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The epigenetic reprogramming of poorly aggressive melanoma cells by a metastatic microenvironment

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Abstract

A dynamic, complex relationship exists between tumor cells and their microenvironment, which plays a pivotal role in cancer progression, yet remains poorly understood. Particularly perplexing is the finding that aggressive melanoma cells express genes associated with multiple cellular phenotypes, in addition to their ability to form vasculogenic-like networks in three-dimensional matrix - called vasculogenic mimicry, which is illustrative of tumor cell plasticity. This study addressed the unique epigenetic effect of the microenvironment of aggressive melanoma cells on the behavior of poorly aggressive melanoma cells exposed to it. The data show significant changes in the global gene expression of the cells exposed to 3-D matrices preconditioned by aggressive melanoma cells, including the acquisition of a vasculogenic cell phenotype, upregulation of ECM remodeling genes, and increased invasive ability - indicative of an epigenetic, microenvironment-induced reprogramming of poorly aggressive melanoma cells. However, this epigenetic effect was completely abrogated when a highly cross-linked collagen matrix was used, which could not be remodeled by the aggressive melanoma cells. These findings offer an unique perspective of the inductive properties associated with an aggressive melanoma microenvironment that might provide new insights into the epigenetic regulation of tumor cell plasticity and differentiation, as well as mechanisms that could be targeted for novel therapeutic strategies.

Keywords: melanoma - plasticity - vasculogenic mimicry - 3-dimensional matrix - epigenetic - tumor microenvironment

Introduction

Cancer pathogenesis involves dynamic interactions within the tumor-host microenvironment. Most noteworthy is cutaneous melanoma which is considered one of the few remaining cancers escalating in incidence [1, 2], and thus represents a growing public health burden worldwide [3; for review, see 4; 5]. In addition, uveal melanoma is considered the most common primary intraocular cancer in adults [6], where metastasis occurs in an unpredictable manner in approximately 50% of patients with a primary tumor originating in the choroid or ciliary body of the eye [6]. Without question, the clinical manage-

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ment of cutaneous and uveal melanoma, and many other types of cancer, would benefit significantly from the identification of valid predictors of disease onset, progression and metastatic potential.

Recent reports directed toward unveiling the molecular signature of melanoma tumor cells have resulted in important classification schemes for cutaneous [7] and uveal [8, 9] melanoma. In fact, translational studies are beginning to emerge that support the promise of microarray technology in melanoma care [10; for review, see 11]; however, the precise biological function of aberrantly expressed genes, their involvement in phenotype diversity, and their role(s) in tumor cell interactions with the microenvironment are yet to be fully understood.

With respect to melanoma, comparative global gene analyses of aggressive and poorly aggressive human cutaneous and uveal melanoma cell lines have revealed the unexpected finding that aggressive tumor cells express genes (and proteins) that are associated with multiple cellular phenotypes [8, 12, 13]. These include genes that are usually expressed by epithelial, endothelial, pericyte, fibroblast, hematopoietic, kidney, neuronal, muscle, and several other cell types, and their respective precursor stem cells. These molecular findings suggest that aggressive melanoma cells revert to an undifferentiated, "plastic" phenotype, a concept that challenges our current thinking of how to identify and target tumor cells that can possibly masquerade as other cell types. A specific example of melanoma cell plasticity is vasculogenic mimicry, which characterizes the unique ability of aggressive melanoma cells (but not poorly aggressive melanoma cells) to express endothelia-associated genes and form extracellular matrix (ECM)-rich vasculogenic-like networks in three-dimensional (3-D) culture [14; for review, see 12]. The formation of these networks recapitulates the embryonic development of vasculogenic networks, and they are associated with the distinctly patterned, ECM-rich networks observed in aggressive tumors of patients with melanoma [8, 14–17]. Additional studies have confirmed vasculogenic mimicry in various tumor types [for review, see 12], including the demonstration of blood flow and fluid exchange between tumor cell-lined vascular spaces and endothelium-lined vasculature [18–20]. Moreover, there is now compelling evidence for the existence of an intratumoral, tumor-cell-lined, ECM-rich, patterned network that can provide an extravascular fluid pathway, referred to as the "fluid-conducting meshwork" [19–21], that may have significant implications for tumor perfusion and dissemination.

The precise etiology of vasculogenic mimicry remains unclear; however, this form of plasticity involves dysregulation of the tumor-specific phenotype and the concomitant transdifferentiation of aggressive tumor cells into other cell types - such as endothelial-like cells [22]. Furthermore, select angiogenesis inhibitors are ineffective in destroying tumor cell vasculogenic mimicry [23, 24], which is an important consideration in the design of antivascular therapies. Therefore, our goal is to identify the molecular mechanisms underlying vasculogenic mimicry and tumor cell plasticity and to elucidate the unique role the tumor microenvironment plays in this process. The current study represents an extension of previous work [25] with an overarching objective to determine the potential epigenetic influence of the microenvironment of aggressive melanoma cells on poorly aggressive melanoma cells. The data reveal profound changes in the global expression of poorly aggressive melanoma cells exposed to 3-D collagen I matrices preconditioned by aggressive melanoma cells - indicative of an epigenetic, microenvironment-induced reprogramming of a tumor cell phenotype. In addition, the poorly aggressive melanoma cells acquired characteristics associated with an aggressive phenotype, including the expression of various cell phenotype associated genes, ECM remodeling genes, and acquisition of increased invasive potential. It is anticipated that these findings will offer an unique perspective of the inductive properties associated with the microenvironment of aggressive tumor cells that might provide new targets and paradigms for therapeutic intervention strategies.

Materials and methods

Cell culture

The primary choroidal or ciliary body human uveal melanoma cell lines (OCM-1A, C918), and cell lines derived from a heterogeneous uveal melanoma liver metastasis (MUM-2B, MUM-2C) have been described previously [8, 26]. These cell lines were maintained in

Table 1 Biological properties of human melanoma

Culture designation	Cell phenotype†	Invasive potential‡	Vasculogenic mimicry§
OCM-1A	vimentin only	Poor (2.0 ± 2.2 0.1)	-
C918 (primary)	vimentin+keratin(s)	High (12.5-13 ± 0.5)	+
MUM-2B	vimentin+keratin(s)	High (13.3-15 ± 0.6)	+
MUM-2C	vimentin only	Poor (2.0-2.3 ± 0.06)	-

† Scoring of tumor cell phenotype using classical pathology markers of vimentin (mesenchymal) and cytokeratins 8 and 18 (epithelial) intermediate filaments (IFs) was based on a positive (+) and negative (-) ranking system, determined by immunohistochemistry and Northern blot analysis.
‡ Invasiveness was calculated as the percentage of cells capable of invading a collagenous matrix-coated polycarbonate membrane over 24 hours within a membrane invasion culture system (MICS) chamber compared with the total number of cells seeded (± SE; n=6 wells per parameter and run in triplicate experiments).
§ Vasculogenic mimicry was assessed based on the ability of cells seeded onto a three-dimensional collagen I matrix to form tubular vasculogenic-like networks over 7 days.
Derived and subsequently cloned from the MUM-2 heterogeneous cell line [8].

RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 0.1% gentamycin sulfate (Gemini Bioproducts, Calabasas, CA). The biological properties of all cell lines used in this study are presented in Table 1. All cultures were determined to be free of *mycoplasma* contamination using a PCR-based detection system (Roche, Indianapolis, IN).

Preconditioned matrices

A critical aspect of this work was the production of defined 3-dimensional (3-D) matrices as follows: Twenty-five microliters of deformable type I collagen (average 3 mg/ml; Discovery Labware, Bedford, MA), or highly cross-linked, rigid, native rat tail (tendon) collagen from adult rats (a kind gift from Dr. Charles Little, Kansas University Medical Center) was dropped into twelve-well culture dishes and polymerized with an application of 100% ethanol at room temperature. After extensive washing with PBS, tumor cells were seeded onto the 3-D matrix in complete medium. The 3-D cultures were then observed after 3-4 days and images captured digitally using a Zeiss Televal inverted microscope (Carl Zeiss, Inc., Thornwood, NY) and Hitachi HV-C20 CCD camera (Hitachi Denshi America, Ltd.). For experiments designed to analyze the transdifferential potential of poorly aggressive melanoma cells (OCM-1A and MUM-2C) when placed on a matrix preconditioned by highly aggressive melanoma cells (C918 or MUM-2B), the aggressive tumor cells which had preconditioned the matrix were removed after 3-4 days with 20 mM NH_4OH followed by thorough washes with water, PBS, and then complete medium. A detailed outline of this experimental protocol is presented in Fig. 1.

Invasion assays

Uveal melanoma cells (5 x 10^4) were harvested from collagen I or collagen I preconditioned by MUM-2B cells using 2 mM EDTA in PBS followed by resuspension in RPMI medium supplemented with 1X Mito+ (Discovery Labware). The cells were then seeded into the upper wells of the MICS (membrane invasion culture system) chamber [26] onto human collagen IV/laminin/gelatin-coated (Sigma Chemical Co., St. Louis, MO) polycarbonate membranes containing 10-mm pores (Osmonics, Livermore, CA). After 24 h of incubation at 37°C, the cells that invaded each membrane were collected, stained and counted as previously described [26]. Percent invasion was corrected for proliferation and calculated as the total number of invading cells divided by the total number of cells seeded X 100. Statistical analyses and student's t test for significance of the data generated from the invasion assays were performed using Microsoft Excel (Microsoft, Redmond, CA). All assays were performed in triplicate.

