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

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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.

Keywords

metastatic melanoma; Smad7; β -catenin; N-cadherin; human skin grafts

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 12–15% of patients diagnosed with metastatic melanoma survive to 5 years (Horner MJ, 2008). Effective treatment modalities for this disease are exceptionally limited. Even in light of multiple clinical trials dacarbazine remains the only approved therapy for metastatic melanoma and its 15% efficacy has not been shown to extend patient survival (Divito et al., 2004; Falkson et al., 1998). Nonetheless, recent studies have employed immunotherapy or small molecule inhibitors, such as imatinib (Gleevec[®]) with some success (Hodi et al., 2008). Additionally, clinical trials targeting other signaling molecules such as TGF- β , whose overexpression is a

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hallmark of many cancers including melanoma, appear promising (Schlingensiepen et al., 2006).

The transforming growth factor-beta (TGF- β) super-family of proteins are 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 (T β R2) resulting in recruitment and phosphorylation of multiple residues along the GS domain of type-1 receptor (T β R1), thereby forming a heterotetrameric TGF- β receptor complex (T β R; 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), Smad2/3, are phosphorylated by T β R1 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 (T β R) or Smad4 binding (Imoto et al., 2003). 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.

Recent evidence has shown that multiple Smad proteins engage in signaling unrelated to the TGF- β superfamily (Hoover and Kubalak, 2008). For example, posttranscriptional modification of microRNA-21 occurs *via* R-Smad-dependent interaction (Davis et al., 2008). Furthermore, Smad7's traditional role as the predominant mechanism by which the T β R is 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 (Edlund et al., 2005). Recently, *Tang et al.* showed that Smad7 directly binds β -catenin, effectively sequestering it, resulting in a loss of both GSK3 β -directed β -catenin phosphorylation and subsequent ubiquitin-mediated degradation (Tang et al., 2008). As a result β -catenin/E-cadherin complexes were stabilized. 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 et al., 2002; Hazan et al., 2000; 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 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).

We have recently shown that stable over-expression of Smad7 in 1205Lu metastatic melanoma cells reduces 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 work, 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 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 blocked 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 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.

RESULTS

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 1A). 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 T β R complex, is depicted in Figure S1.

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 (Figure S2). Animal autofluorescence was corrected through the use of spectral-unmixing. Following a sufficient imaging period, animals were euthanized and tissue was harvested for frozen sectioning ensuring proper preservation of fluorescent proteins.

Small tumors were identified in 1205Lu-Vc grafts within 12 days (Figure 1B), while tumor growth was entirely absent from 1205Lu-Smad7 grafts up to 25 days (Figure 1C). 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 2). 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 2) 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 (left and center portions of tumor; Figure 1C, upper panel) 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 primary melanocytes were grafted and found incorporated into the lower epidermis and were readily identifiable *via* brightfield microscopy (Figure 2, 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 (Figures S3A and B). 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, levels of proteolytically cleaved and activated caspase-3, a converging point for apoptotic pathways, were examined and quantitated *in vitro* as well as *in vivo* (Figures S3C and D). *In vitro* examination of active caspase-3 indicated no statistically significant ($p>0.18$) difference in levels when comparing 1205Lu-Vc versus 1205Lu-Smad7 cells with expression of proteolytically processed caspase-3 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. 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.

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 3A). 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 3B). Day 25 grafts were comparable; 1205Lu-Smad7 cells remained near the dermal-epidermal junction, while 1205Lu-Vc cells formed substantial

tumor masses (Figure S4). The inability of 1205Lu-Smad7 cells to form tumors suggested that Smad7 alone reverts the highly invasive phenotype of 1205Lu.

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 4A); $p < 0.001$. Representative images depict the preference of Smad7 cells for HFF's (Figure 4A; below). Conversely, 1205Lu-Vc cells were incapable of heterotypic interaction with either HFF or HFK (Figures 4A 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 4A), 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 ($p < 0.001$).

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 5A shows immunoblot analysis, while Figure 5B 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. 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, following GAPDH normalization, no change in FL N-cadherin was observed, indicating a TGF- β receptor-independent pathway (Figure S5).

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 5C). The efficiency of β -catenin immunoprecipitation was confirmed by detection of this protein in both cell types following immunoprecipitation (Figure 5C, left) despite lower levels of β -catenin in total cell extract (Figure 5C, right). Likewise, the reciprocal immunoprecipitation with FLAG (fused to Smad7) revealed its interaction with β -catenin in 1205Lu-Smad7 cells (Figures 5D). As expected, immunoblot analysis revealed efficient Smad7 immunoprecipitation in 1205Lu-Smad7, 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 5E).

