Published in final edited form as:

Melanoma Res. 2010 December ; 20(6): 485–495. doi:10.1097/CMR.0b013e32833fafb4.

Evaluation of Molecular Markers of Mesenchymal Phenotype in Melanoma

Leann M. Mikes^{1,*}, Manish Kumar^{2,*}, Gulsun Erdag¹, Kevin T. Hogan^{3,*}, Kerrington R. Molhoek³, Marty W. Mayo², and Craig L. Slingluff Jr.^{3,a}

¹Department of Pathology, University of Virginia, Charlottesville, VA 22908

²Department of Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, VA 22908

³Department of Surgery, University of Virginia, Charlottesville, VA 22908

Abstract

Epithelial to mesenchymal transition is a developmental process allowing epithelial cells to dedifferentiate into cells displaying mesenchymal phenotypes. The pathological role of EMT has been implicated in invasion and metastasis for numerous carcinomas, yet limited data exist addressing whether mesenchymal transition (MT) occurs in malignant melanoma cells. Our group developed an *in vitro* 3D culture system to address MT in melanoma cells upon TGF- β /TNF- α treatment. Loss of E-cadherin is one of the best indicators of MT in epithelial cells. Not surprisingly, E-cadherin was expressed in only three of twelve (25%) melanoma cell lines and all three mesenchymal proteins, N-cadherin, vimentin, and fibronectin, were expressed by seven (58%) lines. However, following cytokine treatment, two or more mesenchymal proteins were elevated in nine (75%) lines. Data support the TGF- β production by melanoma cells which may induce/support MT. Evaluation of E-cadherin, N-cadherin, and Snail expression in melanoma tissue samples are consistent with an inverse coupling of E-cadherin and N-cadherin expression, however, there are also examples suggesting a more complex control of their expression. These results indicate that malignant melanoma cell lines are susceptible to MT following cytokine treatment and highlight the importance of understanding the effects of cytokines on melanoma to undergo MT.

Keywords

Melanoma; Epithelial to Mesenchymal Transition (EMT); Transforming Growth Factor (TGF); Tumor Necrosis Factor (TNF); Cadherin; Vimentin; Fibronectin; Snail

Introduction

Epithelial to mesenchymal transition (EMT) is a process whereby epithelial cells lose epithelial features and acquire properties of mesenchymal cells. EMT is associated with decreased expression of epithelial markers such as E-cadherin, and increased expression of mesenchymal markers such as N-cadherin, vimentin, and fibronectin (Reviewed in [1]). This transition to a more mesenchymal phenotype is also characterized by a loss of cell-cell

^aTo whom correspondence should be sent at Department of Surgery, P.O. Box 801329, University of Virginia, Charlottesville, VA 22908; 434-924-1505, FAX 434-982-5959, cls8h@virginia.edu.

^{*}Current address: Department of Microbiology, University of Virginia, VA 22908 (LMM); Biologics Development Center, Dr. Reddy's Laboratories Ltd., Hyderabad, India (MK); Massey Cancer Center, Virginia Commonwealth University, Richmond, VA 23298 (KTH)

adhesion, cytoskeletal remodeling, and increased cell motility. EMT is a normal process that occurs during many stages of embryonic development, however, there is growing evidence to suggest that the ability of cancer cells to metastasize is facilitated by a mesenchymal morphology (Reviewed in [2]). The change in adhesion molecule expression causes cells to detach from epithelium and to gain the ability to migrate to distant sites. The loss of epithelial cell markers such as E-cadherin has been associated with metastatic potential and poor prognosis of several carcinomas [3-5]. EMT can be induced *in vitro* in carcinoma cells with TGF- β , TNF- α , or other cytokines [6-8]. Treating epithelial cancer cells with both TGF- β and TNF- α induces a more complete EMT than either alone ([9] and Mayo, unpublished data).

Although found embedded within the basal layer of squamous epidermal epithelial cells, melanocytes are not considered epithelial because they do not form a lining. However, their migration from the neural crest ectoderm during embryologic development may be facilitated by a transient mesenchymal phenotype (Reviewed in [10, 11]). Like epithelial cells, adult melanocytes express E-cadherin, which maintains keratinocyte contact and regulation; its loss can contribute to an invasive phenotype [12, 13]. Also, adult melanocytes are negative for N-cadherin, but melanoma cells can express it. N-cadherin allows coupling to dermal fibroblasts and vascular endothelial cells in the tumor stroma, which is thought to facilitate metastasis [13, 14]. The switch from E-cadherin to N-cadherin expression by melanoma cells has been observed, and melanoma cells commonly express the mesenchymal protein vimentin, suggesting that a degree of mesenchymal transition (MT) may be constitutive in some melanomas. E-cadherin expression is negatively regulated by the transcription factor Snail [15]. Expression of N-cadherin is increased in metastatic vs. nonmetastatic melanoma [16], further suggesting a possible association of MT and metastatic potential. However, the range and variability in constitutive expression of epithelial and mesenchymal proteins is not known for human melanomas, and it is not known whether melanoma cells undergo MT like epithelial cells in response to TGF- β and TNF- α .

To assess whether melanoma cells can transition to a more mesenchymal phenotype upon cytokine treatment, we treated melanoma cell lines with TGF- β and TNF- α in a 3D culture system and evaluated changes in expression of E-cadherin, N-cadherin, vimentin, and fibronectin. This system represents a more biologically relevant model than monolayer culture in which to examine the effects of cytokine treatment on melanoma cell lines ([9] and Mayo, unpublished data). In addition, we evaluated cytokine production by the melanoma cell lines prior to TGF- β and TNF- α treatment, and evaluated E-cadherin, N-cadherin, and Snail expression in 197 melanoma tissue samples. The present report reveals a range of epithelial-mesenchymal features among different melanomas, and also suggests that MT may be induced in most human melanoma cell lines.

