Scientific; Pittsburg, PA) according to the manufacturer’s protocol. For immunofluorescence, sections were cut, deparaffinized in xylene, rehydrated through successive washes in decreasing concentrations of ethanol, followed by antigen retrieval in boiling 6.5 mM sodium citrate, pH 6.0 for 6 min, as described previously (Divito et al., 2004). In a humidity chamber, sections were blocked for 20 min at room temperature with SuperBlock (Thermo; Rockford, IL) chamber), incubated at 4°C overnight with antibodies that were diluted to 1 µg/ml in SuperBlock, washed 3x in PBS/0.05% Tween (PBS-T), and further incubated for 1h with either Alexa 488- or Alexa 594-conjugated secondary antibody at 1:500 dilution (Invitrogen). After washing 3x in PBS-T, sections were counterstained with DAPI for 5 min, washed, and mounted using Fluoromount-G (SouthernBiotech; Birmingham, Alabama). Images were captured with an inverted Olympic epifluorescence microscope.
qRT-PCR and primer sequences

qRT-PCR was performed by standard protocols using 2-step RT-PCR (Invitrogen, Carlsbad, CA), 0.75 µg of RNA, and specific primers: MART-1: Forward 5’-
TGCCCACAAGAAGGGTTTGA-3’; Reverse 5’-CCCAATGCTCCACCTGCTTA-3’. MITF:
Forward 5’-TATCAGGTGCAGACCCACCT-3’; Reverse 5’-
GTCCTGAGCTTGCCATGTCCA-3’.

Promoter Assays

Dual luciferase assays were performed as previously described (Trabosh et al., 2009). In brief, a 1.9 kb fragment of the wild-type, or E1/E2 mutant MART-1 promoter cloned into the pGL3-Basic expression vector (Du et al., 2003) were generously provided by Jessica Fuller and David E. Fisher, MD., PhD (Chief of Dermatology, Massachusetts General Hospital). 80% confluent cells were transfected in 12-well plates for 24 h along with pRL-SV40 Renilla luciferase (Promega; Madison, WI) as a control for transfection efficiency. Cell lysates were derived and Firefly/Renilla luciferase activity was assayed on a plate reader according to manufacturer’s protocol (Dual Luciferase Reporter Assay System; Promega). Experiments were performed in triplicate and repeated three times. Data shown is of a representative experiment after normalization to Renilla luciferase with error bars representing ±SD. A Student’s t-test was used to determine statistical significance.
Statistical analyses

Statistical analyses were performed using JMP10 Pro (SAS Institute Inc.; Cary, NC) and SigmaPlot (Systat; San Jose, CA). Error bars represent ± SD. Results are the means ± SD of three biologic replicates of a representative experiment. Each experiment was repeated a minimum of three times. A student’s t-test was used to calculate p-value. p-values of less than 0.05 were considered statistically significant.
CHAPTER I

Smad7 Restricts Melanoma Invasion by Restoring N-cadherin Expression and Establishing Heterotypic Cell-Cell Interactions In Vivo

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**ABSTRACT**

**Summary:** The list of TGF-β-related proteins in non-canonical TGF-β signaling is growing. Examples include receptor-Smads directing micro-RNA processing and inhibitory-Smads, e.g. Smad7, directing cell adhesion. Human skin grafts with fluorescently-tagged melanoma cells revealed Smad7-expressing cells positioned themselves proximal to the dermal-epidermal junction and failed to form tumors, while control cells readily invaded and formed tumors within the dermis. Smad7 significantly inhibited β-catenin T41/S45 phosphorylation associated with degradation and induced a 4.5-fold increase in full-length N-cadherin. Cell adhesion assays confirmed a strong interaction between Smad7-expressing cells and primary dermal fibroblasts mediated via N-cadherin; while control cells were incapable of such interaction. Immunofluorescent analysis of skin grafts indicated N-cadherin homotypic interaction at the surface of both Smad7 cells and primary dermal fibroblasts, in contrast to control melanoma cells. We propose that Smad7 suppresses β-catenin degradation and promotes interaction with N-cadherin, stabilizing association with neighboring dermal fibroblasts, thus mitigating invasion.

**Significance:** DsRed-labeled 1205Lu metastatic melanoma cells, GFP-labeled keratinocytes and human dermal fibroblasts were combined to generate human skin onto the dorsa of nude mice and then monitored for tumor growth via fluorescence imaging. Histology revealed cells expressing Smad7 remained proximal to the dermal-epidermal junction, while control cells invaded. We find both β-catenin and N-cadherin mediate this interaction. We propose that Smad7 can revert the phenotype to that of a radial growth phase melanoma, a significantly lessened state of disease. To our knowledge this is the first report describing a mechanism by which Smad7 directs cell adhesion via N-cadherin and limits cell invasion in vivo.
RESULTS

In vivo Imaging Using Fluorescently Labeled HFK with GFP and 1205Lu cells with DsRed

We have found that in vivo human skin grafts are a useful tool in skin biology capable of addressing keratinocyte, melanocyte and fibroblast transformation and subsequent invasion, a utility not possible with subcutaneous injection. Typically, cell-type identification in vivo can be accomplished via antibody staining, however few markers exist for precise identification of only melanoma cells without expression in neighboring keratinocytes. This is particularly true for melanoma cells, such as 1205Lu, that only weakly express commonly used melanoma markers such as S100 or MART1. Therefore, in order to accurately determine the localization of Smad7-expressing cells, we generated 1205Lu-Smad7 and vector control (1205Lu-Vc) cell lines stably expressing DsRed. Additionally, to determine proper incorporation of grafted human foreskin keratinocytes (HFK), we stably expressed GFP (Figure 2B). Primary human foreskin fibroblasts (HFF) were grafted without a fluorescent tag. Smad7 overexpression as well as the accompanied loss of phosphorylated-Smad3, a known marker for the active TβR complex, is depicted in Figure 2A.

In vivo Fluorescence Imaging

Following grafting, animals were tracked for tumor growth using live animal fluorescence imaging over the course of 25 days. Spectral profiles of GFP-HFK and DsRed-1205Lu grafts exhibited characteristic 498/515nm and 558/583nm excitation/ emission, respectively (Supplemental Figure S1). Animal autofluorescence was corrected through the use of spectral-
Smad7 Inhibits Tumorigenesis Using Human Skin Grafts

Small tumors were identified in 1205Lu-Vc grafts within 12 days (Figure 2C), while tumor growth was entirely absent from 1205Lu-Smad7 grafts up to 25 days (Figure 2D). Representative 20X and 40X hematoxylin and eosin (H&E) staining depicted a large tumor mass in 1205Lu-Vc grafts, while 1205Lu-Smad7 grafts allowed proper epithelial formation and lacked tumor formation (Figure 3A, upper panel). 1205Lu-Vc grafts began to form tumors within one week following removal of silicon domes and in most cases did not allow for the formation of mature epidermis present in 1205Lu-Smad7 grafts. Mature 1205Lu-Smad7 epidermis displayed histology similar to that of human epidermis (Figure 3A, middle panel) as well as normal epidermal immunocytochemical markers including basal (K5/K14) and suprabasal (K1/K10) keratin pairs (not shown). The short time to tumor formation, lack of epithelial development and presence of necrosis (Figure 2D, upper panel, center portion of tumor) are all indicative of the aggressive nature of the 1205Lu line and explain the inability of 1205Lu-Vc grafts to maintain a mature epithelium. Additionally, animals were successfully grafted with primary human foreskin melanocytes (HFM). Pigmented HFM were isolated, with permission at Georgetown University Medical Center, from neonatal human foreskin and cultured as described in Materials and Methods. Once pelleted, melanocytes have the characteristic brown pigment and can be seen visually and under phase-contrast or brightfield microscopy (Figure 3B). Pigmented primary melanocytes were grafted and found incorporated into the lower epidermis and were readily
identifiable via brightfield microscopy (Figure 3A, bottom left). As expected, primary melanocytes do not reside in the dermis, nor do they form tumors and therefore serve as control for cell positioning within the grafting experiments.

Although previous work has shown no appreciable difference in proliferation between 1205Lu-Vc and 1205Lu-Smad7 cells in vitro, in order to determine if this is maintained in vivo Ki-67 immunostaining was performed. Tissue analysis revealed that 13-22% of 1205Lu-Vc cells were Ki-67 positive and proliferative, compared to 3-6% of 1205Lu-Smad7 cells (Supplemental Figures S2A and S2B). These results were consistent with the observed tumorigenesis. In addition, to address whether apoptosis was playing a role in limiting proliferation following introduction of Smad7, cleaved caspase-3 expression was examined and quantitated in vitro as well as in vivo (Supplemental Figures S2C and S2D). In vitro examination of caspase-3 activity indicated no statistically significant (p>0.18) difference in expression when comparing 1205Lu-Vc versus 1205Lu-Smad7 cells, with caspase-3 expression ranging from 2.3-6.8% and 1.1-3.6%, respectively. Likewise, in vivo immunostaining reflected in vitro results with a similarly low level of basal expression of cleaved caspase-3 (Supplemental Figure S2D). Grafted tissue sections from matched day 12 animals were stained for cleaved caspase-3 and no statistical difference (p>0.93) in expression was found when comparing 1205Lu-Vc with 1205Lu-Smad7 each with ranges of 3.4-8.6% and 2.5-8.9%, respectively. These results indicated apoptosis did not play a significant role in limiting proliferation and tumorigenesis in 1205Lu cells expressing Smad7.

**Localization of 1205Lu/S7-expressing cells in Human Skin Grafts**
Frozen sections of 1205Lu-Vc and 1205Lu-Smad7 grafts were then analyzed for localization of 1205Lu cells with respect to the epidermal and dermal layers of the skin grafts. Day 12 grafts showed a readily identifiable tumor mass via DsRed-labeled 1205Lu-Vc cells (Figure 4A). Interestingly, 1205Lu-Smad7 cells localized just below the dermal-epidermal junction and formed a mature epithelium. More striking was, occasionally, 1205Lu-Smad7 cells were found within the epidermis, presumably interacting with keratinocytes much like that of a normal melanocyte (Figure 4B). Day 25 grafts were comparable; 1205Lu-Smad7 cells remained near the dermal-epidermal junction, while 1205Lu-Vc cells formed substantial tumor masses (Supplemental Figure S3). The inability of 1205Lu-Smad7 cells to form tumors suggested that Smad7 alone reverts the highly invasive phenotype of 1205Lu.

**In vitro Cell Adhesion Assays**

To better address whether Smad7 expression may have directed any heterotypic interactions with either primary HFK’s or primary HFF’s, modified in vitro cell adhesion assays were performed. Primary HFK’s or HFF’s (both unlabeled) were grown to confluency in 96-well plates, at which point 1205Lu cells (stained with calcein AM) were seeded over the monolayer and assayed for adherence as previously described (Maretzky et al., 2005). Cell adhesion assays established 1205Lu-Smad7 cells preferentially interacted with primary HFF’s, rather than primary HFK’s. 1205Lu-Smad7 cells exhibited a 14-fold increase in adhesion to HFF’s when compared to 1205Lu-Vc (Figure 5A); p<0.001. Representative images depict the preference of Smad7 cells for HFF’s (Figure 5A; below). Conversely, 1205Lu-Vc cells were incapable of heterotypic interaction with either HFF or HFK (Figures 5A and B). The cell adhesion assay confirmed the
predominant interaction was, in fact, with dermal fibroblasts rather than keratinocytes and indicated the observation of Smad7-expressing cells within the epidermis was not representative of the dermal interaction seen in most grafts.

