

Epigenetic Transdifferentiation of Normal Melanocytes by a Metastatic Melanoma Microenvironment

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Abstract

The clinical management of cutaneous melanoma would benefit significantly from a better understanding of the molecular changes that occur during melanocytic progression to a melanoma phenotype. To gain unique insights into this process, we developed a three-dimensional *in vitro* model that allows observations of normal human melanocytes interacting with a metastatic melanoma matrix to determine whether these normal cells could be reprogrammed by inductive cues in the tumor cell microenvironment. The results show the epigenetic transdifferentiation of the normal melanocytic phenotype to that of an aggressive melanoma-like cell with commensurate increased migratory and invasive ability with no detectable genomic alterations. Removal of the transdifferentiated melanocytes from the inductive metastatic melanoma microenvironment results in a reversion to their normal phenotype. However, a normal melanocyte microenvironment had no epigenetic influence on the phenotype of metastatic melanoma cells. This novel approach identifies specific genes involved in the transdifferentiation of melanocytes to a more aggressive phenotype, which may offer significant therapeutic value. (Cancer Res 2005; 65(22): 10164-9)

Introduction

Cutaneous melanoma is considered one of the few remaining cancers escalating in incidence (1) and thus represents a growing public health burden worldwide (2). Indeed, the clinical management of cutaneous melanoma, as well as of many other types of cancer, would significantly benefit from the identification of valid predictors of disease onset, progression, and metastatic potential. The first report directed toward unveiling the molecular signature of melanoma tumor cells resulted in an important classification scheme for cutaneous melanoma (3), which holds translational promise. The normal precursor cell type of human melanoma is the epidermal melanocyte, a cell of neural crest origin. Using microarray technology, a recent study reported expression profiling of normal melanocytes compared with melanoma cell strains (4) and found discrete pathways that might be involved in the transformation of melanocytes to melanoma, including those involved in

embryonic development and epidermal/mesenchymal transition. Also noteworthy is a study that tested genetically defined elements responsible for human melanocytic neoplasia (5). However, the molecular events leading to melanocytic neoplasia with respect to the dynamic role of the microenvironment in the transition of normal melanocytes into a melanoma phenotype have yet to be addressed.

To date, comparative global gene analyses of cutaneous melanoma have revealed that aggressive tumor cells express genes (and proteins) associated with multiple cellular phenotypes (6), coincident with a down-regulation of melanocyte-associated antigen genes (*Melan-A* and *tyrosinase*; refs. 7, 8). These intriguing findings support the premise that aggressive melanoma cells adopt a multipotent, plastic phenotype. One example of melanoma cell plasticity is vasculogenic mimicry, which characterizes the unique ability of aggressive melanoma cells, but not of poorly aggressive melanoma cells, to express endothelial-associated genes and form extracellular matrix (ECM)-rich vasculogenic-like networks in three-dimensional culture (9). These ECM-rich networks, observed in aggressive tumors of patients with melanoma (10), have now been reported in other tumor types (for review, see ref. 11). The etiology of this important vascular phenotype remains unclear; however, it seems to involve dysregulation of the tumor-specific phenotype and the concomitant transdifferentiation of aggressive tumor cells into other cell types. Furthermore, melanoma cells, but not normal melanocytes, share vascular cell-associated markers with endothelial cells (12).

A better understanding of the molecular changes that occur during melanocytic progression to a melanoma phenotype is needed to develop accurate diagnostic markers at the earliest possible stages of transformation. To gain unique insights into this process, we developed a three-dimensional model that allows observations of the interactions of normal human melanocytes with a metastatic melanoma matrix to determine whether these normal cells could be reprogrammed by inductive cues in the tumor cell microenvironment. The data reveal the transdifferentiation of the normal melanocytic phenotype to that of an aggressive melanoma-like cell with commensurate increased migratory and invasive ability with no detectable genomic alterations, thus showing the epigenetic influence of the metastatic microenvironment. However, removal of the transdifferentiated melanocytes from this inductive microenvironment results in their reversion to a normal phenotype. This novel approach provides new insights into the epigenetic reprogramming of the benign melanocytic phenotype as it responds to metastatic environmental cues and, most importantly, identifies specific genes involved in the transdifferentiation of melanocytes to a more aggressive phenotype that may have therapeutic value.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Materials and Methods

