In vivo Switching of Human Melanoma Cells between Proliferative and Invasive States

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Abstract

Metastatic melanoma represents a complex and heterogeneous disease for which there are no therapies to improve patient survival. Recent expression profiling of melanoma cell lines identified two transcription signatures, respectively, corresponding with proliferative and invasive cellular phenotypes. A model derived from these findings predicts that *in vivo* melanoma cells may switch between these states. Here, DNA microarray–characterized cell lines were subjected to *in vitro* characterization before s.c. injection into immunocompromised mice. Tumor growth rates were measured and postexcision samples were assessed by immunohistochemistry to identify invasive and proliferative signature cells. *In vitro* tests showed that proliferative signature melanoma cells are faster growing but less motile than invasive signature cells. *In vivo* proliferative signature cells initiated tumor growth in 14 ± 3 days postinjection. By comparison, invasive signature cells required a significantly longer ($P < 0.001$) period of 59 ± 11 days. Immunohistochemistry showed that regardless of the seed cell signature, tumors showed evidence for both proliferative and invasive cell types. Furthermore, proliferative signature cell types were detected most frequently in the peripheral margin of growing tumors. These data indicate that melanoma cells undergo transcriptional signature switching *in vivo* likely regulated by local microenvironmental conditions. Our findings challenge previous models of melanoma progression that evoke one-way changes in gene expression. We present a new model for melanoma progression that accounts for transcription signature plasticity and provides a more rational context for explaining observed melanoma biology. [Cancer Res 2008;68(3):650–6]

Introduction

Metastatic stage melanoma is an aggressive disease that few patients survive for >2 years. Compounding this, scores of clinical trials testing different adjuvant therapies have brought no significant improvement in the survival outlook for these patients (1). One possible explanation for this is that melanoma is a heterogeneous collection of different cells, and the differences between them are sufficient that some are missed by targeted therapies. The variety of phenotypic and behavioral features melanomas present range from distinct organ specificities during metastasis to changes in motility and invasiveness (2). Furthermore, melanoma tissues have various morphologies, from assorted macroscopic lesional structures to multiple microscopic cellular forms, which often complicate assessments of diagnosis and prognosis (3). Additionally, immunohistochemical staining regularly yields heterogeneous results. Although most melanoma lesions will stain for a number of melanocytic markers, this is not necessarily true for all melanoma cells within a given lesion (4). Finally, DNA microarray examination of different lesions and melanoma cell line collections reveal among them consistent taxonomies of genomic aberrations and transcriptional signatures (5–7). The source of heterogeneity is thought to rest in the combination of how melanoma cells respond to different microenvironments and the reciprocal influence of their own molecular states. This was an idea first conceptualized in Stephen Paget's "seed and soil" model after his observation that particular cancer cells showed tumorigenic preference for certain tissues over others (8, 9). By comparison, current molecular models for melanoma progression are homogeneous. A generally accepted hypothesis assumes that progression is driven by a steady evolution of molecular changes, and this hypothesis provides the dominant paradigm for molecular studies (10).

Of recent interest has been the activity of the microphthalmia-associated transcription factor (Mitf) in regulating melanoma cell proliferation. In normal melanocytes, Mitf is critical for melanocytic differentiation, expression of melanogenic enzymes, and upregulating cyclin-dependent kinase inhibitors to drive cell cycle exit (11–13). However, in melanoma, Mitf is required for proliferation and has been identified as a "lineage survival" factor prone to amplification (14–16). Although the contrast in the activities of Mitf in normal and transformed cells remains unexplained, there is little doubt concerning its central role in melanoma biology.

We recently explored heterogeneity of gene expression in melanoma cells. Bittner and coworkers (17) first suggested that there may be specific transcriptional signatures delineating melanoma cell subgroups. We characterized two different transcription signatures for melanoma cell lines, which, based on known functions of the genes involved, defined their respective contributions to metastatic potential as either proliferative or invasive (7). We further hypothesized that the transcription signatures represent distinct yet interchangeable states regulated by signaling from the microenvironment. Critically, Mitf expression is a central feature of the proliferative signature that is absent from the invasive form. Others' *in vitro* work concerning *MITF* gene regulation have corroborated the hypothesis that its expression is important for differentiating between proliferative and invasive states (16). To test the validity of the proliferative signature, we examined the role of Mitf in the proliferative signature phenotype and compared the *in vivo* tumorigenicity of these cells against

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

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those with an invasive signature. At the same time, we used immunohistochemistry to monitor Mitf and the Ki67 antigen in the resulting tumors to provide evidence of *in vivo* switching between signatures.

Materials and Methods

Melanoma tissues and lines. Melanoma cell cultures were established from surplus material from cutaneous melanoma metastases removed by surgery after having obtained written informed consent of the patient. Clinical diagnosis was confirmed by histology and immunohistochemistry. Melanoma cells were released from tissue sections and grown as previously described (18). Cell lines were chosen according to their transcription pattern signatures as previously described (7). Two proliferative signature (M980513, M000907) and two invasive signature (M991121, M010308) melanoma lines were used.

***In vitro* motility and proliferation assays.** For the motility assays 2×10^4 melanoma cells were seeded on 8 μm transwell microporous filters (Becton Dickinson) in 200 μL RPMI. As a chemoattractant, RPMI containing 10% FCS was added to the lower chamber. After 18 h of incubation, cells on the upper side of the filter were removed with cotton swab. The membrane was then stained using a standard H&E protocol, and the cells were counted under a light microscope. For the proliferation assay, melanoma cells were seeded to a density of 5×10^4 in each well of a six-well plate. After 24, 72, and 96 h, cells were counted in a Neubauer chamber to estimate cell-doubling times.

