A proliferative melanoma cell phenotype is responsive to RAF/MEK inhibition independent of BRAF mutation status

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Summary
Oncogenic mutations within the MAPK pathway are frequent in melanoma, and targeting of MAPK signaling has yielded spectacular responses in a significant number of patients that last for several months before relapsing. We investigated the effects of two different inhibitors of MAPK signaling in proliferative and invasive melanoma cell cultures with various mutations in the MAPK pathway. Proliferative melanoma cells were more susceptible to pathway inhibition than invasive phenotype cells, irrespective of BRAF mutation status, while invasive phenotype cell response was dependent on BRAF mutation status. Critically, MAPK pathway inhibition of proliferative phenotype cells resulted in acquisition of invasive phenotype characteristics. These results show that melanoma cell phenotype is an important factor in MAPK pathway inhibition response. This suggests that while current therapeutic strategies target proliferative melanoma cells, future approaches should also account for the invasive phenotype population.

Introduction
Melanoma is a heterogeneous malignancy whose cells may present with any of a range of distinct molecular profiles. For example, the mitogen-activated protein kinase (MAPK) pathway is frequently activated in melanoma by RAS/RAF gene mutations (Curtin et al., 2005; Thomas et al., 2007; Davies et al., 2002; Smalley and Flaherty, 2009). This pathway responds to receptor tyrosine kinases including receptors for epidermal and vascular endothelial growth factors (Fecher et al., 2008) and regulates melanoma cell proliferation, survival, and migration. Activating mutations in MAPK pathway kinases account for increases in melanoma cell proliferation and resistance to apoptosis (Russo et al., 2009), and this renders melanoma a viable target for MAPK.

Significance
Recent trials with MAPK inhibitors have shown promising results in many patients with metastatic melanoma; however, nearly all responding patients experience disease relapse. We describe here how melanoma cells respond to MAPK inhibition in a phenotype-specific manner, suggesting that slow-cycling invasive phenotype cells provide a treatment-resistant pool from which disease relapse may be derived. The implication is that while MAPK inhibition may successfully treat proliferating cells, another cell population needs to be addressed at the same time.
pathway-specific drugs. Critically, a significant number of patients have been shown to respond, sometimes profoundly, to such treatment. However, nearly all responding patients relapse within 1 yr. The development of resistance can be explained in part by the occurrence of new mutations as reported for MEK (Dhomen and Marais, 2009; Eggermont et al., 2009; Smalley and Flaherty, 2009; Davies et al., 2002; Emery et al., 2009; Flaherty et al., 2010).

We recently proposed a phenotype switching model of melanoma progression. This model identifies two opposing types of melanoma cell, characterized by gene expression profiling and other in vitro analyses (Hoek et al., 2006), which are distinguished by one being proliferative and the other invasive in phenotype (Hoek et al., 2008). The model hypothesizes that melanoma cells switch between proliferative and invasive states and so drive repeated tumorigenic and disseminating phases of disease progression. We investigated the effects of treating melanoma cells with MAPK pathway inhibitors in the context of both the phenotype switching model and BRAF mutational status.

Results

Genotype inconsistencies between tissue and culture

We investigated gene mutations in melanoma material derived from 14 patients using both short-term cultures derived from primary lesions and metastases of melanoma as well as (in ten cases) corresponding paraffin-embedded tumor material. All mutations detected were restricted to MAPK pathway components including EGFR, H-RAS, N-RAS, K-RAS and BRAF. Interestingly, while six cell culture/biopsy pairs (60%) showed faithful reproduction of their gene mutation profiles, four culture/biopsy pairs (40%) did not (Table 1).

Gene expression signatures of melanoma proliferation, invasion, and Matrigel surface organization

By examining the expression of 105 specific genes (Table S1), we assessed the gene expression profile of melanoma cell cultures to predict phenotype according to methods our group established (Hoek et al., 2006) and identified six cultures, which correlated with the proliferative signature and eight, which correlated with the invasive signature (Figure 1). As we previously identified (Hoek et al., 2006), phenotype-specific expression signatures show no significant relationship with the mutation status of the genes we tested (Table 1). At the same time, we confirm that melanoma cells do show signature-specific characteristics (phenotypes) of proliferation, invasion as well as MITF and fibronectin expression (Figures 2A,B,C). However, for the first time we show that cultured melanoma cells display phenotype-specific organizational characteristics when seeded on a basement membrane-like matrix. Specifically, proliferative phenotype cells formed small and isolated clusters, while invasive phenotype cells formed connected networks or aggregates (Figure 3).