Cell culture. The human metastatic cutaneous melanoma cell line C8161 and its poorly aggressive counterpart, C81-61, were isolated from an abdominal wall metastasis (13). The C8161 cell line was maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 0.1% gentamicin sulfate (Gemini Bioproducts, Calabasas, CA) whereas the C81-61 cell line was maintained in Ham's F-10 medium supplemented with 15% fetal bovine serum, 1× MITO+ (BD Bioscience, Bedford, MA), and gentamicin sulfate. Normal human melanocytes were isolated from neonatal foreskins and maintained in Medium 254 with Human Melanocyte Growth Supplement (Cascade Biologics, Portland, OR) and gentamicin sulfate. Melanocyte cultures were designated as HEMn10, HEMn13, HEMn18, or HEMn20. The cultures were determined to be free of *Mycoplasma* contamination using a PCR-based detection system (Roche, Indianapolis, IN).

Three-dimensional preconditioned matrices. A critical aspect of this work was the production of defined three-dimensional matrices as outlined in Fig. 1. Twenty-five microliters of type I collagen (average 3 mg/mL; BD Bioscience) were dropped into 12-well culture dishes and polymerized with an application of 100% ethanol at room temperature. After extensive washing with PBS followed by medium, tumor cells or normal melanocytes were seeded onto the three-dimensional matrix in complete medium. The three-dimensional cultures were then observed after 3 to 4 days and images were captured digitally using a Zeiss model 25 inverted microscope (Carl Zeiss, Inc., Thornwood, NY) and Hitachi HV-C20 CCD camera (Hitachi Denshi America Ltd., Woodbury, NY). For experiments designed to analyze the transdifferential potential of normal melanocytes when placed on a matrix preconditioned by highly aggressive melanoma cells (C8161), the cells were removed after 3 to 4 days with 20 mmol/L NH₄OH followed by thorough washes with water, PBS, and then complete medium.

Invasion and migration assays. Normal melanocytes (5×10^4) were seeded into the upper wells of the Membrane Invasion Culture System (13) chamber onto human collagen IV/laminin/gelatin-coated (Sigma Chemical Co., St. Louis, MO) polycarbonate membranes (pore size 10 μ m; Osmonics, Livermore, CA) in RPMI 1640 with 1× MITO+ (BD Bioscience). After 24 hours of incubation at 37°C, the cells that invaded each membrane were collected, stained, and counted as previously described (13). Percent invasion was corrected for proliferation and calculated as the total number of invading cells divided by the total number of cells seeded \times 100. A modified protocol was followed for migration assays in which the

polycarbonate membranes were soaked for 1 hour in 0.01% gelatin and the assay was processed as described above after 6 hours. Statistical analyses and Student's *t* test for significance of the data generated from the migration and invasion assays, respectively, were done using Microsoft Excel (Microsoft, Redmond, CA). All assays were done in triplicate.

Microarray analysis and expression microarrays. Total RNA from C8161 melanoma cells, normal melanocytes cultured on a human collagen I three-dimensional matrix, and melanocytes cultured on matrix preconditioned by aggressive melanoma cells (each in duplicate) was isolated using TRIZOL reagent (Invitrogen) followed by a further purification through a RNeasy midi column (Qiagen, Valencia, CA). RNAs were labeled and fragmented using the One Cycle Target Labeling Kit (Affymetrix, Santa Clara, CA), hybridized to Affymetrix HG-U133A 2 arrays, washed, stained, and scanned on a GeneChip Scanner 3000 (Affymetrix) according to the recommendations of the manufacturer. Data were extracted using Affymetrix GCOS software and linearly scaled to a trimmed mean intensity of 1,000 units. Genes for which no sample had detectable expression (called "P" by GCOS) were excluded from subsequent analyses.⁶

Semiquantitative reverse transcription-PCR analysis. Total RNA from normal melanocytes cultured on a human collagen I three-dimensional matrix (nonconditioned) or a matrix preconditioned by aggressive melanoma cells was reverse transcribed and gene expression was determined using PCR as previously described (6).⁷