Methods

Cell lines

Melanoma cell lines VMM1, VMM5A, VMM12, VMM14, VMM15, VMM18, VMM19, VMM39, and VMM1040 were cell lines established from patients with metastatic lesions at the University of Virginia [17-23]. Research involving human subjects was performed with informed consent and approved by the University of Virginia's Institutional Review Board. Melanoma cell line DM93 was obtained from Duke University [24]. Melanoma cell lines SK-MEL-24 and WM115 and a non-small cell lung carcinoma, A549, were obtained from the American Type Culture Collection. Cell lines derived from metastatic melanoma lesions were cultured from tumor-involved lymph nodes (VMM5A, VMM12, VMM14, VMM15, VMM18, VMM19, VMM39, DM93, and SK-MEL-24), a brain metastasis (VMM1), or a

recurrent melanoma lesion on the shoulder (VMM1040). Melanoma VMM1040 was a low passage cell line (P6). WM115 was a cell line derived from a primary tumor that produced metastatic lesions [25]. Cell lines are summarized in Table 1. Melanoma cell lines were grown as adherent cultures in a 2D culture system in RPMI 1640 supplemented with 5% fetal bovine serum (FBS), L-glutamine (2mmol/L), penicillin (100 units/mL), and streptomycin (100µg/mL) at 37 °C in 5% CO₂ prior to introduction to the 3D culture system. A549 was cultured in DMEM (high glucose) supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100µg/mL) at 37 °C in 5% CO₂ in a 2D culture system prior to 3D culture.

Reagents

Transforming growth factor-β1 and tumor necrosis factor-α were purchased from Sigma and used at 2 ng/mL and 10 ng/mL respectively to induce EMT ([9] and Mayo, unpublished data). Poly(2-hydroxyethyl methacrylate) (Poly-HEMA) (Sigma) was used to coat Petri dishes to inhibit cell adhesion to the dish surface. Poly-HEMA (10 mg/mL in EtOH) was dispensed into Petri dishes and allowed to air dry overnight before using for 3D cell culture.

3D Cell Culture and Transition of Cells

Melanoma or lung carcinoma cell lines growing in 2D culture flasks were trypsinized, counted, and resuspended at 800,000 cells/mL in either RPMI 1640 supplemented with 5% FBS, L-glutamine (2mmol/L), penicillin (100 units/mL), and streptomycin (100µg/mL) (melanoma) or DMEM (high glucose) supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100µg/mL) (lung carcinoma). On the lid of a tissue culture plate, 20,000 cells were plated in a single drop and inverted. Cells were incubated at 37 °C in 5% CO₂ for 48-72 hours. After 48-72 hours, cell aggregates that formed in the inverted drop were transferred to a Poly-HEMA treated Petri dish in either RPMI 1640 supplemented with 1% FBS, L-glutamine (2mmol/L), penicillin (100 units/mL), and streptomycin (100µg/mL) (melanoma) or DMEM (high glucose) supplemented with 2% FBS, penicillin (100 units/mL), and streptomycin (100µg/mL) (lung carcinoma). Cells were either left untreated or were treated with TGF-β and TNF-α. After a 48 hour incubation at 37 °C in 5% CO₂, additional TGF-β and TNF-α were added to the treated cells. Cells were incubated at 37 °C in 5% CO₂ for an additional 48 hours. After 4 days with TGF-β and TNF-α, cells were harvested for analysis. Select cell lines were analyzed by flow cytometry using 7-Amino-actinomycin D (7-AAD) (Calbiochem, San Diego, CA) to determine cell viability.

SDS-PAGE and Western Blot Analysis

After 4 days of 3D culture, untreated and TGF-β/TNF-α treated cells were harvested for western blot analysis. Briefly, cells were rinsed with PBS and lysed in RIPA lysis buffer (ice-cold 150mM NaCl, 1% NP-40, 12mM deoxycholate, 0.1% SDS, 50mM Tris, pH 7.6, 4mM EDTA, 0.01% glycerol, and Roche complete mini protease inhibitor cocktail). The lysate was centrifuged for 5 minutes at 10,000 × g at 4 °C and the supernatant was stored at -80 °C for further analysis. Protein yield was determined by BCA protein assay according to the manufacturer's protocol (Thermo Scientific). The protein lysate was resuspended in SDS-containing sample buffer and heated for 10 min at 70°C. The lysate (20 µg/lane) was electrophoresed using 4-12% gradient Bis-Tris SDS gels (Invitrogen) and transferred to a PVDF membrane (Immobilon-P, Millipore). Membranes were blocked in 3% non-fat dry milk, 50 mM Tris-Cl, pH 7.5, 0.9% NaCl, and 0.1% Tween-20 at room temperature for one hour. Membranes were probed with antibodies at 4 °C overnight or at room temperature for 1 hour. Proteins were detected with either SuperSignal West Pico (Thermo Scientific) or Immobilon Western (Millipore) Chemiluminescent HRP substrate and exposed to Kodak BioMax film. Semiquantitative analysis was performed based on a band intensity grading scale of 0-4 (Figure 1) and the following film exposure times: E-cadherin and vimentin 2

minute exposure to film, N-cadherin 5-15 second exposure to film, fibronectin 15 second exposure or 2 minute exposure with diluted chemiluminescent reagent. Equal loading of protein was assessed based on GAPDH protein expression. Protein expression that appeared to be equal in the untreated and TGF- β /TNF- α treated samples were reanalyzed by western blot at 10 μ g/lane, 5 μ g/lane, and/or 2.5 μ g/lane to confirm western blot intensity was in the linear range.