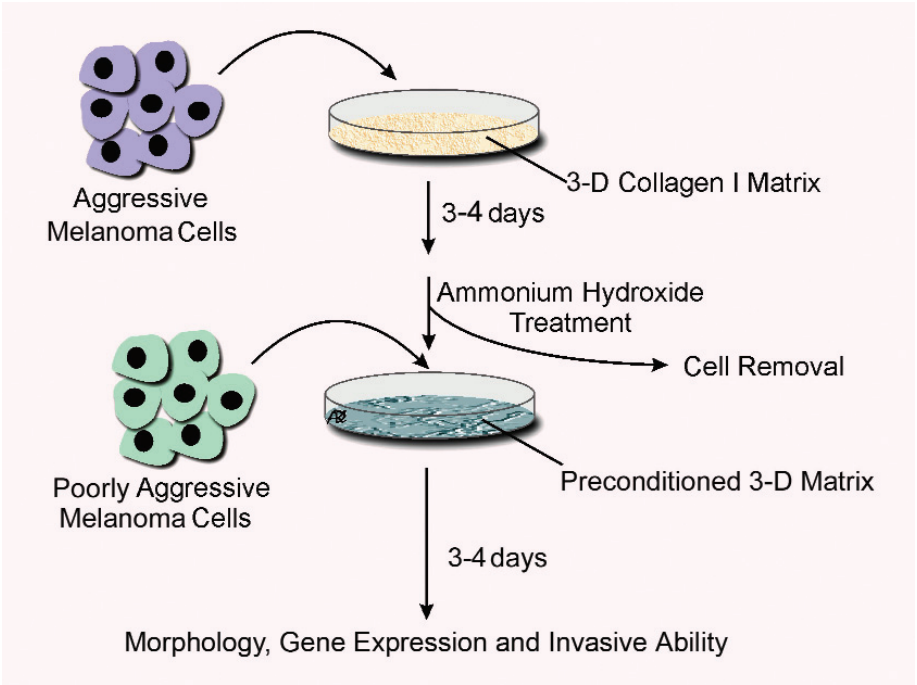


Fig. 1 Experimental plan. As shown in this model, aggressive melanoma cells are cultured on a 3-D, collagen I matrix for 3-4 days, after which the cells are removed using ammonium hydroxide followed by extensive washing of the matrix. This was followed by seeding the poorly aggressive melanoma cells onto the preconditioned matrix, then analyzing the cells for changes in morphology, gene expression and invasive ability compared to the poorly aggressive cells cultured on a non-inductive matrix.

Microarray analysis

Total RNA was labeled with either Cy3-dUTP or Cy5-dUTP by reverse transcription and hybridized to 15,000 element sequence verified human cDNA spotted microarrays as previously described [27]. Images were captured by laser scanner (Agilent Technologies, Palo Alto, CA), and processed with DeArray software. Genes were selected for analysis which showed a ratio of >1.4 or <0.6 in one or more of the conditioned matrix culture experiments. Hierarchical clustering was done using the average linkage method.

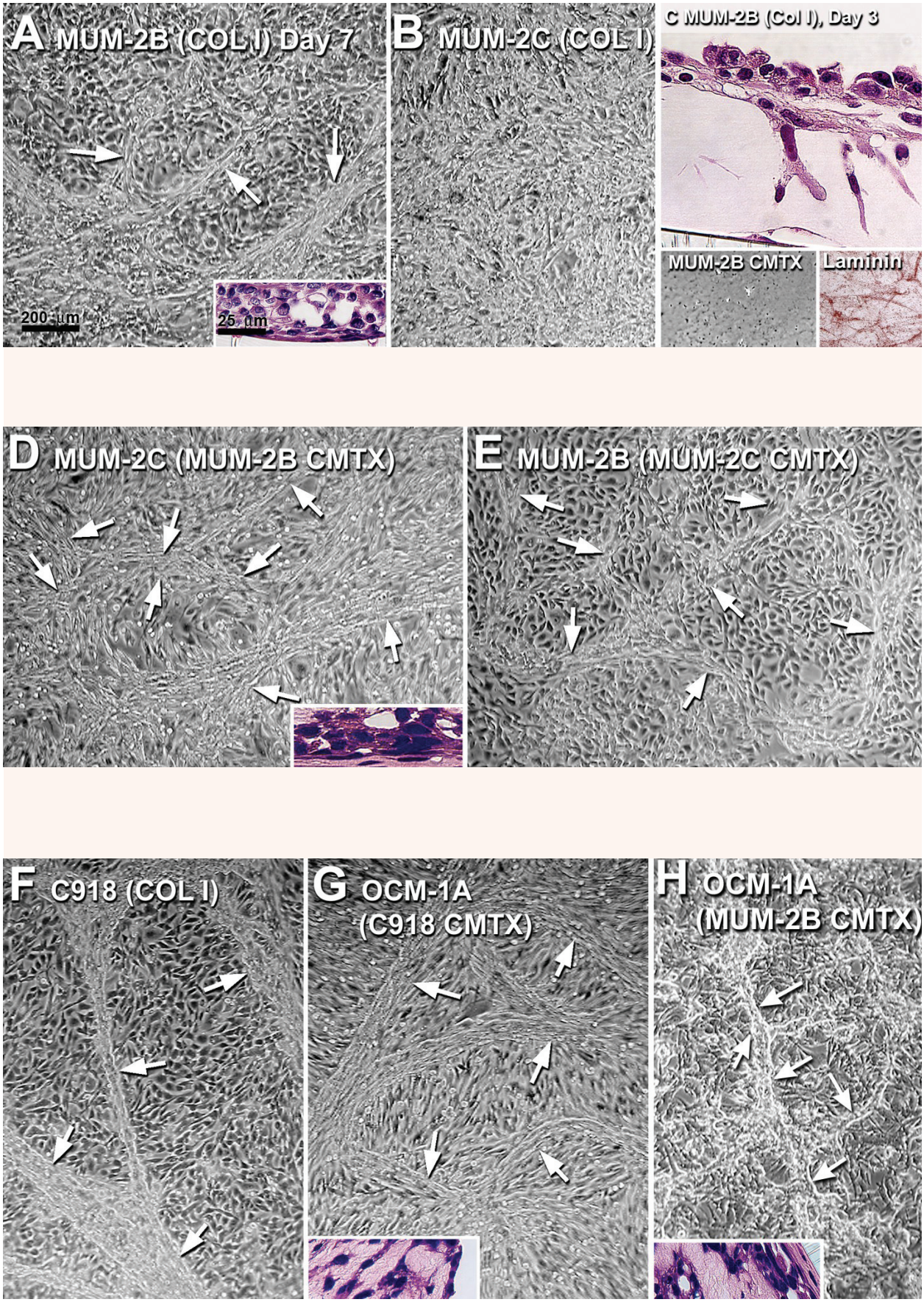
Confirmation of microarray gene expression

Selected differentially expressed genes were further evaluated by semiquantitative RT-PCR analysis. Total RNA from uveal melanoma cell lines cultured on a human collagen I 3-D matrix or a matrix preconditioned by aggressive melanoma cells (Trizol reagent, Invitrogen) was reverse transcribed using the Advantage PCR kit as per the manufacturer's protocol (Clontech Laboratories, Palo Alto, CA). PCR amplifications were performed with gene-specific primers (listed at http://www.childrensmrc.org/hendrix/supplemental/JCMM). Annealing temperature and number of amplification cycles were optimized using cDNA from MUM-2B and MUM-2C cells. PCR amplification reactions were performed in an Infinity Robocycler thermocycler (Stratagene, La Jolla, CA): 1 cycle: 94°C, 1 min; 27–30 cycles: 94°C, 1 min; 62°C or 68°C, 2.5 min, 72°C, 1 min; and 1 cycle: 72°C, 5 min. GAPDH primers (Clontech) were used as controls for PCR amplification. PCR fragments were ligated into the pCR2.1-TOPO sequencing vector as per the manufacturer's protocol (Invitrogen). Plasmid DNA was isolated and subjected to DNA sequencing analysis using fluorescent Sanger-based dideoxy sequencing on an ABI 373A Automated Sequencer (University of Iowa DNA Facility). Two plasmids from each primer set were sequenced and shown to contain 100% identity to the expected DNA sequence.

Results

Acquisition of a vasculogenic phenotype in poorly aggressive melanoma cells exposed to a microenvironment preconditioned by aggressive metastatic melanoma cells

We sought to determine whether the microenvironment of aggressive melanoma tumor cells could influence the phenotype, gene expression profile,


A MUM-2B (COL I) Day 7
200 µm
25 µm
B MUM-2C (COL I)
C MUM-2B (Col I), Day 3
MUM-2B CMTX
Laminin
D MUM-2C (MUM-2B CMTX)
E MUM-2B (MUM-2C CMTX)
F C918 (COL I)
G OCM-1A (C918 CMTX)
H OCM-1A (MUM-2B CMTX)

and invasive potential of poorly aggressive melanoma cells exposed to a "preconditioned" ECM microenvironment as outlined in Fig. 1. The biological properties of the human melanoma tumor cells used in this study are listed in Table 1. The cell phenotype was determined by immunohistochemistry and Northern blot analyses for the expression of cell-type-specific intermediate filament markers: vimentin for the mesenchymal phenotype, and keratins 8, 18 for an epithelial phenotype. Additional assessments were conducted for *in vitro* invasive potential (through basement membrane-coated filters) and for the ability of these different cells to form vasculogenic-like networks (vasculogenic mimicry) in 3-D collagen I matrices. The data presented in Table 1 revealed that the human melanoma cells could be classified into two distinctive categories (based on their biological behavior) - poorly aggressive and aggressive. Specifically, the poorly aggressive OCM-1A cells (derived from a primary tumor), and the MUM-2C cells (derived from the MUM-2 heterogeneous cell line) both expressed vimentin only, a classical melanoma mesenchymal marker, were poorly invasive *in vitro*, and did not engage in vasculogenic mimicry. In contrast, the aggressive C918 cells (derived from a primary tumor), and the MUM-2B cells (also derived from the heterogeneous MUM-2 cell line) coexpressed vimentin and keratin(s), indicative of a dedifferentiated, interconverted phenotype, previously described by our laboratory [8, 26]. In addition, these aggressive melanoma cells were highly invasive *in vitro* and formed vasculogenic-like networks in 3-D collagen I matrix.