Phenotype-specific responses to MAPK pathway inhibition

In proliferative phenotype melanoma cells, MAPK pathway inhibition reduced proliferation by as much as 80% independently of BRAF mutation status. In contrast, invasive phenotype cells were significantly more resistant (Figure 4A,B). Furthermore, invasive phenotype cell susceptibility to RAF265 (and to a lesser degree AZD6244, data not shown) varied between BRAF mutant and wild-type cells, with BRAF mutants being more susceptible (Figure 4C). In contrast, we found that proliferative and invasive phenotype cells were equally susceptible to treatment with the receptor tyrosine kinase inhibitor TKI258 (Figure 4D).

Proliferative to invasive phenotype switching induced by MAPK inhibition

We found that treatment of proliferative phenotype cells with MAPK inhibitors induced, in addition to growth rate reduction, MITF down-regulation. Furthermore, MAPK inhibitor-treated proliferative phenotype cells (regardless of BRAF mutant status) adopted an invasive phenotype organization when seeded on basement membrane matrix (Figure 5A). Interestingly, when the inhibitor was removed from growth medium the cells soon reverted back to their proliferative phenotype behavior (Figure 5A). Additionally, MITF activity is critical for determining melanoma cell phenotype (Carreira et al., 2006) and Western blot experiments show that MITF-target melan-A expression was down-regulated by MAPK inhibitor treatment in a dose-dependent manner but this returned to normal levels after cells were removed from inhibitor (Figure 5B). However, while there was a trend toward increased in vitro invasiveness, in some samples this was not significant (data not shown).

Discussion

The observation that cell culture genotype is not always the same as that of the tissue suggests intraslesional heterogeneity. This mirrors a study, which found that within-nevi melanocytes are heterogeneous for the BRAF mutation (Lin et al., 2009). The implication is that the mutations are not always founder events but may also be driven by host-specific conditions and, as was recently published, therapy (Emery et al., 2009).

In previous publications, our laboratory introduced the hypothesis that melanoma progression is driven by cells switching between phenotypes of proliferation and invasion (Hoek et al., 2006, 2008). The earlier study found no correlation between BRAF and NRAS mutation status and the gene signatures, which were associated with...
Table 1. Cell culture origin and characterization

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age</th>
<th>Primary type</th>
<th>Culture</th>
<th>Culture origin</th>
<th>TNM stage at culture</th>
<th>Phenotype</th>
<th>Cell culture</th>
<th>Paraffin material</th>
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<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>29</td>
<td>SSM</td>
<td>M980513</td>
<td>Lymph node metastasis iliacal</td>
<td>T2aN3M1c</td>
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<td>V600E wt</td>
<td>HRAS (Q61K)</td>
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<td>2</td>
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<td>NM</td>
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<tr>
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<td>n.a.</td>
<td>MaMe065</td>
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<td>Invasive</td>
<td>V600E wt</td>
<td>n.a. n.a.</td>
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</table>

F, female; M, male; SSM, superficial spreading melanoma; NM, nodular melanoma; LMM, lentigo maligna melanoma; n.a., not applicable.
phenotype, and we confirm this lack of correlation here. Interestingly, in addition to confirming the association between gene expression and characteristics of proliferation and invasion, we uncovered a correlation between gene expression and the capacity to form cellular networks on a basement membrane-like matrix. This network formation by invasive phenotype cells is reminiscent of the behavior previously described for invasive melanoma cell lines (e.g. C8161) and interpreted by others as vascular mimicry behavior (Hendrix et al., 2002, 2003). In previous experiments, we injected phenotypes subcutaneously into the flanks of nude mice and made two critical observations: first, while proliferative phenotype cells initiate tumorigenesis relatively quickly, it takes weeks for invasive phenotype cells to do the same; and second, no matter which phenotype was used, the end-state tumors contained cells of both phenotype, indicating that switching had occurred (Hoek et al., 2008). Together our combined findings continue to support two distinct states, which melanoma cells may switch between to drive metastatic progression of the disease.