Western Analysis Antibodies

Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Chemicon International and used at 1:5000. Anti-E-cadherin antibody (clone 34/E-cadherin) was purchased from BD Biosciences and used at 1:700 or 1:250. Anti-N-cadherin antibody (clone 32/N-cadherin) was purchased from BD Biosciences and used at 1:700. Anti-fibronectin antibody (clone 10/fibronectin) was purchased from BD Biosciences and used at 1:700. Anti-vimentin antibody (clone 2Q1123) was purchased from SantaCruz Biotechnology and used at 1:1000. Secondary antibody, ECL anti-mouse IgG, horseradish peroxidase-linked whole antibody from sheep was purchased from Amersham Biosciences and used at 1:5000.

Melanoma Tissue Microarray

Formalin-fixed, paraffin embedded tumor blocks were retrieved from the archives of Department of Pathology, University of Virginia Health System. The melanoma tissue microarray (TMA) blocks were constructed by obtaining 1.0 mm tissue cores from tumor areas of each block and transferring into a recipient paraffin block. Nine TMA blocks were created with quadruplicate or triplicate cores from 197 surgical pathology specimens represented by 695 melanoma cores. Nine specimens were from primary melanoma and 188 specimens were from metastatic melanoma. Multiple 4 μ m sections were cut and consecutive sections were used for H&E and immunohistochemical staining.

Immunohistochemistry

After reviewing the hemotoxylin and eosin stained TMA slides, immunohistochemical stains were performed on the TMA tissue sections using antibodies against human E-cadherin (1:4000 dilution; Epitomics Inc, Burlingame, CA), human N-cadherin (1:100 dilution; Invitrogen, Camarillo, CA), and human Snail (1:100 dilution; Cell Signaling Technology, Danvers, MA) using a DAKO Automated Immunostainer (Dako Corp, Carpinteria, CA) at the University of Virginia Biorepository and Tissue Research Facility. Briefly, paraffin embedded TMA tissue sections were deparaffinized in xylene and rehydrated by sequential incubation in ETOH/water solutions. Antigen retrieval was done in DAKO Pascal Pressure Chamber treating the slides in citrate buffer at 125°C, 22 psi pressure for 30 seconds. After blocking endogenous peroxidase activity with Dako Dual Endogeneous Enzyme Block, slides were incubated with primary antibodies, anti-E-cadherin, anti-N-cadherin, and anti-Snail for 30 minutes, 60 minutes and 30 minutes respectively using DAKO EnVision Dual Link HRP kit (Dako Corp, Carpinteria, CA). Sections were treated with secondary antibody for 30 minutes. Immunohistochemical reactions were developed with DAB substrate (Dako Corp, Carpinteria, CA) and counterstained with hemotoxylin. Appropriate negative and positive controls were included.

Intensity and extent of the immunohistochemical stainings were evaluated and scored by a pathologist (G.E.). The proportion of stained melanoma cells was assessed semiquantitatively and graded as: 0, 0% cells stained; 1, 1-25% cells stained; 2, 26-50% cells stained, 3, 51-75% cells stained, and 4, 76-100% cells stained.

Images were obtained using an Olympus BX51 microscope coupled to an Olympus BP70 digital camera (Olympus America Inc, Center Valley, PA), and software Image ProPlus 4.5 for Windows.

ELISA

Media was collected following a 72 hour incubation from cell aggregates grown in a 3D culture system after they were transferred to a Poly-HEMA treated Petri dish (before the addition of cytokines to induce MT). Supernatants were centrifuged and aliquots were removed and stored at -80°C until analyzed. Quantitative cytokine production was measured using a chemiluminescent ELISA (TNF- α) or a colorimetric ELISA (TGF- β 1) according to manufacturer's instructions (R&D Systems). A standard curve was generated using the supplied reagents. TGF- β 1 samples were activated to detect free and latent TGF- β 1. Background levels of TNF- α or TGF- β 1 from the serum containing media was subtracted from the samples. The concentration of cytokine produced by the cells in 3D culture was calculated using the average value from duplicate samples.

Statistical Methods

Relative intensity on western blot analysis was determined for E-cadherin, N-cadherin, vimentin and fibronectin protein expression at baseline as described above. A linear regression analysis was performed with E-cadherin vs. each of the three mesenchymal proteins and N-cadherin vs. fibronectin and vimentin. For each graph, a linear regression analysis was performed and r^2 values were calculated using GraphPad Prism 4 software.

Results

Baseline Epithelial/Mesenchymal Protein Expression in Melanoma Cell Lines

We evaluated baseline expression of epithelial and mesenchymal proteins in 12 human melanoma cell lines (11 derived from metastatic melanoma and 1 from primary melanoma) (Table 1) and compared them to A549, a non-small cell lung carcinoma that undergoes EMT upon cytokine treatment ([9] and Mayo, unpublished data). Cells were cultured in a 3D culture system, which is more physiologically relevant than monolayer culture and allows cells to organize into structures that resemble their *in vivo* architecture. Cells were harvested, and evaluated for protein expression by western blot, with semiquantitative measures.

As expected, E-cadherin was detected in the lung cancer cell line A549 (X) at a relative expression level of 2.0, but was expressed in only three of twelve melanoma cell lines (A-C, Figure 1A) at relative expression levels of 1.5 to 2.5. No correlations were observed between levels of E-cadherin and any of the three mesenchymal proteins N-cadherin, fibronectin, or vimentin (Figure 1B-D) ($r^2 = 0.06, 0.04, \text{ and } 0.24$ respectively).

N-cadherin was undetectable in two lines (VMM5A and VMM1040), low (intensity<2) in three lines, moderate in three lines ($2 \geq \text{intensity} < 3$), and high (intensity ≥ 3) in four lines (Figure 1E,F). Fibronectin was undetectable in two lines, low in three, moderate in three, and high in four (Figure 1E). Vimentin was undetectable in one line (DM93), moderate in five lines, and high in six lines (Figure 1F). Seven of the twelve melanoma cell lines express all three mesenchymal proteins at baseline, whereas the other five melanoma cell lines express two of the three mesenchymal proteins at baseline. No correlations were observed between the expression of mesenchymal proteins N-cadherin and fibronectin or vimentin (Figure 1E-F) ($r^2 = 0.02 \text{ and } 0.03$, respectively).