Quantitative real-time PCR analysis. Total RNA (2 μ g) was reverse transcribed using the Advantage PCR kit in accordance with the instructions of the manufacturer (Clontech, Palo Alto, CA). Real-time PCR was done on a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA) using TaqMan gene expression human primer/probe sets for the following genes: α_3 -integrin subunit (Hs00233722_ml), *CD13* (Hs00174265_ml), *c-met* (Hs00179845_ml), *EphA2* (Hs00171656_ml), *keratin 7* (Hs00818825_ml), *Ki-67* (Hs00606991_ml), *laminin 5 γ 2 chain* (Hs00194345_ml), *Melan-A* (Hs00194133_ml), *MITF* (Hs00165156_ml), *PAX8* (Hs00243335_ml), *TIE-1* (Hs00175800_ml), and *urokinase* (Hs00170782_ml). cDNA (5 μ L), 1.25 μ L of 20× Gene Expression Assay Mix, and 12.5 μ L of 2× TaqMan Universal PCR Master Mix were mixed and 20 μ L were amplified with the following thermocycler protocol: 1 cycle at 50°C for 2 minutes, 1 cycle at 95°C for 10 minutes, and 40 cycles at 95°C for 15 seconds/60°C for 1 minute. Target gene expression was normalized to the endogenous control gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; 4333764F). Data were analyzed using Applied Biosystems Sequence Detections Software (version 1.2.3).

Array-based comparative genomic hybridization analysis. For array-based comparative genomic hybridization (array-CGH), genomic DNA was fragmented and random prime labeled as previously published (14). Labeled DNAs were hybridized to human oligonucleotides microarrays containing 22,500 elements designed for expression profiling (Agilent Technologies, Human 1A V2), for which 16,097 unique map positions were defined (National Center for Biotechnology Information, Build 35). The median interval between mapped elements is 54.8 kb; 96.7% of intervals of are <1 Mb and 99.5% are <3 Mb. Fluorescence ratios of scanned images were calculated as the average of two paired (dye swap), and copy number profile was generated based on a segmentation algorithm that uses permutation to determine the significance of change points in the raw data. The segmented CGH profile identifies statically significant transitions in copy number, and copy number value for each segment in the profile is assigned the median of the contained probes. CGH profiles from different samples were compared using the segmentation profiles containing only statistical significant copy number changes.⁸

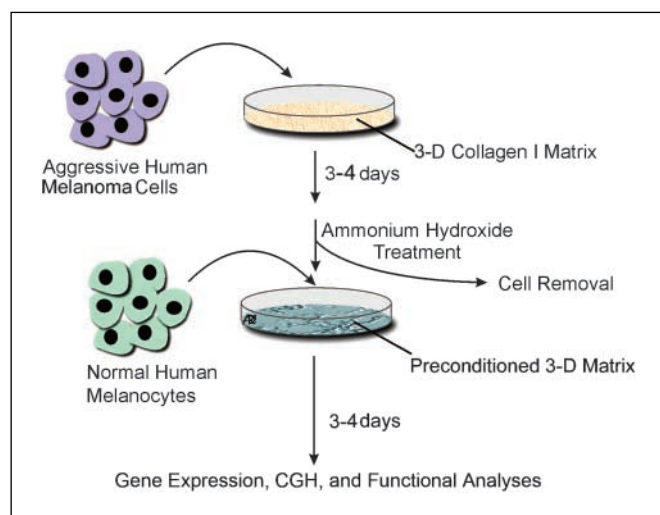


Figure 1. Experimental flow chart. Human metastatic melanoma cells are plated on collagen 1 three-dimensional matrices for 3 to 4 days. After removal of the melanoma cells, human normal melanocytes are plated onto the preconditioned matrix for 3 to 4 days. At the end of this period, gene and protein expression as well as CGH are analyzed and compared with results from human normal melanocytes plated onto collagen I that has not been preconditioned (control).

⁶ Further details about data analysis are available at <http://www.tgen.com>.

⁷ Primers, annealing temperatures, and number of amplification cycles for each gene that was evaluated are listed at <http://www.childrensmrc.org/hendrix/supplemental/>.

⁸ Array-CGH profiles were done at the Center for Applied Cancer Science at Dana-Farber Cancer Institute.

Results and Discussion

Experimental model to study epigenetic changes in normal melanocytes. To gain insight into the molecular changes that occur during melanocytic progression to a melanoma phenotype, we developed a three-dimensional model that allows observations of the interactions of normal melanocytes with a metastatic melanoma matrix microenvironment to determine the possible epigenetic influence of this microenvironment on melanocyte behavior. Specifically, our study tested the hypothesis that the microenvironment of metastatic melanoma cells could induce a transdifferentiated phenotype in benign human epidermal melanocytes. The model and outline of the experimental approach are depicted in Fig. 1. C8161 human metastatic melanoma cells were seeded onto a three-dimensional collagen I matrix and allowed to precondition this microenvironment for up to 4 days, and then the cells were removed while leaving the matrix intact. Subsequently, normal human epidermal melanocytes were seeded onto the melanoma-preconditioned matrix microenvironment for up to 4 days, followed by analyses of potential changes in global gene expression, array-CGH of genome-wide chromosomal numerical alterations, and migratory and invasive ability.