To investigate the potential epigenetic effect(s) of the tumor cell microenvironment (associated with aggressive melanoma cells) on the possible reprogramming of poorly aggressive melanoma cells, we allowed the aggressive MUM-2B cells to "precondition" a defined 3-D microenvironment consisting of a deformable collagen I matrix for up to 3-4 days, then removed the tumor cells and seeded the poorly aggressive MUM-2C cells on the "preconditioned matrix". Additional experimental combinations tested included the poorly aggressive OCM-1A cells incubated on 3-D matrices preconditioned by either the aggressive C918 or MUM-2B melanoma cells, and MUM-2B cells incubated on a 3-D matrix preconditioned by poorly aggressive MUM-2C cells. The results from these experiments are shown in Fig. 2. The aggressive MUM-2B cells formed mature, patterned, vasculogenic-like networks by 7 days on 3-D collagen I matrix, and histological cross-sections of these cultures revealed the tubular nature of the network structures (Figure 2A, inset). By comparison, the poorly aggressive MUM-2C cells were unable to engage in vasculogenic mimicry under similar experimental conditions (Fig. 2B). In preparation for the next set of experiments involving the incubation of poorly aggressive melanoma cells on 3-D matrices preconditioned by aggressive tumor cells, it was important to first determine the morphological status of the aggressive melanoma cell cultures after 3-4 days of

Fig. 2 Phase contrast microscopy of human melanoma cells cultured on collagen I 3-D matrices under varying conditions. (A) Highly aggressive, metastatic MUM-2B cells cultured on a collagen I (COL I) 3-D matrix for seven days form patterned, vasculogenic-like networks that appear tubular when examined by cross-section and stained with H&E (inset). (B) Poorly aggressive, metastatic MUM-2C cells do not form these networks when cultured under the same conditions for seven days. (C) Cross-section of an H&E stained culture of MUM-2B cells on a 3-D matrix after three days and phase contrast image of the matrix after removal of the cells (left inset) and subsequent staining of the preconditioned matrix for laminin (right inset). (D) Poorly aggressive MUM-2C cells form patterned, vasculogenic-like networks when cultured on a matrix preconditioned by the MUM-2B cells (MUM-2B CMTX) and the structures appear tubular in cross-section when stained with H&E (inset). (E) MUM-2B cells cultured on a MUM-2C preconditioned matrix are not inhibited in the formation of the patterned networks by the poorly aggressive cell preconditioned matrix. (F) Highly aggressive primary uveal melanoma C918 cells form patterned, vasculogenic-like networks when cultured on a collagen I 3-D matrix. (G) Poorly aggressive, primary uveal melanoma OCM-1A cells form patterned tracks when cultured on a matrix preconditioned by the C918 cells (C918 CMTX), although the structures do not appear to be tubular in cross-section when stained with H&E (inset). However, in (H), OCM-1A cells form patterned, vasculogenic-like networks when cultured on a matrix preconditioned by the MUM-2B cells (MUM-2B CMTX) which appear tubular in nature when examined in cross-section and stained with H&E (inset). (The 200 m size bar in A also corresponds to B, D, E, F, G and H; the 25 m size bar in A inset also corresponds to panel C plus insets, and insets in D, G and H.)

interacting with the 3-D collagen I matrix. As shown in Fig. 2C, a representative example of aggressive melanoma cells (MUM-2B) interacting for 3 days with a 3-D collagen I matrix demonstrates that the tumor cells are invading into the 3-D matrix, but have not yet formed mature, tubular, vasculogenic-like networks. The two insets in Fig. 2C show that ammonium hydroxide treatment efficiently removed tumor cells from their respective 3-D matrices; and proteins, such as laminin, can be detected deposited in tracks within the preconditioned matrix. When the poorly aggressive MUM-2C cells were incubated on the 3-D matrix preconditioned by the aggressive MUM-2B cells, they formed patterned vasculogenic-like networks - for the first time - some of which were tubular in nature as shown by cross-sectional histological analysis (Fig. 2D, inset). We then tested whether the preconditioning of a 3-D matrix by poorly aggressive MUM-2C cells could affect the vasculogenic mimicry potential of MUM-2B cells, and it did not (Fig. 2E). Additional parameters tested demonstrated that the aggressive C918 melanoma cells (derived from a primary tumor) formed vasculogenic-like networks on 3-D collagen I matrix (Fig. 2F), and poorly aggressive OCM-1A cells incubated on 3-D matrices preconditioned by C918 cells formed patterned tracks, but not tubular networks (Fig. 2G, inset). Most intriguing was the observation (shown in Fig. 2H, inset) that OCM-1A cells incubated on a 3-D matrix preconditioned by aggressive MUM-2B cells (derived from a metastatic tumor) formed vasculogenic-like networks, some of which were tubular in histological cross-section. Collectively, these data support the hypothesis that the microenvironment preconditioned by aggressive metastatic melanoma tumor cells can induce a vasculogenic phenotype in poorly aggressive melanoma cells.

Induction of differential gene expression in poorly aggressive melanoma cells exposed to the microenvironment of aggressive metastatic melanoma cells

To achieve a global gene analysis of the epigenetic changes associated with poorly aggressive melanoma cells exposed to 3-D collagen I matrices preconditioned by aggressive melanoma cells, we employed microarray analysis followed with hierarchical clustering using the average linkage method. Highlights of these data are shown in Fig. 3 as hierarchical clustering of melanoma gene expression patterns under varying culture conditions. The experimental parameters consisted of: **1**) Aggressive MUM-2B cells, poorly aggressive MUM-2C and poorly aggressive OCM-1A cells -each grown on tissue culture plastic compared with their respective incubation on control, unconditioned 3-D collagen I matrix (lanes 1, 2, 3); **2**) MUM-2B compared with MUM-2C cells cultured on either tissue culture plastic or on unconditioned 3-D collagen I matrix (lanes 4, 5); **3**) MUM-2C cells grown on 3-D collagen I matrix preconditioned by MUM-2B cells compared with MUM-2C cells grown on an unconditioned collagen I matrix (lanes 6, 7); and **4**) OCM-1A cells grown on a 3-D collagen I matrix preconditioned by MUM-2B (lane 8) or C918 cells compared with OCM-1A cells grown on control collagen I matrix (lane 9). The complete global gene analysis is available at http://www.childrensmrc.org/hendrix/supplemental/JCMM/; selected genes from the hierarchical clustering that were upregulated under these various experimental parameters are presented in Table 2, and specific down-regulated genes are listed in Table 3. Comparative analysis of genes from the various cell lines grown on plastic relative to unconditioned collagen I matrix showed minimal to no change in their genotype. However, the microarray data demonstrate that the matrix microenvironment preconditioned by aggressive MUM-2B melanoma cells, but not C918 cells, exerted (in most instances) a profound, epigenetic reprogramming of gene expression in the poorly aggressive MUM-2C and OCM-1A cells exposed to these preconditioned 3-D collagen I matrices. There are several categories of genes that are shown to be upregulated in the poorly aggressive melanoma cells indicative of a transdifferentiated phenotype, including genes associated with the ECM and remodeling, endothelial, epithelial, neuronal, and bone marrow mesenchymal stem cell phenotypes, growth factors, signal transduction and transcription factors, and a highly invasive/aggressive cell phenotype. The change in gene expression resulting from the epigenetic influence of the aggressive metastatic melanoma microenvironment on poorly aggressive cells coincides with many of the constitutively upregulated genes in the MUM-2B cells (relative to MUM-2C or OCM-1A). Interestingly, many of the down-regulated genes observed in the poorly aggressive melanoma

Table 2 Microarray analysis of aggressive *versus* poorly aggressive uveal melanoma cells on preconditioned versus control collagen I matrices: Upregulated genes