Mesenchymal Transition Associated Protein Changes Induced by TGF/TNF Treatment

To evaluate if TGF- β and TNF- α could induce MT of melanoma cell lines, we treated 12 melanoma cell lines with TGF- β and TNF- α for 4d in a 3D culture system. This treatment induced decreases in E-cadherin and increases in N-cadherin, fibronectin, and vimentin, both for the lung carcinoma A549 (positive control X, Figure 2A) and for one melanoma cell line DM93 (A, Figure 2A). In contrast, for the melanoma cell line VMM18 (B), the cytokines induced decreases in E-cadherin and vimentin and increases in N-cadherin and fibronectin (Figure 2A). Repeat testing for A549, VMM18, and others were performed several times with consistent findings. Repeat testing for VMM18 is shown (Figure 2B). Flow cytometry data indicate no increase in cell death with cells treated with TGF- β /TNF- α (data not shown).

Of the 12 melanoma cell lines evaluated, cytokine treatment decreased E-cadherin in all 3 cell lines in which it was detectable at baseline (DM93, VMM15, and VMM18) (A-C, Figure 3A). N-cadherin was increased by cytokine treatment in 8 of the 12 melanoma cell lines; however, its expression remained stable in 3 lines (VMM12, WM115, and SK-MEL-24). In one, VMM5A, N-cadherin expression was undetectable in the untreated cells or in the TGF- β /TNF- α treated cells (Figure 3B). Fibronectin expression increased in all melanoma lines except VMM15 (C), whose relative expression level was already 4 pretreatment (Figure 3C). Vimentin expression increased in 5 lines, stayed the same in 4, and interestingly, decreased in 3 upon TGF- β /TNF- α treatment (Figure 3D).

Based on these findings, it appeared that responses to TGF- β /TNF- α treatment in the twelve melanoma cell lines could be grouped into four categories (I-IV) based on a combination of constitutive expression and cytokine-induced changes in E-cadherin and N-cadherin expression (Figure 3E). Category I (3 lines) were E-cadherin expression at baseline and had expected results for epithelial cells undergoing EMT, with decreased E-cadherin and increased N-cadherin upon cytokine treatment. The other nine melanoma lines had no detectable E-cadherin protein expression at baseline: Five, in category II, had increased N-cadherin with treatment. Three, in category III, had high N-cadherin expression that did not change after cytokine treatment. Just one cell line fell in the last category (IV), where neither E-cadherin nor N-cadherin are expressed in the untreated cells and neither were increased with cytokine treatment.

TNF- α and TGF- β 1 Cytokine Production

In the interest of evaluating whether melanoma cells themselves may secrete cytokines capable of inducing mesenchymal transition, the production of TNF- α and TGF- β 1 by melanoma cells was determined for three melanoma cell lines in 3D culture (one from each category, I-III) prior to cytokine treatment to induce MT. In supernatants of cell lines B (VMM18), D (VMM1), and I (VMM12), TGF- β 1 was detected at 169, 1188, and 1704 pg/mL, respectively. TNF- α was undetectable in supernatants of all three lines.

E-cadherin, N-cadherin and Snail Expression in Melanoma Tissue

Protein expression by cell lines may or may not reflect *in situ* expression patterns. We were interested in expression of E-cadherin and N-cadherin *in situ* in melanoma, and whether expression of Snail corresponds with N-cadherin expression. Thus, expression of these proteins (E-cadherin, N-cadherin, and Snail) were each assessed by immunohistochemical analysis on a melanoma tissue microarray (TMA) representing 197 melanoma tissue samples, with 3-4 cores each. E-cadherin and N-cadherin were mostly localized to the cell membrane, while Snail staining was nuclear. Proportions of cells staining positive for E-cadherin, N-cadherin and Snail were scored on a scale from 0-4. For E-cadherin, 154 samples (78%) had one or more cores that were positive (score of 1 or more), while 128

samples (65%) were positive for N-cadherin. Only 8 of 197 samples (4%) had detectable Snail. An example in Figure 4 is a melanoma sample expressing E-cadherin but negative for N-cadherin and Snail (Figure 4A-C). Another core is shown in Figure 4D-F that is negative for E-cadherin and positive for both N-cadherin and Snail. In Figure 4G-I is a core that expresses E-cadherin and N-cadherin but is negative for Snail. E-cadherin and N-cadherin staining in Figure 4G-H are mirror images of each other.

Four staining patterns for E-cadherin and N-cadherin were discerned among the 695 melanoma tissue cores and accounted for 87% of the cores (Figure 5A). Thirty six percent of the cores were N-cadherin negative or low (scores 0-1) but expressed E-cadherin in 51-100% of the cells (scores 3-4); in 23%, E-cadherin was absent or low (0-1), but most melanoma cells expressed N-cadherin (3-4). In 17% of the cores, both E-cadherin and N-cadherin were expressed in 51-100% of the cells (3-4), while in 11%, E-cadherin and N-cadherin was absent or low (0-1). Of the 25 cores positive for Snail (score 1 or higher), the vast majority (21/25, 84%) were also highly positive for N-cadherin (score 3-4) and 15/25 (60%) were negative or low (0-1) for E-cadherin (Figure 5B).