Recombinant adenovirus vector and small interfering RNA. Recombinant first-generation, E1/E3–deleted Ad5-based vectors Ad-H1-siMitf and Ad-H1-siControl were generated as described previously (19). Briefly, homologous recombination was performed in human embryonic retinoblast line 911 cells between a transfer plasmid pAd-H1-siMitf encoding the Mitf-specific small interfering RNA (siRNA) sequence under the control of the H1 promoter and a genomic *Cla*I DNA fragment isolated from AdMLP-*lacZ*. To construct pAd-H1-siMitf, the cytomegalovirus promoter of pAd-CMVΔlacZ-lnk1 was replaced with the H1 promoter (20). The H1 promoter fragment was PCR amplified from genomic DNA of human 293T cells and cloned into *Sfi*I/*Bam*HI–restricted pAd-CMVΔlacZ-Ink1. Subsequently, oligonucleotides for the silencing cassette (21) containing a 19-nucleotide siRNA sequence targeting Mitf (22) were cloned into *Nhe*I/*Sal*I–restricted pAd-H1ΔlacZ-lnk1 (Ad-H1-siMitf). For a mock control (Ad-H1-siControl), the siRNA sequence of Mitf was scrambled and blasted to ensure no human sequence is targeted. Recombinant adenoviruses were plaque purified, amplified, and CsCl purified. Viral titers were determined by plaque assay, using 911 cells, and were 1.8×10^{10} plaque-forming units (pfu)/mL for Ad-H1-siMitf and 1.3×10^{10} pfu/mL for Ad-H1-siControl.

Transfection and transforming growth factor-β challenge assay. Melanoma cells were seeded to a density of 4×10^4 in a 24-well plate 1 day before infection. The next day, medium was changed to RPMI containing 2% FCS and cells were either infected with virus particles carrying the pAd-H1-siMitf or pAd-H1-siControl. For assessment of susceptibility to growth inhibition by transforming growth factor-β (TGF-β), cells were challenged with 5 ng/mL recombinant TGF-β (Biosource) 24 h after virus transduction. After a further 56 h, cell growth was estimated using a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

Western blot analyses. Cells were solubilized in lysis buffer containing 20 mmol/L Tris-HCl (pH 7.5), 1% Triton X-100, 150 mmol/L NaCl, 10% glycerol, and Complete mini protease inhibitor (Roche Diagnostics GmbH). Proteins were separated on a NuPAGE 10% Bis-Tris gel (Invitrogen) under denaturing and reducing conditions followed by transfer onto a Nitrocellulose membrane (Invitrogen). Mitf protein was detected with a mouse anti-Mitf monoclonal antibody (clone C5; LabVision) diluted 1:100 in 3% bovine serum albumin at 4°C overnight. Secondary rabbit–anti-mouse antibodies (Abcam) conjugated with peroxidase was used at a dilution of 1:10,000. Detection by chemiluminescence used an enhanced chemiluminescence system (GE Healthcare).

Xenografts. For each melanoma line, a total of 3×10^6 cells were injected into both flanks of 8-week-old female athymic nude mice. Mice were kept in individually ventilated cages for a maximum of 75 days postinjection. Volume of tumors was measured using vernier calipers ($V = W^2 \times L \times 0.5$) once every 3 to 7 days until linear growth was detected, after which measurements were taken every 1 to 2 days. If at least one xenograft tumor reached 1 cm^3, the mouse was sacrificed and tumors were removed. If the condition of the mouse deteriorated (e.g., listlessness, loss of weight), the mouse was sacrificed and tumors were removed. All remaining mice were sacrificed on the 75th day and tumors were removed. Effective tumor initiation time was calculated on the day tumor volume reached 100 mm^3. Tumors not reaching 100 mm^3 within 75 days were not considered.

Immunohistochemistry. Cell lines were prepared for immunohistochemistry as follows. Briefly, cells were cultured, washed in PBS (Biochrom), and then put into suspension by incubating in 2 mL trypsin/EDTA solution (Biochrom) at 37°C. Trypsin was inactivated by adding 18 mL of FCS-containing growth medium. Cell suspensions were centrifuged for 5 min at 2,000 rpm. After removing the supernatant, four drops of plasma were added to the pellet and the solution was mixed. One drop of thrombin was added and after 5 min, the coagulated material was encapsulated for fixation in 4% formalin and embedded in paraffin. Excised xenograft samples were fixed in 4% formalin and embedded in paraffin. Slides were cut from paraffin blocks and immunohistochemically stained using the alkaline phosphatase–anti-alkaline phosphatase technique and counterstained using hematoxylin. Antibodies used were directed against Mitf (clone D5; DakoCytomation) or Ki-67 (clone MIB-1; DakoCytomation). Counting of stained and unstained nuclei was done on a PC using the free UTHSCSA ImageTool program (developed at the University of Texas Health Science Center at San Antonio, Texas, and available from the Internet by anonymous FTP).[4]

Statistical analysis. For all quantitative sample comparisons, Student's two-sample heteroscedastic t test was used to calculate a t statistic for comparison against a significance cutoff of $P = 0.05$.

Results

Phenotypic assignment of cell lines. To study the *in vivo* tumorigenic behavior of melanoma cell lines with different transcriptional signatures, we selected pairs of proliferative and invasive signature melanoma cell lines based on previous genome-wide transcription profiling experiments (7). We did supervised hierarchical clustering of these samples using normalized signal intensity data from 105 genes shown to be tightly linked to signature (Fig. 1*A*; Supplementary Fig. S1). Earlier experiments had shown that proliferative signature lines were significantly less motile than invasive signature lines. Also, TGF-β challenge showed that proliferative signature cells were significantly more susceptible to TGF-β–mediated growth inhibition than invasive signature cells (7). We performed additional motility and proliferation experiments to expand this range of *in vitro* characterizations. Cell growth experiments showed a significant ($P < 0.001$) difference in proliferation rates between proliferative and invasive signature cell lines (Fig. 1*B*). Conversely, invasive signature cell lines plated at subconfluent densities on microporous transwell filters migrated in significantly ($P < 0.001$) higher numbers toward the lower chamber than identically plated proliferative cell lines (Fig. 1*C*). With these experiments, we concluded that signature assignments given to cell lines according to their gene expression signature correlate with *in vitro* data in the context of our model.