Gene Name	MUM-2B/ MUM-2C†	MUM-2C on MUM-2B cmtx/ MUM-2C Col I‡	OCM-1A on C918 cmtx/ OCM-1A Col I‡	OCM-1A on MUM-2B cmtx/ OCM-1A Col I‡
Cell phenotype associated genes				
Aminopeptidase N, CD13	18.8	11	1.1	4.2
Vascular endothelial (VE)-cadherin	3.6	1.5	0.9	1.2
Melanoma cell adhesion molecule	>20	6.2	0.8	5.7
EphA2 protein tyrosine kinase	6.5	1.3	1.0	3.0
Keratin 7	9.8	2.6	1.0	2.1
Epithelial membrane protein 1	9.3	1.9	1.0	3.9
Putative lymphocyte G0/G1 switch gene	>20	7.8	1.1	10.1
ECM-related genes				
Fibronectin 1	>20	11.6	1.2	6.5
Laminin 5β3	18.6	7.5	1.0	4.5
Laminin 5γ2	8.9	2.5	1.1	3.1
Integrin, α_3 -subunit	>20	2.8	1.1	5.5
Lysyl oxidase-like 2	5.5	2.4	1.1	1.8
Matrix metalloproteinase-2 (MMP-2)	4.2	1.5	1.1	1.5
Matrix metalloproteinase-14 (MT1-MP)	3.3	1.7	0.8	1.5
Urokinase	9.5	1.5	1.2	4.9
Transcription/signal transduction/growth factor-related genes				
c-met protooncogene	3.2	2.8	1.0	2.9
Interleukin-8	9.7	>20	1.3	4.1
Paired box gene 8	8.7	4.5	1.2	2.0
Colony stimulating factor 3	> 20	5.2	1.0	9.7
Cysteine-rich, angiogenic inducer, 61	16.9	2.0	1.2	2.8
GRO I oncogene	11.0	1.8	1.0	2.5
Interleukin-1β	>20	5.8	0.9	11.6
Rho GDP dissociation inhibitor (GDI) β	7.9	2.4	1.2	2.3
Urokinase Receptor	4.5	2.9	1.0	2.1
Inhibin, β A	15.7	6.1	1.0	2.0
Zinc finger protein 41	>20	8.4	1.6	6.3
Transmembrane 4 superfamily member 1	11.7	4.4	1.1	3.2
Thymosin, β 4, X chromosome	>20	4.8	1.3	7.6
Thymosin, β 4, Y chromosome	>20	3.4	1.2	7.2
Cytochrome P450, subfamily IIIA (niphedipine oxidase), polypeptide 4	>20	4.6	1.1	14.6

Altered gene expression in human uveal melanoma cells was identified by cDNA microarray analysis.
† Selected genes with a differential expression of 1.4-fold or greater are reported as a ratio of aggressive to poorly aggressive uveal melanoma cells.
‡ Differential expression of selected genes reported as a ratio of poorly aggressive uveal melanoma cells grown on a Col I matrix (cmtx) preconditioned by aggressive uveal melanoma cells compared to cells grown on an unconditioned control Col I matrix.

Table 3 Microarray analysis of aggressive *versus* poorly aggressive uveal melanoma cells on preconditioned versus control collagen I matrices: Down-regulated genes

Gene Name	MUM-2B/ MUM-2C †	MUM-2C on MUM-2B cmtx/ MUM-2C Col I ‡	OCM-1A on C918 cmtx/ OCM-1A Col I ‡	OCM-1A on MUM-2B cmtx/ OCM-1A Col I ‡
Melanoma antigen, family A, 8	0.53	0.49	0.97	0.29
Melanoma antigen, family D, 2	0.65	0.66	0.93	0.48
Preferred expression in melanoma	0.09	0.79	0.97	0.47
Tyrosinase-related protein I	0.05	0.62	1.00	0.45
Melan-A	0.25	0.41	0.65	0.35

Altered gene expression in human uveal melanoma cells was identified by cDNA microarray analysis.
† Selected genes with a differential expression of 1.4-fold or greater are reported as a ratio of aggressive to poorly aggressive uveal melanoma cells.
‡ Differential expression of selected genes reported as a ratio of poorly aggressive uveal melanoma cells grown on a Col I matrix (cmtx) preconditioned by aggressive uveal melanoma cells compared to cells grown on an unconditioned control Col I matrix.

homology domains-1), VEGF-C (vascular endothelial growth factor-C)]; ECM proteins associated with an aggressive cancer cell phenotype (laminin 5γ2 chain and an associated receptor, α_3-containing integrin); a homeobox gene (PAX 8); an epithelial phenotype-specific gene (keratin 7); a matrix remodeling gene (urokinase); a mesenchymal stem cell-associated gene (CD13); and a proto-oncogene associated with a migratory cell phenotype (c-met). Most noteworthy is the upregulation of genes (mentioned above) by the poorly aggressive OCM-1A and MUM-2C cells exposed to matrices preconditioned by the aggressive, metastasis-derived MUM-2B cells, thus validating select data presented in Fig. 3 and Table 2. In most cases, these genes are not expressed by the poorly aggressive melanoma cells grown on the control 3-D collagen I matrices, but their induced expression is similar (in many instances) to the genes expressed constitutively by the aggressive melanoma cells. Of particular significance in this comparative analysis is the difference in gene expression between the OCM-1A cells exposed to the microenvironment preconditioned by the aggressive, metastasis-derived MUM-2B cells, which induced a profound upregulation of all selected genes, relative to an unremarkable epigenetic effect on OCM-1A cells exposed to the microenvironment preconditioned by the C918 cells (derived from a primary tumor).

The next question we addressed pertained to the stability of the epigenetic induction of the transdifferentiated phenotype. We focused our observations on the poorly aggressive MUM-2C cells exposed to the matrix microenvironment preconditioned by the aggressive MUM-2B cells, as shown in Fig. 4B. Following exposure to the preconditioned matrices, the MUM-2C cells were replated onto tissue culture plastic from 1 day to 21 days. By 21 days, expression of VE-cadherin, TIE-1, urokinase, and the integrin α_3-subunit appears to be gone; and expression of EphA2, VEGF-C, laminin 5 γ2 chain, PAX 8, keratin 7, CD13, and c-met is dramatically reduced. These data indicate that the epigenetic effect of the aggressive metastatic tumor cell microenvironment on poorly aggressive cells is dependent on continuous exposure to the preconditioned matrices with respect to the induction and maintenance of epigenetic changes in gene expression.

Changes in invasive ability of poorly aggressive melanoma cells grown on inductive matrices preconditioned by aggressive metastatic melanoma cells

Poorly aggressive MUM-2C and OCM-1A melanoma cells were grown for 3-4 days on either a 3-D unconditioned collagen I matrix directly, or on a matrix preconditioned by aggressive metastatic MUM-2B cells, then assessed for their ability to invade *in vitro*. In both assays (Fig. 4C),

