Influence of the Microenvironment on Melanoma Cell Fate Determination and Phenotype

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Abstract
Tumor cells communicate bidirectionally with the surrounding microenvironment, sending and receiving topographical and molecular cues that direct diverse cellular phenomena, including differentiation, growth, and invasion. The microenvironment has long been acknowledged as a facilitator of melanoma progression, and recent studies have illuminated tumor-associated factors, including hypoxia and the extracellular matrix, as important mediators of melanocyte transformation and transdifferentiation. Although these findings portray the microenvironment as a perilous obstacle to the successful treatment of advanced melanomas, it is important to note that certain molecular milieus may be capitalized on as potential treatment modalities. Indeed, our group and others have elucidated the unique ability of embryonic microenvironments to normalize aggressive melanoma cells toward a more benign melanocytic phenotype. The microenvironment therefore presents a novel target for the treatment and ultimately the prevention of melanoma progression and metastasis. (Cancer Res 2006; 66(16): 7833-6)

Introduction
The incidence of melanoma has increased dramatically over the last 50 years. This is of particular concern because of the aggressive nature of the disease. Although melanoma accounts for only 10% of all skin cancers, it is responsible for >80% of skin cancer deaths, and the 5-year survival rate for patients with metastatic disease is only 16% (1, 2). Hence, understanding factors that promote melanocyte transformation and melanoma progression is of extreme importance.

The normal precursor of melanoma is the melanocyte, a pigment-producing cell of neural crest origin. To better understand pathways that might be involved in the transformation of melanocytes to melanoma, studies have used gene array analyses to decipher some of the major gene expression differences between these cell types, both in purified cell preparations and in whole tissue samples (3, 4). The effects of specific mutations on melanomagenesis have also been systematically explored (5, 6). Collectively, these studies have elucidated factors, including activating B-RAF mutations and activation of the mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways as key mediators of melanocyte transformation (6). More recently, our group and others have extended these findings by investigating the dynamic role of the microenvironment and the epigenetic changes in the transition between melanocytic and melanoma cell phenotypes (summarized in Fig. 1).

The Microenvironment and Melanoma Progression
The tumor microenvironment is extremely complex, consisting of elements as diverse as extracellular matrix (ECM) components, such as laminin and collagen, growth factors, including vascular endothelial growth factor, nutrients, such as glucose, and varying concentrations of oxygen. Collectively, these components provide the architecture necessary for invasion and supply signals that control cellular phenomena, including differentiation, growth, and survival. In addition to a purely auxiliary role as a storage compartment for mitogenic signals and a scaffold for cellular invasion, the microenvironment has emerged as a major player in melanocyte transformation and transdifferentiation. For example, recent studies have determined that low oxygen levels (hypoxia) may be essential for melanocyte transformation (7) and that ECM condition by aggressive melanoma cells can epigenetically transdifferentiate normal melanocytes toward an invasive melanoma cell-like phenotype (8).

Hypoxia characterizes the microenvironment of many solid tumors and has been shown to promote melanoma cell invasion and metastasis, largely through the up-regulation of proteins, such as the urokinase receptor (9). Recently, Bedogni et al. showed that hypoxia also contributes to melanocyte transformation. This study revealed that constitutively active Akt, which is observed in a high percentage of melanomas, can transform melanocytes exclusively when oxygen levels are low. Furthermore, the distance of the skin from superficial blood vessels renders it mildly hypoxic with oxygen levels between 1% and 5%. This microenvironmental milieu permits melanocytes to stabilize the transcriptional cofactor hypoxia-inducible factor-1α (HIF-1α), which promotes hypoxia-associated gene expression. It was discovered that this up-regulation of HIF-1α enhances melanocyte transformation by synergizing with constitutively active Akt to promote anchorage-independent growth in vitro and tumor formation in vivo (7). This finding exemplifies how the microenvironment can complement aberrant genetic changes to promote melanomagenesis.

Aggressive melanoma cells manifest a transdifferentiated phenotype characterized by the aberrant expression of genes normally restricted to other cell lineages concomitant with the loss of melanocyte-specific factors, such as Tyrosinase and Melan-A (10). Furthermore, aggressive melanoma cells acquire a vascular phenotype and are able to form patterned ECM-rich networks reminiscent of the structures produced by endothelial cells during vasculogenesis. This process, termed “vasculogenic mimicry,” is an example of tumor plasticity, is correlated with a poor prognosis clinically, and has been described in a variety of other tumor types (10). Using an unique three-dimensional culture system, we
recently revealed that normal epidermal melanocytes exposed to an ECM conditioned by metastatic cutaneous melanoma cells for 4 days were reprogrammed to similarly express genes associated with a multipotent plastic phenotype concomitant with the ability to form vasculogenic-like networks (8). Of note, the melanocytes seemed transdifferentiated, expressing genes, such as erythropoietin-producing hepatocarcinoma-A2 (EphA2), vascular endothelial-cadherin (VE-Cadherin), and tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 1 (Tie-1). Genes normally associated with ECM remodeling and invasion, including laminin 5 γ2 (Ln-5 γ2) and Urokinase, were also up-regulated, with a commensurate increase in migratory and invasive ability (8). Interestingly, melanocytes exposed to the metastatic matrix still retained the expression of pigment pathway-associated genes (MITF, Melan-A, and Tyrosinase), suggesting that certain aspects of the melanocyte phenotype are maintained through the initial steps of transdifferentiation (8). Remarkably, the changes in gene expression were transient, and array comparative genomic hybridization analysis revealed no significant genomic changes in DNA between melanocytes cultured on unconditioned ECM and melanocytes exposed to the metastatic melanoma microenvironment, at least after 4 days of exposure (8). These results illuminate the ability of the microenvironment to induce the transdifferentiation of melanocytes independent of genetic mutations. However, ongoing studies will determine whether a longer exposure period results in genomic changes.