The 197 melanoma specimens in the TMA included three that were source tumors for three of the melanoma cell lines used for the MT cultures (VMM15, VMM19, and VMM39). The VMM15 cell line (C) is in group I, expressing E-cadherin and a low level of N-cadherin by western, which corresponds well to the corresponding tissue on the TMA, where over 75% of the cells express E-cadherin, N-cadherin is not expressed, and Snail is not detected. Cell lines VMM19 (G) and VMM39 (E) are in group II (E-cadherin^{neg} and N-cadherin⁺ by Western). The corresponding melanoma tissue for VMM19 also matches the cell line well, as a low expresser of E-cadherin (score 1) in three of the four cores (the fourth core = score 4) and a high expresser of N-cadherin (expressed in over 75% of the melanoma cells, score 4). The corresponding tissue for cell line VMM39 expresses both E-cadherin and N-cadherin in over 75% of the cells. None of the three tissues expressed Snail.

Discussion

Unlike most epithelial cells, melanocytes as well as melanoma cells typically express the mesenchymal protein vimentin [26]. A majority of melanoma cells have also lost E-cadherin expression [13, 15, 27]. Baseline protein expression patterns in melanoma cell lines suggest there is a range of “epithelialness” and “mesenchymalness” of melanoma cell lines. Surprisingly, there was no direct relationship between amount of E-cadherin protein expression and expression of any of the mesenchymal proteins or among the mesenchymal proteins in the untreated cells. Thus, there likely is a complex spectrum of epithelial to mesenchymal phenotypes in melanoma prior to TGF/TNF treatment. In addition, among the proteins we evaluated, there does not appear to be one marker that predicts how melanoma cells will respond to cytokine treatment.

EMT has been demonstrated for some epithelial cells in standard two-dimensional cultures in plastic dishes, however, cell polarization is impaired and growth factors may not be able to access the basolateral side of the monolayer. Three-dimensional culture systems allow cells to organize into structures that resemble their *in vivo* architecture which may promote a more rapid and synchronous EMT (Reviewed in [9]). The 3D culture system we report here represents a more biologically relevant model than monolayer culture in which to examine the effects of cytokine treatment on melanoma cell lines (Mayo, unpublished data).

Melanocytes express E-cadherin [28]. Loss of E-cadherin is a hallmark of EMT. TGF- β can downregulate E-cadherin [29], and loss of E-cadherin in melanoma can be associated with increased N-cadherin expression [30]. Many of the findings in this study are consistent with

an inverse coupling of N-cadherin and E-cadherin expression, with one negatively regulating the other. However, there also are counter-examples in which both are absent or both are expressed, suggesting more complex control of their expression. Thus an important area for future study is the molecular and epigenetic control of mesenchymal transition. Although it is theoretically conceivable the effects of TGF and TNF treatment on cell lines represent selection of more mesenchymal cells, these well established melanoma cell lines are relatively homogeneous and flow cytometry data support that cell death is not increased in the cytokine treated cells.

Interestingly E-cadherin is undetectable at baseline in 9 of the 12 melanoma cell lines analyzed in this study, but they may or may not express mesenchymal proteins; thus, some melanoma cells may have a partially mesenchymal phenotype. In the VMM5 cell line, (category IV) there is no expression of either E-cadherin or N-cadherin, which suggests that the inverse coupling of these two proteins is not absolute. This and other melanomas could possibly include other cadherins such as dysadherin or vascular endothelial (VE)-cadherin, which have been associated with aggressive melanoma and indicate a poor prognosis [31, 32]. The expression of dysadherin can downregulate E-cadherin [33], so it is possible that this or other cadherins are counter-regulated in ways that extend beyond the mesenchymal transition.

With cell lines VMM18, VMM19, and VMM14, vimentin expression decreases upon TGF- β and TNF- α treatment. Vimentin is one of 70 known intermediate filaments. Vimentin is a good marker of EMT, however, its levels are regulated by NF- κ B. The TGF- β /SMAD7 signaling pathway has been shown to repress NF- κ B transcriptional activity. Another intermediate filament most likely compensates for the lack of robust vimentin expression.

Multiple cytokines can induce EMT in epithelial cell lines or in epithelial cancers, with variable effects on individual cell lines ([29, 34] and reviewed in [2, 35]). TGF- β can induce EMT [29, 36, 37], while other cytokines such as TNF- α can enhance the effects of TGF- β in EMT ([9] and Mayo, unpublished data). TGF- β is found in the melanoma microenvironment [38, 39] and the production of TGF- β is increased in melanoma cells expressing Snail [40]. We measured TGF- β and TNF- α produced by three melanoma cell lines cultured without exogenous cytokines. Group I is thought to be the most “epithelial-like” while Group III is thought to be the most “mesenchymal like”. Data from these three lines are consistent with this subsetting of the melanomas based on “epithelialness” and “mesenchymalness,” though a larger study may be necessary to test this association more definitively. We suspected that TGF- β produced by the tumor cells may induce or support mesenchymal transition; so we would expect that TGF- β -producing cell lines would have a more mesenchymal phenotype.

TNF, a mediator of EMT, was not detected in these 3 cell lines but has been reported to be secreted by a minority of melanoma cells [41]. TNF can be produced by other cells found in the tumor microenvironment including host immune cells. Thus, both TNF- α and TGF- β may be present in the tumor microenvironment and may be able to induce a mesenchymal phenotype spontaneously *in vivo*. The mesenchymal phenotype has been shown to induce multiple immunosuppressive and immunoresistance mechanisms [40]. This correlation suggests an interesting balance and link among cytokines, EMT, the tumor microenvironment, immune response, tumor invasiveness, and metastatic potential in cancer. It also supports targeting these cytokines and especially TGF β , in the tumor microenvironment in novel therapies.

Data from the melanoma tissue microarray also support subsetting melanomas based on E-cadherin and N-cadherin expression. Snail was expressed in only 4% of the melanoma tissue samples. We suspect this transcription factor is only transiently expressed during the

transition to a more mesenchymal phenotype. In the 3 cases in which both cell lines and the original tumor were evaluated, there was correspondence of E- and N-cadherin expression, but in another case, there were differences between the tumor and the cell line, which may be explained either by the effects of stromal cells on the tumor *in vivo* or by selection of a subset of melanoma cells in culture.