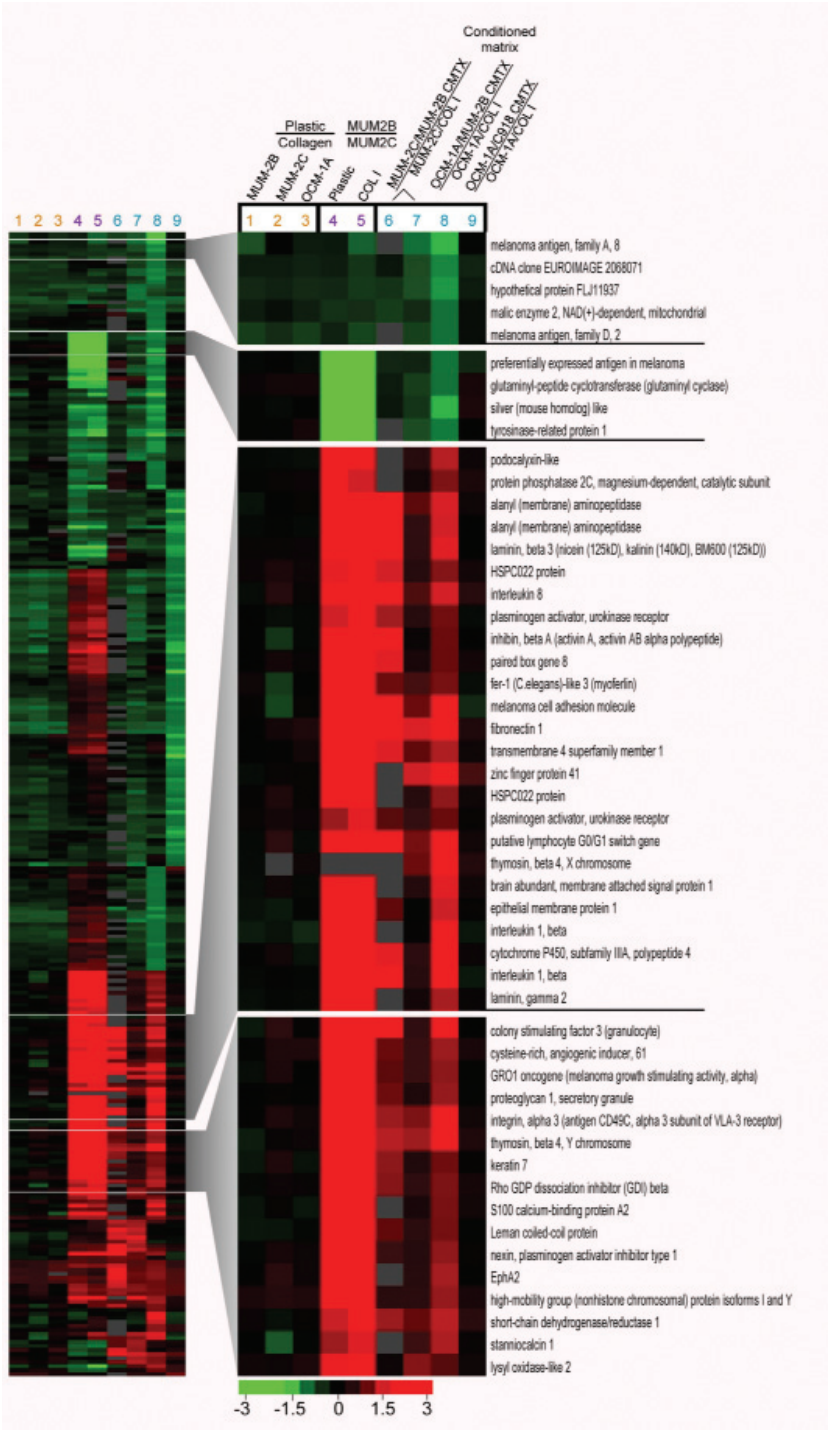
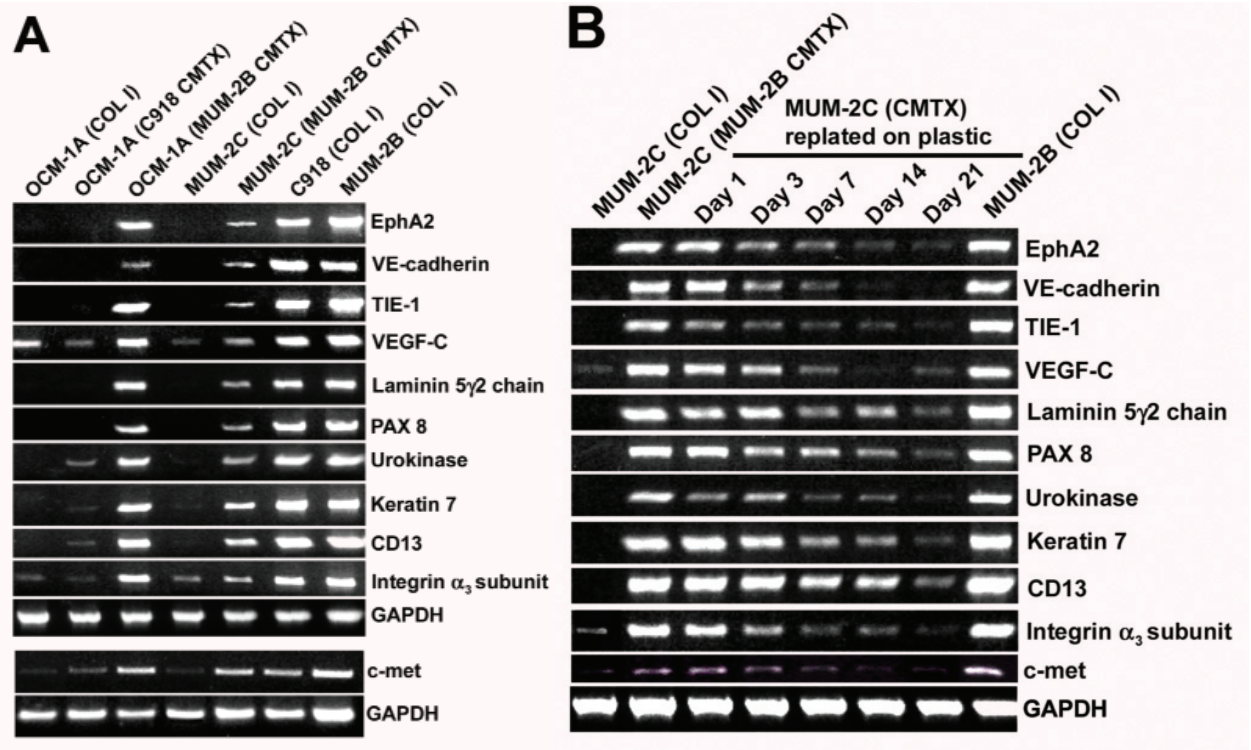


Fig. 3 Hierarchical clustering of melanoma gene expression patterns under varying culture conditions. The clustering data shown on the left represent the significantly differentially expressed genes (greater than 1.4-fold) in various experimental parameters from the entire assay. The clustering data on the right demonstrate highlighted genes of interest from the same experimental parameters. Lanes 1 (MUM-2B), 2 (MUM-2C) and 3 (OCM-1A) serve as controls demonstrating minimal changes between cells cultured on plastic or collagen I 3-D matrices. Lanes 4 and 5 illustrate multiple differences between more aggressive (MUM-2B) and poorly aggressive (MUM-2C) uveal melanoma cells when cultured on either plastic (lane 4) or collagen I 3-D matrices (lane 5). Lanes 6-8 demonstrate that some of the gene expression profile of the MUM-2B cells is recapitulated in the poorly aggressive melanoma cells grown on matrices preconditioned by the aggressive cells. Lanes 6 and 7: MUM-2C cells cultured on MUM-2B cell preconditioned matrix relative to MUM-2C cells cultured on a collagen I 3-D matrix from two replicate cultures. Lane 8: OCM-1A cells cultured on a MUM-2B cell preconditioned matrix relative to OCM-1A cultured on a collagen I 3-D matrix. Lane 9: OCM-1A cells cultured on a C918 preconditioned matrix relative to OCM-1A cells cultured on a collagen I 3-D matrix. Red indicates increased expression; green indicates decreased expression; grey indicates measurements of low quality; and black represents no change in expression.

cells exposed to the matrix microenvironment preconditioned by aggressive metastatic melanoma cells are associated with melanoma and melanocyte-specific antigens - similar to their constitutive down-regulation observed in the aggressive MUM-2B cells.

Confirmation of select differentially expressed genes was accomplished by semi-quantitative RT-PCR analysis of human melanoma cells under various experimental conditions that underwent microarray analysis (Fig. 4A). The categories of genes tested consisted of: endothelial/vascular-associated genes EphA2 (erythropoietin-producing hepatocellular carcinoma-A2), VE-cadherin (vascular endothelial), TIE-1 (tyrosine kinase with Ig and epidermal growth factor



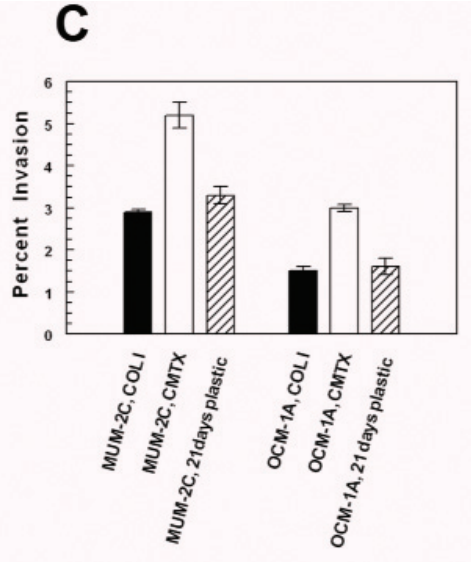


Fig. 4 Confirmation of differentially expressed genes by semi-quantitative RT-PCR analysis in human melanoma cells that underwent microarray analyses (shown in Fig. 3) and functional analysis of invasive ability *in vitro*. Gene-specific primers (available at http://www.childrensmrc.org/hendrix/supplemental/ JCMM) were used to evaluate the expression of (A) EphA2, VE-cadherin, TIE-1, VEGF-C, laminin 5γ2 chain, PAX8, urokinase, keratin 7, CD13, the integrin α_3-subunit and c-met in the following experimental categories: Poorly aggressive melanoma cells isolated from a primary tumor (OCM-1A) or a liver metastasis (MUM-2C), grown on either a 3-D collagen I matrix (COLI) or on a 3-D matrix preconditioned by aggressive melanoma cells isolated from either a primary tumor (C918 CMTX) or liver metastasis (MUM-2B CMTX). Total RNA was isolated using Trizol reagent. GAPDH-specific primers were used as a control for equal loading. (B) Select genes were studied further by RT-PCR to determine the longevity of EphA2, VE-cadherin, TIE-1, VEGF-C, laminin 5γ2 chain, PAX8, urokinase, keratin 7, CD13, integrin α_3-subunit and c-met gene expression in MUM-2C cells grown on a 3-D collagen I matrix preconditioned by MUM-2B cells (MUM-2B CMTX), and subsequently removed from this "inductive" matrix and replated onto tissue culture plastic for 1, 3, 7, 14 and 21 days, respectively, and then compared with the gene expression profiles of MUM-2C or MUM-2B cells grown on collagen I only (COLI). (C) Poorly aggressive OCM-1A and MUM-2C uveal melanoma cells were grown for 3 days on either a 3-D collagen I matrix (COLI) or a collagen I matrix preconditioned by aggressive MUM-2B cells (CMTX), or then replated onto tissue culture plastic for 21 days, and then seeded into the upper wells of the MICS (membrane invasion culture system; 5 x 10^4 cells/well) chamber. For the invasion assay, the chamber utilized a polycarbonate membrane containing 10 ?m pores coated with a matrix comprised of human collagen IV/human laminin/gelatin and cells loaded into the chamber in RPMI 1640 with 1X Mito+. After 24 h of incubation at 37°C, the cells that had invaded through the membrane were collected, stained and counted. 1.5% of the OCM-1A and 2.9% of the MUM-2C cells cultured on the collagen I matrix were found to invade during the course of the invasion assay, while 3.0% of the OCM-1A and 5.2% of the MUM-2C cells invaded over the same period of time after they were cultured on the collagen I matrix preconditioned by the aggressive MUM-2B cells. After these cells were replated on plastic for 21 days, invasion returned to baseline levels. Percent invasion was corrected for proliferation and calculated as total number of cells seeded times 100. (n=6 wells per measurement ± SE run in duplicate experiments; $p<0.01$ invasion assays.)

