Paracrine Effect of NRG1 and HGF Drives Resistance to MEK Inhibitors in Metastatic Uveal Melanoma

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Abstract

Uveal melanoma patients with metastatic disease usually die within one year, emphasizing an urgent need to develop new treatment strategies for this cancer. MEK inhibitors improve survival in cutaneous melanoma patients but show only modest efficacy in metastatic uveal melanoma patients. In this study, we screened for growth factors that elicited resistance in newly characterized metastatic uveal melanoma cell lines to clinical-grade MEK inhibitors, trametinib and selumetinib. We show that neuregulin 1 (NRG1) and hepatocyte growth factor (HGF) provide resistance to MEK inhibition. Mechanistically, trametinib enhances the responsiveness to NRG1 and sustained HGF-mediated activation of AKT. Individually targeting ERBB3 and cMET, the receptors for NRG1 and HGF, respectively, overcome resistance to trametinib provided by these growth factors and by conditioned medium from fibroblasts that produce NRG1 and HGF. Inhibition of AKT also effectively reverses the protective effect of NRG1 and HGF in trametinib-treated cells. Uveal melanoma xenografts growing in the liver in vivo and a subset of liver metastases of uveal melanoma patients express activated forms of ERBB2 (the coreceptor for ERBB3) and cMET. Together, these results provide preclinical evidence for the use of MEK inhibitors in combination with clinical-grade anti-ERBB3 or anti-cMET monoclonal antibodies in metastatic uveal melanoma.

Introduction

Uveal melanoma originates from the melanocytes within the iris, choroid, and ciliary body (1). Each year, approximately 2,500 new patients will be diagnosed with this disease in the United States. Half of these patients will develop metastases, typically in the liver, within 15 years of initial diagnosis with a peak of metastasis between 2 and 5 years. Although there are effective therapeutic strategies to prevent local recurrence and to eradicate primary uveal melanoma, patients with metastatic disease are found to be refractory to current chemotherapies and immune checkpoint blockers and usually die within a year (2).

Recent advances have identified genetic alterations in uveal melanoma. In contrast to its cutaneous counterpart, oncogenic BRAF mutations are infrequent in uveal melanoma (3–6). Activating mutations in two \( \alpha \) subunits of the heterotrimeric G proteins, GNAQ and GNA11, are found in 80% of uveal melanomas in mutually exclusive manner and are believed to occur at an early stage of disease (7–11). The GNAQ and GNA11 mutations are typically in Q209 but less frequently in R183. Other studies have also identified recurrent mutations in SF3B1 (12–14), a RNA splicing factor, and EIF1AX (12) in primary uveal melanomas in mutually exclusive manner and associate with low metastatic potential. Inactivating mutations in the tumor-suppressor BRCA1-associated protein 1 (BAP1) on chromosome 3 are found in 32% to 50% of primary uveal melanoma and associate with a more aggressive/higher likelihood of metastasis (15–17).

Oncogenic mutations in GNAQ and GNA11 abrogate their intrinsic GTPase activities, resulting in activation of the RAF/MEK/ERK1/2 and protein kinase C (PKC) signaling, JNK, and p38 via regulation of the small GTPases of RhoA and Rac1 (18). These signaling pathways promote tumor proliferation and growth. Knockdown of GNAQ in mutant, but not wild-type, uveal melanoma cell lines diminishes ERK1/2 activation and induces cell-cycle arrest (8, 19) and AMP-activated protein kinase–dependent autophagic cell death (20). Although these findings emphasize the potential of targeted therapy in uveal melanoma, directly targeting mutant GNAQ and GNA11 has proved to be structurally challenging.

Targeting MEK with small-molecule inhibitors, such as trametinib (GSK1120212) and selumetinib (AZD6244), has been pursued in clinical trials for melanoma. Trametinib monotherapy has achieved 25% to 40% partial/complete response rates in BRAF V600E/K cutaneous melanoma patients (21). In contrast, while trametinib is recently FDA-approved for cutaneous melanoma, it is largely ineffective in uveal tumors. In a phase I trial containing...
16 uveal melanoma patients, 8 patients had stable disease but no partial or complete responses were observed (21). In a phase II trial, selumetinib improved progression-free survival compared with standard chemotherapy (15.9 vs. 7.0 weeks; ref. 22). Although overall survival was improved with selumetinib, the improvement did not reach statistical significance, possibly due to the crossover study design. Thus, targeting MEK alone in uveal melanoma patients has limited clinical benefit. The crossover study design. Thus, targeting MEK alone in uveal melanoma patients has limited clinical benefit. The crossover study design. Thus, targeting MEK alone in uveal melanoma patients has limited clinical benefit. The crossover study design. Thus, targeting MEK alone in uveal melanoma patients has limited clinical benefit. The crossover study design. Thus, targeting MEK alone in uveal melanoma patients has limited clinical benefit. The crossover study design. Thus, targeting MEK alone in uveal melanoma patients has limited clinical benefit. The crossover study design. Thus, targeting MEK alone in uveal melanoma patients has limited clinical benefit.