While BRAF/NRAS mutation status was phenotype independent, we were still interested in the response of these phenotypes to MAPK pathway inhibitors. The identification of distinct phenotypic differences among melanoma cells (Hoek et al., 2006) and the discovery of their capacity to switch between these phenotypes, as monitored by observing immunohistochemical staining pattern changes of MITF, which is tightly specific for the proliferative phenotype (Hoek et al., 2008; Eichhoff et al., 2010), suggested to us a possible explanation for relapse from initially successful MAPK pathway inhibition therapy.

That proliferative phenotype cells are significantly more susceptible to MAPK pathway inhibition is a striking finding which, in the light of the phenotype switching model, suggests that while proliferative phenotype cells within metastases are suitably susceptible to
inhibitor treatments (and probably reflect observed tumor mass reductions), invasive phenotype cells largely survive treatment to permit later relapse and continued disease progression. Supporting this, we note a recent study conducted by other researchers who used PLX4032 (specific for mutant BRAF) against a range of cell lines for which they had also obtained gene expression profiling data (Tap et al., 2010). Like us, they found that samples with a melanocytic gene signature were significantly more susceptible to inhibition, although they did not make the connection this has with melanoma cell phenotype switching. Interestingly, we found that inhibitor response in invasive phenotype samples was dependent upon whether BRAF was wild type or mutated. It has been shown that such a response difference is because of the higher affinity of RAF265 for mutant BRAF (Mordant et al., 2010), and this is also supported by the PLX4032 study (Tap et al., 2010). However, the BRAF mutation status dependency of invasive phenotype cell response contrasts with the absence of a similar dependency in proliferative phenotype samples. Why this may be so is not yet clear.

While no previous study investigating MAPK pathway inhibitors has acknowledged a melanoma cell phenotype switching context, there have been some interesting results. Haass et al. (2008) assessed tumor cell proliferation with AZD6244 in nine melanoma cell lines (five BRAF wild type and four BRAFV600E), finding that AZD6244 was more effective against BRAFV600E melanoma lines. Hoflich et al. (2009) investigated seventeen melanoma cell lines and their response to treatment with GDC-0879, a specific RAF inhibitor,
reporting that BRAFV600E mutation predicts higher sensitivity of melanoma cell lines to the RAF inhibitor. Sondergaard et al. (2010), using the specific RAF inhibitor PLX4032, described that while BRAF wild types were resistant to treatment there was differential sensitivity to the drug within the BRAFV600E subset of melanoma lines they used. These studies all showed variation in inhibitor response, even within BRAF mutant subsets. We speculate that the differentiation of the cultures these groups used into proliferative and invasive phenotypes may resolve at least some of the observed variation.

In contrast to the MAPK inhibitor treatments, TKI258 treatment was equally effective against the phenotypes. However, this may be explained by its capacity to target a wider variety of different receptors including those for fibroblast, vascular endothelial and platelet-derived growth factors. However, having a broader target molecule population for a drug is often correlated with increased drug-related side effects and while at least one clinical safety trial had been completed in melanoma patients, the results had not yet been made public at the time this manuscript was submitted (ClinicalTrials.gov Identifier NCT00303251).

The observation that proliferative cells responded to MAPK inhibition by down-regulating MITF activity and reducing proliferation agrees with other studies showing that suppression of both MITF and BRAF inhibits proliferation (Kido et al., 2009). However, we also found that MAPK inhibition of proliferative cells induced invasive phenotype-like organization when the cells were plated onto a basement membrane-like matrix. Additional gene expression analyses showed that treated proliferative phenotype cells expressed a gene signature, which strongly resembled that of invasive phenotype cells (data not shown). This leads us to speculate that switching from a proliferative phenotype to an invasive one may be facilitated by a reduction in MAPK signaling.

The differential susceptibility of melanoma cell phenotypes to MAPK inhibition and the apparent relationship between MAPK signaling and phenotype switching has potential relevance to clinical therapy. Interpretation of our findings in the context of the phenotype switching model for melanoma progression agrees that MAPK inhibitor-based treatments would initially drive disease stabilization and regression. However, we also suggest that invasive phenotype cells could be much less affected (and perhaps even increased in number). Surviving invasive phenotype cells, providing a pool from which cells carrying resistance mutations could arise, would be free to later switch back to the proliferative phenotype and precipitate relapse of disease, contributing to the limited response duration, which has been observed in several clinical trials.