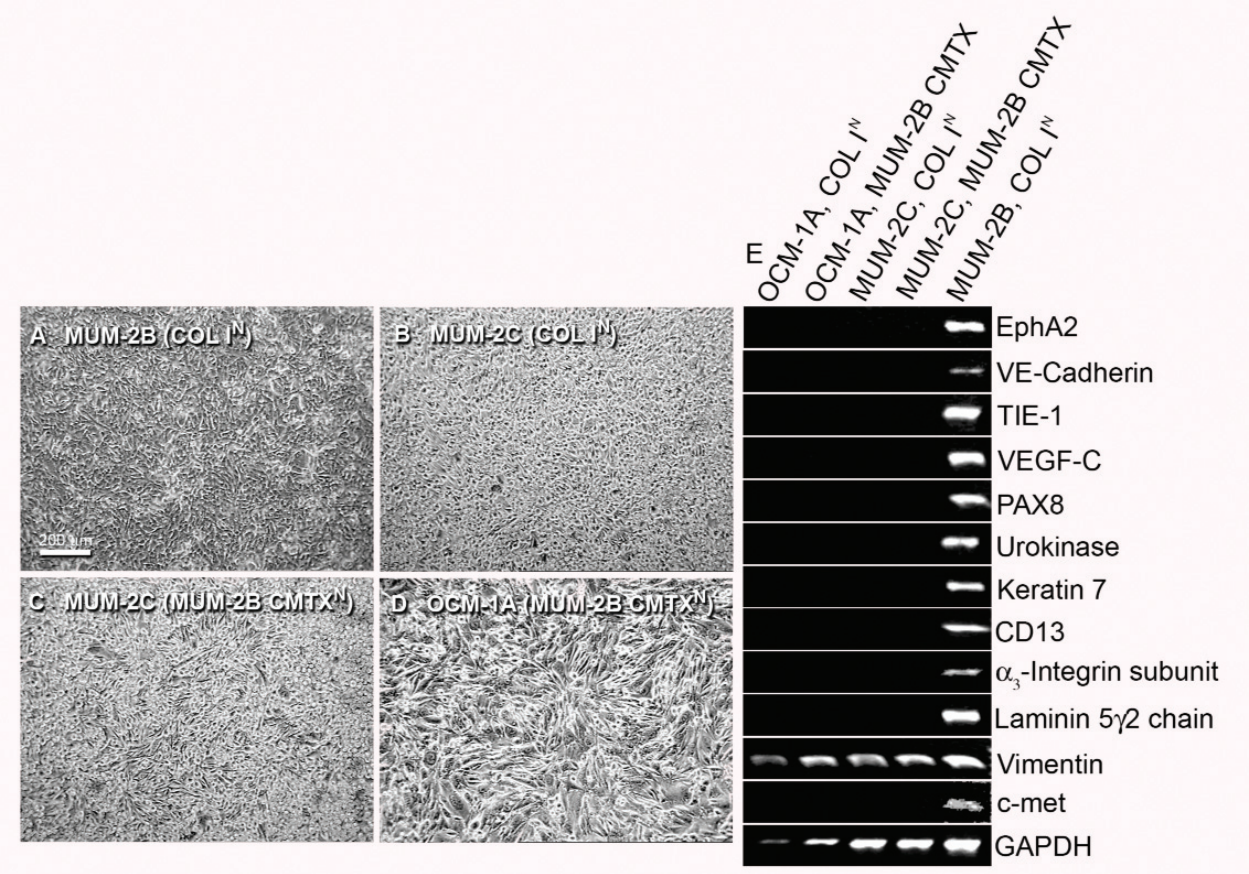


Fig. 5 Morphological and molecular analyses of melanoma cells exposed to a rigid, highly cross-linked collagen matrix. Phase contrast microscopy of: (A) aggressive MUM-2B melanoma cells cultured on a native, highly cross-linked (non-deformable) collagen I 3-D matrix (COL IN) for up to 4 days, showing the inability of these tumor cells to form vasculogenic-like networks; B) poorly aggressive MUM-2C melanoma cells cultured on the same matrix, unable to form vasculogenic structures; C, D) MUM-2C and OCM-1A poorly aggressive melanoma cells, respectively, exposed to the rigid, highly cross-linked 3-D collagen I matrices preconditioned by MUM-2B metastatic melanoma cells. (The 200 m scale bar in A also corresponds to B, C, and D.) (E) Confirmation of differentially expressed genes by semi-quantitative RT-PCR analysis in human melanoma cells exposed to the rigid, highly cross-linked, 3-D collagen I matrices for up to 4 days. GAPDH was used to demonstrate equal loading of samples.

there was a significant increase in the percent invasion (79%) of the MUM-2C and (94%) of the OCM-1A cells exposed to the MUM-2B preconditioned matrix *versus* their respective controls grown on unconditioned collagen I. These data demonstrate the epigenetic effect of an aggressive metastatic melanoma cell microenvironment on the biological activity of poorly aggressive tumor cells, with respect to invasive potential. However, when the MUM-2C and OCM-1A cells (that had been exposed to the metastatic melanoma matrix) were replated onto tissue culture plastic for 21 days, their invasive ability *in vitro* returned to baseline levels prior their exposure to the metastatic microenvironment.

Abrogation of the epigenetic induction potential of the metastatic melanoma microenvironment

Lastly, we investigated whether the substitution of a highly cross-linked, rigid collagen I matrix (that was potentially non-deformable) might affect the ability of the highly aggressive MUM-2B cells to remodel their microenvironment and epigenetically

induce changes in the poorly aggressive MUM-2C and OCM-1A cells exposed to it. As shown in Fig. 5, the MUM-2B cells were unable to form vasculogenic-like networks on this matrix (Fig. 5A), which was similar to the MUM-2C cells (Fig. 5B). Furthermore, the highly cross-linked matrices preconditioned by the MUM-2B cells were unable to epigenetically induce any changes in the phenotype or gene expression profile of the MUM-2C nor the OCM-1A cells exposed to them (Fig. 5 C-E). These data indicate that the biomechanical interactions between the tumor cells and their microenvironment(s) are a critical component in the epigenetic reprogramming of other cells.

Discussion

The tumor microenvironment plays a critical role in cancer progression [28–30]. The current study is an extension of a previous report that was designed to introduce an innovative model to investigate the potential epigenetic influence of the microenvironment of the MUM-2B aggressive melanoma cells on the poorly aggressive MUM-2C melanoma cells exposed to it (25), and paved the way for a global molecular and mechanistic analysis of additional melanoma cells lines in the present report. The findings generated from this strategic approach showed profound changes in the phenotype, gene expression, and biological activity of poorly aggressive melanoma cells exposed to a metastatic melanoma microenvironment, and indicated their transdifferentiation into an aggressive melanoma cell phenotype. Particularly noteworthy was the observation that the majority of genes induced in the poorly aggressive melanoma cells through this epigenetic event were dramatically reduced and several absent by 21 days after the cells were removed from an inductive microenvironment and maintained on tissue culture plastic, suggesting a transient response to epigenetic induction by the microenvironment.

The cell lines selected for a major portion of the study allowed an in depth comparative analyses of the vasculogenic phenotype, molecular profile, and invasive potential associated with aggressive *versus* poorly aggressive human uveal melanoma cells. Although previous studies from our laboratory had confirmed that both aggressive cutaneous and uveal melanoma cells formed vasculogenic-like networks in 3-D collagen I matrices, whereas poorly aggressive melanoma cells were incapable of vasculogenic mimicry [13, 14, 31], a global comparative molecular analysis of the possible epigenetic effect(s) of the microenvironment of several aggressive melanoma cell lines had not been attempted. Prior microarray analyses of human melanoma cell lines (grown on tissue culture plastic) derived from cutaneous and uveal melanomas showed comparable gene expression profiles [7, 8]. However, a major focus of the current study utilized uveal melanoma as the primary experimental model because of the advantage it offered in comparing cell lines isolated from primary tumors and metastatic lesions, in addition to aggressive and poorly aggressive clones isolated from the same patient.

Specifically, the morphological analyses presented in Fig. 2 clearly demonstrate that by 3-4 days, aggressive melanoma cells had formed cord-like structures with evidence of highly migratory cells invading the 3-D collagen I matrices. Furthermore, these cells deposited ECM proteins, such as laminin, in tracks throughout the 3-D matrix. Removal of cells from the preconditioned matrices was efficient and did not compromise the integrity of the collagen I matrix. Most noteworthy was the observation that poorly aggressive melanoma cells (MUM-2C and OCM-1A) acquired a vasculogenic phenotype and formed tubular vasculogenic-like networks in response to a metastatic microenvironment (preconditioned by MUM-2B), but not to a microenvironment preconditioned by melanoma cells derived from a primary tumor (C918) - suggesting the possibility of different ECM-remodeling capabilities by the C918 cells relative to MUM-2B cells, or a difference in their ability to produce various inductive factors necessary to epigenetically reprogram the poorly aggressive tumor cells. Also intriguing was the finding that the matrix microenvironment preconditioned by the poorly aggressive MUM-2C cells offered no apparent informative cues with respect to inhibiting or enhancing the vasculogenic phenotype of the aggressive MUM-2B cells that demonstrated the predominance of the aggressive cell phenotype under these experimental conditions.