In this study, we show that two growth factors, neuregulin 1 (NRG1) and hepatocyte growth factor (HGF), mediate resistance to the MEK inhibitors trametinib (23) and selumetinib (24) in metastatic human uveal melanoma cells. Mechanistically, MEK inhibition enhances responsiveness to NRG1 and promotes sustained HGF-induced phosphorylation of cMET. Targeting NRG1–ERBB3 or HGF–cMET signaling overcomes the respective growth factor–mediated resistance. In addition, fibroblast-derived factors act in a paracrine manner to induce resistance to trametinib through activating NRG1–ERBB3 and HGF–cMET signaling in uveal melanoma cells. Finally, ERBB2 (the co-receptor for ERBB3) and cMET were activated in an orthotopic metastatic uveal melanoma mouse model and in a subset of liver metastases of uveal melanoma. Together, these data suggest that cotargeting MEK with ERBB3 and/or cMET may enhance the efficacy of MEK inhibitor in advanced-stage uveal melanoma patients.

Materials and Methods

Metastatic uveal melanoma cell lines and cell culture

UM001, UM003, and UM004 were derived from liver, retroperitoneal and orbital metastases of human uveal melanoma, respectively. The mutational status of UM001 and UM003 cells was described previously (25). Mutational analysis of GNAQ was performed by Sanger sequencing. UM001 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 10% nonessential amino acids, 2.5 mmol/L L-glutamine, and 50 mg/mL streptomycin. UM003 and UM004 cells were maintained in MEM medium containing 15% (UM003 cells) or 10% (UM004 cells) heat-inactivated FBS and penicillin–streptomycin. Human telomerase reverse transcriptase (hTERT)–immortalized foreskin fibroblastic BJ1 cells were provided by Dr. Ubaldo Martinez-Outschoorn (Thomas Jefferson University, Philadelphia, PA). The human embryonic lung fibroblastic WI-38 cell line was provided from the ATCC. HT-BJ1 and WI-38 cell lines were maintained in DMEM medium supplemented with 10% heat-inactivated FBS and penicillin–streptomycin. To collect conditioned medium from HT-BJ1 and WI-38, cells were cultured in UM001 or UM003/4 medium for 3 days.

Inhibitors, growth factors, and function-blocking antibodies

Trametinib (GSK1120212), selumetinib (AZD6244), laptinib, crizotinib, and MK2206 were purchased from Selleck Chemicals. Recombinant human NRG1, PDGF-BB, and insulin-like growth factor 1 (IGF1) were purchased from Cell Signaling Technology; recombinant human epidermal growth factor (EGF) was purchased from Lonza Walkersville Inc.; recombinant human HGF was provided by Michael P. Lisanti (University of Manchester, Manchester, United Kingdom). Humanized ERBB3 monoclonal antibody U3-1287 was provided by U3 Pharma Gmbh.

Short-interfering RNA and transfection

UM001 and UM004 cells (3 × 104) were seeded in 6-well plates. The next day, cells were transfected for 4 to 6 hours with chemically synthesized short-interfering RNAs (siRNA) at a final concentration of 25 nmol/L using Lipofectamine RNAiMAX (Invitrogen) as previously described (26). cMET siRNAs (#1: GAAGAAUUCGCGAAGUAUA; #2: CGCGAUCUGGACCAAUUG; #2: CAAGGAUUCUGGACCAAUUG) were purchased from Dharmacon Inc. The nontargeting siRNA (UAGCGACUAAAACACAUU) was used as a control.