Although the mechanism by which the metastatic ECM induced melanocyte transdifferentiation was not fully elucidated in this study, our array comparative genomic hybridization analyses strongly suggest that melanocytes may be regulated epigenetically. Unlike genetic changes, epigenetic modifications are reversible and are mediated by such processes as methylation/demethylation of DNA and acetylation/deacetylation of histones. These types of modifications occur during development and guide differentiation by stably activating and silencing gene expression (11). The melanocytes in our study may have been showing a comparable type of epigenetic regulation. Previous studies in our laboratory determined that in melanoma Ln-5 γ2 chain, matrix metalloproteinase (MMP)-2, and membrane type 1-MMP (MT1-MMP) are required for vasculogenic-like network formation, a measure of tumor cell plasticity (12). Furthermore, poorly aggressive melanoma cells seeded onto an ECM preconditioned by metastatic melanoma cells engage in vasculogenic mimicry, mediated by promigratory Ln-5 γ2 chain fragments. These fragments, generated in an aggressive...
tumor cell conditioned ECM by MMP-2 and MT1-MMP proteolysis (12), may similarly contribute to melanocyte transdifferentiation. In contrast to hypoxia, it is unlikely that exposure to the metastatic ECM induced a "true" melanocyte transformation: The melanocytes did not increase the proliferation marker Ki-67 and did not gain any detectable genomic alterations (8). It would however be interesting to examine whether the metastatic ECM could synergize with microenvironmental factors, such as hypoxia, to induce melanocyte transformation. Interestingly, hypoxia has been shown to promote vascular tube formation and to up-regulate the expression of vasculogenic mimicry genes (including Ln-5γ2, Tie-1, and EphA2; refs. 13, 14). As we have presented, cues deposited into the ECM by metastatic melanomas induce normal melanocytes to assume aggressive tumor-associated phenotypes. Hence, by promoting the deposition of metastatic matrices, low oxygen levels in skin may exacerbate the transdifferentiation and, possibly, transformation of normal melanocytes in the earliest stages of melanocytic neoplasia.

Because melanocytes can undergo transdifferentiation in response to microenvironmental cues deposited by aggressive melanoma cells, we questioned whether melanocytes could reciprocally reprogram melanoma cells toward a more "normal" melanocytic phenotype. Interestingly, exposure of aggressive melanoma cells to ECM conditioned by normal melanocytes (from neonatal foreskins) did not alter the plastic, dedifferentiated gene expression pattern associated with the aggressive cells (8, 15). This brings to light an important question: how can an aggressive cancer cell be reprogrammed to a more normal cell type?

Pierce and colleagues suggested that cancer is a problem of developmental biology; hence, an embryonic microenvironment capable of differentiating a stem cell lineage should be able to reprogram cancers derived from that lineage (16). In support of this concept, implantation of B16 murine melanoma cells into the embryonic skin of a developing mouse during the time of premelanocyte migration was shown to inhibit melanoma tumor formation. Furthermore, medium conditioned from skin explanted at the time of premelanocyte arrival irreversibly inhibited melanoma cell proliferation and promoted the acquisition of a neuronal morphology (16).

More recently, we used an embryonic chick model to specifically explore the possibility of reverting melanoma cells toward their neural crest–derived cells of origin (17). In this study, we implemented confocal imaging to track the behavior of green fluorescent protein–labeled metastatic melanoma cells following transplantation in ovo adjacent to host chick premigratory neural crest cells. The transplanted melanoma cells migrated into the surrounding host tissue in a programmed manner, suggesting that they were able to respond to host neural crest migratory cues (17). Furthermore, the melanoma cells displayed neural crest–like morphologies and, in a manner similar to neural crest cells, were able to populate structures, such as the brachial arches, sympathetic ganglia, and dorsal root (17). Interestingly, a subpopulation of melanoma cells that invaded the chick periphery was reprogrammed to express the melanocyte–associated protein Mart-1/Melan-A (17). These results confirm that melanoma cells can respond to developmental cues and suggest that factors unique to the neural crest embryonic milieu may be implemented to reprogram aggressive melanoma cells to a more benign melanocytic cell type.