To investigate the mechanism by which 1205Lu-Smad7 cells associate with fibroblasts, we pretreated 1205Lu cells with the calcium chelator EGTA, which reduced the heterotypic interaction 3.3-fold (p<0.001), indicating the association is Ca$^{2+}$-dependent (Figure 5A), suggesting a cadherin-dependent interaction. WM793, from which the metastatic 1205Lu’s are derived, express N-cadherin but not cadherin 11, cadherin 6, P-cadherin, VE-cadherin, or desmosomal cadherins (Schmitt et al., 2007; Windoffer et al., 2002). To determine if N-cadherin mediates the Ca$^{2+}$-dependent interaction, cells were pretreated with neutralizing antibody (MAb GC4) directed against the extracellular portion of N-cadherin. GC4 significantly diminished 1205Lu-Smad7/ HFF interaction compared to either untreated or IgG control-treated cells (p<0.001).

**Assessing Cell Adhesion Proteins in 1205Lu/S7 Cells**

In light of *in vitro* confirmation of a heterotypic cell-cell interaction, the mechanism behind this interaction was further investigated. Changes in expression and localization of the adhesion-related catenins and cadherins have been implicated in cellular invasion and advancing disease; as such we explored the expression patterns of these proteins. Figure 6A shows immunoblot analysis, while Figure 6B depicts quantification of proteins examined. Protein expression profiles indicated a significant (p<0.05) increase in total β-catenin protein when comparing 1205Lu-Smad7 cells to 1205Lu-Vc. Primary HFK total cell lysate was used as control. 1205Lu-
Vc and 1205Lu-Smad7 cells did not exhibit differences in expression of α-catenin and do not express E-cadherin. Conversely, a significant increase (4.5-fold; p<0.001) in levels of full length (FL) N-cadherin was observed in 1205Lu-Smad7 cells compared to 1205Lu-Vc. In addition, vimentin, an intermediate filament commonly used in diagnosing malignant melanoma and whose upregulation correlates with poorer prognosis (Hendrix et al., 1992), was significantly reduced in cells expressing Smad7 (p<0.001). Since loss of N-cadherin has been identified as a hallmark of cell migration and advanced disease in other cell types (Maretzky et al., 2005; Mochizuki and Okada, 2007; Van Hoorde et al., 1999), the increase in FL N-cadherin expression suggested a mechanism by which 1205Lu-Smad7 cells remain adjacent to the dermal-epidermal junction, while 1205Lu-Vc cells readily invaded the dermis. Taken together, stabilized N-cadherin and depleted vimentin suggested a less migratory phenotype. In addition, putative N-cadherin cleavage products detected by N-cadherin immunoblot (not shown) were further examined by immunoprecipitation followed by mass spectrometry and, unfortunately, were determined to not be of N-cadherin origin. Ectodomain shedding or cleavage products of N-cadherin have been associated with cell migration and invasion (Figure 6C, N-cadherin domains detected with antibody clones) (Kohutek et al., 2009; Reiss et al., 2005). Yet, mass spectrometry determined the additional products detected by N-cadherin antibody were in fact cross-reactivity with the intermediate filament, vimentin (Figure 6D, results mass spectrometry data table). To determine if increased FL N-cadherin was a function of TGF-β inhibition, cells were treated with 10 µM SB431542 (Sigma-Aldrich St Louis, MO), a TGF-β receptor kinase inhibitor, for either 24 or 48 h. SB431542 blocked phosphorylation of Smad3, indicating that the compound was
inhibiting TGF-β activation. However, no change, in fact a slight reduction in FL N-cadherin was observed, indicating a TGF-β receptor-independent pathway (Supplemental Figure S4).

We next explored the TGF-β receptor-independent role of Smad7 on FL N-cadherin protein levels. Previous studies have shown that Smad7 binds and stabilizes E-cadherin/β-catenin complexes, promoting cell-cell interaction (Tang et al., 2008). We therefore investigated if a similar mechanism could be enhancing 1205Lu-Smad7/ HFF interactions, in this case via N-cadherin by performing coimmunoprecipitation experiments. Immunoprecipitation of endogenous β-catenin revealed its interaction with FL N-cadherin and Smad7 in 1205Lu-Smad7 but not 1205Lu-Vc cells, as determined by immunoblot analysis (Figure 7A). The efficiency of β-catenin immunoprecipitation was confirmed by detection of this protein in both cell types following immunoprecipitation (Figure 7A, left) despite lower levels of β-catenin in total cell extract (Figure 7A, right). Likewise, the reciprocal immunoprecipitation with FLAG (fused to Smad7) revealed its interaction with β-catenin in 1205Lu-Smad7 cells (Figures 7B). As expected, immunoblot analysis revealed efficient Smad7 immunoprecipitation in 1205Lu-Smad, but not 1205Lu-Vc cells. These data suggest direct interactions between Smad7, β-catenin and FL-N cadherin. To confirm that the increases in the levels of β-catenin and FL N-cadherin were in fact due to ectopic Smad7 expression and not an artifact of culture conditions and/or additional genetic changes, we reduced Smad7 levels by transfecting cells with pooled siRNAs specific for Smad7. siRNAs specific for Smad7 reduced levels of Smad7, β-catenin and FL N-cadherin, but not GAPDH control, compared to cells transfected with scrambled siRNA (Figure 7C).
Since β-catenin levels were reduced in 1205Lu-Vc cells, we examined phosphorylation of β-catenin at threonine residue 41 and serine residue 45, which target β-catenin for ubiquitin-mediated degradation (Nelson and Nusse, 2004; Peifer and Polakis, 2000). Immunoblot analysis revealed a significant increase in β-catenin T41/ S45 phosphorylation in 1205Lu-Vc cells, compared to 1205Lu-Smad7 cells (Figure 7D, E; p<0.01). Taken together, the increased levels of β-catenin (Figure 6A), direct Smad7/ β-catenin/FL N cadherin interactions (Figure 7A, B), as well as decreased β-catenin phosphorylation in response to Smad7 (Figure 7D) indicate a novel role for Smad7 in N-cadherin-mediated adhesion in melanoma, similar to that reported for E-cadherin in breast cancer models (Edlund et al., 2005; Tang et al., 2008).

Subcellular localization of β-catenin and N-cadherin in vitro and in vivo

Potential differences in subcellular localization of β-catenin and N-cadherin were then assessed in 1205Lu-Vc versus 1205Lu-Smad7 cells. Confocal immunofluorescence identified significant changes in localization of both β-catenin and N-cadherin when comparing 1205Lu-Vc versus 1205Lu-Smad7 cells. Here, redistribution of β-catenin from punctate, cytoplasmic and nuclear localization seen in 1205Lu-Vc is in contrast to the nearly exclusive membranous expression in 1205Lu-Smad7 cells (Figure 8A). Immunoblot blot showed that while total β-catenin levels were higher in 1205Lu-Smad7 cells, β-catenin expression was minimal in the nucleus of 1205Lu-Smad7 cells (Figures 6A, 8B). Additionally, N-cadherin localization reflected that of β-catenin, as both proteins appear co-localized at the cell surface in overlaid images of 1205Lu-Smad7 cells. Redistribution of catenin and cadherin to the cell surface in 1205Lu-Smad7 cells suggests these proteins are present and available for cell-cell interactions. N-cadherin
immunofluorescence was then used to address whether these stable homo- and heterotypic interactions are preserved in vivo. Tissue from matched day 9 1205Lu-Vc and 1205Lu-Smad7 grafts identified that N-cadherin was noticeably reduced in 1205Lu-Vc cells, yet beyond the tumor border N-cadherin was found expressed in dermal fibroblasts (Figure 8C left panel, images iii and v respectively). In contrast, 1205Lu-Smad7 cells, as well as interacting fibroblasts in the microenvironment maintained N-cadherin expression. Data presented in Figure 8A identified N-cadherin found at the periphery of 1205Lu-Smad7 cells supported homotypic interaction; high resolution analysis shown in Figure 8D shows a similar pattern for N-cadherin in vivo, whereby peripheral N-cadherin expression coordinated both heterotypic and homotypic interactions. These results confirmed 1205Lu-Smad7 grafts retained the N-cadherin-directed interaction with dermal fibroblasts in vivo. These data therefore illustrate a mechanism for mitigated tumorigenesis. The above observations support the notion that Smad7 can suppress the invasive/ metastatic phenotype of the 1205Lu line through preservation of β-catenin and, subsequently, N-cadherin.
**DISCUSSION**

From a clinical standpoint, *in situ* radial growth phase (RGP) melanoma is restricted to the epidermis, while invasive-RGP has a unique presentation defined by a small non-expansile cluster of transformed melanocytes present in the upper dermis that have little proliferative capacity (Guerry et al., 1993). Progression to vertical growth phase (VGP) melanoma is characterized by highly proliferative, neoplastic nests found migrating throughout the dermis, which may then advance to metastatic melanoma. We propose that overexpression of Smad7 in the metastatic 1205Lu line results in reversion from that of a metastatic phenotype, to an invasive-RGP melanoma, a significantly lessened stage of disease. Progression from invasive-RGP to VGP correlates with a significant drop in patient survival (Clark et al., 1989). Therefore, identifying mechanisms associated with this process are essential for improving patient outcome. Here, we sought to determine the localization of Smad7 expressing cells within the human epidermis; in so doing we provide mechanistic insight for how Smad7 adversely effects the invasive nature of melanoma cells. Our previous studies (Javelaud et al., 2005; Javelaud et al., 2007) showed a TGF-β-dependent pathway was necessary for subcutaneous tumor formation and metastasis. We utilized a skin graft system in the current study to examine an additional TGF-β-independent pathway through which β-catenin becomes stabilized and redirected to the cell surface in response to Smad7. We propose this redistribution promotes interaction with FL N-cadherin and contributes to stable cell-cell contacts thereby abrogating tumor formation. Further, we propose that both pathways (TGF-β-dependent and -independent) are necessary but not sufficient for tumorigenesis. Thus, inhibition of TGF-β prevents tumorigenesis (Javelaud et al.,
2005; Javelaud et al., 2007), while blocking N-cadherin inhibits cell adhesion (Figure 4) which appears to be necessary for heterotypic interactions of 1205Lu-Smad7 \textit{in vivo} (Figures 3 and 6). Loss of E-cadherin, coupled with upregulation of N-cadherin is an important event in melanoma progression. Studies using multiple melanoma cell lines show that restoring E-cadherin re-establishes contact with keratinocytes and inhibits melanoma invasion (Hsu et al., 2000). Further, ectopic expression of N-cadherin has been shown to increase motility in cultured melanoma cells (Ebisawa et al., 2001). Therefore, it was surprising that we found an association between increased levels of N-cadherin and loss of tumorigenicity in the current study. However, it has been proposed that subsequent to its upregulation, reduction in N-cadherin can result in disruption of cell-cell adhesion and enhanced invasion (Dwivedi et al., 2009; Mochizuki and Okada, 2007; Okada, 2007). For example, depleted FL N-cadherin is required for cell migration in human glioblastoma cells as well as mouse embryonic fibroblasts (Kohutek et al., 2009; Maretzky et al., 2005). Although another study shows that blocking N-cadherin via the small molecule inhibitor ADH-1 prevents melanoma metastasis, however melanoma migration within the dermis was untested (Augustine et al., 2008). Moreover, while ADH-1 sensitizes tumors to chemotherapy, it also increases tumor growth on its own. For transformed melanocytes within the dermis, blocking N-cadherin may in fact exacerbate disease by eliminating the stable, heterotypic interactions governed by cadherins as suggested by \textit{Nakagawa and Takeichi} (Nakagawa and Takeichi, 1998). Furthermore, elevated N-cadherin protein levels in patient-derived melanomas can correlate with improved survival, while depleted N-cadherin was associated with a less favorable patient outcome (Kreizenbeck et al., 2008).
We extend these findings by describing that reduced FL N-cadherin expression correlated with melanoma invasion seen in the 1205Lu-Vc grafts. Conversely, accumulation of FL N-cadherin was associated with the non-tumorigenic 1205Lu-Smad7 graft (Figures 2 and 5A). Furthermore, using cell adhesion assays we find a stable interaction among Smad7-expressing melanoma cells and primary dermal fibroblasts that is governed through N-cadherin (Figure 4A), an effect previously unreported in response to Smad7 expression. Moreover, N-cadherin’s role in adhesion is underscored through *in vivo* N-cadherin immunofluorescence, which detected N-cadherin in both 1205Lu-Smad7 cells and surrounding fibroblasts. However, N-cadherin staining of vector control tissue showed no expression within the tumor microenvironment and was only found in dermal fibroblasts outside the tumor border (Figures 6C and D). To our knowledge this is the first *in vivo* report describing preservation of heterotypic cell-cell interactions in response to Smad7. Our findings with Smad7 reinforce the proposal that tight cell-cell adhesion, *via* a mature cadherin/catenin interaction, is detrimental to cell migration (Nakagawa and Takeichi, 1998).