Altered phenotype of normal human melanocytes exposed to the microenvironment of metastatic cutaneous melanoma. The morphologic analyses of the melanoma cells and melanocytes are shown in a side-by-side comparison in Fig. 2. Aggressive C8161 human cutaneous metastatic melanoma cells form vasculogenic-like networks on a three-dimensional collagen I matrix (Fig. 2A) whereas low passage, normal human epidermal melanocytes (from several different primary cultures) grown for 4 days on a three-dimensional collagen I matrix form a homogeneous dense cellular sheet with no networks (Fig. 2B). When the normal melanocytes were seeded onto the three-dimensional matrices preconditioned by the C8161 metastatic melanoma cells, they acquired an altered phenotype manifested by the formation of vasculogenic-like networks (Fig. 2C), similar to those formed by the metastatic melanoma cells (Fig. 2A). By comparison, when normal melanocytes were seeded onto three-dimensional collagen I matrices preconditioned by poorly aggressive, genetically matched C81-61 human melanoma cells, neither the melanoma cells (Fig. 2D, *inset*) nor the melanocytes (Fig. 2D) showed a vasculogenic phenotype. These data indicate that the three-dimensional matrices preconditioned by the aggressive metastatic melanoma, but not by the poorly aggressive melanoma (derived from the same patient), exert an epigenetic effect on normal melanocytes, leading to their transdifferentiation to a phenotype resembling the aggressive melanoma cells. These observations are further supported by the molecular signature(s) of C8161 versus C81-61 melanoma cells showing the expression of multiple cellular phenotypes with augmented ECM remodeling capabilities by the aggressive C8161 cells, but not by the poorly aggressive C81-61 cells (6). Additional evidence showing an altered melanocyte phenotype is shown in Fig. 2E where the melanocytes cultured on the metastatic C8161 melanoma-preconditioned matrix were 2.2-fold more migratory and 3.6-fold more invasive than melanocytes cultured on a control three-dimensional collagen I matrix. (Similar results were achieved with other melanocyte cultures exposed to the metastatic microenvironment, as shown in the Supplementary data).

Epigenetic induction of differential gene expression in normal human melanocytes exposed to the microenvironment of metastatic cutaneous melanoma cells. To characterize the effect of microenvironment on gene expression, we expression profiled human metastatic C8161 melanoma cells (in duplicate),