Mitf is a marker of proliferative phenotype. To follow cell signatures *in vivo*, we selected immunohistochemical markers according to their signature specificity. Previous analysis indicated

[4] ftp://maxrad6.uthscsa.edu

that Mitf mRNA and protein levels are high in proliferative signature lines and at low or undetectable levels in invasive signature samples (7). We confirmed this by performing immunohistochemistry on paraffin-embedded cultures of proliferative and invasive signature melanoma lines. Immunohistochemical staining of the different signature cell line pellets with anti-Mitf antibodies showed that in proliferative signature cell lines, 93% of cells were positive for nuclear staining for Mitf whereas invasive signature cell lines showed no positivity (Fig. 2; Supplementary Fig. S2). Because invasive melanoma cells have down-regulated genes responsible for the melanocytic phenotype observable in proliferative signature melanoma cells, there are no immunohistochemical markers that unequivocally identify them. Instead, the differential in growth rates for the signatures indicated that a general proliferation marker may be useful for immunohistochemical identification of signature type *in vivo*. Although examination of previously published gene expression data shows that between transcriptional signature types, there is no significant differential in the expression of mRNA encoding the proliferation marker Ki67 antigen, the significant difference in *in vitro* proliferation rates suggest that Ki67 antigen is likely to show a difference at the protein level. Accordingly, staining for Ki67 antigen showed that 94% of proliferative signature cells and 45% of invasive signature cells had positively stained nuclei (Fig. 2). These results indicate that Mitf is a good marker for specific identification of proliferative signature cells and that Ki67 antigen is a suitable marker for identifying regions undergoing differential rates of proliferation.

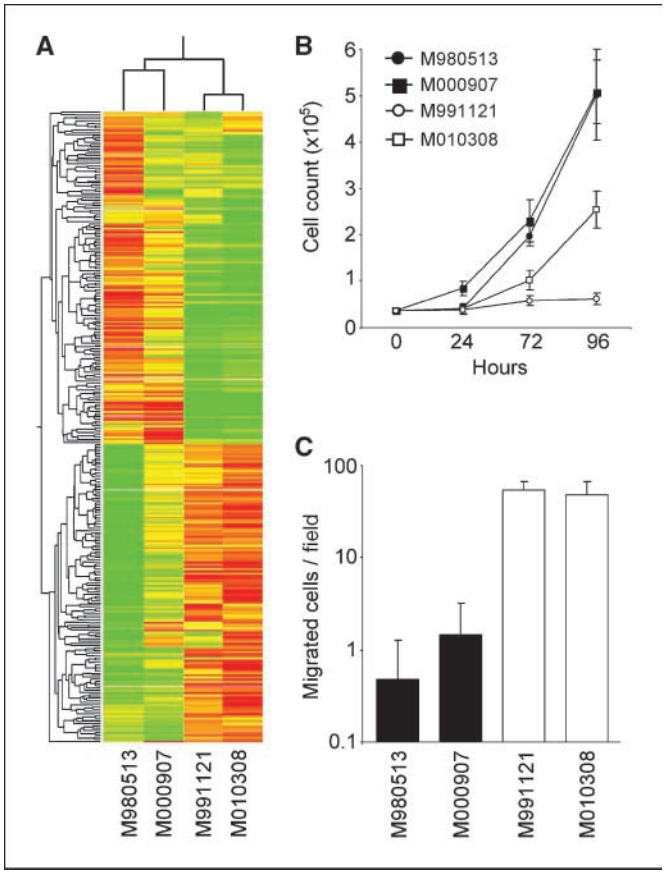

Figure 1. *In vitro* correlations with gene expression signatures. M980513 and M000907 proliferative signature melanoma lines, as well as M991121 and M010308 invasive signature melanoma lines, were chosen for this study. *A,* a gene expression heat map, generated by clustering samples based on the normalized expression of 105 metastatic potential genes (Supplementary Fig. S1), highlights subtype-specific signatures. *In vitro* growth (*B*) and motility (*C*) experiments correlate appropriately with proliferative and invasive signature assignments. *Bars,* SD.

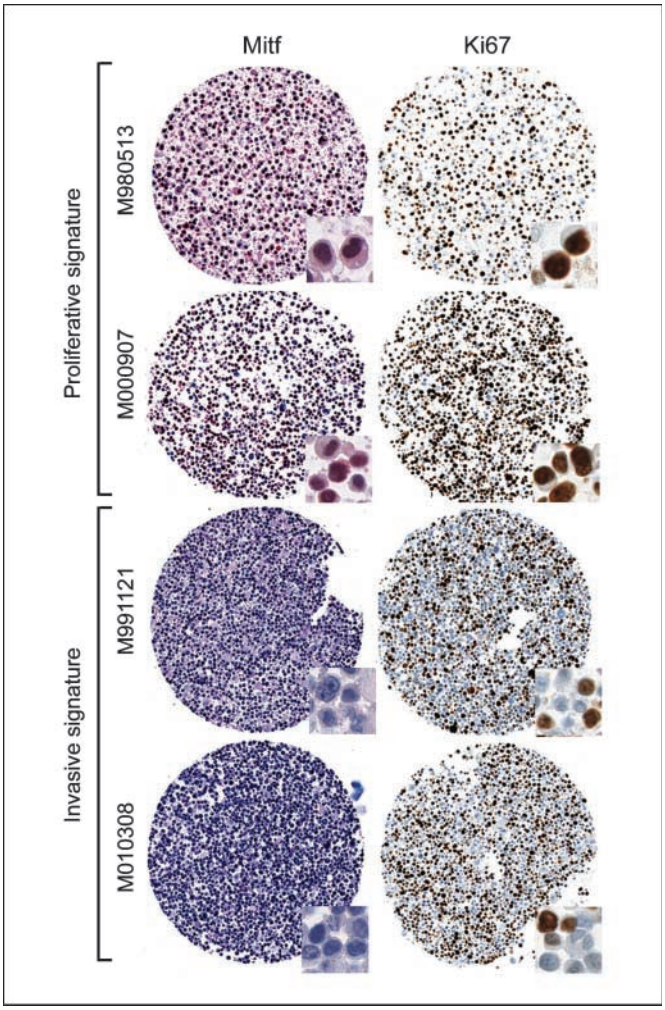

Figure 2. Immunohistochemical marker correlations with gene expression signatures. Immunohistochemical analysis of paraffin-embedded cell lines shows that proliferative and invasive signature lines have differential staining for Mitf (93% and 0%, respectively) and Ki67 antigen (94% and 45%, respectively). See Supplementary Fig. S2 for a higher-resolution image.