Based upon these results, we propose a model for cadherin switching in the context of Smad7 in the progression of metastatic melanoma (Figure 7). Downregulation of E-cadherin results in a loss of anchorage of normal melanocytes to keratinocytes in the upper epithelium. As melanocytes undergo transformation, downregulation of E-cadherin coupled with an increased N-cadherin allows transformed melanocytes access to fibroblasts. Data presented here and elsewhere support the notion that cells harboring stabilized N-cadherin, in this case *via* Smad7, are limited in their capacity to invade. However, cellular populations with reduced levels of N-cadherin effectively lose stable heterotypic interactions with dermal fibroblasts, resulting in
contribution to advanced disease, at which time N-cadherin may be again upregulated at distant metastatic sites.
FIGURES, GRAPHS AND DATA TABLES
(CHapter I)
Figure 2: Expressing Smad7 in 1205Lu cells and Stable GFP-HFK's and DsRed-1205Lu Metastatic Melanoma Cells. (A) Blocking TGF-β in 1205Lu cells results in loss of Smad3 phosphorylation. Expression of Smad7 and p-Smad3 are shown; GAPDH is the loading control. (B) Primary HFKs stably expressing GFP (top); 1205Lu-Vc and 1205Lu-Smad7 melanoma cell lines stably expressing DsRed, respectively (middle and lower panels). Each figure contains 20X phase-contrast insets.
Figure 2: 1205Lu-Vc Grafts Form Tumors Suppressed by Smad7. (C) Day 12 Grafts. Left-to-right: grayscale images, DsRed-expressing 1205Lu cells, GFP-expressing primary HFKs and overlaid images far right (animal autofluorescence is depicted in pink). Small palpable tumors present in 1205Lu-Vc animals were detectable by day 12, while 1205Lu-Smad7 expansion appeared minimal. (D) Day 25 Grafts. 1205Lu-Vc animals had a large tumor mass depicted via DsRed, while 1205Lu-Smad7 growth remained comparable to day 12. In addition, 1205Lu-Vc animals frequently contained localized necrosis (upper panel, arrowhead). Four animals were used per condition.
Figure 3: Histology of Human Skin Grafts. (A) Left, representative H&E of day 12 1205Lu-Vc (top panel) and 1205Lu-Smad7 (middle panel) grafts. Day 12 (left panel) shows 1205Lu-Smad7 grafts formed a mature epidermal layer; while 1205Lu-Vc showed a dense, proliferating melanoma largely devoid of epithelia. Day 25 (right panel) shows representative H&E of day 25 1205Lu-Vc grafts showed a dense invading melanoma, while 1205Lu-Smad7 formed normal epithelia and lacked tumor formation. Left, bottom image, primary melanocytes correctly positioned themselves within the lower epidermis and were identifiable via brightfield microscopy (arrow). In order to identify the epidermis, HFK-GFP cells are shown as an overlay in primary melanocyte graft; 40X inset depicts melanocyte localization. As expected, primary melanocytes did not result in tumor formation and therefore serve as control for the graft itself as well as melanocyte localization. Four animals were used per condition.
Figure 3: Isolating Primary melanocytes. (B) Primary melanocytes were derived from neonatal human foreskin; top panel shows pelleted melanocytes after trypsinization. Middle panel shows plated melanocytes on day 4 with a mixture of differentiating primary keratinocytes; keratinocytes can be differentiated and removed from the culture in the presence of CaCl$_2$. Bottom panel, phase contrast and bright field images of primary melanocytes at day 10 (unpublished data).
Figure 4: Grafted 1205Lu-Smad7 Cells Reside Proximal to the Dermal-Epidermal Junction, while 1205Lu-Vc Cells Invade the Dermis. (A) 20X epifluorescence microscopy. 1205Lu-Smad7 graft showed Smad7-expressing cells reside just below the dermal-epidermal junction (arrow), while 1205Lu-Vc grafts depict an invading melanoma. Overlaid images are shown far right. Epidermis and dermis are depicted in the right-hand margin. (B) 40X confocal microscopy, via DsRed, showed the predominant localization of 1205Lu-Smad7 cells (upper panel) seen in most grafts. Occasionally, 1205Lu-Smad7 cells were found residing in the upper-epidermal layer (lower panel). Overlaid images are shown right and depict: GFP-keratinocytes (green); DsRed-Smad7 cells (red) and DAPI-stained nuclei (blue). White arrowheads identify the positioning of 1205Lu-Smad7 cells within the grafts.
Figure 5: 1205Lu-Smad7 Cells Form Stable Associations with Dermal Fibroblasts. (A) HFF monolayers were overlaid with either 1205Lu-Vc or 1205Lu-Smad7 cells. 1205Lu cells stained with calcein-AM were either left untreated or treated with EGTA; neutralizing MAb GC4 N-cadherin antibody; or matched IgG isotype control. (B) Same as above, though HFK’s were used in place of HFF’s. 1205Lu-Smad7 cells did not form adhesions with primary HFK; instead distinct adhesions with primary dermal fibroblasts were formed. No adhesions were present using 1205Lu-Vc cells with either HFF or HFK. Percent adherent cells are plotted, 1205Lu-Vc are depicted in black bars; Smad7 are shown in gray. Representative images of HFF cells overlaid with either 1205Lu-Vc or 1205Lu-Smad7 cells are shown below. *Note: a small portion of labeled 1205Lu-Vc cells can be seen at top left of image, while in 1205Lu-Smad7 the cells appear attached throughout. This experiment was performed three independent times. Results are the means ± SDs of three biological replicates of a representative experiment.
Figure 6: Smad7 Restores Full-Length N-cadherin Protein Expression. (A) From top to bottom: 30µg of total cell lysate was probed for β-catenin, α-catenin, E-cadherin, full-length (FL) N-cadherin and vimentin. HFK total cell lysate was used as a positive control. Total β-catenin appeared increased in Smad7 cells (p<0.05). Full-length N-cadherin (~130kDa) was also significantly enhanced in 1205Lu-Smad7 cells when compared to 1205Lu-Vc and represented a 4.5 fold increase over vector control (p< 0.001). Full-length N-cadherin was detected using monoclonal N-cadherin clone-32 antibody (BD Biosciences). Vimentin, an intermediate filament commonly used to diagnose malignant melanoma, was significantly reduced in cells expressing Smad7 when compared to control (p<0.001). (B) Densitometry for each of the cell adhesion proteins assayed: β-catenin, N-cadherin and vimentin each reached statistical significance.
Figure 6: Structure of N-cadherin and Mass Spectrometry Data. (C) Image shows the ectodomain composed of cadherin repeats (shown in black), detected by GC4 N-cadherin antibody clone, the region cleaved by ADAM-10 proteases. The C32 antibody clone detects the intracellular portion of N-cadherin following cleavage, resulting in 76kDa and 58kDa fragments. Both antibodies also recognize full-length N-cadherin. (D) Mass-spectrometry data following N-cadherin immunoprecipitation. N-cadherin was immunoprecipitated using the C32 antibody, separated on SDS-PAGE and stained using silver stain. Bands representing 58kDa fragment was excised from the gel and ran on MALDI-TOF MS. The results indicate these fragments were not of N-cadherin origin, but rather were cross-reactivity with vimentin (unpublished data).
**Figure 7: Smad7 Interacts with β-catenin in vitro.** (A) Endogenous β-catenin immunoprecipitation was performed and followed by immunoblot with: anti-N-cadherin, anti-β-catenin, and anti-Smad7. β-catenin immunoprecipitated Smad7; Smad7 is shown on left panel below the IgG heavy chain (IgG_HC). β-catenin also immunoprecipitated a proportional amount of N-cadherin in Smad7 cells. Molecular weights are indicated. 15% Inputs are shown on right in each figure. (B) anti-Smad7-FLAG immunoprecipitated Smad7 bound to endogenous β-catenin, confirmed by probing with both anti-β-catenin and anti-Smad7. (C) 120nM of Smad7-siRNA reduced both β-catenin and N-cadherin, indicating the effect was Smad7 specific. (D + E) 50µg protein was probed for phosphorylated-β-catenin T41/ S45. Smad7 expression reduced the amount of β-catenin targeted for degradation (p<0.001). Experiments were performed three independent times. Results are the means ± SDs of three biological replicates of a representative experiment.
Figure 8: Smad7 Redistributes β-catenin and N-cadherin to the Cell Surface in vitro. (A) Upper panel, 40X confocal immunofluorescence shows cells co-stained for β-catenin and N-cadherin (H-63 Santa Cruz Biotechnology Santa Cruz, CA). Neither β-catenin nor N-cadherin was appreciably expressed at the cell surface in 1205Lu-Vc cells, demonstrating the inability of 1205Lu-Vc cells to engage in cell-cell adhesion. Lower panel, 1205Lu-Smad7 cells showed membranous co-localization of both β-catenin and N-cadherin, indicating both proteins are present at the cell surface. (B) Cells were subjected to nuclear and cytoplasmic fractionation; 50µg of each fraction was analyzed. 1205Lu-Vc cells showed an increased proportion of β-catenin in the nucleus, while cells expressing Smad7 showed cytoplasmic accumulation and limited nuclear expression.
Figure 8: Promotes Heterotypic Cell-Cell Interactions via N-cadherin in vivo. (C) Representative tissue collected from day 9 grafts was stained for N-cadherin (DAPI in blue; DsRed-1205Lu in red; GFP-HFK in green; N-cadherin in light blue). Left panel, 1205Lu-Vc cells lacked N-cadherin expression (panel iii) when overlaid, confirming, in vivo, 1205Lu-Vc cells do not engage in cell-cell adhesion via N-cadherin. Lowest left panel showed fibroblasts outside the tumor border expressed N-cadherin. Right panel, N-cadherin expression was observed at the cell periphery of both 1205Lu-Smad7 cells and fibroblasts. (D) DAPI is shown in blue; DsRed-1205Lu in red; and N-cadherin in green) 100X confocal immunofluorescence shows 1205Lu-Vc cells lacked N-cadherin expression in vivo. Lower image, both 1205Lu-Smad7 cells and dermal fibroblasts show N-cadherin expression. White boxes isolate shared 1205Lu-Smad7/fibroblast border and arrowheads identify peripheral N-cadherin localization.
Figure 9: Melanoma Progression Model. 1) E-cadherin (shown in black) directs cell-cell adhesion between normal melanocytes and normal keratinocytes. 2) In melanoma, E-cadherin expression is often reduced resulting in a loss of attachment to keratinocytes. 3) A concomitant increase in N-cadherin (shown in light blue) establishes linkage to dermal fibroblasts. 4) During invasion, reduction of N-cadherin can permit transformed cells to migrate through the dermis. This results in loss of a stable interaction between melanoma and fibroblasts thereby contributing to disease progression. Smad7 expression can limit invasion by stabilizing the cell-adhesion related proteins β-catenin and subsequently N-cadherin, while 1205Lu cells lacking Smad7 continue to invade and show reduced N-cadherin expression at their surface, although N-cadherin may be re-upregulated at distant metastatic sites.
SUPPLEMENTARY FIGURES
(CHAPTER I)
**Figure S1:** *Representative Spectral Plots.* Left and right panels compare HFK-GFP versus 1205Lu-DsRed target signal (top panel), followed by autofluorescence intensity (middle panel). Correction following spectra-unmixing shows enhanced, true signal devoid of autofluorescence (lower panel). Target signal emission following spectral correction was plotted in dashed lines along with the corrected autofluorescence (dashed white curve).
Figure S2: 1205Lu-Vc Grafts are Proliferative. Matched 1205Lu-DsRed grafts were stained for the proliferation marker Ki-67. (A) Top right panel, 1205Lu-Vc cells positive for Ki-67. Lower right panel, 1205Lu-Smad7 cells were predominantly Ki-67 negative. (B), Quantitating Ki-67 expression. Results indicated approximately 13-22% of 1205Lu-Vc cells express Ki-67, compared to only 3-6% of 1205Lu-Smad7 cells. (C) Assaying Apoptosis. Caspase-3 cleavage was addressed by immunofluorescence (DAPI is shown in blue; caspase-3 in green) and western blot. An average of 4.2% of 1205Lu-Vc cells showed activated caspase-3 compared to an average of 2.6% of 1205-Smad7 cells. As positive control, cells were treated with 1µM Staurosporine for 4h and then assayed for caspase-3 cleavage. No statistical difference (p>0.19) in caspase-3 activity was found when comparing 1205-Lu-Vc versus 1205Lu-Smad7 cells; this was confirmed by western blot.
Figure S2: Activated caspase-3 in vivo (20X). (D) Tissue from matched day 12 grafts also showed no difference (p>0.93) in caspase-3 activation, quantification is shown on the right. Five high-power fields representing >500 cells were used to assess both Ki-67 and caspase-3 activation. Bottom panel shows caspase-3 staining in human squamous cell carcinoma and was used as a positive control for active caspase-3 expression. Apoptotic areas can readily be seen in the center of each image while surrounding tissue lacks caspase-3 activation.
Figure S3: *Grafted 1205Lu-Smad7 Cells Remain Near the Dermal-Epidermal Junction at Day 25.* Tissue from matched day 25 tissue sections of 1205Lu-Smad7 grafts show Smad7-expressing cells remain just below the dermal-epidermal junction and do not invade, in contrast to the large tumor masses found in vector control. Overlaid images are shown far right. Epidermis and dermis are depicted in the right-hand margin.