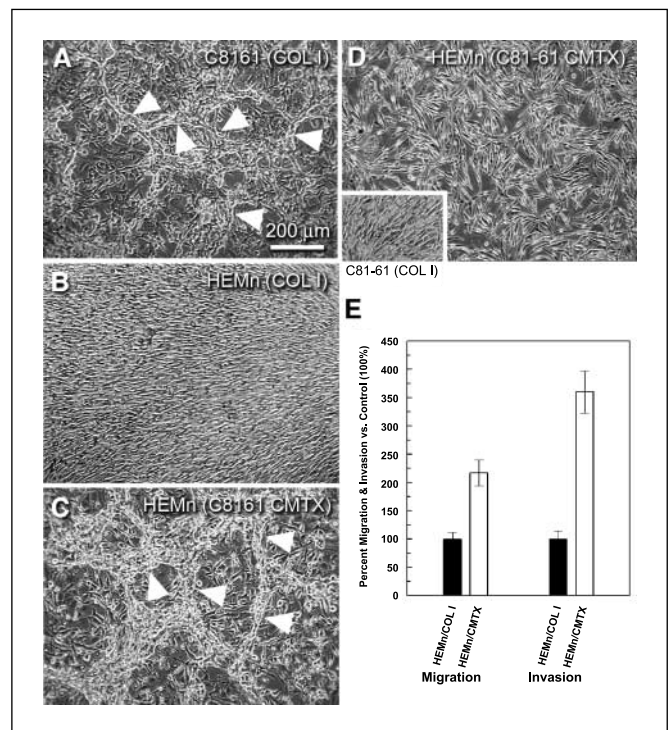
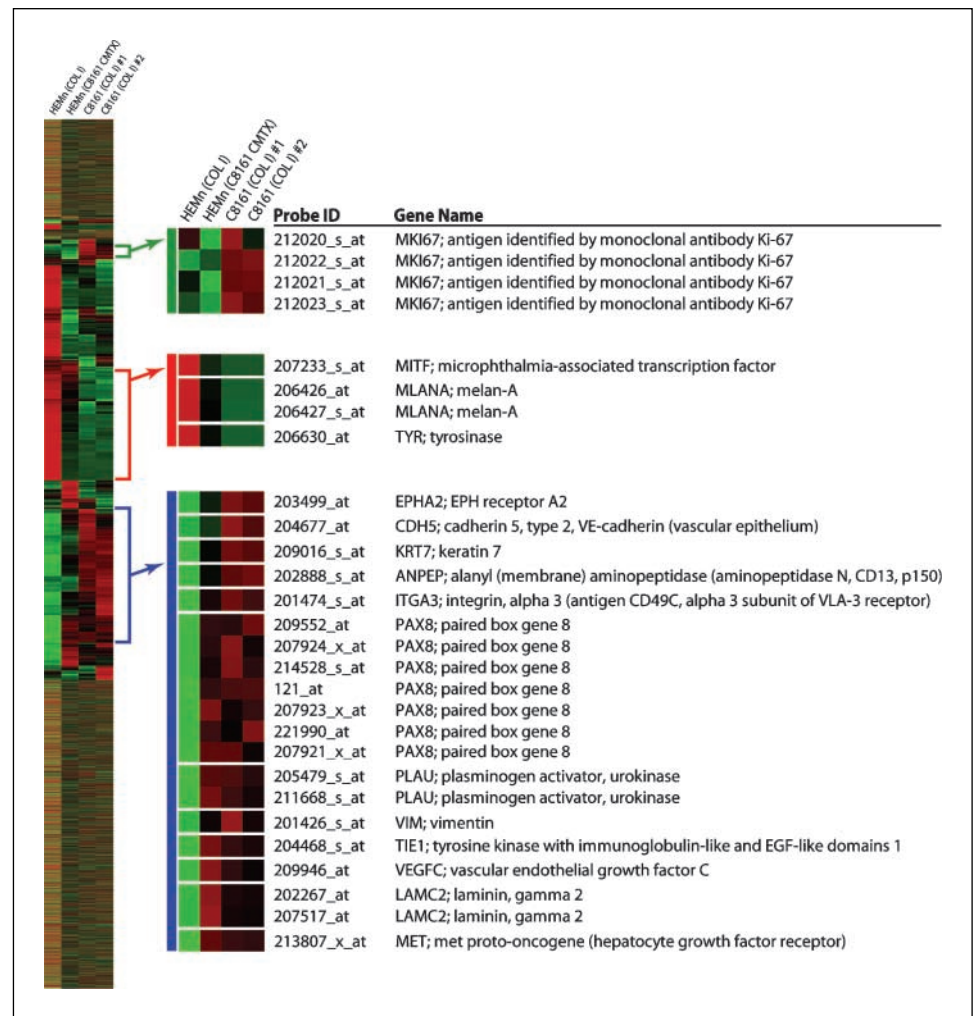


Figure 2. Epigenetic changes in normal melanocytes exposed to a metastatic melanoma microenvironment. A, aggressive C8161 human cutaneous metastatic melanoma cells form vasculogenic-like networks (arrowheads) when grown on a three-dimensional collagen I matrix (COL I). B, human epidermal melanocytes (HEMn18) cultured on three-dimensional collagen I matrix do not form vasculogenic-like networks. C, HEMn18 cultured on a C8161 melanoma cell-preconditioned matrix (C8161 CMTX) after removal of the C8161 melanoma cells are induced to form vasculogenic-like networks (arrowheads). D, HEMn18 cultured on a poorly aggressive C81-61 human cutaneous melanoma cell-preconditioned matrix (after removal of the cells; C81-61 CMTX) do not form vasculogenic-like networks. *Inset*, C81-61 cells do not form vasculogenic-like networks when cultured on a three-dimensional collagen I matrix. A-D, phase contrast microscopy; bar, 200 μ m. E, percent migration and invasion of HEMn18 *in vitro* after culturing on a control, three-dimensional collagen I matrix (each normalized to 100%) relative to their percent migration and invasion after culturing on a three-dimensional collagen I matrix preconditioned by aggressive C8161 melanoma cells (HEMn/CMTX) for 4 days (after removal of the C8161 cells). HEMn18 cultured on the C8161 melanoma cell-preconditioned matrix were 2.2-fold more migratory and 3.6-fold more invasive than HEMn18 cells cultured on a control three-dimensional collagen I matrix for 4 days.