**Methods**

**Cell culture and media**

Surplus material from cutaneous melanoma and melanoma metas-tases removed by surgery were obtained after written informed consent approved by the local IRB (EK647 and EK800). Clinical diagnosis was confirmed by histology and immunohistochemistry. Melanoma cells were released from tissue sections and grown as previously described (Geertsen et al., 1998). Patient origin was confirmed through genotyping of patient-derived paraffin-embedded tissues and peripheral blood mononuclear cells using 11 different gene loci (AmpFlSTR SGM Plus PCR Amplification Kit; Applied Biosystems, Foster City, CA, USA).

**Genotyping**

DNA was extracted from each cell culture and paraffin punch using a QIAamp DNA Mini kit (Qiagen, Valencia, CA, USA). OncoCarta™ Panel v1.0 workflow was used to investigate 238 known mutations in 19 oncogenes using a PCR and primer extension method followed by mass spectrometry readout (Sequenom, San Diego, CA, USA). The oncogenes were ABL1, AKT1, AKT2, BRAF, CDK, EGFR, ERBB2, FGFR1, FGFR3, FLT3, HRAS, JAK2, KIT, KRAS, MET, NRAS, PDGFRA, PIK3CA, and RET, the full list of mutations investigated is available for download from the Sequenom website (http://www.sequenom.com/OncoCarta).
Gene expression profiling
Melanoma cell culture RNA extraction and gene expression profiling, including normalization and analyses, were performed as previously described (Hoek et al., 2006). Phenotype-specific profile identification was performed by hierarchically clustering sample data using 105 genes previously established to show melanoma phenotype-specific expression patterns (Hoek et al., 2006). Cultures with profiles not clearly assignable to either proliferative or invasive signatures (i.e., their profiles were intermediate-type) were not selected.

In vitro invasion and proliferation assays
For invasion analyses, cells were seeded on 8-µm pore PET filters with a uniform layer of BD Matrigel® basement membrane matrix (BD Biosciences, Heidelberg, Germany). RPMI containing 10% FCS was used as chemotactant. After 22 h of incubation, the membrane was stained using a standard H&E protocol, and cells were counted under a light microscope. Invasion values were calculated by dividing the number of cells migrating through matrix-coated inserts by the number of cells migrating through uncoated inserts. For the proliferation assay, melanoma cells were seeded to a density of 2 × 10^4 cells in each well of a twenty-four-well plate. After 96 h, proliferation was estimated using a colorimetric (MTT) assay. To assess proliferation during treatment with MAPK pathway targeting inhibitors, cells were seeded in order to achieve confluency after 96 h (up to 4 × 10^6 cells) in triplicate. After 24 h, cells were treated with RPMI complete containing AZD6244 (Astra Zeneca, Mereside, UK), RAF265 (Novartis, Basel, Switzerland) or multitriggered receptor tyrosine kinase inhibitor TKI258 (Novartis) at various concentrations (1 nM–10 µM). Proliferation was measured using MTT assay after a further 72 h.

Matrigel surface organization
Empty 48-well plates were coated with Matrigel® and seeded with 4 × 10^4 melanoma cells in 400 µl of RPMI complete medium. Cell morphology and organization were assessed using phase contrast microscopy after incubation for 24 h.

Western blotting
Cells were washed twice with cold phosphate-buffered saline (PBS) and lysed at 4°C in lysis buffer containing 20 mM Tris–HCl (pH 7.5), 1% Triton X-100 (Sigma-Aldrich, St Louis, MO, USA), 137 mM NaCl, 10% glycerol and protease inhibitors (Roche, Basel, Switzerland). Proteins were separated by SDS–PAGE under reducing conditions and then transferred onto nitrocellulose membranes (Innolugen, Basel, Switzerland). Membranes were probed with a mouse anti-melan-A monoclonal antibody (Abcam, Cambridge, UK) followed by horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody (Bio-Rad, Reinach, Switzerland). Bound antibodies were detected by chemiluminescence (ECL; GE Healthcare, Buckinghamshire, UK).

Statistical analyses
For all quantitative sample comparisons, Prism (GraphPad Software, La Jolla, CA) was used to perform two-way ANOVA.

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References
Simultaneous suppression of MITF and BRAF V600E enhanced inhibition of melanoma cell proliferation. Cancer Sci. 100, 1863–1869.


Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. A specific group of 105 genes for which expression is significantly associated with melanoma cell phenotype.

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