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.

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).

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

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Melanoma Switches between Growth and Invasion

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 × 10^5 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 × 10^3 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 CiaI DNA fragment isolated from AdMLP-lacZ. To construct pAd-H1-siMitf, the cytomegalovirus promoter of pAd-CMVlacZ-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 SfiI/BamHI-restricted pAd-CMVlacZ-lnk1. The H1 promoter fragment was PCR amplified from genomic DNA of human 293T cells and cloned into SfiI/BamHI-restricted pAd-CMVlacZ-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 adenviruses 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).

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 genomewide 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. 1A; 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 cell lines 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. 1B). 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. 1C). 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...
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 immuno-histochemistry 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.

**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. 3A). We found that Mitf depletion from proliferative signature melanoma cells made them less susceptible to TGF-β-mediated growth inhibition (Fig. 3B), showing that Mitf mediates the growth-inhibitory effect of TGF-β. Further, we showed that

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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.
TGF-\(\beta\) treatment reduces Mitf mRNA and protein expression (Fig. 3C), suggesting that TGF-\(\beta\)-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\(^3\), \(\sim 14 \pm 3\) 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 \(\pm\) 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.

**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. 5A–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. 5F–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
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, TPAPI, 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 that Mitf-positive lines proliferate faster (Fig. 1) and express Ki67 antigen with greater frequency than Mitf-negative lines (Fig. 2).

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.
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 tumorogenesis. 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 passed 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 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...
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-mediated 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.

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