Our next step determined the molecular epigenetic effects induced by the matrix microenvironment preconditioned by aggressive uveal melanoma cells, utilizing a nonbiased global gene analysis

approach. Highlighted genes from the 14,000 cDNA element microarray are presented in Fig. 3, and the complete gene list is available at http://www.childrensmrc.org/hendrix/supplemental/JCMM/. Critical to the performance of the comparative analysis was the establishment of multiple controls, including the differential gene expression of all uveal melanoma cell lines grown on tissue culture plastic relative to unconditioned 3-D collagen I matrices relative to preconditioned 3-D collagen I matrices. The selected genes presented in Fig. 3, 4, and Tables 2, 3 were subdivided into the following categories to better appreciate their respective biological significance: Cell phenotype associated genes; ECM-related genes; and transcription/signal transduction/growth factor-related genes.

With respect to the cell phenotype associated genes, the poorly aggressive MUM-2C and OCM-1A melanoma cells exposed to the metastatic-derived MUM-2B cell microenvironment showed a significant down-regulation of several melanoma-specific markers — similar to those constitutively down-regulated in the aggressive MUM-2B (and C918) tumor cells. For example, melanoma antigen (family A,8 and family D,2), preferred expression in melanoma, tyrosinase-related protein 1, and Melan-A were all down-regulated in MUM-2B (and C918) aggressive melanoma cells, but not in MUM-2C or OCM-1A poorly aggressive cells — until they were exposed to the MUM-2B preconditioned matrix microenvironment. The expression of melanoma associated antigen genes has been linked to a favorable disease outcome in advanced stage melanoma [32], and several of these antigens are used as important markers in the diagnosis of melanoma [33, 34]. Collectively, these data suggest that melanoma cells appear to dedifferentiate as they acquire an aggressive phenotype, which might make them more difficult to identify using routine histopathological markers for diagnosis. However, it is interesting to note that melanoma cell adhesion molecule, upregulated constitutively in the aggressive melanoma cells, was epigenetically induced in the poorly aggressive MUM-2C and OCM-1A cells exposed to the MUM-2B-preconditioned matrix microenvironment. This adhesion molecule, also referred to as MCAM, MUC18, and CD146, is a member of the immunoglobulin supergene family and has been shown to mediate melanoma-endothelial cell heterophilic ligand adhesion integral in the metastatic cascade and can facilitate cell-host interactions [35]. Other cell phenotype associated genes that were upregulated in the aggressive melanoma cells and epigenetically induced in the poorly aggressive melanoma cells exposed to the MUM-2B microenvironment are involved in angiogenesis, lymphangiogenesis and vasculogenesis, including EphA2, VE-cadherin, TIE-1, and VEGF-C. These molecules, with their binding partners, are a few of the factors that are required for the formation and maintenance of the vasculature[36–39]. At the protein level, VE-cadherin and EphA2 are expressed only by aggressive melanoma cells, and not by poorly aggressive melanoma cells [40, 41]. Furthermore, down-regulation of VE-cadherin or EphA2 expression results in the complete inability of aggressive melanoma cells to form vasculogenic-like networks in 3-D culture. These previous observations from our laboratory, together with the current findings, suggest that the metastasis-derived MUM-2B cells preconditioned their matrix microenvironment in a manner that induced both cell lines to acquire a vascular phenotype as demonstrated by their expression of vascular cell-associated genes and ability to form vasculogenic-like networks for the first time in 3-D culture. Additional intriguing vascular cell phenotype associated genes that were induced epigenetically in the poorly aggressive melanoma cells are CD13 and putative lymphocyte G0/G1 switch gene. CD13, a surface-bound metallopeptidase, also referred to as alanyl aminopeptidase (APN), has been identified as the leukocyte surface differentiation antigen (predominantly expressed on cells of myelo-monocytic lineage), and most recently implicated as an angiogenic regulator and transcriptional target of Ras signaling pathways in endothelial morphogenesis and a prime target for anti-angiogenic tumor-homing peptides [42–45]. Of particular note in the current study was the observation that the epigenetically induced expression of CD13 endured 21 days after the poorly aggressive melanoma cells were removed from their inductive matrix preconditioned by MUM-2B cells. Thus, it is tempting to speculate that the upregulation of this gene might represent an early event in the differentiation pathway of vascular cells, possibly upstream of VE-cadherin, EphA2, and TIE-1. Coincident with the strong epigenetically induced expression of CD13 is a similar induction of the putative lymphocyte

G0/G1 switch gene. Although this gene was originally thought to be the lectin-induced switch of lymphocytes from the G0 to the G1 phase of the cell cycle [46], more recent findings indicate its involvement in mesenchymal progenitor developmental events leading to the differentiation of osteogenic, chondrogenic and predominantly adipogenic lineages [47]. The strong induction of the putative lymphocyte G0/G1 switch gene in the poorly aggressive melanoma cells supports the hypothesis that these cells acquire a dedifferentiated, plastic phenotype, similar to that associated with the aggressive melanoma cells.

The epithelial-associated genes - epithelial membrane protein 1 (EMP1) and keratin 7, were also upregulated in the poorly aggressive melanoma cells exposed to the MUM-2B preconditioned matrix microenvironment. EMP1, alternatively referred to as tumor-associated membrane protein, has been detected in embryonic kidney, brain, gut and is linked to cell-cell interactions and the regulation of cell proliferation, in addition to neuronal differentiation and neurite outgrowth [48–50]. Similarly, the induced expression of keratin 7, indicative of a simple epithelial cell type, in the mesenchymally-derived poorly aggressive melanoma cells, strongly suggests their transition to a dedifferentiated, interconverted phenotype, previously shown by our laboratory and others to be closely associated with aggressive behavior and metastatic disease [26, 51–53].

Although the current study indicates that poorly aggressive melanoma cells exposed to the metastatic cell-derived microenvironment were induced to express genes associated with a vascular cell phenotype and form vasculogenic-like networks, it is unknown whether they are fully competent to provide a vascular function similar to that demonstrated previously by aggressive melanoma cells [22]. We are just beginning to appreciate the complexity of cell fate restriction and transdifferentiation [54–56], and the data generated in the present study strongly suggest that epigenetic regulation of cell phenotype specific genes plays a critical role in transdifferentiation and dedifferentiation. Understanding the lineage of tumor cells and the significance of their altered circuitry is critical in elucidating cancer as a disease of altered cellular behavior [57].

The epigenetic induction of ECM-related genes in poorly aggressive melanoma cells exposed to the matrix preconditioned by metastasis-derived aggressive MUM-2B cells (highlighted in Table 2) suggests a newly acquired potential to remodel the microenvironment. It is well accepted that the cellular microenvironment can directly modulate cell fate [58, 59], but the molecular details governing this dynamic interplay remain poorly understood. However, we are gaining a greater appreciation of the cooperative roles of specific ECM components and matrix remodeling proteases involved in the interactions between tumor cells and their microenvironment [30, 60, 61]. In the present study, the most robust epigenetic induction of an ECM-related gene observed in poorly aggressive melanoma cells was fibronectin. Emerging evidence points to fibronectin as a critical regulator of ECM organization and stability with broader implications in cellular migration, differentiation, and metastasis [62–64]. Of additional significance is earlier work uncovering the metalloproteinase stimulating ability of cleaved fragments of fibronectin [65, 66], which may provide a biological mechanism for the increased migratory and invasive potential acquired by poorly aggressive melanoma cells exposed to a metastatic melanoma cell preconditioned microenvironment, shown in Fig. 4. Other genes that were upregulated in the poorly aggressive melanoma cells exposed to the microenvironment preconditioned by metastasis-derived MUM-2B cells are associated with matrix remodeling: urokinase, lysyl oxidase-like 2, and matrix metalloproteinases-2 and -14 (MT1-MMP) [31, 67–72]. The epigenetic upregulation of laminin 5 and an associated α_3-containing integrin observed in the poorly aggressive melanoma cells was of particular interest based on previous studies from our laboratory demonstrating the requirement for cooperative interactions of laminin 5 γ2 chain, MMP-2 and MT1-MMP for vasculogenic mimicry by aggressive melanoma cells [73, 74]. Laminins are important components of basement membranes that are involved in regulating differentiation, tumor metastasis, cell attachment, migration and angiogenesis [75–78]. Proteolytic cleavage of the laminin 5 γ2 chain by MT1-MMP and MMP-2 results in the formation of laminin 5 γ2' and γ2x promigratory fragments [74, 79, 80]. Laminin is also an integral component of vasculogenic-like networks or fluid-conducting meshwork formed by aggressive melanoma cells *in vitro* and *in vivo*, respectively [12, 19, 21, 74]. Thus, it is tempting to speculate that the acquired

ability of poorly aggressive melanoma cells to upregulate laminin 5 (and an associated α_3-containing integrin) and the MMPs necessary to cleave it into promigratory signals in their microenvironment provides additional evidence that they have acquired a transdifferentiated phenotype that resembles a more aggressive melanoma cell, with possible implications in altered signaling capabilities as well [81]. Quite interestingly, the inductive potential of the microenvironment preconditioned by aggressive metastatic melanoma cells can be neutralized by treatment with a chemically modified tetracycline (CMT-3 or COL-3), which is a potent inhibitor of MMP activity, inhibits the cleavage of laminin 5 chain to promigratory fragments, and down-regulates MMP-2, MMP-9, MT1-MMP, VE-cadherin, VEGF-C, and TIE-1 [31]. Indeed, these are important biological findings that may be useful in targeting molecular cues in the microenvironment of aggressive tumors, ultimately inhibiting the triggering of the angiogenic/vasculogenic switch thought to initiate critical control pathways [82, 83].