As a corollary of these findings, we used a three-dimensional model to examine whether the microenvironment of human embryonic stem cells (hESC) could similarly reprogram the metastatic melanoma cell phenotype (15). Using this approach, we determined that, unlike melanocytes, hESC conditioned matrices can dramatically influence the phenotype of metastatic melanoma cells. Specifically, exposure of melanoma cells to hESC microenvironments resulted in the reexpression of the melanocyte–specific marker Mart-1/Melan-A as well as a reduction in invasive potential (15). In contrast to the study by Pierce and colleagues, the conditioned medium of hESCs did not alter the aggressive melanoma phenotype, suggesting that the hESC transdifferentiating factor(s) is preferentially deposited or stabilized in the matrix (15). Collectively, these findings suggest that embryonic microenvironments contain cues not contained by differentiated tissues, which may be harnessed to epigenetically reprogram metastatic melanoma cells.

### Targeting the Melanoma Microenvironment: A Novel Treatment Strategy

The microenvironment plays an active role in tumor progression and therefore presents a novel target for therapy (18, 19). As we have presented, cues deposited by metastatic melanomas remain in the ECM even after the cells have been removed. Hence, treatment regimes that specifically target aggressive tumor cells may fail to account for changes in the extracellular microenvironment that persist after removal or destruction of an aggressive tumor. By inducing the transdifferentiation or transformation of melanocytes, this microenvironmental signature could result in a recurrence or continuance of the tumor. Therefore, a successful anticancer regimen should target the tumor microenvironment as an adjunct to more classic therapies, such as radiation and surgery.

The deposition of Ln-5γ2 chain promigratory fragments into the ECM by aggressive melanoma cells can act as a latent trigger, inducing poorly aggressive melanoma cells (and perhaps melanocytes) to assume a more aggressive, transdifferentiated phenotype. As a caveat, the activities of MT1-MMP and MMP-2 are required for the generation of these promigratory fragments (12). Thus, MMP inhibition could be a viable therapeutic strategy for the treatment of melanoma in combination with other therapeutic modalities. To assess the use of MMP inhibition, we recently examined the consequences of adding a chemically modified tetracycline (CMT; COL-3) to metastatic melanoma cells in three-dimensional culture (20). We found that COL-3 effectively inhibited MMP-2 and MMP-9 activity and that it down-regulated MMP-2, MMP-9, and MT1-MMP gene expression. As expected, by inhibiting MMP activity, COL-3 prevented the proteolysis of Ln-5γ2 chain into promigratory fragments, thereby inhibiting vascular network formation and VE-Cadherin expression in highly aggressive melanoma cells. Perhaps most noteworthy, ECMs conditioned by aggressive melanoma cells treated with COL-3 were no longer able to epigenetically induce Ln-5γ2 chain expression or vasculogenic mimicry in poorly aggressive cells (20). It is very likely that COL-3 could similarly inhibit the transdifferentiation of normal melanocytes. Although clinical trials have reported some success of modified tetracyclines in the treatment of cancer, particularly metastatic cancer and HIV-related Kaposi's sarcoma (21, 22), several serious side effects have been noted (23, 24). Our studies suggest that the effective dose of CMT necessary to inhibit melanocyte transdifferentiation could be well below those used in these clinical trials. Hence, at these lower more tolerable doses,
tetracyclines (such as COL-3) may be a potential adjunct therapy designed to prevent melanoma recurrence.

Our work indicates that, in addition to preventing the trans-differentiation of melanocytes, tumor microenvironments may also be modified to promote the reversion of melanoma cells toward a more melanocytic phenotype. This strategy of differentiation, in addition to tumor ablation, could lead to less toxic treatments for melanoma. Our novel developmental models suggest that it is possible to reprogram aggressive melanoma cells and that the secret to this phenomenon lies in factors that are distinctive to embryonic environments. For example, exposure of aggressive melanoma cells to the ECM of hESCs or the microenvironment of the developing neural crest results in the reexpression of Mart-1/Melan-A (15, 17). The presence of this pigment-associated protein correlates with a favorable disease outcome in melanoma patients, perhaps by enabling T cells to better recognize and target tumor cells (25, 26).

Hence, by reversioning highly aggressive metastatic melanoma cells to a less aggressive melanocytic-like phenotype, exposure to micro-environments generated by embryonic stem cells may be a viable therapeutic strategy.

Due to its effect on cell fate, the microenvironment is a significant mediator of melanoma progression and metastasis. Indeed, factors, such as hypoxia and matrix deposition, play a profound role throughout melanoma progression, actively promoting the earliest steps of melanocyte transformation and later enhancing aggressive phenotypes, including invasion, vasogenic mimicry, and angiogenesis. Although the microenvironment often facilitates aggressive melanoma phenotypes, we have shown that certain microenvironments, particularly those associated with embryonic cells, can reprogram aggressive melanoma cells to a less aggressive phenotype. As such, manipulating the microenvironment may lead to novel treatment strategies designed to both prevent and reverse melanoma progression.

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