MTS assay

UM001, UM003, and UM004 cells (3–6 × 104 cells/well) were plated overnight into 96-well plates and treated with trametinib (0.003–0.4 μmol/L) or vehicle (DMSO) for 72 hours. Each assay was performed in triplicate. Cell growth inhibition was assayed by the MTS (Promega Corporation) according to the manufacturer’s instructions (error bars reflect ±SEM of three independent experiments). IC50 and IC25 values were calculated using GraphPad Prism.

Western blotting

Cells were washed in cold PBS and lysed directly in Laemmli sample buffer. Lysates were resolved by SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 1% BSA and incubated with indicated primary antibodies overnight at 4°C. Western blotting assays were detected using the horseradish peroxidase–conjugated secondary antibodies followed by development using chemiluminescence substrate (Pierce). Primary antibodies used were: ERK2 (D-2) from Santa Cruz Biotechnology Inc.; ERBB3 (B2E), phospho-ERBB3 Y1197 (C56E4), phospho-ERBB3 Y1189 (21D3), ERBB2 (DBF12), phospho-ERBB2 Y1196 (D66B7), MET, phospho-MET Y1234/1235 (D26), PDGFR, phospho-PDGFRβ Y751 (C63G6), EGFR, phospho-EGFR Y845, IGF1R, phospho-IGF1R Y1131, AKT, phospho-AKT T308 (C31E5E), phospho-AKT S473 (D9E), phospho-ERK1/2 (D13.14.4E), and phospho-TC2 T1462 from Cell Signaling Technology; and actin from Sigma-Aldrich. Chemiluminescence was visualized on a Versadoc Multilimeter and quantitated using Quantity-One software (Bio-Rad).

Flow cytometry

Cells were trypsinized, washed with cold PBS, and resuspended in 0.5 mL of cold PBS to achieve single-cell suspension. Cells were then fixed in 4.5 mL of 70% ethanol for 2 hours, followed by centrifugation at 800 g for 5 minutes. Cell pellets were washed with cold PBS and resuspended in 1 mL of 0.1% (v/v) Triton X-100 staining solution containing 100 ng/mL of RNase and 40 μg/mL of propidium iodide (PI) for 30 minutes at room temperature. Staining was then analyzed by flow cytometry on a BD FACS Calibur flow cytometer (BD Biosciences). Data were analyzed by the FlowJo software (TreeStar, Inc.). To determine cell surface expression of ERBB3 and cMET, uveal melanoma cells were incubated in PBS with 2% BSA and 50 μL of phycoerythrin (PE)-conjugated anti-ERBB3 antibody.
(R&D Systems), PE-conjugated anti-cMET antibody, or isotype control IgG antibody on ice for 45 minutes. Washed cells were analyzed by flow cytometry and data were analyzed by the FlowJo software.

Cell viability assays
Cells were plated at a confluence of $3 \times 10^5$ per well in 6-well plates. The next day, growth factors, drugs, or function-blocking antibodies were added as indicated. Cells were cultured for additional 3 days (UM001 cells) or 5 days (UM003 cells, for which medium and additives were replenished once), at which time AlamarBlue (Invitrogen) was added to each well and allowed to reduce for approximately 1 hour. Medium (120 μL) was collected in triplicate from each condition and absorbance readings for oxidized and reduced AlamarBlue were taken at wavelengths 600 and 570 nm, respectively, in a Multiskan Spectrum spectrophotometer (Thermo Scientific). The change in viability was calculated from the resulting absorbance values using the manufacturer's guidelines. All conditions were normalized to the DMSO control.

Cell growth assays
Cells were plated as for viability assays and stained with crystal violet solution (1% crystal violet, 10% buffered formalin) for 30 minutes. After decanting the staining solution, wells were thoroughly washed in distilled water and air-dried. Plates were imaged by scanning while colonies were imaged on a Nikon Eclipse Ti inverted microscope (Nikon) with NIS-Elements AR 3.00 software (Nikon).

Immunohistochemistry
UM001 cells (1 × 10^6) were injected into the liver of NSG mice or hHGFki mice (STOCK Hgfl^+/+ (HGF)Arvo Prkd^−/−; The Jackson Laboratory) and allowed to colonize for 4 to 5 weeks (NSG mice) or 8 weeks (hHGFki mice). Tissue samples from UM001 xenografts were fixed in formalin overnight. Paraffin-embedded tissue sections were deparaffinized and antigen retrieval was accomplished using high pH conditional buffer. Sections were incubated with anti-phospho-ERBB2 Y1221/Y1222 (6B12) and anti-phospho-cMET Y1234/Y1235 (D26) antibodies (Cell Signaling Technology) overnight. The next day, sections were incubated for 30 minutes in ImmPRESS UNIVERSAL Reagent (Vector Laboratories), followed by incubating for 2 to 5 minutes in ImmPACT NOVA-RED (Vector Laboratories). Sections were counterstained with hematoxylin.