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 5F, G; $p < 0.01$). Taken together, the increased levels of β -catenin (Figure 5A), direct Smad7/ β -catenin/FL N cadherin interactions (Figure 5C, D), as well as decreased β -catenin phosphorylation in response to Smad7 (Figure 5F) 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).

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 6A). Immunoblot blot showed that while total β -catenin levels were higher in 1205Lu-Smad7 cells, β -catenin was not detectable in the nucleus of 1205Lu-Smad7 cells (Figures 5A, 6B). 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 6C 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 6A identified N-cadherin found at the periphery of 1205Lu-Smad7 cells supported homotypic interaction; high resolution analysis shown in Figure 6D 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 *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). 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 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.

METHODS

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 ϕ NX 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, 5×10^4 primary human foreskin fibroblasts (HFF) or primary human foreskin keratinocytes (HFK) were seeded onto 96-well plates and grown to confluence. 1.2×10^6 1205Lu-Vc or 1205Lu-Smad7 melanoma cells were re-suspended in 1x PBS/0.1%BSA and labeled with 2.5 μ M calcein AM (Invitrogen Carlsbad, CA) for 30 min, 37°C. Cells were washed in 1x PBS to remove excess dye. 1205Lu-Vc and 1205Lu-Smad7 cells were pre-incubated with either N-cadherin (GC4) antibody (50 μ g/mL), IgG control (50 μ g/mL) each 2 hrs; EGTA (5mM) for 20 min; or left untreated. The cells were added to confluent monolayers at 5×10^4 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 $\pm$ 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, 250 μ g of total protein was incubated with either: 0.5 μ g/mL β -catenin antibody or 40 μ l 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 20 μ g 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 $\pm$ 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 20×10^3 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 (1 μ g/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 protocols (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 8×10^6 , 5×10^6 and 1×10^6 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 $5 \mu\text{m}$ using a cryostat and placed onto SuperFrost glass slides (Fisher Scientific Pittsburgh, PA).

Statistical Analyses

Bar graphs and statistical analysis were performed using SigmaStat and SigmaPlot (Systat San Jose, CA). Error bars represent $\pm$ SD. Student's t-test was used to calculate p-values. P-values of less than 0.05 were considered statistically significant.

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*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Stable GFP-HFK's and DsRed-1205Lu Metastatic Melanoma Cells/1205Lu-Vc Grafts Form Tumors Suppressed by Smad7

1A top, primary HFKs stably expressing GFP; *middle and lower panel*, 1205Lu-Vc and 1205Lu-Smad7 melanoma cell lines stably expressing DsRed, respectively. Each figure contains 20X phase-contrast insets. *1B, 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. *1C, 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 2. Histology of Human Skin Grafts

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. Grafted 1205Lu-Smad7 Cells Reside Near the Dermal-Epidermal Junction, while 1205Lu-Vc Cells Invade the Dermis

3A, 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. 3B, 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 4. 1205Lu-Smad7 Cells Form Stable Associations with Dermal Fibroblasts

4A, 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. 4B, 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 $\pm$ SDs of three biological replicates of a representative experiment.

5A. DiVito KA **5B. DiVito KA**

5C. DiVito KA

5D. DiVito KA

Figure 5. Smad7 Restores Full-Length N-cadherin Protein Expression

5A, 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 ($p < 0.001$). Figure 5C, D densitometry for each of the cell adhesion proteins assayed: β-catenin, N-cadherin and vimentin each reached statistical significance. 5C, 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. 5D, anti-Smad7-FLAG immunoprecipitated Smad7 bound to endogenous β-catenin, confirmed by probing with both anti-β-catenin and anti-Smad7. 5E, 120nM of Smad7-siRNA reduced both β-catenin and N-cadherin, indicating the effect was Smad7-specific. 5F and G, 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 $\pm$ SDs of three biological replicates of a representative experiment.

Figure 6. Smad7 Redistributes β -catenin and N-cadherin to the Cell Surface in vitro and Promotes Heterotypic Cell-Cell Interactions via N-cadherin in vivo

6A, upper panel, 40X confocal immunofluorescence shows cells co-stained for β -catenin and N-cadherin (H-63 SantaCruz 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. 6B, Cells were subjected to nuclear and cytoplasmic fractionation; 50 μ g 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. 6C, 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. 6D, (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 7. 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.