Mitf expression reflects signature phenotype. To confirm that Mitf expression is functionally linked to signature phenotype, we used siRNA to knockdown Mitf protein levels and assessed the effects *in vitro*. One *in vitro* characteristic that distinguishes between proliferative and invasive signature melanoma cells is a differential in susceptibility to TGF-β–mediated inhibition of proliferation, with proliferative signature cells being more sensitive to TGF-β than invasive signature cells (7). Because proliferative signature cells express Mitf and invasive signature cells do not, we hypothesized that Mitf expression mediated the growth-inhibitory effect of TGF-β on proliferative signature cells. We did anti-Mitf siRNA knockdown experiments in a proliferative signature melanoma line and confirmed knockdown by Western blot analyses (Fig. 3*A*). We found that Mitf depletion from proliferative signature melanoma cells made them less susceptible to TGF-β–mediated growth inhibition (Fig. 3*B*), showing that Mitf mediates the growth-inhibitory effect of TGF-β. Further, we showed that

TGF-β treatment reduces Mitf mRNA and protein expression (Fig. 3*C*), suggesting that TGF-β–mediated growth inhibition may be effected by reduction of *MITF* expression. This shows that Mitf function is closely linked to the relationship between transcription signature and *in vitro* phenotype, confirming it as a useful *in vivo* marker for identifying different signature cells.

Proliferative cells form fast-growing tumors sooner than invasive cells. To test the relationship of cell line signature assignments with *in vivo* behavior, we performed s.c. injection of cell lines into the flanks of immunocompromised mice and recorded tumor growth characteristics. We found that proliferative melanoma lines consistently initiated tumors, measured as the time at which tumor volume exceeded 100 mm^3, ~14 ± 3 days after being injected into the flanks of athymic nude mice. This was significantly ($P < 0.001$) shorter than for invasive lines, which took 59 ± 11 days (Fig. 4). These data provide *in vivo* evidence for the significance of a proliferative signature in melanoma cells as predicted by *in vitro* experiments. The proliferative signature–seeded tumors all initiated growth at nearly the same time point. Contrasting this, initiation times for the invasive signature–seeded tumors were spread over a wider period. This suggests that, unlike proliferative signature–seeded initiation, invasive signature-seeded initiation may be dependent on microenvironmental variation.

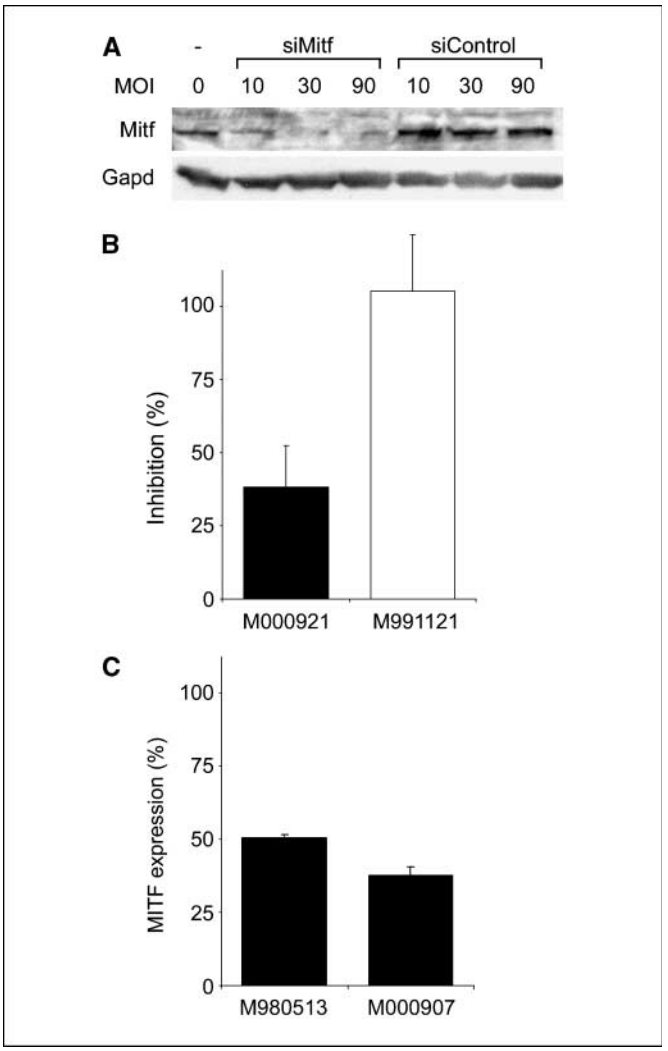

Figure 3. siRNA knockdown of Mitf protects against TGF-β–mediated growth inhibition. *A,* siRNA-mediated knockdown of Mitf in a proliferative signature melanoma cell line (M000921) was confirmed by Western blot analysis. *B,* the ratio of TGF-β–mediated inhibition of growth in cells treated with siRNA targeting Mitf over cells treated with a control siRNA is compared between proliferative (M000921) and invasive (M991121) signature melanoma cell lines. This shows that Mitf knockdown promotes resistance to TGF-β–mediated growth inhibition in a proliferative signature melanoma cell line, whereas identical treatment does not change susceptibility in an invasive signature line. TGF-β treatment of proliferative signature lines (M980513, M000907) results in reduction of Mitf mRNA (*C*).

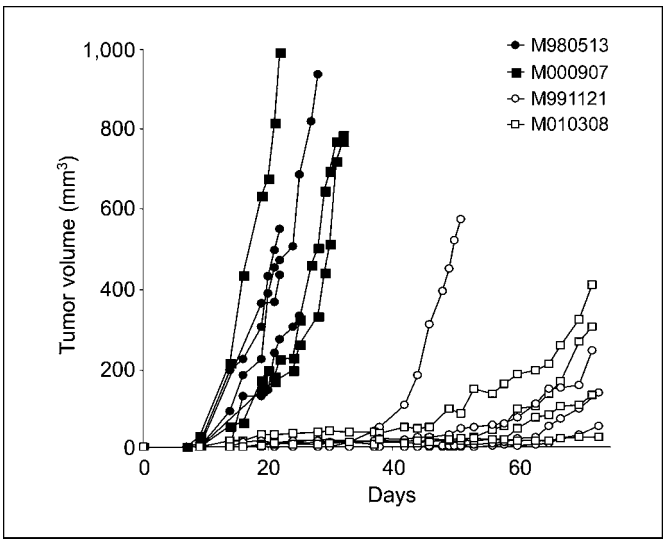

Figure 4. Xenograft tumor growth. Human melanoma cell lines (M980513, M000907, M991121, M010308) were injected into both flanks of immunocompromised nude mice. Proliferation of melanoma cells led to tumor growth, which was monitored daily. Proliferative melanoma cells (M980513, M000907) rapidly formed tumors, whereas invasive melanoma cells (M991121, M010308) took weeks longer to initiate tumor growth.