normal human epidermal melanocytes grown on collagen I, and normal human epidermal melanocytes grown on collagen I matrices preconditioned by C8161 melanoma cells. Expression values for genes with detectable expression in the data set were subjected to hierarchical clustering (Fig. 3). Matrix preconditioned by aggressive melanoma cells greatly alters the pattern of gene expression of normal human epidermal melanocytes, both inducing (cluster denoted in blue) and repressing (cluster denoted in red) expression of many genes in a manner consistent with the pattern of gene expression observed for aggressive C8161 melanoma cells themselves. Unsupervised sample classification algorithms including principal components analysis, multidimensional scaling, and hierarchical clustering all suggest that the gene expression pattern of melanocytes grown on C8161-preconditioned matrix is more similar to that of C8161 cells than that of normal human melanocytes grown on control, nonconditioned collagen I (data not shown).⁹

⁹ The complete global gene analysis is available at <http://www.tgen.com>.

Figure 3. Hierarchical clustering of all genes with detectable gene expression via oligonucleotide microarray. Microarray data for human epidermal melanocytes (HEMn18) grown on a three-dimensional collagen I matrix, HEMn18 grown on a three-dimensional collagen I matrix preconditioned by human cutaneous metastatic melanoma C8161 cells, and two replicates of C8161 cells grown on a three-dimensional collagen I matrix (#1 and #2) were normalized on a per-gene basis. Genes were clustered using Cluster and TreeView software (<http://rana.lbl.gov/EisenSoftware.htm>). Black, average expression levels for a given gene are represented; red, above average expression; green, below average expression. Select genes chosen for RT-PCR validation are highlighted.



Confirmation of selected differentially expressed genes was accomplished by semiquantitative reverse transcription-PCR (RT-PCR), shown in Fig. 4A. (An additional melanocyte culture exposed to the metastatic melanoma matrix is shown in the Supplementary data.) The categories of genes tested consisted of differentiation/phenotype-associated genes [erythropoietin-producing hepatocellular carcinoma-A2 (*EphA2*); vascular endothelial-cadherin (*VE-cadherin*); tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 1 (*TIE-1*), associated with blood vessel formation and maintenance (11); paired box gene 8 (*PAX8*), a transcription factor important in thyroid cell differentiation (15); vascular endothelial growth factor-C (*VEGF-C*); *keratin 7*, an epithelial marker; *CD13*, a mesenchymal stem cell-associated gene; and *vimentin*, a mesenchymal marker]; ECM remodeling-, proliferation-, and migration/invasion-associated genes [*laminin 5* $\gamma 2$ chain and an associated receptor, $\alpha 3$ -*integrin subunit*; *urokinase* and *Ki-67*]; melanocyte pathway-associated genes [*MITF* (a basic/helix-loop-helix/leucine-zipper transcription factor), *Melan-A*, and *tyrosinase*]. These data indicate that the normal melanocytes exposed to a metastatic melanoma matrix for 4 days were reprogrammed to express genes associated with a multipotent, plastic phenotype similar to the aggressive C8161 melanoma cells. Specifically, the melanocytes up-regulated genes associated with (a) a transdifferentiated phenotype (*EphA2*, *VE-cadherin*, *TIE-1*,

VEGF-C, *PAX8*, *keratin 7*, and *CD13*) and (b) an ECM remodeling, migratory, and invasive phenotype (*laminin 5* $\gamma 2$ chain, *urokinase*, $\alpha 3$ -*integrin subunit*, and *c-met*). However, there were two notable exceptions pertinent to the confirmed genes tested. The first exception is that the melanocytes did not acquire the Ki-67 proliferation marker, which is strongly expressed by the metastatic melanoma cells. Another important exception is that the melanocytes did not lose their characteristic expression of pigmentation pathway-associated genes (*MITF*, *Melan-A*, and *tyrosinase*). *MITF* is a melanoma-specific transcription factor that regulates the expression of the melanoma antigen recognized by T cells 1 (*MART1*) gene encoding Melan-A (8) and tyrosinase is a critical enzyme in the melanin biosynthesis pathway (16). Previous reports have shown the favorable disease outcome in melanoma patients in whom these differentiation melanocyte-associated antigen genes are expressed (7). Thus, it is tempting to speculate that efforts to reexpress these genes in patients with metastatic disease could serve as a viable strategy for redifferentiating a highly aggressive, plastic, melanoma tumor cell phenotype to a melanocytic phenotype. Our data suggest that the melanocyte phenotype is maintained at least through the initial stage of epigenetic transition. However, the robust induction of c-Met expression—associated with the augmented invasive and metastatic potential (17, 18)—in the benign melanocytes exposed to metastatic