The transcriptional/signal transduction/growth factor-related genes have been categorized separately from those related to the cell phenotype and the ECM, but it is plausible to assume that they are interrelated. For example, as a tumor cell transdifferentiates and acquires different matrix remodeling capabilities, it may also gain the ability to activate poorly diffusible matrix-sequestered growth factors that regulate biological function and trigger various signal transduction pathways, as previously suggested in other models [60]. There was a robust response by poorly aggressive melanoma cells, exposed to the matrix microenvironment preconditioned by metastatic MUM-2B cells, resulting in the upregulation of genes involved in proliferation/survival, motility, and activation of important signaling pathways. Previous work from our laboratory correlated the expression of c-met proto-oncogene (receptor for hepatocyte growth factor/scatter factor; HGF/SF) in aggressive uveal melanoma cells expressing an interconverted/dedifferentiated phenotype (cells coexpressing vimentin and keratins), and suggested that HGF/SF may play an important role in the metastatic dissemination of this tumor [84]. The significance of c-met as a regulator of mitogenesis, motility, and morphogenesis and its critical role in metastasis has been demonstrated in other studies as well [85, 86]. Furthermore, the c-met/HGF/SF signaling pathway holds great promise as a therapeutic target for intervention strategies [87, 88]. Interestingly, our previous study measuring c-met in MUM-2C cells removed from the MUM-2B preconditioned matrix for up to 21 days showed sustained expression throughout this time period (25). However, a repeat of this experiment along with the additional cell lines indicated that c-met had diminished considerably by 21 days post-matrix-exposure (Fig. 4B), although the initial epigenetic induction of the MUM-2C cells exposed to the MUM-2B matrix is less robust than the experimental data shown in Fig. 4A. This may be explained by the slightly longer epigenetic exposure of the poorly aggressive melanoma cells to their respective matrices (shown in Fig. 4A) than occurred in Fig. 4B.

Additional genes in this category that have significant implications in cellular migration and invasion include Rho GDP dissociation inhibitor, the thymosin β4 family and the transmembrane 4 superfamily [89–92]. The urokinase receptor (uPAR), also upregulated in the poorly aggressive melanoma cells exposed to inductive matrices, has been linked to cellular migration through its ability to promote pericellular proteolysis, mediate cell signaling, and regulate integrin function, and most recently has been identified as a preferential binding partner for α3β1 [93, 94]. The upregulation of uPAR coincides with the increased expression of the α3-containing integrin - also considered a binding partner for laminin. uPAR also activates α5β1 (a fibronectin-specific integrin), which coincides with the upregulation of fibronectin by these cells [95]. Another potentially related upregulated gene that could provide a transduction role is cysteine-rich, angiogenic inducer, 61 (CYR61), which acts as an ECM-associated signaling molecule and promotes endothelial cell adhesion and neovascularization through an integrin-dependent pathway [96]. Thus, the potential for multiple signaling interactions and down-stream events associated with the upregulation of key molecules may help to elucidate the pathways underlying the transdifferentiation of poorly aggressive melanoma cells. In conjunction with the upregulated signaling molecules were genes associated with potent cytokines and growth factors, including interleukin 8 (IL8), interleukin 1-β (IL1β), colony stimulating factor 3 (CSF3; granulocyte colony-stimulating factor,

GCSF), inhibin, β A, and the GRO1 oncogene (melanoma growth stimulatory activity). IL8 is a member of the CXC chemokine family and is a proinflammatory mediator of neutrophil activation and migration [97]. IL8 expression has been shown to be stimulated by IL1β - primarily produced by blood monocytes and most recently implicated in the development of hepatic metastases of melanoma [98]. CSF3 (also called GCSF), is known to stimulate the proliferation and differentiation of the progenitor cells for granulocytes [99, 100], in a manner similar to the biological activity on inhibin, (β A, also called activin A), whose ligands act as growth and differentiation factors in many cells [101]. The GRO1 oncogene, formerly called melanoma growth stimulatory activity, is a mitogenic polypeptide secreted by human melanoma cells and important to their growth and survival [102]. Also intriguing was the upregulation (by poorly aggressive melanoma cells exposed to metastatic preconditioned matrices) of zinc finger protein 41 that encodes regulatory proteins [103], paired box gene 8 important in differentiation [104], and cytochrome P450, subfamily 3A4 which plays a central role in the metabolism of drugs [105]. It is interesting to note that a previous study comparing gene expression profiles between metastatic derivatives and their poorly metastatic parental cells implicated genes involved in matrix remodeling and signal transduction as well [106]. Collectively, the upregulation of this important category of transcription/signal transduction/growth factor-related genes by poorly aggressive melanoma cells represents the emergence of a tumor cell phenotype transdifferentiating into a more aggressive phenotype, similar to the metastatic cell that imprinted its microenvironment.

Overall, the cellular and molecular analyses employed in this study to measure the effects of a microenvironment preconditioned by aggressive uveal melanoma cells (isolated from a metastasis) on poorly aggressive melanoma cells revealed an intriguing epigenetic induction of a transdifferentiated phenotype and a reprogramming of gene expression in poorly aggressive tumor cells. Of special significance were the results showing a complete abrogation of the epigenetic influence of the metastatic melanoma matrix by neutralizing the ability of these tumor cells to sufficiently remodel their microenvironment. These observations coincide with those of Paszek and colleagues [107] showing the critical nature of tensional homeostasis between tumor cells and their microenvironment. The implications of these findings pose important clinical challenges involving: **1**) the detection of tumor cells - as they may phenotypically mimic other cell types; **2**) the targeting of aggressive tumor cells within a heterogeneous tumor that have the potential to modify their microenvironment is such a manner as to epigenetically induce transdifferentiation of other tumor cells and possibly normal cells, and **3**) the development of new clinical strategies to neutralize the epigenetic influence of the tumor microenvironment. Also germane to the interpretation of results from the current study are previous reports highlighting the molecular signature of metastasis-associated genes in primary tumors [108] and also raising questions about the concept of metastasis genes [109]. Although our investigation did not directly address this interesting debate, experimental evidence from our work underscores the important differences in the epigenetic influence by aggressive melanoma cells isolated from a primary uveal melanoma compared with the metastatic cell-derived inductive matrices. Since the molecular signatures are similar for both of these aggressive melanoma cell types, it is tempting to speculate that the interactions of tumor cells with their respective microenvironments may be more revealing of their metastatic propensity than a molecular profile of cell lines grown on tissue culture plastic. Furthermore, transdifferentiation is emerging as an important phenomenon that adds a new level of complexity to developing rational therapeutic strategies [54, 110–112].

Recent work from our laboratory has shown the epigenetic transdifferentiation of normal human melanocytes by a metastatic cutaneous melanoma microenvironment, which allowed the identification of genes associated with the earliest transformation of melanocytes to a neoplastic phenotype [113]**.** Further to this point, during the development of Kaposi's sarcoma, endothelial cells transdifferentiate into tumor cells [114] with a lymphatic endothelial signature [115], whereas aggressive melanoma cells as well as melanocytes or poorly aggressive melanoma cells (exposed to metastatic inductive matrices), transdifferentiate to an endothelial-like phenotype. These observations raise the intriguing possibility that these two tumor

cell types could share a common epigenetic reprogramming resulting in the emergence of a new phenotype. In our recently published study examining the epigenetic transdifferentiation of normal melanocytes by a metastatic cutaneous melanoma microenvironment (which used Affymetrix arrays; 113), many of the genes that we observed to be epigenetically induced in the melanocytes are similar to the upregulated genes in the poorly aggressive melanoma cells in the current study (using cDNA microarrays). In addition, removal of the transdifferentiated melanocytes from the inductive melanoma microenvironment resulted in a reversion to their normal phenotype, similar to the outcome of the poorly aggressive melanoma cells in the present study. The global gene analyses for the melanocyte work is available at http://www.tgen.com, which can be compared with the data from the current study at http://www.childrensmrc.org/hendrix/supplemental/JCMM/. In our efforts to manage melanoma, one of the major problems to address is drug resistance [116]. Compounding these efforts are recent *in vitro* and *in vivo* data showing that aggressive melanoma cells engaged in vasculogenic mimicry are relatively unaffected by select angiogenesis inhibitors [23, 24]. Additional evidence indicates that tumor cells may remodel their microenvironment with extra ECM to increase their survival in the presence of therapeutic agents [117], which has been shown to adversely affect interstitial transport in solid tumors [118]. MMP inhibitors have also experienced challenges in clinical trials, but these proteinases are still worth consideration in the development of strategies to target the tumor microenvironment [29, 30, 31, 119]. As we elucidate the inductive potential of proteolytically cleaved fragments of the ECM, it is clear that these partially degraded molecules could be prime targets for therapeutic intervention - potentially for the use in a combinatorial manner with other therapies [77]. Successful management of malignant melanoma and other cancers will benefit from the identification of essential regulatory pathways and molecular switches underlying the plastic tumor cell phenotype and its unique interactions with the microenvironment. Indeed, the experimental approach used in this study allows the identification of early markers associated with melanoma pathogenesis, which may provide the basis for new therapeutic targets.

Acknowledgments

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