Tumors derived from proliferative or invasive lines are indistinguishable. Because both transcription signature melanoma cell types yielded tumors, we were interested in examining these for signature-specific differences. Upon excision, the tumors were stained for Mitf and Ki67 antigen expression. Tumors derived from invasive signature cell lines, which did not stain for Mitf, revealed melanoma cells with nuclei that were Mitf positive and melanoma cells with nuclei that were Mitf negative (Figs. 5*A–E*; Supplementary Fig. S3). Tumors derived from proliferative signature cell lines, which stained for Mitf, showed the same patterning of stained and unstained melanoma cell nuclei (Fig. 5*F-J*; Supplementary Fig. S4). Additionally, we found that Mitf-stained nuclei tended to concentrate within the peripheral margins of the tumors. Ki67 antigen staining patterns were similarly indistinguishable in tumors derived from proliferative or invasive signature lines. Also, it was apparent that tumor regions showing Mitf-positive nuclei were also enriched for Ki67-positive nuclei. These findings showed that after removal, tumors seeded with invasive or proliferative signature cell lines were not distinguishable and that homogeneous *in vitro* staining patterns yielded strikingly heterogeneous patterns *in vivo*, showing that signature patterns of melanoma cells change bidirectionally.

Discussion

A feature of current models for gene expression involvement in melanoma progression is their explicitly one-way nature. It is typical to present gene expression changes proceeding concomitantly with stage progression, where a gene either increases or

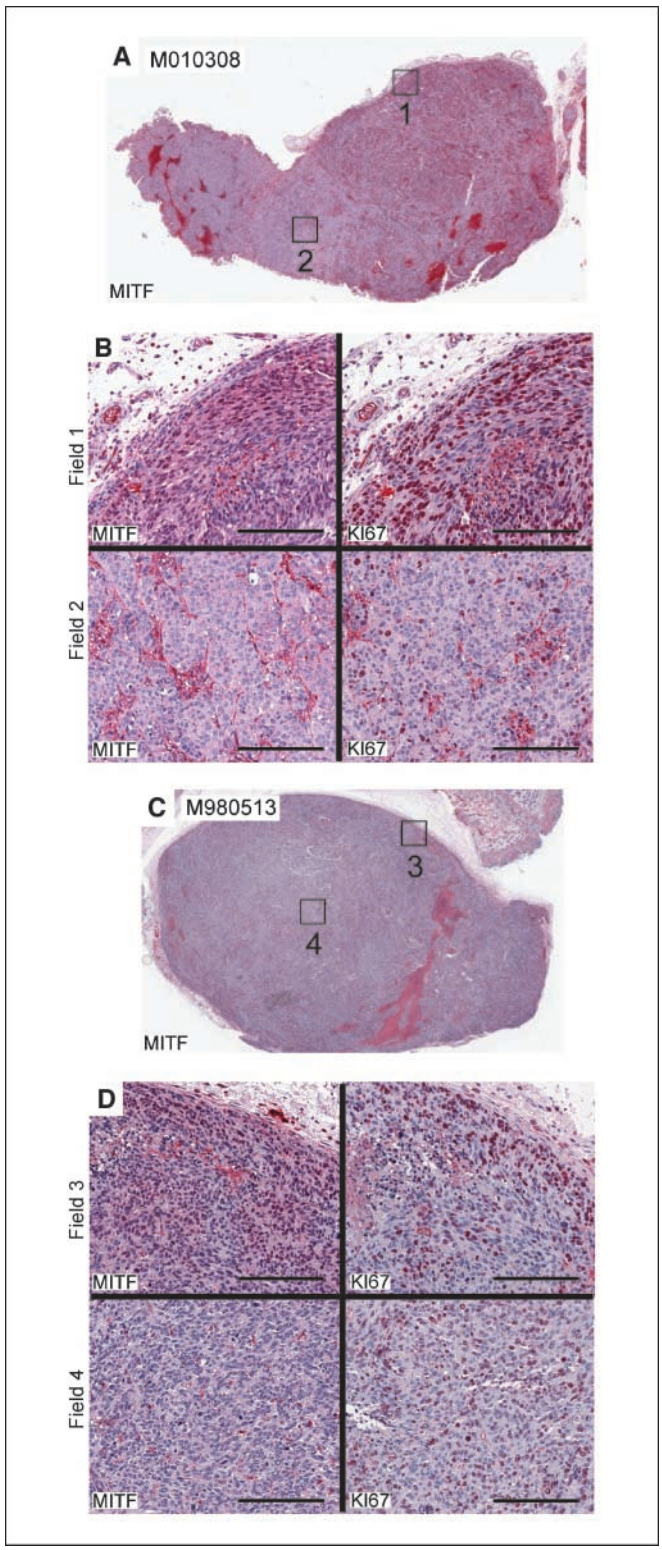

Figure 5. Immunohistochemistry of melanoma xenograft tumors. Human melanoma cell lines (M980513, M000907, M991121, M010308) were injected into the flanks of immunocompromised nude mice and allowed to grow tumors for a maximum of 75 d. After a tumor had formed, it was removed and subjected to immunohistochemical analysis. *A,* a day 75 tumor resulting from an invasive signature melanoma (M010308); see Supplementary Fig. S3 for a higher-resolution image. *B,* Mitf and Ki67 stains of fields 1 and 2. *C,* a day 22 tumor resulting from a proliferative signature melanoma (M980513); see Supplementary Fig. S4 for a higher-resolution image. *D,* Mitf and Ki67 stains of fields 3 and 4. *Black horizontal bars,* 200 μm.

decreases expression as the disease evolves through clinically recognized stages to metastasis (10). However, models of this design do not account for the broad molecular heterogeneity apparent in melanoma. Indeed, it is clear that, in many melanoma cells within a given lesion, genes expected to be down-regulated in late stages are found active and others expected to be up-regulated are not. One possible answer is that many genes associated with metastatic potential do not undergo one-way modification of regulation and instead retain the potential to reverse changes in their expression.