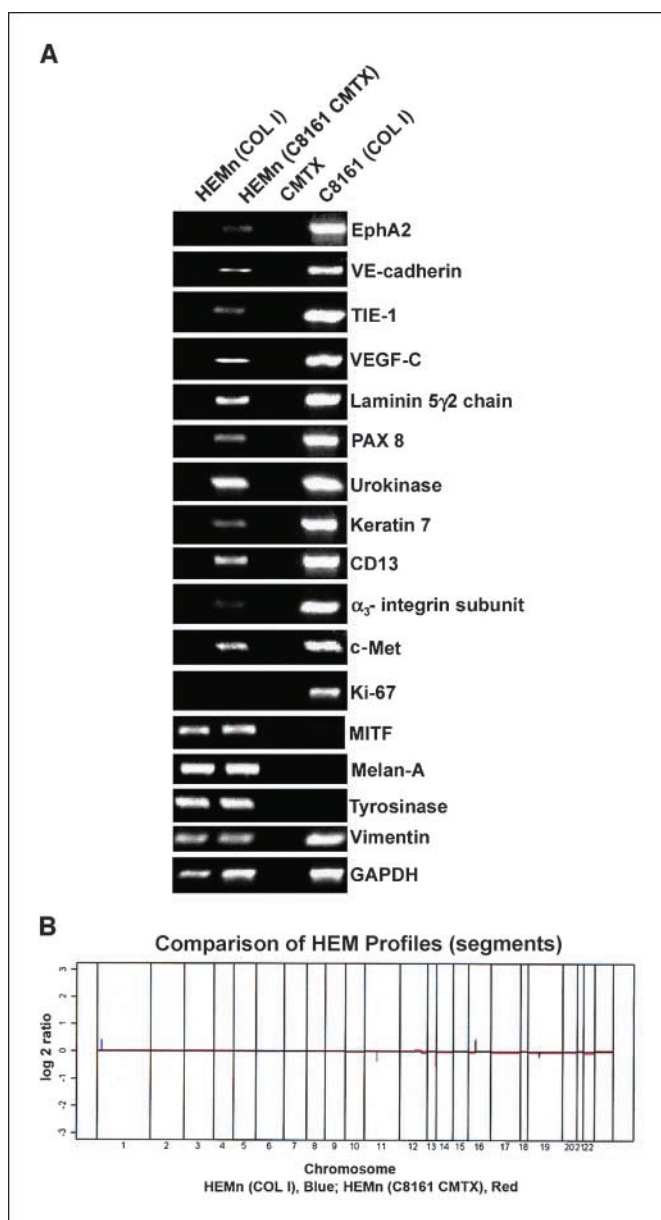


Figure 4. Epigenetic transdifferentiation of normal melanocytes exposed to a metastatic melanoma microenvironment. *A*, confirmation of differentially expressed genes in human epidermal melanocytes (HEMn18) cultured on a collagen I three-dimensional matrix compared with HEMn18 cultured on a C8161 metastatic melanoma–preconditioned matrix by semiquantitative RT-PCR analysis. Gene-specific primers (available at <http://www.childrensmrc.org/hendrix/supplemental/>) were used to evaluate the expression of EphA2, VE-cadherin, TIE-1, VEGF-C, laminin 5 γ 2 chain, PAX8, urokinase, keratin 7, CD13, α_3 -integrin subunit, c-Met, Ki-67, MITF, Melan-A, tyrosinase, and vimentin. The CMTX lane serves as a control showing the complete removal of the tumor cells from the preconditioned matrix before subsequent HEMn plating. *B*, array-CGH profiles of HEMn18 DNA from cells cultured on a three-dimensional collagen I matrix (blue) compared with HEMn18 DNA from cells cultured on a C8161-preconditioned matrix (red) referenced against male DNA with the x-axis coordinate representing the oligonucleotide probes ordered by genomic map position and displayed by segmented data.