Expression of phospho-ERBB2 and phospho-cMET in the liver biopsy specimens from human uveal melanoma patients was carried out in a Ventana Ultra stainer (Ventana Medical Systems). In brief, heat-induced epitope retrieval was performed using cell conditioner I buffer. Sections were then incubated with prediluted primary antibodies, following by Alkaline Phosphatase Multimer (Ventana Medical Systems) incubation. The fast red chromogen was applied for bright fuchsia color development. The sections were counterstained with hematoxylin for microscopic evaluation. The histologic evaluation of individual tumor specimens was carried out with slides stained with hematoxylin and eosin (H&E). The intensity of staining and the percentage of positive cells were semi-quantitatively evaluated by a board-certified pathologist (P.A. McCue) without clinical information. Staining intensity was scaled as 0 (negative), 1 (weak to moderate positive), and 2 (strong positive).

Patient samples
Uveal melanoma liver metastatic biopsies were formalin-fixed and paraffin-embedded immediately following isolation. IHC was performed using anti-phospho-ERBB2 Y1221/Y1222 (6B12) and anti-phospho-cMET Y1234/Y1235 (D26). Staining was scored in a blinded manner, as above. Patient samples were collected under a protocol approved by the Institutional Review Board (IRB) at Thomas Jefferson University (IRB protocol number: Control # 11E.548). All patients gave informed consent.

Results

NRG1 and HGF rescue the growth-inhibitory effect of MEK inhibitors in uveal melanoma cells
We determined the effects of clinically relevant, small-molecule MEK inhibitors in three genetically characterized cell lines derived from uveal melanoma metastases: UM001 was established from liver metastasis and harbors a GNAQ Q209P mutation; UM003 was generated from retroperitoneal metastasis and harbors a GNAQ Q209L mutation (25), and UM004 was established from orbital metastasis and harbors a GNAQ Q209P mutation (Supplementary Fig. S1A). On the basis of initial dose response experiments (Supplementary Fig. S1B), UM001, UM003, and UM004 cells were treated with 100 nmol/L of the MEK inhibitor, trametinib, in subsequent experiments. Trametinib treatment rapidly and persistently blocked ERK1/2 phosphorylation in all cell lines (Fig. 1A). To determine whether MEK inhibition led to growth arrest or a cytotoxic effect, we treated cells with trametinib for 3 days (UM001 and UM004 cells) or 5 days (UM003 cells) and analyzed the cell-cycle profile by PI staining (Fig. 1B). In all three cell lines, trametinib caused a strong accumulation of the sub-G1 population (trametinib vs. DMSO: 65% vs. 3% in UM001 cells; 58% vs. 5% in UM003 cells; 23% vs. 0.5% in UM004 cells) and a significant reduction of S-phase population (trametinib vs. DMSO: 1.3% vs. 7% in UM001 cells; 3.5% vs. 1% in UM003 cells; 7.5% vs. 2% in UM004 cells). These results indicate that MEK inhibition elicits cytotoxicity and growth arrest in monocultures of metastatic uveal melanoma cells.