Investigations into the gene expression signatures of melanoma cell lines taken from late-stage tumors show that a given cell line will usually express one of two major transcription programs. It was also determined that the genes whose expression patterns respectively delineated the two signatures were likely involved in melanoma metastatic potential (7, 17). One of these signatures (identified by us as proliferative) has MITF and other melanocytic genes (e.g., *TYR, DCT, MLANA*) up-regulated along with a number of additional neural crest-related factors (e.g., *SOX10, TFAP1A*, and *EDNRB*). This signature is associated with high rates of proliferation, low motility, and sensitivity to growth inhibition by TGF-β. A second signature (identified by us as invasive) down-regulates these genes and instead up-regulates others whose secreted products (e.g., *INHBA, COL5A1*, and *SERPINE1*) are involved in modifying the extracellular environment. This signature is associated with lower rates of proliferation, high motility, and resistance to growth inhibition by TGF-β. Having identified these genes, we found that many of the proliferative signature were frequent responders to Wnt signaling, and those of the invasive signature were commonly TGF-β signal driven, and we proposed that the balance in activity of these signaling pathways is responsible for the different transcription signatures observed (7). Among genes comprising the invasive signature are several (e.g., *WNT5A, DKK1*, and *CTGF*) known to negatively regulate Wnt signaling (23–25), suggesting that activation of TGF-β signaling may precipitate deactivation of Wnt signaling. Similar cross-talk opposition between TGF-β and Wnt signaling has already been noted in gastrointestinal cancer (26). This possible link between the signatures indicated that they may be reversible given appropriate signals and further suggested that proliferation and invasion are program states that melanoma cells activate according to microenvironmental cues (7).

The results of our *in vitro* proliferation and motility analyses were consistent with signature assignments inferred from earlier DNA microarray experiments (7). To immunohistochemically differentiate signatures *in vivo*, we used nuclear Mitf as our marker for the proliferative signature and nuclear Ki67 antigen as a general indicator for proliferation activity. We found *in vitro* that although both invasive and proliferative signature melanoma cells expressed Ki67 antigen, less than half of the invasive signature cells expressed it, correlating with the relative growth differences observed between proliferative and invasive cells *in vitro*. Because Ki67 antigen is not detected in G_0 (27, 28), and it may be an absolute requirement for cell proliferation (29), we conclude that invasive signature cells spend more time in G_0 (quiescence). Increasing Mitf expression in melanoma has been shown to be a proliferative factor and involved in Cdk2 production and activity (16, 30, 31). Our data also support a proliferative role for Mitf *in vitro* as we find that Mitf-positive lines proliferate faster (Fig. 1) and express Ki67 antigen with greater frequency than Mitf-negative lines (Fig. 2).

We did siRNA knockdown of Mitf in a proliferative signature cell line to show that this confers a TGF-β resistance phenotype that others have shown is characteristic of invasive signature line melanomas (32–34). Our DNA microarray data suggested that *Mitf* gene expression is central to the proliferative signature and may therefore have a role in mediating the growth-inhibitory response to TGF-β. After knockdown of Mitf expression in a proliferative signature line, the cells gained resistance to TGF-β–mediated inhibition of proliferation (Fig. 3). This correlates with experiments by others who have shown that invasive characteristics are increased in melanoma cells treated with siRNA targeting Mitf expression (16, 35). Together, these combined findings indicate that regulation of Mitf expression is critical to signature membership and supports our contention that *in vivo* changes in nuclear Mitf staining indicate proliferative/invasive signature switching.

Our xenograft experiments showed that tumor growth patterns correlated appropriately with the gene expression signatures. Proliferative signature lines initiated tumors ~2 weeks after injection, whereas invasive signature lines lay dormant for an average of 8 weeks before tumor growth began (Fig. 4). Whereas these and the *in vitro* experiments further support the different signature assignments to different melanoma cell lines, immunohistochemical examination of the tumors showed evidence for signature switching during tumorigenesis. Comparison of *in vitro* Mitf staining patterns with *in vivo* Mitf staining patterns shows that resultant tumors deriving from either proliferative signature or invasive signature lines reveal the presence of both Mitf-positive and Mitf-negative melanoma cells (Fig. 5). Concurrently, Ki67 antigen staining, although found throughout the tumors, was more frequent in regions positive for Mitf staining than in regions absent of Mitf. Furthermore, the distribution of Mitf staining and increased frequency of Ki67 antigen positivity shows a distinctly peripheral pattern. This confirms that, as expected, melanoma cells proximal to the interface between host tissues and the tumor are actively undergoing increased rates of proliferation. To address concerns that our invasive signature lines were contaminated with proliferative signature cells, we monitored *in vitro* proliferation rates for invasive signature cells serially passaged over the same time frame as conducted for the xenograft experiments. We observed no change in proliferation rates (data not shown), and therefore we believe that the change in tumor growth in invasive signature line–seeded xenografts is not due to prior contamination with proliferative signature cells.

The long lag time for tumor initiation observed with invasive signature cells and the presence of Mitf-positive nuclei in resulting tumors indicate that tumor growth was probably preceded by a switch in some cells to the proliferative signature type. Similar microenvironment-driven signature switching has been previously shown in other experiments. Recent studies investigating the effect of embryonic environments on melanoma cells has shown how these environments can affect the aggressive phenotype. Kulesa and coworkers (36), using the aggressive C8161 melanoma line, showed that transplantation of C8161 cells into chick embryonic tissues stimulated reexpression of melanocytic markers similar to poorly aggressive cells. Complimentary *in vitro* studies in which poorly aggressive cells, grown on three-dimensional matrices preconditioned by aggressive lines, showed up-regulation of extracellular matrix modifying genes and increased invasive ability (37). Additional experiments in zebrafish revealed that in embryonic environments, inhibition of the morphogen Nodal switched melanoma cells to a less aggressive phenotype, suggesting that Nodal signaling (which acts through TGF-β family receptors) was important to maintaining aggressive phenotypes in melanoma cells (38). Signature switching of cells in response to the microenvironment would explain why our xenograft tumors deriving from different signature lines were immunohistochemically indistinguishable.