melanoma microenvironments strongly indicates that these cells are acquiring invasive properties as further shown by their increased invasive and migratory ability. The Supplementary data showing the RT-PCR analysis (*A*) and confirmation of select genes using an additional normal human melanocyte cell culture exposed

to a C8161-preconditioned matrix, as well as the migration and invasion data (*B*), are generally overlapping with the data presented in Figs. 2 and 4. However, it is important to note that the melanocytes exposed to the melanoma matrix for 4 days (Fig. 4A) show a more robust gene expression than the melanocytes exposed to a similar matrix for only 3 days (Supplementary data A), thus illustrating a direct correlation between the time of exposure and epigenetic induction by the microenvironment. To determine the stability and longevity of the epigenetic induction of the transdifferentiated melanocytes, the cells were removed from the metastatic melanoma–preconditioned matrices and cultured on tissue culture plastic for 7, 14, and 21 days, respectively. As shown in Table 1, the expression of key genes associated with an aggressive melanoma phenotype is remarkably reduced by 7 days and almost undetectable by 21 days, indicating that the epigenetic effect is transient in nature. Thus, removal of the melanocytes from the inductive metastatic melanoma matrix results in a reversion to their normal phenotype. Another noteworthy observation presented in the Supplementary data (*C*) is the RT-PCR analysis of C8161 cells exposed to three-dimensional collagen I matrices preconditioned by two independent cultures of normal human melanocytes showing no change in the expression of genes tested. These data indicate that a benign melanocyte microenvironment does not epigenetically influence metastatic melanoma cells to change their plastic, molecular phenotype.

Table 1. Quantitative PCR of longevity of C8161-conditioned matrix–induced gene expression in HEMn

Gene name	Col I	CMTX	Post CMTX, day 7	Post CMTX, day 14	Post CMTX, day 21
Laminin 5 γ 2 chain	(1.0) ^{*,†}	>100	24.5	10.7	8.0
Keratin 7	(1.0) [†]	>100	18.1	8.8	5.8
CD13	(1.0) [†]	>100	15.3	5.8	4.0
α_3 -Integrin subunit	1.0	18.6	4.2	4.4	5.0
Ki-67	1.0	7.4	0.8	0.9	0.7
MITF	1.0	2.3	2.1	3.2	2.4
Melan-A	1.0	0.6	1.3	1.5	2.1
Vimentin	1.0	1.5	2.3	2.5	2.9
EphA2	1.0	42.1	1.4	1.4	2.0
VEGF-C	(1.0) [†]	>100	8.9	4.5	5.1
VE-cadherin	(0) ^{†,‡}	>100	3.5	(0.6) [†]	(1.0) [†]
TIE-1	(0) ^{†,‡}	94	2.9	(1.1) [†]	(1.0) [†]
PAX8	(0) ^{†,‡}	>100	8.9	1.9	(1.0) [†]
Urokinase	(0) ^{†,‡}	>100	4.9	2.4	1.0

Abbreviations: Col I, three-dimensional collagen I matrix; CMTX, C8161-conditioned matrix.

*Quantitative real-time PCR analysis of fold difference in gene expression of melanocytes (HEMn) exposed to C8161-conditioned matrix, replated onto tissue culture plastic for 7, 14, and 21 days, respectively, and then compared with HEMn grown on a three-dimensional collagen I matrix (value normalized to 1.0).

† $C_t > 34$ cycles.

‡Fold difference in gene expression relative to post CMTX day 21 value normalized to 1.0 due to the undetectable levels of gene expression of HEMn grown on a three-dimensional collagen I matrix.

Lastly, array-CGH analysis of genome-wide chromosomal numerical aberrations, particularly regional amplification and deletions, was done to determine whether any genomic changes had occurred in the normal melanocytes exposed to metastatic melanoma matrix microenvironments (Fig. 4B). Comparison of the DNA profiles of melanocytes grown on a three-dimensional control collagen I matrix and melanocytes exposed to a matrix preconditioned by human metastatic C8161 melanoma cells revealed no significant genomic changes between these DNA samples, further supporting the epigenetic induction of changes in melanocyte gene expression directly related to exposure to the metastatic microenvironment.

Transdifferentiation is emerging as an important phenomenon that adds a new level of complexity to developing rational therapeutic strategies (11, 19). Pertinent to this theme and our work is the observation that during the development of Kaposi's sarcoma, endothelial cells transdifferentiate into tumor cells (20) whereas aggressive melanoma cells and melanocytes exposed to a metastatic melanoma matrix transdifferentiate to form endothelial-

like networks known as vasculogenic mimicry. Our current study used a novel experimental approach that revealed an epigenetic induction of a transdifferentiated phenotype in normal melanocytes exposed to a microenvironment of metastatic cutaneous melanoma cells. The three-dimensional microenvironment model presented here may serve as a viable tool to address essential signaling pathways and epigenetic mechanisms critical to melanocytic transformation to melanoma. These findings offer new insights into microenvironmental influence on cellular differentiation and plasticity, as well as mechanisms that could be targeted for novel therapeutic strategies in preventing melanocytic neoplasia.

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