Figure S4: *Pharmacological Inhibition of TGF-β Does Not Affect N-cadherin Expression.* 1205Lu cells were grown in serum free media for 24h then treated with 10 µM TGF-β receptor kinase inhibitor SB3431542 (Sigma-Aldrich St. Louis, MO) for 24 and 48h. 30µg of total cell lysate was separated on 8% SDS-PAGE and then probed for N-cadherin (clone-32, BD Biosciences). Loss of phospho-Smad3 was used to show TGF-β inhibition. Neither 24h nor 48h of treatment effected N-cadherin resulted in appreciable changes in N-cadherin.
CHAPTER II

Id2, Id3 and Id4 Overcome a Smad7-Mediated Block in Tumorigenesis, Generating TGF-β-Independent Melanoma

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The role for the inhibitors of differentiation (Id) proteins in melanomagenesis has been poorly explored. In other cell types, Ids have been shown to contribute to cell proliferation, migration and angiogenesis and, along with a number of other genes, are direct downstream targets of the TGF-β pathway. Expression of Smad7, which suppress TGF-β signaling, or synthetic TGF-β inhibitors, were shown to potently suppress melanomagenesis. We found that endogenous Id2, Id3 and Id4 expression was elevated in 1205Lu versus 1205Lu cells constitutively expressing Smad7, indicating Ids may play a role in melanomagenesis. Therefore, the effects of Tet-inducible expression of Id2, Id3, or Id4 along with Smad7 in TGF-β-dependent 1205Lu human melanoma cells were explored in vitro and in vivo. 1205Lu cells formed subcutaneous tumors in athymic mice, whereas cells expressing Smad7 failed to form tumors. However 1205Lu cells expressing Smad7 along with Doxycycline-induced Id2, Id3 or Id4, were able to overcome the potent tumorigenic block mediated by S7, to varying degrees. Histology of tumors from 1205Lu cells expressing Smad7+Id4 revealed an average of 31% necrosis, compared to 5.2% in tumors from 1205Lu with vector only. The amount of necrosis was consistent with expression of the proliferation marker Ki-67, regardless of tumor size. Downstream, Ids re-upregulated invasion and metastasis-related genes MMP2, MMP9, CXCR4 and osteopontin, previously shown to be downregulated in response to Smad7. This study shows that Id2, Id3, and Id4 are each able to overcome TGF-β dependence, and establish a role for Ids as key mediators of TGF-β melanomagenesis.
RESULTS

Upregulation of Id2, 3, and 4 in 1205Lu Metastatic Melanoma Cells

Endogenous Id protein expression was examined in TGF-β-dependent and -independent melanoma cell lines (Figure 10). Immunoblots were probed for Id2, Id3, or Id4. In general, Id protein expression was variable across the multiple melanoma cell lines examined, this was not unexpected as certain Ids share some overlapping functions, for example Id2 with Id4 (Coppe et al., 2003; Lyden et al., 1999; Samanta and Kessler, 2004). Id2 was weakly expressed in human foreskin melanocytes (HFM) and increased in melanoma. Id3, was not observed in HFM, yet was expressed in MNT1 and 1205Lu, and at higher levels in the more proliferative Mel501, Sk-28 and WM852 melanoma cells. Interestingly, Id4 was expressed in pigmented HFMs and at low levels in 1205Lu/Vc, or not detected in other melanoma lines. The loss of Id4 protein expression may be due to promoter methylation, as has been observed in other cancer types (Yu et al., 2005). Importantly, Id2, Id3 and Id4 each appeared to be TGF-β-dependent, as they were observed elevated in 1205Lu/Vc when compared to 1205Lu cells expressing Smad7 (1205Lu/S7) (Figure 10, lanes 6 and 7). These results suggested that Ids may revert 1205Lu/S7 cells back to the tumorigenic phenotype observed with 1205Lu/Vc cells.

Role of Ids in Proliferation

Effects of Id2-4 on cell cycle were first assessed in normal HFM (Figure 11A). To determine if Id mediated changes in cell proliferation in vitro, HFM expressing GFP were compared to cells expressing Id2 (top), Id3 (middle) or Id4 (bottom). In HFM expressing either Id2 or Id3, S-phase
was significantly increased indicating an alteration to cell proliferation, with a more robust increase (1.94-fold; p<0.001) detected in cells expressing Id3. Id3 also resulted in a concomitant drop in G1-phase (p<0.001) and increased G2/M (p<0.001). HFM cells expressing Id4 did not result in statistically significant changes to the percentage of cells in the cell cycle, suggesting that Id4 may not significantly alter proliferation or division in melanocytes.

To investigate if Id2, Id3, or Id4 expression alters proliferation or tumorigenesis in Smad7-expressing 1205Lu melanoma cells, stable transfectants of each Id, in a tetracycline-on (tet-on) expression system, were generated in 1205Lu/S7 cells (Materials and Methods). Individual cell colonies were isolated following the introduction of each construct (S7/Id2, S7/Id3, and S7/Id4), and assayed for expression of Ids (Figure 11C), as well as Smad7 and p-Smad3 (Figure 11B). TGF-β signaling was completely inhibited. Since we previously showed Id2 increases both S-phase and contributes to increased tumorigenesis in mouse fibroblasts (Trabosh et al., 2009), we predicted a similar effect in melanoma and therefore performed cell cycle analysis of 1205Lu/S7/Id-expressing cells in the presence or absence of Tet. However, no changes in S-phase were observed in S7/Id2, S7/Id3 or S7/Id4 (Figure 11E). Previous work showing the same 1205Lu cells expressing Smad7 which mitigated tumorigenesis in vivo also did not result in changes to cell growth in vitro (Javelaud et al., 2005). Yet, a 1.8-fold increase (p<0.01) in G2/M was observed in S7/Id2 cells at 72 and 120 h after Tet induction (Figure 11D). S7/Id3 cells did not exhibit any significant changes in the cell cycle. However, similar to the S7/Id2-expressing cells, S7/Id4 cells showed a 1.3-fold increase (p<0.01) in the G2/M population 120 h after tet induction (Figure 11D), suggesting an overlapping relationship in cell division between Id2 and Id4 expression in 1205Lu melanoma cells.
Since G2/M was elevated in cells expressing both Id2 and Id4 after 72 and/or 120h in culture we began to investigate proteins involved in the G2/M pathway (Figure 11F). Phosphorylation of histone H3 at serine 10 has been used to detected entry into M-phase as increased phosphorylation is strongly associated with chromatin condensation (Preuss et al., 2003). Phosphorylation begins late in G2 but levels of p-histone H3 (S10) are highest during prophase/metaphase when the chromosomes are highly condensed and phosphorylation persists until anaphase when condensation is relieved and serine 10 phosphorylation is no longer detected (Hendzel et al., 1997). Here, we focused on Id2 and induced gene expression for 120h in order to replicate the observations seen in total cell cycle analysis described above (Figure 11D). We also used colcemid (24h) as a positive control to induce a G2 block in 1205Lu cells and then stain cells with p-histone H3 (S10) (Cell Signaling) followed by flow cytometry. Colcemid treatment functioned as a appropriate positive control as treated cells increased from an average of 12% to 27% in G2 phase and from an average of 2% to 11% in M-phase. However, Id2 did not result in any detectable changes to cells in M-phase, potentially indicating the effects of Id2 expression in 1205Lu cells may be directed at G2 rather than M-phase. Therefore, using immunoblot we assessed proteins integral to the G2 checkpoint using total cell lysate. Proteins were analyzed following Id2 induction over a time course of 48, 72 and 120h either in the presence or absence of tetracycline. However, only a slight increase in total cyclin B1 levels was observed at the 72h time point (Figure 11H). We next addressed G2 protein activity by immunoblot with p-cdk1 T14/Y15. De-phosphorylation of cdk1 at T14/Y15 is performed through activity of the dual-specificity phosphatase cdc25 B/C and de-phosphorylation is an integral step for cells to progress into G2 (Jin et al., 1998). We find increased T14/Y15
phosphorylation of cdk-1 at 72h and 120h, while total levels of cdk-1 did not change (Figure 11I). This suggests cells expressing Id2 are not permitted entry into G2 as the same rate as cells lacking Id2 expression. We then assessed Wee1 kinase, the kinase responsible for opposing cdc25 de-phosphorylation and find Wee1 also increased at 72 and 120h, potentially explaining the elevated levels of p-cdk-1 observed. We next investigate cyclin B1 localization and find cells expressing Id2 restrict cyclin B1 to the nucleus, as it is well known cyclin B1 nuclear exit is require for cells to progress through G2-phase and enter M-phase (Jin et al., 1998). Finally, we observe an increase in the number of cells containing multinucleation when expressing Id2 for 120h (Figure 11K and 11L). Ten low power (20x) images were randomly collected from cells expressing Id2 after 120h, and cells harboring 2 or more nuclei were quantitated as multi-nucleated and plotted. We find a statistically significant increase in the number of cells with multinucleation at both the 72h (p<0.001, 1.70 fold increase) and 120h (p<.01; 1.73 fold increase) time points. Unfortunately, attempts to further elucidate the mechanism associated with increased p-cdk-1 and increased Wee1 kinase expression were not fruitful. Wee1 and Myt1 kinase as well as cdc25 B/C have multiple residues associated with activating and inactivating phosphorylation and the mechanism involved could not be explored, especially since cdc25B/C antibodies were unreliable in our hands. Therefore, since multi-nucleation and cell cycle alterations were observed we re-focused efforts upon examining alteration to the tumorigenic potential of 1205Lu cells co-expressing Smad7 and Ids.
Role of Id2-4 in Melanomagenesis

We then sought to examine whether Id2, Id3 or Id4 can bypass the TGF-β requirement for tumorigenesis in 1205Lu cells expressing Smad7. Therefore, 1205Lu/S7/Id cells were subcutaneously injected into athymic mice and maintained ad-libitum with doxycycline-supplemented feed to induce Id expression. 1205Lu/Vc formed vigorously growing tumors, while 1205Lu/S7 did not, as we previously reported (DiVito et al., 2010). However, 1205Lu cells harboring Smad7 along with Id2, Id3 or Id4 (S7/Id2, S7/Id3, or S7/Id4) were each able to establish subcutaneous tumors only in the presence of doxycycline. The ability these cells to form tumors in the absence of TGF-β signaling (Figure 12A), completely bypasses the previously established requirement for the TGF-β pathway for melanomagenesis in 1205Lu cells (Javelaud et al., 2005; Javelaud et al., 2007). Animals xenografted with S7/Id3 or S7/Id4 had the largest tumors when compared to either S7 or S7/Id2 (Figure 12B and Table 1). In the absence of doxycycline, animals xenografted with the same cells did not develop a single tumor, showing the absolute requirement for Ids in these cells (Figs. 12A, right panel and 12C). These results support the hypothesis that these Ids are able to overcome the Smad7-mediated block in tumorigenesis and strongly support a role for Ids in melanomagenesis.