That rapidly proliferating cells are found closer to the periphery of growing tumors also supports a role for the microenvironmental determination of activity. However, this location of proliferative phenotype melanoma cells at the tumor periphery directly contradicts the long-held assumption that these cells represent the tumor invasive front. This assumption is primarily based on observations linking primary tumor thickness and the frequency of subsequent metastatic disease (39). We offer that the thickness of the primary tumor may, rather than serve to bring its peripheral cells closer to vascular egress, determine the extent to which cells deeper within it experience microenvironmental change, which (as we contend) drives the switch to a more invasive phenotype.

Perhaps more interesting is the comparison of our model with the identification of *MITF* as a lineage-specific oncogene. Garraway and coworkers (15) have shown *MITF* gene amplification in a fraction of melanomas and show its correlation with a poorer prognosis. They speculated that increased *MITF* gene dosage may in part compensate affected melanoma cells in settings where Mitf activity is normally lost. This suggests that with amplification of its gene, Mitf expression in our xenograft model would be preserved in all cells. However, whereas increased gene dosage may play a role when the gene is activated (and precipitate a worsened prognosis in patients), this does not mean the gene is necessarily immune from otherwise normal signaling responses.

These data are critical pieces of the melanoma progression puzzle because they suggest not only that invasion and proliferation are divisible aspects of metastatic potential, but that these different transcriptional states are interchangeable programs between which melanoma cells oscillate during progression in response to changing microenvironmental cues (Fig. 6). What these microenvironmental cues precisely are remains unknown, but there is growing evidence that hypoxia may be one (40) and

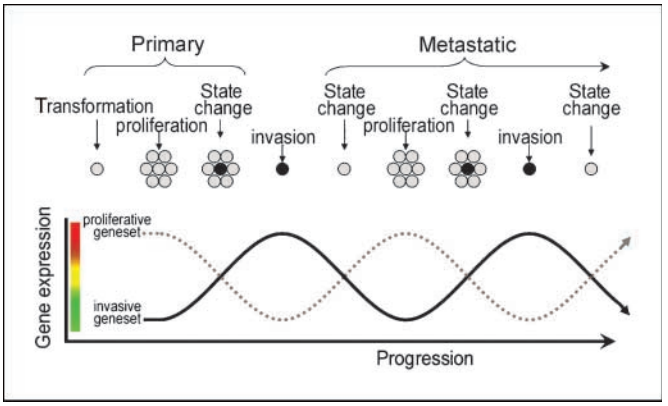

Figure 6. An integrated model for gene regulation of melanoma metastatic potential and progression. Early-phase melanoma cells expressing the proliferative signature gene set proliferate to form the primary lesion. Following this, an unknown signal switch, likely brought about by altered microenvironmental conditions (e.g., hypoxia or inflammation), gives rise to cells with a significantly different invasive signature gene set. Invasive signature cells escape and, upon reaching a suitable distal site, revert to the proliferative state and nucleate a new metastasis where the cycle is repeated. Each switch in phenotype (*state change*) is accompanied by an exchange in expressed gene sets from proliferative to invasive and vice versa.

inflammation another (41). The model we use to describe melanoma progression is presented in binary terms of invasive versus proliferative. However, this is not to deny that intermediate transcription signatures exist between the described archetypes and we have indeed characterized other melanoma lines with intermediate signatures in our earlier study (7). Therefore, we believe that the two signatures discussed here represent opposite ends of a signature continuum between which melanoma cells slide in response to microenvironment-linked changes in signaling.

The relevance of this model to clinical aspects of melanoma is that it may explain why metastatic melanoma is so refractory to chemotherapeutic and immunotherapeutic strategies. During treatment of metastatic melanoma, the so-called mixed responses are often observed and this phenomenon may derive from a heterogeneous distribution of proliferative and invasive signature cells within metastases. The heterogeneity inherent in tumors containing melanoma cells with biological activities dependent on the microenvironment suggests that whereas proliferating cells are susceptible to chemotherapy, there are populations of cells which, although not proliferating, have the capacity to switch back to a proliferative program and successfully drive tumor progression once therapy has ceased. Finally, although our state-switching model for melanoma progression offers attractive answers for why the disease behaves as it does, we acknowledge the caveat that much of our hypothesis rests on expression signatures obtained *in vitro* and thus may not fully recapitulate the *in vivo* biology of melanoma. Therefore, until the *in vivo* situation is resolved, we cannot yet advocate the abandonment of models that favor stepwise accumulation of genetic lesions as a driver for melanoma progression.