A limitation of this study is the semi-quantitative analysis of western blot data for epithelial and mesenchymal protein markers. However, several consistent patterns were observed, especially regarding the presence or absence of epithelial or mesenchymal proteins and regarding changes in protein expression upon TGF- β and TNF- α treatment. Also, the *in situ* studies support findings that mesenchymal transition can occur in human melanoma, either spontaneously or upon exposure to TNF- α and TGF- β . Future studies evaluating and understanding the tumor microenvironment, the cytokines that are produced, and their effects on mesenchymal transition may shed further light on the role they play *in vivo*. If MT of melanoma cells does support invasion and metastasis as it does in carcinomas, there may be a role in inhibiting the causative factors, either by blocking TGF- β or by inhibiting some downstream signaling from TNF- α within the tumor microenvironment.

Acknowledgments

We would like to thank Dr. David Bruns for his mentorship and support to LMM during her fellowship and Dr. Jochen Schaefer for developing the melanoma tissue microarray. We would also like to acknowledge the University of Virginia Biorepository and Tissue Research Facility (BTRF) for the immunohistochemical staining of the melanoma tissue microarray.

Grant Support: Leann M. Mikesh, Ph.D. was supported by the American Association for Clinical Chemistry Past President's Scholarship. Kerrington R. Molhoek, Ph.D. was supported by the American Cancer Society, California Division Campaign for Research 2007 Postdoctoral Fellowship. This work was also partly supported by grant CA132580 to Dr. Marty Mayo from NIH/NCI and from the University of Virginia Cancer Center and the Commonwealth Foundation for Cancer Research to Dr. Craig L. Slingluff, Jr. and the Human Immune Therapy Center, University of Virginia.

References

1. Turley EA, Veishe M, Radisky DC, Bissell MJ. Mechanisms of Disease: epithelial-mesenchymal transition does cellular plasticity fuel neoplastic progression? *Nat Clin Prac Oncol*. 2008; 5:280–290.
2. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer*. 2002; 2:442–454. [PubMed: 12189386]
3. Lee M-Y, Chou C-Y, Tang M-J, Shen M-R. Epithelial-Mesenchymal Transition in Cervical Cancer: Correlation with Tumor Progression, Epidermal Growth Factor Receptor Overexpression, and Snail Up-Regulation. *Clin Cancer Res*. 2008; 14:4743–4750. [PubMed: 18676743]
4. Frixen U, Behrens J, Sachs M, Eberle G, Voss B, Warda A, et al. E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. *J Cell Biol*. 1991; 113:173–185. [PubMed: 2007622]
5. Tamura S. The E-cadherin-mediated cell-cell adhesion system in human cancers. *Br J Surg*. 1997; 84:899–900. [PubMed: 9240126]
6. Mani SA, Guo W, Liao M-J, Eaton EN, Ayyanan A, Zhou AY, et al. The Epithelial-Mesenchymal Transition Generates Cells with Properties of Stem Cells. *Cell*. 2008; 133:704–715. [PubMed: 18485877]
7. Pon YL, Zhou HY, Cheung ANY, Ngan HYS, Wong AST. p70 S6 Kinase Promotes Epithelial to Mesenchymal Transition through Snail Induction in Ovarian Cancer Cells. *Cancer Res*. 2008; 68:6524–6532. [PubMed: 18701475]
8. Arima Y, Inoue Y, Shibata T, Hayashi H, Nagano O, Saya H, et al. Rb Depletion Results in Deregulation of E-Cadherin and Induction of Cellular Phenotypic Changes that Are Characteristic. *Cancer Res*. 2008; 68:5104–5112. [PubMed: 18593909]