To assess tumor proliferative potential, tissues were harvested and stained with the Ki-67 proliferation marker. 1205Lu/Vc and S7/Id3 tissues had the highest percentage of Ki-67 cell positivity, with averages of 15.3% and 14.5%, respectively. Interestingly, S7/Id2 and S7/Id4 had fewer Ki-67 positive cells (6.1% and 6.9%, respectively; Figs. 13A+B). This was unexpected as S7/Id4 tumors had substantial growth observed via fluorescence imaging. However, after examining H&E sections an extensive amount of multi-focal necrosis was found within S7/Id4
tumors, explaining the low number of Ki-67 positive cells observed. To quantify necrosis, ten low power (20x) images of H&E sections were analyzed per tumor, and necrotic areas were analyzed using Image J analysis software (NIH). Necrotic areas were then expressed as a percentage of total area per image. S7/Id4 tumors had extensive necrosis, with an average of 31% (Figure 13C + D). By contrast, 1205Lu/Vc tumors exhibited an average of only 5.2% necrosis, while S7/Id2 and S7/Id3 showed minimal necrosis with 0.13% and 0.95%, respectively. Even the aggressive 1205Lu/Vc tumors, which had substantially more tumor growth than those expressing Id4, did not show the degree of necrosis observed in Id4 sections, suggesting an active process rather than one that arose due to an exhaustion of tumor resources.

To investigate the mechanism by which Ids bypass TGF-β-mediated tumorigenesis, Id2, Id3, or Id4 were transiently expressed in both 1205Lu/Vc and 1205Lu/S7-expressing cells; stable downregulation was not performed, as Ids have overlapping functions. qRT-PCR was then performed to examine four tumor-promoting genes previously shown to be down-regulated in response to Smad7 in 1205Lu cells (Javelaud et al., 2007). Id2, Id3 and Id4 strikingly derepressed MMP2, MMP9, CXCR-4, and osteopontin (Figs. 14A-C), suggesting that Id2, Id3 and Id4 play a role downstream of TGF-β-mediated tumorigenesis. In fact, Id2, Id3 and Id4 even upregulated MMP2, MMP9, osteopontin and CXCR4 in 1205Lu parental cells. CXCR4 was observed to have the most robust upregulation in response to Id expression across each of the different Ids examined.
**DISCUSSION**

Id2, Id3 and Id4 are each able to overcome the potent tumorigenic block provided by Smad7 in 1205Lu metastatic melanoma cells, thus generating TGF-β independent tumors in the previously TGF-β-dependent 1205Lu melanoma cells. Previous work has shown that both in the presence of Smad7, the endogenous TGF-β antagonist, as well as synthetic TGF-β inhibitors, melanomagenesis is blocked in 1205Lu cells, establishing the requirement for TGF-β in transformation (Javelaud et al., 2005; Javelaud et al., 2007). Observations shown here suggest an Id-related tumorigenic mechanism acting downstream or independently of TGF-β. We believe that Ids mediate tumorigenesis primarily downstream of TGF-β (Figure 14D, thick arrows) because 1) Smad7 blocks Id expression, and 2) Re-expression of Ids overcome Smad7-mediated repression of tumorigenesis. We further hypothesize that the mechanism responsible was due, in part, to elevated MMP2, MMP9, CXCR4 and osteopontin expression previously shown to be downregulated in response to Smad7 (Javelaud et al., 2007). In our model, these four genes are re-upregulated, to varying degrees, in response to Id2, Id3 and Id4 expression, with CXCR4 demonstrating the most robust response. MMPs have a strong link to invasion and tumorigenic progression and are Id-dependent, as Id2 and Id3 silencing results in a potent loss of MMP gene expression (Asirvatham et al., 2007); however the association between MMPs and Id4 was unknown prior to this work. Ectopic Id4 expression has also been shown to elevate USF-1 expression levels in cervical cancer cells (Pagliuca et al., 1998). Increased CXCR4 expression in response to Id2-4 in the current study may therefore be due to upregulation of upstream stimulatory factor-1 (USF-1), which can associate with an E-box at -260bp in the CXCR4
promoter, stimulating its expression (Moriuchi et al., 1999). The induction of osteopontin that we observed may be due to the interaction of Ids with their known partner E47. This would disrupt E47/Twist bHLH heterodimers, relieving repression of osteopontin. This was shown to be the mechanism by which ectopic Id expression in mesenchymal cells induces osteopontin (Hayashi et al., 2007).

In addition, increased necrosis (Figs 13C + D) was observed in tumors expressing S7/Id4 (31%) when compared to 1205Lu (5%), yet 1205Lu had even larger tumors than mice xenografted with S7/Id4 (Figs 12A + B). Further analysis of S7/Id4 tumor histology revealed an immune cell infiltration composed of tissue histocytes. We believe this phagocytic component found in S7/Id4 tumors may have also contributed to the observed necrosis and therefore reduced proliferation detected by Ki-67 staining; this mechanism is currently under investigation.

In conclusion, we find that Id2, Id3 and Id4 are each able to contribute significantly to melanomagenesis independent of TGF-β in 1205Lu melanoma cells. Further work is required to address whether TGF-β inhibitors might inadvertently select for Id-positive tumors, and could be used as adjuvants with Id-antagonists, such as Id aptamers, which are currently under study (Lahn et al., 2005; Mern et al., 2010). At present, only anti-Id4 appears to be a reliable measure of Id protein levels for use on paraffin sections in our hands. However, in order to fully address any relationship between Id expression and prognosis in melanoma, future studies will need to examine Id status in patient-derived tumors and determine correlation with overall survival.
Figures, Graphs and Data Tables
(Chapter II)
Figure 10: Increased expression of Id2, Id3 and Id4 in 1205Lu/Vc versus 1205Lu/Smad7. Endogenous Id2 (top; 15 kDa), Id3 (middle; 15 kDa) and Id4 (bottom; 19 kDa) protein were probed across multiple melanoma cell lines using immunoblot analysis with 80 µg of total cell lysate and specific anti-sera against Id proteins. Id expression was variable across melanoma cell lines with Id2 and Id3 expression more prominent in the more proliferative MNT1, Sk-28, 501Mel and WM852 cells. In 1205Lu cells, increased endogenous expression of Id2, Id3 and Id4 were detected when 1205Lu was compared to 1205Lu cells expressing Smad7. Endogenous Id4 was observed only in primary HFM and 1205Lu cells lacking Smad7-expression. GAPDH (34 kDa) is used as a loading control.
Figure 11: Id2 and Id3 increase S-phase in primary HFM, but not in 1205Lu. (A) HFM were transiently transfected with Id2 (top), Id3 (middle) or Id4 (bottom) and subjected to cell cycle analysis after 48 h; the percentage of cells in each cell cycle phase are plotted. HFM cells expressing either Id2 or Id3 showed a statistically significant increase in percentage of cells in S-phase of the cell cycle; while Id4 resulted in no significant changes. (B) Smad7 blocks TGF-β expression in 1205Lu cells. Cells were subjected to immunoblot with antibodies to Smad7 (upper panel; 51 kDa) or p-Smad3 (middle panel; 52 kDa) to confirm Smad7 expression and inhibition of TGF-β signaling. (C) Stable clones of 1205Lu/S7 melanoma cells were subjected to immunoblot with antibodies to Id2 (top), Id3 (middle), or Id4 (bottom) to confirm Id protein expression.
**Figure 11: Id2 and Id4 Increase G2/M in 1205Lu cells.** Stable S7/Id2 (top), S7/Id3 (middle) or S7/Id4 (bottom) clones were grown in presence or absence of tetracycline over a 48, 72, and 120 h time course showed increased percentage of cells in G2/M phase in both Id2 and Id4-expressing cells. Statistically significant changes are depicted with asterisk(s) and were compared to matched un-induced populations. No changes in S-phase were detected in S7 cells expressing Id2, Id3, or Id4. Analyses were performed in triplicate and represent biological replicates. Error bars represent ±SD. (F) G2/M pathway. Image shows proteins involved in the G2/M pathway. Important mediators of this checkpoint are cyclin B1 and cdk-1 (also known as cdc2). Cdc25B/C is known as the dual specificity phosphatase removing inhibitory phosphorylation from cdk-1. Wee1 and Myt1 kinase oppose cdc25 through re-phosphorylation.
**Figure 11:** Assessing the G2/M Pathway. (G) Id2 expression does not affect M-phase in 1205Lu cells. Cells were grown in the presence of absence of tetracycline for 120h, then stained for p-histone H3 (serine 10). Cells were treated with colcemid (0.05ug/ml; 24h) as a positive control from G2 arrest. No changes to histone H3 were detected in the presence of Id2. (H) Immunoblot analysis of G2/M related proteins. Total lysate was harvest at the indicated time point and assayed for cyclin B1 and cdk-1, however only a slight increase in total cyclin B1 was detected (unpublished results).
I. Assaying cdk-1 Activity. P-ck1 T14/Y15 was assessed by immunoblot. (I) P-cdk-1 was found increased in cells expressing Id2 both at 72 and 120h (+tet lanes). Wee1 kinase levels were not reproducibly changed (unpublished results). (J) Cyclin B1 localization. Subcellular localization of cyclin B1 was assessed in cells expressing Id2 after 120hr. Nuclear accumulation of cyclin B1 was evident in cells expressing Id2 (right) (unpublished results).