Acknowledgments

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References

1. Sasse AD, Sasse EC, Clark LG, Ulloa L, Clark OA. Chemoimmunotherapy versus chemotherapy for metastatic malignant melanoma. Cochrane database of systematic reviews (Online) 2007(1):CD005413.
2. Fidler IJ, Kripke ML. Metastasis results from preexisting variant cells within a malignant tumor. Science 1977; 197:893–5.
3. Levene A. On the histological diagnosis and prognosis of malignant melanoma. J Clin Pathol 1980;33: 101–24.
4. Banerjee SS, Harris M. Morphological and immunophenotypic variations in malignant melanoma. Histopathology 2000;36:387–402.
5. Curtin JA, Fridlyand J, Kageshita T, et al. Distinct sets of genetic alterations in melanoma. N Engl J Med 2005; 353:2135–47.
6. Haqq C, Nosrati M, Sudilovsky D, et al. The gene expression signatures of melanoma progression. Proc Natl Acad Sci U S A 2005;102:6092–7.
7. Hoek KS, Schlegel NC, Brafford P, et al. Metastatic potential of melanomas defined by specific gene expression profiles with no BRAF signature. Pigment Cell Res 2006;19:290–302.
8. Paget S. The distribution of secondary growths in cancer of the breast. Lancet 1889;1:571–3.
9. Ribatti D, Mangialardi G, Vacca A. Stephen Paget and the 'seed and soil' theory of metastatic dissemination. Clin Exp Med 2006;6:145–9.
10. Miller AJ, Mihm MC, Jr. Melanoma. N Engl J Med 2006;355:51–65.
11. Steingrimsson E, Copeland NG, Jenkins NA. Melanocytes and the microphthalmia transcription factor network. Annu Rev Genet 2004;38:365–411.
12. Carreira S, Goodall J, Aksan I, et al. Mitf cooperates with Rb1 and activates p21Cip1 expression to regulate cell cycle progression. Nature 2005;433:764–9.
13. Loercher AE, Tank EM, Delston RB, Harbour JW. MITF links differentiation with cell cycle arrest in melanocytes by transcriptional activation of INK4A. J Cell Biol 2005;168:35–40.
14. Du J, Widlund HR, Horstmann MA, et al. Critical role of CDK2 for melanoma growth linked to its melanocyte-specific transcriptional regulation by MITF. Cancer Cell 2004;6:565–76.
15. Garraway LA, Widlund HR, Rubin MA, et al. Integrative genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma. Nature 2005;436:117–22.
16. Carreira S, Goodall J, Denat L, et al. Mitf regulation of Dia1 controls melanoma proliferation and invasiveness. Genes Dev 2006;20:3426–39.
17. Bittner M, Meltzer P, Chen Y, et al. Molecular classification of cutaneous malignant melanoma by gene expression profiling. Nature 2000;406:536–40.
18. Geertsen RC, Hofbauer GF, Yue FY, Manolio S, Burg G, Dummer R. Higher frequency of selective losses of HLA-A and -B allospecificities in metastasis than in primary melanoma lesions. J Invest Dermatol 1998;111: 497–502.
19. Hemmi S, Geertsen R, Mezzacasa A, Peter I, Dummer R. The presence of human coxsackievirus and adenovirus receptor is associated with efficient adenovirus-mediated transgene expression in human melanoma cell cultures. Hum Gene Ther 1998;9:2363–73.
20. Hasuwa H, Kaseda K, Einarsdottir T, Okabe M. Small interfering RNA and gene silencing in transgenic mice and rats. FEBS Lett 2002;532:227–30.
21. Saydam O, Glauser DL, Heid I, et al. Herpes simplex virus 1 amplicon vector-mediated siRNA targeting epidermal growth factor receptor inhibits growth of human glioma cells *in vivo*. Mol Ther 2005;12:803–12.
22. Busca R, Berra E, Gaggioli C, et al. Hypoxia-inducible factor 1{α} is a new target of microphthalmia-associated transcription factor (MITF) in melanoma cells. J Cell Biol 2005;170:49–59.
23. Ishitani T, Kishida S, Hyodo-Miura J, et al. The TAK1-NLK mitogen-activated protein kinase cascade functions in the Wnt-5a/Ca(2+) pathway to antagonize Wnt/β-catenin signaling. Mol Cell Biol 2003;23:131–9.
24. Zorn AM. Wnt signalling: antagonistic Dickkopfs. Curr Biol 2001;11:R592–5.
25. Mercurio S, Latinkic B, Itasaki N, Krumlauf R, Smith JC. Connective-tissue growth factor modulates WNT signalling and interacts with the WNT receptor complex. Development 2004;131:2137–47.
26. Mishra L, Shetty K, Tang Y, Stuart A, Byers SW. The role of TGF-β and Wnt signaling in gastrointestinal stem cells and cancer. Oncogene 2005;24:5775–89.
27. Braun N, Papadopoulos T, Muller-Hermelink HK. Cell cycle dependent distribution of the proliferation-associated Ki-67 antigen in human embryonic lung cells. Virchows Arch 1988;56:25–33.
28. Bruno S, Darzynkiewicz Z. Cell cycle dependent expression and stability of the nuclear protein detected by Ki-67 antibody in HL-60 cells. Cell Prolif 1992;25: 31–40.
29. Schluter C, Duchrow M, Wohlenberg C, et al. The cell proliferation-associated antigen of antibody Ki-67: a very large, ubiquitous nuclear protein with numerous repeated elements, representing a new kind of cell cycle-maintaining proteins. J Cell Biol 1993;123:513–22.
30. Widlund HR, Horstmann MA, Price ER, et al. β-Catenin-induced melanoma growth requires the downstream target Microphthalmia-associated transcription factor. J Cell Biol 2002;158:1079–87.
31. Du J, Fisher DE. Identification of Aim-1 as the underwhite mouse mutant and its transcriptional regulation by MITF. J Biol Chem 2002;277:402–6.
32. Heredia A, Villena J, Romaris M, Molist A, Bassols A. The effect of TGF-β1 on cell proliferation and proteoglycan production in human melanoma cells depends on the degree of cell differentiation. Cancer Lett 1996;109:39–47.
33. Krasagakis K, Garbe C, Schrier PI, Orfanos CE. Paracrine and autocrine regulation of human melanocyte and melanoma cell growth by transforming growth factor β *in vitro*. Anticancer Res 1994;14:2565–71.
34. Roberts AB, Anzano MA, Wakefield LM, Roche NS, Stern DF, Sporn MB. Type β transforming growth factor: a bifunctional regulator of cellular growth. Proc Natl Acad Sci U S A 1985;82:119–23.
35. Lekmine F, Chang CK, Sethakorn N, Das Gupta TK, Salti GI. Role of microphthalmia transcription factor (Mitf) in melanoma differentiation. Biochem Biophys Res Commun 2007;354:830–5.
36. Kulesa PM, Kasemeier-Kulesa JC, Teddy JM, et al. Reprogramming metastatic melanoma cells to assume a neural crest cell-like phenotype in an embryonic microenvironment. Proc Natl Acad Sci U S A 2006;103: 3752–7.
37. Seftor EA, Meltzer PS, Kirschmann DA, Margaryan NV, Seftor RE, Hendrix MJ. The epigenetic reprogramming of poorly aggressive melanoma cells by a metastatic microenvironment. J Cell Mol Med 2006;10: 174–96.
38. Topczewska JM, Postovit LM, Margaryan NV, et al. Embryonic and tumorigenic pathways converge via Nodal signaling: role in melanoma aggressiveness. Nat Med 2006;12:925–32.
39. Breslow A. Tumor thickness in evaluating prognosis of cutaneous melanoma. Ann Surg 1978;187:440.
40. Holmquist L, Lofstedt T, Pahlman S. Effect of hypoxia on the tumor phenotype: the neuroblastoma and breast cancer models. Adv Exp Med Biol 2006;587: 179–93.
41. de Visser KE, Eichten A, Coussens LM. Paradoxical roles of the immune system during cancer development. Nat Rev Cancer 2006;6:24–37.

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