9. Liu X. Inflammatory cytokines augments TGF-beta1-induced epithelial-mesenchymal transition in A549 cells by up-regulating TbetaR-I. *Cell Motility and the Cytoskeleton*. 2008; 65:935–944. [PubMed: 18792103]
10. Tolleson WH. Human Melanocyte Biology, Toxicology, and Pathology. *Journal of Environmental Science and Health, Part C: Environmental Carcinogenesis and Ecotoxicology Reviews*. 2005; 23:105–161.
11. White RM, Zon LI. Melanocytes in Development, Regeneration, and Cancer. *Cell Stem Cell*. 2008; 3:242–252. [PubMed: 18786412]
12. Hsu M, Andl T, Li G, Meinkoth J, Herlyn M. Cadherin repertoire determines partner-specific gap junctional communication during melanoma progression. *J Cell Sci*. 2000; 113:1535–1542. [PubMed: 10751145]
13. Hsu M-Y, Meier FE, Nesbit M, Hsu J-Y, Van Belle P, Elder DE, et al. E-Cadherin Expression in Melanoma Cells Restores Keratinocyte-Mediated Growth Control and Down-Regulates Expression of Invasion-Related Adhesion Receptors. *Am J Pathol*. 2000; 156:1515–1525. [PubMed: 10793063]
14. Li G, Satyamoorthy K, Herlyn M. N-Cadherin-mediated Intercellular Interactions Promote Survival and Migration of Melanoma Cells. *Cancer Res*. 2001; 61:3819–3825. [PubMed: 11325858]
15. Poser I, Dominguez D, de Herreros AG, Varnai A, Buettner R, Bosserhoff AK. Loss of E-cadherin Expression in Melanoma Cells Involves Up-regulation of the Transcriptional Repressor Snail. *J Biol Chem*. 2001; 276:24661–24666. [PubMed: 11323412]
16. Alonso SR, Tracey L, Ortiz P, Perez-Gomez B, Palacios J, Pollan M, et al. A High-Throughput Study in Melanoma Identifies Epithelial-Mesenchymal Transition as a Major Determinant of Metastasis. *Cancer Res*. 2007; 67:3450–3460. [PubMed: 17409456]
17. Hogan KT, Sutton JN, Chu KU, Busby JAC, Shabanowitz J, Hunt DF, et al. Use of selected reaction monitoring mass spectrometry for the detection of specific MHC class I peptide antigens on A3 supertype family members. *Cancer Immunology, Immunotherapy*. 2005; 54:359–371.
18. Slingluff C Jr, Cox A, Henderson R, Hunt D, Engelhard V. Recognition of human melanoma cells by HLA-A2.1-restricted cytotoxic T lymphocytes is mediated by at least six shared peptide epitopes. *J Immunol*. 1993; 150:2955–2963. [PubMed: 7681084]
19. Molhoek KR, Griesemann H, Shu J, Gershenwald JE, Brautigan DL, Slingluff CL Jr. Human Melanoma Cytolysis by Combined Inhibition of Mammalian Target of Rapamycin and Vascular Endothelial Growth Factor/Vascular Endothelial Growth Factor Receptor-2. *Cancer Res*. 2008; 68:4392–4397. [PubMed: 18519701]
20. Yamshchikov G, Thompson L, Ross W, Galavotti H, Aquila W, Deacon D, et al. Analysis of a natural immune response against tumor antigens in a melanoma survivor: lessons applicable to clinical trial evaluations. *Clin Cancer Res*. 2001; 7:909s–916s. [PubMed: 11300491]
21. Yamshchikov GV, Mullins DW, Chang C-C, Ogino T, Thompson L, Presley J, et al. Sequential Immune Escape and Shifting of T Cell Responses in a Long-Term Survivor of Melanoma. *J Immunol*. 2005; 174:6863–6871. [PubMed: 15905528]
22. Kittlesen DJ, Thompson LW, Gulden PH, Skipper JCA, Colella TA, Shabanowitz JA, et al. Human Melanoma Patients Recognize an HLA-A1-Restricted CTL Epitope from Tyrosinase Containing Two Cysteine Residues: Implications for Tumor Vaccine Development. *J Immunol*. 1998; 160:2099–2106. [PubMed: 9498746]
23. Huntington JT, Shields JM, Der CJ, Wyatt CA, Benbow U, Slingluff CL Jr, et al. Overexpression of Collagenase 1 (MMP-1) Is Mediated by the ERK Pathway in Invasive Melanoma Cells: ROLE OF BRAF MUTATION AND FIBROBLAST GROWTH FACTOR SIGNALING. *J Biol Chem*. 2004; 279:33168–33176. [PubMed: 15184373]
24. Darrow T, Slingluff C Jr, Seigler H. The role of HLA class I antigens in recognition of melanoma cells by tumor-specific cytotoxic T lymphocytes. Evidence for shared tumor antigens. *J Immunol*. 1989; 142:3329–3335. [PubMed: 2785141]
25. Herlyn M, Thurin J, Balaban G, Benniselli JL, Herlyn D, Elder DE, et al. Characteristics of Cultured Human Melanocytes Isolated from Different Stages of Tumor Progression. *Cancer Res*. 1985; 45:5670–5676. [PubMed: 4053039]

26. Miettinen M, Lehto V-P, Virtanen I. Presence of fibroblast-type intermediate filaments (vimentin) and absence of neurofilaments in pigmented nevi and malignant melanomas. *Journal of Cutaneous Pathology*. 1983; 10:188–192. [PubMed: 6190849]
27. Danen E, de Vries T, Morandini R, Ghanem G, Ruiter D, van Muijen G. E-cadherin expression in human melanoma. *Melanoma Research*. 1996; 6:127–131. [PubMed: 8791270]
28. Tang A, Eller M, Hara M, Yaar M, Hirohashi S, Gilchrist B. E-cadherin is the major mediator of human melanocyte adhesion to keratinocytes in vitro. *J Cell Sci*. 1994; 107:983–992. [PubMed: 8056851]
29. Peinado H, Quintanilla M, Cano A. Transforming Growth Factor {beta}-1 Induces Snail Transcription Factor in Epithelial Cell Lines: MECHANISMS FOR EPITHELIAL MESENCHYMAL TRANSITIONS. *J Biol Chem*. 2003; 278:21113–21123. [PubMed: 12665527]
30. Kuphal S, Bosserhoff AK. Influence of the cytoplasmic domain of E-cadherin on endogenous N-cadherin expression in malignant melanoma. *Oncogene*. 2006; 25:248–259. [PubMed: 16132038]
31. Nishizawa A, Nakanishi Y, Yoshimura K, Sasajima Y, Yamazaki N, Yamamoto A, et al. Clinicopathologic significance of dysadherin expression in cutaneous malignant melanoma. *Cancer*. 2005; 103:1693–1700. [PubMed: 15751018]
32. Hendrix MJC, Seftor EA, Meltzer PS, Gardner LMG, Hess AR, Kirschmann DA, et al. Expression and functional significance of VE-cadherin in aggressive human melanoma cells: Role in vasculogenic mimicry. *Proceedings of the National Academy of Sciences of the United States of America*. 2001; 98:8018–8023. [PubMed: 11416160]
33. Ino Y, Gotoh M, Sakamoto M, Tsukagoshi K, Hirohashi S. Dysadherin, a cancer-associated cell membrane glycoprotein, down-regulates E-cadherin and promotes metastasis. *Proceedings of the National Academy of Sciences of the United States of America*. 2002; 99:365–370. [PubMed: 11756660]
34. Oft M, Peli J, Rudaz C, Schwarz H, Beug H, Reichmann E. TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. *Genes & Development*. 1996; 10:2462–2477. [PubMed: 8843198]
35. Grunert S, Jechlinger M, Beug H. Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis. *Nat Rev Mol Cell Biol*. 2003; 4:657–665. [PubMed: 12923528]
36. Kim J, Jang YS, Eom KS, Hwang Y, Kang HR, Jang SH, et al. Transforming Growth Factor beta1 Induces Epithelial-to-Mesenchymal Transition of A549 Cells. *J Korean Med Sci*. 2007; 22:898–904. [PubMed: 17982242]
37. Kasai H, Allen J, Mason R, Kamimura T, Zhang Z. TGF-beta1 induces human alveolar epithelial to mesenchymal cell transition (EMT). *Respiratory Research*. 2005; 6:56. [PubMed: 15946381]
38. Reed JA, McNutt NS, Prieto VG, Albino AP. Expression of transforming growth factor-beta 2 in malignant melanoma correlates with the depth of tumor invasion. Implications for tumor progression. *Am J Pathol*. 1994; 145:97–104. [PubMed: 8030760]
39. Moretti S, Pinzi C, Spallanzani A, Berti E, Chiarugi A, Mazzoli S, et al. Immunohistochemical evidence of cytokine networks during progression of human melanocytic lesions. *International Journal of Cancer*. 1999; 84:160–168.
40. Kudo-Saito C, Shirako H, Takeuchi T, Kawakami Y. Cancer Metastasis Is Accelerated through Immunosuppression during Snail-Induced EMT of Cancer Cells. *Cancer Cell*. 2009; 15:195–206. [PubMed: 19249678]
41. Colombo MP, Maccalli C, Mattei S, Melani C, Radrizzani M, Parmiani G. Expression of cytokine genes, including IL-6, in human malignant melanoma cell lines. *Melanoma Research*. 1992; 2:181–190. [PubMed: 1450672]