Figure 11: Assaying cdk-1 Activity. P-cdk1 T14/Y15 was assessed by immunoblot. (I) P-cdk-1 was found increased in cells expressing Id2 both at 72 and 120h (+tet lanes). Wee1 kinase levels were not reproducibly changed (unpublished results). (J) Cyclin B1 localization. Subcellular localization of cyclin B1 was assessed in cells expressing Id2 after 120hr. Nuclear accumulation of cyclin B1 was evident in cells expressing Id2 (right) (unpublished results).
Figure 11: Multinucleation in 1205Lu cells expressing Id2. (K) Cells grown in the presence of tetracycline over a 120h time course contained multinucleation at both the 72 and 120h time point. Images from the 120h time point are shown and multinucleated cells are indicated in blue asterisks. (L) Quantitating Multinucleation. Ten random low power (20X) images were captured after 120h of Id2 expression. Cells containing two or more nuclei were counted as multinucleated, cells from both the 72 and 120h time point reached statistical significance (unpublished results). Error bars represent ± standard deviation, where 0.05=*, 0.01= **; 0.001=***.
Figure 12: Id2, Id3 or Id4 expression bypasses Smad7-mediated block in tumorigenesis. (A) S7/Id cells were xenografted into athymic mice (n=8) continuously fed with doxycycline-supplemented feed to induce Ids and were subjected to live-animal fluorescence imaging. Representative images of animals taken at days 7, 19, 33, 44 and 60 are shown; no tumor growth in S7/Vc animals (top) and prominent tumor formation in all 1205Lu/S7 cells expressing Ids (lower panels). Right panel shows representative images of animals lacking tumor formation after being injected with the same S7/Id clones grown in the absence of doxycycline (B) Tumor size was monitored over the time course and plotted for mice fed with doxycycline. (C) No tumor growth was observed in animals maintained with feed lacking doxycycline, 1205Lu/Vc cells are shown for comparison.
Figure 12: Tumor lysates were probed for Id2, Id3 or Id4 expression. (D.) Protein expression indicates Ids were expressed in vivo and GAPDH was used as a loading control. Statistical analyses using analysis of variance (ANOVA) across all pairs was performed and are shown in Table 1 above.
Figure 13. Assaying tumor Proliferation and Necrosis. (A+B) Ki-67 immunofluorescence was performed to assess in vivo proliferation; Ki-67 (red), DAPI (blue). Five high-power fields (40X) representing >300 cells/field were used to count the number of Ki-67-positive cells; % Ki-67 positive cells in tumors are shown. Ki-67 expression is quantitated and compared to 1205Lu/S7. All Id expressing tumors were more proliferative than non-tumorigenic S7-expressing cells. Error bars represent ±SD. (C+D) Examining necrosis in xenografts. Ten low power (20X) images were randomly captured across each tumor section; necrosis was readily detected and encircled using Image J software (NIH). Representative images are shown. Tumors expressing S7/Id4 had an average of 31% necrosis, while other S7/Id expressing tumors had little observed necrosis.
A. Id2

**Figure 14.** Id2-4 Increases Expression of Tumor-Promoting Genes. Id2 (A), Id3 (B), Id4 (C) were transiently expressed in 1205Lu/Vc 1205Lu/S7 cells and subjected to qRT-PCR using primers specific to MMP2, MMP9, osteopontin, and CXCR4. Fold-change in expression was calculated after normalization against ß-actin. Sk28 cells with low metastatic potential were used as a negative control. Transfections were performed in biological triplicate, cells were collected after 48 hours and representative results are shown. Error bars represent ±SD.
Figure 14. *Id2-4 Increases Expression of Tumor-Promoting Genes.* *Id2* (A), *Id3* (B), *Id4* (C) were transiently expressed in 1205Lu/Vc 1205Lu/S7 cells and subjected to qRT-PCR using primers specific to MMP2, MMP9, osteopontin, and CXCR4. Fold-change in expression was calculated after normalization against β-actin. Sk28 cells with low metastatic potential were used a negative control. Transfections were performed in biological triplicate, cells were collected after 48 hours and representative results are shown. Error bars represent ±SD.
Figure 14: Model of alternate pathways for the role of Ids in TGF-β-mediated melanomagenesis.
CHAPTER III

Inhibitor of Differentiation-4 (Id4) Induces Tumorigenesis and Pigmentation in Advanced Melanoma Leading to Histiocyte Infiltration and Tumor Necrosis

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TGF-β signaling, Id proteins and tumorigenesis are all interrelated. Current chemotherapeutics for the treatment of melanoma are only marginally effective. Immunotherapy provides the most promise, yet innate immunity is often overlooked. Smad7 (S7) and other TGF-β inhibitors have been shown to potently suppress melanomagenesis; however, little work examining the proliferation-associated inhibitors of differentiation (Id) in melanoma has been reported, particularly for Id4. Subcutaneous injection of TGF-β-dependent 1205Lu human melanoma cells co-expressing Id2, Id3, or Id4, with Smad7 bypassed the tumorigenic block mediated by S7, generating TGF-β-independent tumors and questioning the role of Ids as co-oncogenes as they have been referred. Most surprisingly, 1205Lu/S7 cells expressing Id4, but not Id2 nor Id3, activated robust melanin production in amelanotic 1205Lu cells, confirmed by Fontana-Masson stain and de-novo expression of MART-1 and tyrosinase protein. In human tumors, melanin correlated with Id4 localization. Mechanistic investigation revealed increased nuclear M-MITF and MART-1 promoter activation in both 1205Lu and WM852 melanoma cells, suggesting broader implications for Id4 in melanoma. Additionally, pigment-laden CD163+ mouse histiocytes, with areas of extensive necrosis were found throughout 1205Lu/S7/Id4 tumors, but not in parental, 1205Lu/S7/Id2 or 1205/S7Id3-derived tumors. This work better defines the role of the inhibitors of differentiation, particularly Id4, in advanced melanoma and may have implications for new pathways in developing immunotherapeutics in the treatment of melanoma.
RESULTS

Upregulation of Ids and correlation of Id4 with pigment in human melanoma

A small cohort of human melanomas (n=4) was examined for Id expression by immunofluorescence analysis. Id2 and Id3 antisera were not reliable since cells transfected with Id2 or Id3 stained similarly to untransfected populations (Figure S5A, B). The Id4 antibody was validated for immunofluorescence using ectopic expression of Id4 in 1205Lu and WM852 cell lines. Id4 expression was observed in 2/4 human melanomas, mostly localized to pigmented areas (Figure 15A), suggesting that Id4 may play a role in melanin synthesis.

Endogenous Id protein expression was then examined in TGF-β-dependent and -independent melanoma cell lines (Figure 15B). Immunoblots were probed for Id2, Id3, or Id4; Id1 was not examined in this study. Id2 was weakly expressed in HFM and increased in melanoma. Id3, not observed in HFM, was expressed in MNT1 and 1205Lu, and at higher levels in the more proliferative Mel501, Sk-28 and WM852 melanoma cells. Id4 was expressed in pigmented HFMs, and at low levels in 1205Lu-vector control cells (1205Lu/Vc), or not detected in other melanoma lines. The loss of Id4 protein expression may be due to promoter methylation, as has been observed in other cancer types (Yu et al., 2005). Id2, Id3 and Id4 were TGF-β-dependent, since they were elevated in (1205Lu/Vc) compared to 1205Lu expressing Smad7 (1205Lu/S7) (Figure 15B, lanes 6 and 7). These results suggest that Ids could revert 1205Lu/S7 cells to the tumorigenic phenotype observed with 1205Lu/Vc.
**Role of Ids in pigmentation and proliferation in cultured cells**

We found that Id4 associates with MITF, a protein integrally associated with both pigmentation and proliferation (Figure 15D) (Lee et al., 2006). While Id4 is associated with pigmentation in tumors, ectopic expression of Id4 did not seem to be able to alter expression of pigment-associated proteins in HFM (Figure 15C).

**Role of Id4 in Pigmentation**

1205Lu metastatic cells were originally derived in the 1980’s by Dr. Meenhard Herlyn’s laboratory from the WM793 vertical growth phase (VGP) cell line. Both cell lines are amelanotic and do not produce melanin in culture, nor pigmented lesions in vivo. Remarkably, then, were what appeared to be darkly pigmented tumors in all 1205Lu/S7/Id4 animals (Figure 16A-C). In contrast, no trace of pigment was detected in 1205Lu/Vc, 1205Lu/S7, S7/Id2 or S7/Id3 tissues. To confirm melanin deposits, sections were stained with Fontana-Masson. 1205Lu/S7/Vc, 1205Lu/S7/Id2 and S7/Id3 tissues were stained in parallel with pigmented normal human skin as a positive control. Melanin identified by Fontana-Masson stain was readily detected via light microscopy and appears as black deposits in both the S7/Id4 tumors and positive control human skin (Figure 16D, lower panel); H&E’s were used for comparison (Figure 16D, upper panel). Thus, S7/Id4 co-expression not only overcomes the tumorigenic block provided by Smad7, but at the same time, the tumors appeared “differentiated”, as melanin production was re-established.

When tumor lysates were subjected to immunoblot analysis, expression of Id2, Id3, or Id4 was confirmed (Figure 17A). Immunoblotting with antisera specific for pigment-associated proteins revealed TYR expression only in S7/Id4 and S7/Id2 tissues (Figure 17B). Strong
MART-1 was also readily detected in S7/Id4 and S7/Id2 tumors, and inhibition of TGF-β signaling in Smad7-expressing tumors was confirmed by decreased p-Smad3. In the absence of Id4 or Id2, endogenous MART-1 protein is undetectable in 1205Lu cells. A significant accumulation of total MITF in S7/Id3 and Id4 tumors was also noted, compared with that of 1205Lu/S7 tissue, similar to the levels observed in 1205Lu/Vc (Figure 17B). Interestingly, elevated levels of MITF alone do not appear to be sufficient to re-establish pigment synthesis, as S7/Id3 tumors did not contain melanin yet had comparatively high levels of MITF expression. MITF RNA levels were also examined in cultured cells following Id4 transfection. However, Id4 did not result in changes in MITF transcription (Figure S7), suggesting that the mechanism of upregulation is at the protein level. MITF subcellular localization was then assessed using cell fractionation followed by immunoblot analysis with polyclonal antisera that identifies both A- and M-MITF. An accumulation of nuclear M-MITF (Figure 17C) was observed in both 1205Lu/Vc and 1205Lu/S7 transfected with Id4. An accumulation of phosphorylated nuclear M-MITF was also observed in WM852 cells, thus broadening the role for Id4 in process of pigmentation (Figure 17D). Taken together, these observations indicate that melanin synthesis was initiated de novo in response to Id4 induction, as TYR and MART-1 were not detected in parental 1205Lu/Vc, 1205Lu/S7/Vc, nor S7/Id3-derived tumors. Furthermore, while 1205Lu/Vc cells express endogenous MITF, it appears non-functional in terms of activating pigment production in the absence of Id4 and Smad7.

S7/Id2, S7/Id3 and S7/Id4 paraffin sections were subjected to further analysis using immunofluorescence with S100- and MART-1-specific antisera in order to verify melanoma origin of each cell, and to identify MART-1 subcellular localization. S7/Id2 and S7/Id4 tissue
sections were positive for both MART-1 and S100, while S7/Id3 tissue was positive only for S100 (Figure 17E). The localization of both S100 and MART-1 was cytoplasmic, as expected.

**MART-1 Expression In Vitro**

We focused further on MART-1 for two reasons. First, it is an early gene product involved in pigmentation. Secondly, MART-1 antigen has been used to prime patient-derived T-cells in the treatment of melanoma. To elucidate the mechanism involved in Id4-induced pigmentation, 1205Lu/Vc and 1205Lu/S7 cells were transfected with empty vector or Id4 for 24 and 48 h. Immunoblot analysis of cell extracts using monospecific antibody revealed that MART1 expression is induced in both 1205Lu/Vc/Id4 and S7/Id4 cells by 24h and more strongly after 48h (Figure 17F). S7/Id4 cells induced MART-1 more robustly at 24 h than 1205Lu/Vc/Id4 cells, although Id4 alone was sufficient to induce expression. Lysates were also probed for Id4, Smad7 and p-Smad3 and confirmed TGF-β inhibition in S7 cells in culture, similar to that observed in Id4 tumors (Figure 17F). Interestingly, TYR was not induced in vitro as it was in tumors (Supplemental Figure S8). To determine if induction was at the level of RNA, semi-quantitative RT-PCR was performed. MART-1 RNA was also elevated in both 1205Lu/Vc/Id4 and S7/Id4 cells (Figure 17G).

To address whether Id4 affects MART-1 promoter activity, 1205Lu/Vc or 1205Lu/S7 cells were transiently transfected with empty vector (Vc) or Id4, in the presence of either the 1.9 kb MART-1 promoter containing E-boxes important for MITF binding (wtMART-1), or the same promoter with inactivating E-box point mutations (E1/E2/MART-1), linked to a luciferase reporter (Du et al., 2003). A *Renilla* luciferase construct was included for normalization. Dual
luciferase assays of 1205Lu/Vc and S7 cells transfected with Id4 and wtMART-1 promoter revealed a 1.9 fold induction of the MART-1 promoter (p<0.01) (Figure 17H). In contrast, the double E-box mutant (E1/E2/MART-1) promoter was not Id4-responsive (p<0.05). To examine if these results were 1205Lu cell-specific, another human melanoma line was examined. WM852 cells are also amelanotic and have low levels of endogenous MART-1 protein. Id4 transfection in WM852 cells resulted in a similar induction, with a 2.3-fold increase in promoter activity (p<0.001); when the E1/E2 mutant replaced the wt MART-1, a significant (p<0.05) loss of activity was observed (Figure 17I).

Finally, chromatin immunoprecipitation (ChIP) was used to determine whether MITF binds the MART-1 promoter more readily in the presence of Id4. Here, 1205Lu cells were again transfected for 48h, cells were harvested and ChIP was performed, followed by semi-quantitative PCR according to established protocols (Du et al., 2003). We find Id4 enhances the binding of MITF protein to both the upstream and downstream MART-1 promoter regions previously described (Figure 17J + K) (Du et al., 2003). Taken together these results indicate that Id4 can induce MART-1 promoter in vitro in the two different melanoma cell lines examined, and provides mechanistic insight for the observations in vivo.

**Histiocyte Infiltration in 1205Lu/S7/Id4**

While re-starting pigment production was an interesting and unexpected finding, further analysis of tumor sections revealed another noteworthy aspect of these lesions. Pathologist examination of S7/Id4 tissue H&E sections suggested the presence of tumor-associated histiocytes and multifocal areas of necrosis. Histiocytes are of the monocyte/macrophage lineage and have been
previously observed in melanoma via CD163 expression (Bronkhorst et al., 2011; Jensen et al., 2009). Sections were co-stained with MART-1 and CD163 to differentiate melanoma cells from histiocytes. Cytoplasmic/membranous localization of CD163 on S7/Id4 sections confirmed clusters of histiocytes concentrated around areas of intense pigmentation (Figure 18A). Mouse-specific CD163 immunoreactivity confirmed that these cells must be host-derived as the antibody does not cross-react with human CD163 antigen. Furthermore, co-localization of MART-1 with CD163 in cells containing melanin indicated that histiocytes were likely phagocytosing Id4-expressing melanoma cells (Figure 18A, far right). Adjacent tumor cells that were MART-1+/CD163− exhibited reduced melanin production as determined by brightfield microscopy, suggesting that these cells may be in the initial phases of melanin production. As control, S7/Id2 and S7/Id3 sections were co-stained with MART-1 and CD163. Id2 tissues were MART-1+ and CD163−, while Id3 tissues were MART-1− and CD163− (Figure 18A, left and middle). Additionally, CD163+/ MART-1+ S7/Id4 cells contained multiple pyknotic nuclei, indicating that melanoma cells were phagocytosed and then became necrotic (Figure 18B). We hypothesized that necrotic areas may have been pigment-producing locales targeted by histiocytes, and, indeed, Fontana-Masson staining revealed necrotic areas containing remnants of melanin (Figure 18C).

Taken together, the data suggest the following sequence: 1) Id4 induction, 2) de novo MART-1 expression, followed by 3) melanin production, 4) an innate immune response driven by histiocyte recruitment and triggered by melanin, and 5) phagocytosis of melanin-producing S7/Id4 cells by mouse-derived histiocytes. To examine if human melanomas recruit histiocytes, sections of human melanomas were stained using human-specific antisera to CD163. Three of 4
tumors examined were observed positive for CD163 (Figure 18D). Similar to Id4 expression (Figure 1A), CD163 expression appears prominent around pigmented areas.

We have proposed a model for the role of Ids in pigmentation in melanoma (Figure 19). Here we suggest normal melanocytes which typically express MART-1 and are capable of presenting MART-1 antigen (Ag) at the cell surface undergo transformation through a multitude of mechanisms. Some of these mechanisms involve TGF-β overexpression together with any combination of increased MMP2, MMP9, osteopontin or CXCR4. Through data presented here we find Id2 and Id4 are capable of influencing the pigmentation pathway by contributing to re-expression of MART-1 and tyrosinase, while Id3 does not appear to play a role in this process. We also find enhanced MITF binding to MART-1 promoter via ChIP, which likely contributes to the enhanced MART-1 expression. Further, it appears melanin synthesis and/or release from S7/Id4 cells in vivo, triggers an innate immune response composed of tissue macrophages (histiocytes) which also coincided with extensive multi-focal areas of necrosis. Whether histiocyte activation can contribute to an adaptive immune response via activation of T-cells is yet to be determined.
**DISCUSSION**

Id4 expression in human tumors coincided with melanin synthesis, and based upon those initial observations, we further examined the roles of Id2, Id3 and Id4 in metastatic melanoma (Figure 1A). In human melanoma cells, Id2, Id3 and Id4 expression was upregulated in 1205Lu/Vc cells compared to 1205Lu cells expressing Smad7 (Figure 1B), suggesting that, in 1205Lu/Vc melanoma cells, TGF-β stimulates Id expression, in contrast to its role in normal cells where Ids are typically repressed. In fact, Id2 has been shown to be an important gene in determining melanoma aggressiveness by suppressing TGF-β activation of p15^Ink4b^ (Cummings et al., 2008; Schlegel et al., 2009). Furthermore, Id4 was found to directly associate with MITF (Figure 1D). Ids altered the cell cycle in both melanocytes and melanoma cells (Figure 2A,C). Id2 and Id4, but not Id3, increased G2/M phase cells (Figure 2C) and multinucleation (Figure S1), potentially linking these two genes in melanocyte function, and suggesting a role for Ids in cell proliferation and possibly tumorigenesis.

We and others have previously reported that Smad7 inhibits melanomagenesis by down-regulating tumorigenic/metastatic related genes, as well as by promoting cell adhesion via β-catenin and N-cadherin (DiVito et al., 2010; Javelaud et al., 2005; Javelaud et al., 2007). In the current study, Id4 bypassed the Smad7-mediated reduction in MMP2, MMP9, osteopontin, and CXCR4 in cultured cells (Figure 3E), and induced tumors *in vivo* (Figure 3A). MMPs have a strong link to tumorigenic progression and are Id1-3 dependent, as Id silencing results in a potent loss of MMP gene expression (Asirvatham et al., 2007). Increased CXCR4 expression in response to Id4 may be E-box dependent, since upstream stimulatory factor-1 (USF-1) can
associate with an E-box at -260bp along the CXCR4 promoter, stimulating its expression. Furthermore, ectopic Id4 expression maintains elevated USF-1 expression in cervical cancer cells (Moriuchi et al., 1999; Pagliuca et al., 1998), which could potentially explain the observed CXCR4 response. Similarly, osteopontin activation requires TGF-β/BMP, R-Smad, and Smad4. Additionally, although Twist, a bHLH transcription factor stabilized by E47, functions as a negative regulator of the BMP pathway by inhibiting Smad4, Id1 induction in mesenchymal cells has been shown to overcome Twist-mediated repression of Smad4 (Hayashi et al., 2007).

Id4 expression induces pigmented lesions in vivo (Figure 4), and upregulates MART-1 protein, RNA, and the gene promoter in an E-box-dependent manner, in both 1205Lu/Vc and 1205Lu/S7 cells (Figure 5). This appears to be a general phenomenon, since Id4 also upregulates the MART-1 promoter in WM852 melanoma cells, which are derived from a different patient (Figure 5H). These results suggest a novel Id4-MITF-MART1 pathway. While MITF RNA levels were unchanged by Id4 (Figure S4), Id4 elevated MITF protein levels in nuclear fractions (Figure 5C). This nuclear MITF appears as a lower molecular weight doublet, with sizes corresponding to those of melanocyte-specific MITF (M-MITF), previously shown to be restricted to the nucleus due to the loss of the N-terminal nuclear export signal (NES) (Lu et al.). Id4 therefore either alters MITF gene promoter choice, or preferentially stabilizes the smaller M-MITF forms. Given that Ids function by sequestering other bHLH proteins, together with the direct binding we observe in HFM, Id4 may directly stabilize M-MITF leading to its nuclear accumulation. In any case, MITF regulates nearly all gene products involved in melanocyte differentiation, and the levels of nuclear MITF determine which gene sets become activated (Goding, 2011; Goodall et al., 2008). MITF is also an oncogene due to mutations/amplifications.
described in patients (Garraway and Sellers, 2006), which is consistent with the increased tumorigenesis observed in this study. Id4 therefore appears to promote both early growth as well as later melanogenesis, in part, via MITF accumulation (Carreira et al., 2006; Giuliano et al., 2010; Goding, 2011). Whether this pathway also interacts with the TGF-β-Id-MMP/osteopontin/CXCR4 pathway remains to be determined.

Most studies are consistent with Ids blocking differentiation. In myeloid cells, however, Id2 mRNA was elevated when cells were stimulated to differentiate (Ishiguro et al., 1996; Park et al., 2008). Similarly, Id2 was found to stimulate PPARγ and promote adipocyte differentiation (Ishiguro et al., 1996; Park et al., 2008). Thus, Ids do not always sequester proteins in order to block differentiation, potentially diversifying the role of Ids in development.

Finally, xenografts showed CD163⁺ histiocytic infiltration and necrosis in pigmented areas (Figure 6A-C). While both S7/Id2 and S7/Id4 lesions re-expressed MART-1, only Id4 tumors contained histiocytes, indicating that the immune trigger was melanin production rather than MART-1 expression (Figure 6A). Furthermore, extensive necrosis was only observed in Id4-expressing lesions (Figure S3). Even aggressive 1205Lu/Vc tumors, which had substantially more tumor growth than those expressing Id4, did not show the degree of necrosis observed in Id4 sections, suggesting an active process rather than one that arose due to an exhaustion of tumor resources. Together with the observation that histiocytes co-stained for MART-1 and CD163, our results suggest that mouse histiocytes phagocytose melanin-producing cells and target them for removal. It is currently unknown whether the observed expression of CD163 in human melanomas (Figure 6D) is due to the same sequence of events as in the xenografts, or rather the result of melanoma transdifferentiation (Shabo and Svanvik).
Recent findings have shown that macrophages can recruit additional immune cells and present antigen to T-cells (Barrio et al., 2012). Id4 may thus amplify the adaptive response in immunocompetent animals. It should be noted that CD163 expression appears to have mixed reports as far as a correlation with patient survival. In uveal melanoma, CD163 is associated with poorer overall survival, while CD163 expression in lymphoma appears to have no correlation with disease recurrence (Harris et al., 2012; Jensen et al., 2009). One study examining CD163 in Stage I/II melanoma reports stromal histiocyte infiltration leads to poorer overall survival, however when other factors, such as tumor size, were assessed using multivariate analysis no correlation was found (Jensen et al., 2009). The above study also did not correlate CD163 expression with that of melanoma-specific markers such as MART-1. Retrospective/prospective studies will be required to expand the size of the current human melanoma cohort and examine whether Id4, MART-1, and CD163+ histiocytes are predictive of overall survival in melanoma.
FIGURES, GRAPHS AND DATA TABLES
(CHAPTER III)
Figure 15: Id4 correlates with pigment in human melanoma and endogenous Id2, Id3 and Id4 are elevated in 1205Lu cells. (A) Fixed-paraffin sections of human melanomas (n=4) were examined for Id4 expression by immunofluorescence; Left panel: Id4, red; DAPI, blue; right panel: corresponding brightfield images; dashed lines separate areas of higher pigment from lower pigment. (B) Immunoblot of endogenous Ids. Id2 (top; 15 kDa), Id3 (middle; 15 kDa) and Id4 (bottom; 19 kDa) protein in multiple melanoma cell lines; GAPDH (34 kDa) is a loading control. (C) Id4 does not alter endogenous MITF, MART-1, TRP-1 or TYR in primary HFM. Primary HFM were transiently transfected with Id4 and subjected to immunoblot with antibodies to MITF (59 kDa), MART-1 (18 kDa), TRP-1 (75 kDa) or TYR (75 kDa). Immortalized human keratinocytes (HaCaT) were used as negative control. (D) Immunoprecipitation shows Id4 associates with MITF. Transfected HFM were subjected to immunoprecipitation with anti-FLAG. Top left, MITF immunoprecipitated via Id4-FLAG; lower left, Id4-FLAG; right panels show 10% input.
Figure 16: *Id4 re-establishes melanin production in vivo.* Representative animals at Day 60 are shown. Left to right: S7/Vc, S7/Id2, S7/Id3 and S7/Id4. (B + C) Extensive pigmentation in S7/Id4 tumors before (B) and after (C) paraffin embedding.
Figure 16: Fontana-Masson Staining reveals Melanin deposits and Id expression is confirmed in vivo. (D) Fixed tumor sections were stained with Fontana-Masson stain to detect the presence of melanin, which appear as brown/black deposits throughout S7/Id4 tissue and positive control normal human skin, but not in the other xenografts. Pigmented human skin is used as positive control where melanin can be seen deposited in basal keratinocytes. Top panel, H&E; bottom panel, Fontanna-Masson stained sections.