Figure 1. Baseline E-cadherin and Mesenchymal Protein Expression in Melanoma Cell Lines
 Melanoma cell lines are indicated as A-L. Control, lung carcinoma cell line, A549 is indicated as X. A=DM93, B=VMM18, C=VMM15, D=VMM1, E=VMM39, F=VMM14, G=VMM19, H=VMM1040, I=VMM12, J=WM115, K=SkMel24, L=VMM5A, X=A549. Western blot analysis for baseline protein expression of E-cadherin is shown from individual experiments for cell lines X and A-E. GAPDH was used as a loading control. Protein expression from western blot analysis based on semi-quantitative analysis on a 0-4 scale as shown. Results, including linear regression analysis, are plotted for B): E-cadherin vs. N-cadherin, C): E-cadherin vs. vimentin, D): E-cadherin vs. fibronectin, E): N-cadherin vs. fibronectin ($r^2=0.02$), and F) N-cadherin vs. vimentin ($r^2=0.03$).

Figure 2. Western Blot of EMT Related Proteins in Transitioned and Non-transitioned Cells
 Shown in (A) are the baseline protein expression levels of E-cadherin, N-cadherin, vimentin, and fibronectin and protein expression in TGF- β and TNF- α treated cells by western analysis of lung carcinoma, A549, and a melanoma cell lines, DM93 and VMM18. Graphed in (B) is the reproducibility of E-cadherin, N-cadherin, fibronectin, and vimentin protein levels in cell line B, VMM18. Individual experiments performed over several months. Red circles indicate baseline protein expression and blue squares indicate protein expression after TGF- β and TNF- α treatment.

Figure 3. Relative Expression of EMT Markers in Melanoma Cell Lines upon TGF- β and TNF- α Treatment

Illustrated in (A) are the changes in relative protein expression from baseline after TGF- β and TNF- α treatment in a) E-cadherin, b) N-cadherin, c) fibronectin, d) vimentin on a 0-4 scale as illustrated. GAPDH was used as a loading control (data not shown). Red circles indicate baseline protein expression and blue squares indicate protein expression after TGF- β and TNF- α treatment. In (B), melanoma cell lines were grouped into four categories (I-IV) based on changes in relative protein expression of E-cadherin and N-cadherin after treatment with TGF- β /TNF- α treatment. An “*” indicates no protein was detected at baseline and after TGF/TNF treatment, positive control worked. A “—” indicates protein was detected, but no change in expression observed after TGF- β /TNF- α treatment of cells. A=DM93, B=VMM18, C=VMM15, D=VMM1, E=VMM39, F=VMM14, G=VMM19, H=VMM1040, I=VMM12, J=WM115, K=SkMel24, L=VMM5A, X=A549.

Figure 4. E-cadherin, N-cadherin, and Snail Immunohistochemical Staining on Melanoma Tissue Microarray

Shown in A-I are immunohistochemical staining of three different cores from melanoma tissue on the TMA. The melanoma tissue sample in A-C stains positive for E-cadherin (A) and negative for N-cadherin (B) and Snail (C). The melanoma tissue sample in D-F stains negative for E-cadherin (D) and positive for N-cadherin (E) and Snail (F). In G-I, the melanoma tissue sample stains positive for E-cadherin (G) and N-cadherin (H) (note the mirror image) and negative for Snail (I).

Figure 5. Overall distribution of melanoma tissue expressing E-cadherin, N-cadherin, and Snail Shown in A) are the percent of cores on the melanoma tissue microarray with expression of N-cadherin and/or E-cadherin; B) the number of cores with N-cadherin and/or E-cadherin expression that also express Snail (scores 1 or greater).

Table 1

Melanoma cell lines and their origin

Cell line	Origin of melanoma cell line
WM115	Primary melanoma
VMM1040	Subcutaneous and dermal nodule
DM93	TIN
SK-MEL-24	TIN
VMM5A	TIN
VMM12	TIN
VMM14	TIN
VMM15	TIN
VMM18	TIN
VMM19	TIN
VMM39	TIN
VMM1	Brain metastasis

TIN = tumor involved nodes