Figure 17: Immunoblotting for Id2, Id3 and Id4. (A) Immunoblot analysis of tumor lysates with antibodies to Id2-4 was performed to confirm protein expression; Vc and S7/Vc are shown for comparison.
Figure 17: Id4 induces MART-1 and TYR in vivo and modulates MITF localization and MART-1 promoter activity in vitro. (A) Immunoblot analysis with antibodies to pigment-related proteins TYR, MITF and MART-1; densitometry to quantify MITF expression (bottom). Id4 expression strongly upregulates MART-1 and tyrosinase in vivo, both proteins were also upregulated, though to a lesser extent, in animals expressing Id2, but not Id3. Smad7 and p-Smad3 serve as controls for TGF-β inhibition in tumor lysate.
Figure 17: *MITF Subcellular Localization.* (C+D) Transient transfection with Id4 or empty vector (Vc), followed by cell fractionation and immunoblotting with antibodies to cytoplasmic (MITF-A) and nuclear (MITF-M). (D) WM852 cells also show increased MITF-M in the presence of Id4 expression.
Figure 17: S100 and MART-1 immunofluorescence overlays (40X). S100, red; MART-1, green; DAPI, blue. Insets, secondary antibody controls. Bottom image. Same as above, yet showing individual images without overlays.
Figure 17: MART-1 Expression in vitro. (F+G) Cells were transiently transfected with either Id4 or Vc and subjected to immunoblot analysis with anti-MART1 (F) or RT-PCR to assess MART-1 RNA levels (G). p-Smad3, Smad7, Id4 and GAPDH serve as controls, and β-Actin as RT-PCR loading control.
Figure 17: MART-1 Promoter Assays. (H) Cells were co-transfected with Id4 or Vc and the MART-1 promoter containing E-boxes for MITF binding (wtMART-1) linked to a luciferase reporter, or the same promoter with inactivating E-box point mutations (E1/E2/MART-1). Results of dual luciferase assays are shown, with Renilla luciferase used for normalization.
Figure 17: MART-1 Promoter Assay. (I) Again, experiments were repeated in WM852 cells to examine the broader role Id4 may be playing in melanoma. Id4 was found to upregulate the MART-1 promoter in wt constructs and not in E1/E2 mutant constructs. Left panel, wtMART1 or E1/E2 MART with point mutations depicted as dashed lines. 
Figure 17: MART-1 Chromatin immunoprecipitation (ChIP) shows MITF binds MART-1 promoter following Id4 expression. Both the upstream (left) and downstream (right) MART-1 E-boxes were assessed for MITF binding by ChIP. Id4 was found to enhance the binding of MITF to the MART-1 promoter at both sites. Sk-28 was immunoprecipitated and used as a positive control for the IP. Sk-28 genomic DNA (gDNA) was used as a positive control for the PCR. Water was used as a negative control for the PCR.
Figure 18: *Id4-induces melanin production and recruits CD163*^+ histiocyte in vivo. (A) Tumor sections were co-immunostained for MART-1 and CD163 to detect the presence of histiocytes. MART-1, green; mouse specific CD163, red; DAPI, blue. Left panel, S7/Id2, middle panel, S7/Id3; right panel, S7/Id4. (B) Multiple pyknotic nuclei observed in CD163 staining. CD163, red; DAPI, blue. (C) Necrotic areas reveal remnants of pigment in S7/Id4 as detected by Fontanna-Masson stain.
Figure 18: CD163 Immunofluorescence in human melanomas. (D) Human melanomas were subjected to immunofluorescence analysis with antibodies to human CD163. Left panel, CD163 (red), DAPI (blue); right panel, corresponding brightfield images.
Figure 19: Proposed model for tumorigenesis, pigmentation and immune response in melanoma cells expressing Ids. Top to bottom, pigmented melanocytes normally secrete melanin to adjacent keratinocytes in response to UV exposure. Normal melanocytes respond to TGF-β by downregulating Ids, limiting their tumorigenic potential. (A) Transformed melanoma cells bypass the TGF-β growth inhibition, in part through Id expression. In some cases melanin synthesis is downregulated or halted potentially by mutations in genes involved in pigmentation or through multiple aberrant steps in melanin signaling, generating amelanotic melanomas. (B) Elevated levels of CXCR4, osteopontin, MMPs and MITF, in response to Ids, likely contribute to tumorigenesis. (C) However, Id2 and Id4 expression stimulates members of the pigment pathway and melanin synthesis is established resulting in a differentiated phenotype. (D) Id3 expression restrains differentiation and favors proliferation. Id4 results in secretion of melanin, triggers an immune response in athymic mice and recruits histiocytes that engulf melanin and phagocytose melanoma cells.
SUPPLEMENTARY FIGURES
(CHAPTER III)
**Figure S5. Id4 Antibody Validation for use in Immunofluorescence.** WM852 (A) or 1205Lu/S7 (B) melanoma cells were transfected for 24 h with Id2-4 and subjected to immunofluorescence staining with antibodies to Id2-4 (Santa Cruz Biotech). Anti-Id4 specifically labels Id4 transfected cells, while antibodies to Id2 or Id3 were nonspecific and showed no difference in Id levels between transfected and empty vector control cells.
Figure S6: Spectral Plots of DsRed-expressing S7/Id cells \textit{in vivo}. Cells expressing S7 and either Id2, Id3 or Id4 were selected to constitutively express DsRed with a target signal of 583nm (top); autofluorescence is detected and plotted (middle); following spectral correction target signal still remains at 583nm (bottom).
**Figure S7:** Id4 does not alter MITF RNA expression. 1205Lu cells were transfected with either Id4 or empty vector for 24h, RNA was isolated and qRT-PCR was performed. Changes in MITF RNA expression were undetectable following Id4 transfection.

**Figure S8:** Id4 was unable to induce TYR in 1205Lu/S7 cells. Stable S7/Id4 cells were induced with tetracycline and, after 1, 7, and 14 days, subjected to immunoblot analysis with antibodies to TYR and Id4; GAPDH was used as loading control.
CHAPTER IV

The Evolving Relationship between Transforming Growth Factor-Beta and The Inhibitors of Differentiation – A Discussion

Since its discovery, in the early 1980’s, by Anita Roberts of the National Institutes of Health (NIH) and in the many years since, the role of transforming growth factor–β (TGF-β) has become ever expanding. As mentioned earlier in this work, TGF-β is the founding member of a large family of transmembrane receptors called the TGF-β superfamily; this family includes other members such as the bone morphogenic proteins (BMP), Nodal, as well as Activin-like kinase receptors (ALK). If we consider only the TGF-β and BMP members of this family for a moment, as their expression/activity directly applies to the regulation of expression of the inhibitors of differentiation (Id). Research has shown TGF-β functions as a negative regulator of cell proliferation, while BMP opposes that function, with respect to Ids, by promoting proliferation and stimulating Id expression. The above generality is true in the case of most normal, non-transformed cells (Ruzinova and Benezra, 2003). For example, in immune (B-cells), epithelial and endothelial cells TGF-β expression, through the Smad family of proteins will suppress the expression of Id1-3 genes, often through activity of the ATF3 transcription factor (Figure 1). Smad2 and Smad3 typically govern the TGF-β response mentioned above, resulting in limited Id gene expression. Yet, the BMP activity opposes that of the TGF-β family by stimulating Id gene expression, ultimately and potently influencing cell fate, much of this work has been done in immune cells such as B-cells. For example, ectopic Id2 gene expression in hematopoietic progenitor cells results in a drastic downregulation in B-cell differentiation
implicating Ids as repressors of terminal differentiation in immune cells (Ji et al., 2008; Li et al.), as mentioned this effect seems to be restricted to normal cells.

In the transformed state, such as in melanoma, the role of the TGF-β family seems to be counterintuitive, as the response to TGF-β growth inhibition becomes impotent. Melanoma cells secrete large amounts of TGF-β ligand to which cells become unresponsive, resulting in no delay in cell proliferation and desensitized cyclin dependent kinases inhibitors (CDKI), nor a reduction in Id gene expression (as we have observed, Figure 10, page 68). This had also been observed in other melanoma studies in which the lack of a response to TGF-β has largely been attributed to expression of the Id2 gene and downregulation of the CDKI p15<sup>Ink4b</sup> (Schlegel et al., 2009). Here, investigators used previously reported cell lines that have been characterized as either proliferative melanoma cells or invasive melanoma cells. As mentioned above, previous work has shown resistance to TGF-β-mediate growth suppression is a turning point in melanoma. Cells that are resistant to TGF-β are more invasive and thus are linked to a poorer overall survival in patients. When Id was examined in established TGF-β resistant melanoma cell lines, the Id2 gene was observed to be more strongly expressed in those cells which had the invasive phenotype. To refer back to my work described in this thesis, the 1205Lu melanoma cell line, which was used throughout, produces high levels of TGF-β (as determined by Smad3 phosphorylation and Smad2/3 luciferase activity assays), high levels of Ids2-4 in the absence of Smad7 and are highly metastatic (Javelaud et al., 2007). Schlegel et al also determined that when Id2 was repressed by TGF-β, the resulting cell line had a proliferative phenotype rather than that of an invasive signature. Furthermore, the mechanism of Id2 repression in these proliferative melanoma cell lines was attributed to induction of p15<sup>Ink4b</sup>.
In my work, Id2-4 expression results in re-establishment of tumorigenesis in melanoma cells expressing Smad7, we find the mechanism behind this observation is linked to upregulation of metastasis-related genes MMP2, MMP9, osteopontin, and CXCR4. When probed for p15\textsuperscript{ink4b}, we also find reduced expression in cells expressing ectopic Ids2-4, indicating suppression of p15\textsuperscript{ink4b} is important in suppressing melanomagenesis (Figure 2). Other CDKIs such as p27 and p19 were also slightly reduced in response to Id2, though not to the same extent as the reduction observed with p15. Unfortunately, there is limited data exploring the relationship of TGF-β and Ids in melanoma, which is one of the main reasons why we chose to explore this potential cooperative relationship and indeed we find a positive correlation with TGF-β, Ids and tumorigenesis in 1205Lu melanoma cells.
**Figure 1.** TGF-β and the inhibitors of Differentiation (Id) in Immune, Epithelial and Endothelial cells. In, normal cells TGF-β functions to downregulate id expression (occasionally) through ATF3, while BMP activation functions to upregulate Id expression.

**Figure 2:** CDKI expression in 1205Lu-Smad7 expressing cells. p15 Ink4b was reduced in response to Ids2-4, while other CDKIs were only marginally reduced.
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