Because drug resistance to targeted therapies may be mediated by the tumor microenvironment, we sought to identify growth factors that are able to protect metastatic uveal melanoma cells from MEK inhibition. We screened five growth factors: EGF, platelet-derived growth factor (PDGF); HGF; NRG1, and IGF1 for their capacity to rescue uveal melanoma cells from MEK inhibition. These growth factors did not enhance proliferation and viability of UM001, UM003, and UM004 cells compared with vehicle-treated cells (Fig. 1C and D, left; Supplementary Fig. S2A, left; Supplementary Fig. S2B). Treatment of uveal melanoma cells with trametinib dramatically decreased proliferation and viability, an effect that was blocked by NRG1 and HGF but not EGF and PDGF (Fig. 1C and D, right; Supplementary Fig. S2A, right; Supplementary Fig. S2B). IGF1 enhanced proliferation and viability of trametinib-treated UM003 cells, albeit to a lesser extent than NRG1 and HGF (Fig. 1D, right; Supplementary Fig. S2B, right). Next, we tested a second MEK inhibitor, selumetinib/AZD6244, on uveal melanoma cells. Selumetinib effectively blocked ERK1/2 phosphorylation in these cells (Supplementary Fig. S3A). NRG1 and HGF also rescued uveal melanoma cell survival from...
NRG1 and HGF rescue growth abrogation induced by MEK inhibitors in uveal melanoma cells. A, UM001, UM003, and UM004 cells were treated with 100 nmol/L of trametinib (GSK1120212) for the indicated times. Cell lysates were probed with phospho ERK1/2, total ERK2, and actin antibodies. B, UM001, UM003, and UM004 cells were treated with DMSO or trametinib for 3 days (UM001 and UM004 cells) or 5 days (UM003). Cells were then fixed, permeabilized, and subjected to PI staining. Cell-cycle analysis was performed with the FlowJo software. ***; *P < 0.01; **P < 0.001, based on the two-tailed Student t test assuming unequal variance. C, UM001 cells were treated with vehicle control, 10 ng/mL of EGF, PDGF-B, HGF, NRG1, and IGF1 alone or together with 100 nmol/L trametinib. After 72 hours, cells were subjected to crystal violet staining. Representative microscopic images of the cells at ×200 magnification are shown. Scale bar, 50 μm. D, UM003 cells were treated as in C for a total of 5 days. Drugs and growth factors were replenished on day 3. Cells were stained with crystal violet.
Selumetinib treatment (Supplementary Fig. S3B and S3C). These data demonstrate that NRG1 and HGF partially restore growth and viability of metastatic uveal melanoma cells treated with MEK inhibitors.

MEK inhibition enhances responsiveness to NRG1 through ERBB3 and ERBB2 in uveal melanoma cells

On the basis of the upregulation of NRG1–ERBB3 signaling in BRAF V600E cutaneous melanoma cells following RAF inhibitor treatment (27), we tested the impact of trametinib on NRG1-induced ERBB3 signaling in uveal melanoma cells. Treatment with trametinib sensitized UM001 and UM003 cells to NRG1-stimulated ERBB3 phosphorylation at Y1197 and Y1289 in dose and time course experiments (Fig. 2A–C). Interestingly, UM003, but not UM001 and UM004 cells, showed enhanced expression of ERBB3 in response to trametinib (Supplementary Fig. S4A). Phosphorylated Y1197 and Y1298 in ERBB3 are within YXXM-motifs, which dock phosphoinositide 3-kinase (PI3K), leading to AKT phosphorylation (28). In line with enhanced phospho-ERBB3 levels, AKT phosphorylation at S473 and T308 was elevated following NRG1 stimulation in trametinib-treated UM001 and UM003 cells (Fig. 2A–C). ERBB3 exhibits low intrinsic kinase activity and uses a coreceptor, typically another ERBB family member, to signal. UM001, UM003, and UM004 cells express ERBB2 but undetectable levels of EGFR and ERBB4 in either basal or trametinib-treated conditions (Supplementary Fig. S4B).

Targeting NRG1 signaling overcomes resistance to MEK inhibitors in metastatic uveal melanoma cells

To determine whether blocking ERBB3 prevents NRG1-mediated resistance to trametinib, we took two distinct strategies. First, we used U3-1287, a humanized ERBB3 monoclonal antibody that is being used in clinical setting (29). U3-1287 effectively blocked NRG1-stimulated phosphorylation of ERBB3 and downstream AKT activation in UM001 cells (Fig. 3A). Second, we examined the effect of U3-1287 on NRG1-induced resistance to trametinib. As above, cell proliferation (Fig. 3B) and viability (Fig. 3C) of trametinib-treated UM001 cells were partially restored in the presence of NRG1. U3-1287 alone did not suppress the cell growth and viability of UM001 cells but did dramatically impair the ability of NRG1 to restore growth and viability of UM001 cells treated with trametinib (Supplementary Fig. S3B and S3C). These data demonstrate that NRG1 and HGF partially restore growth and viability of metastatic uveal melanoma cells treated with MEK inhibitors.

NRG1 stimulated ERBB2 phosphorylation in trametinib-treated UM001 and UM003 cells (Fig. 2A–C). Furthermore, silencing ERBB2 effectively inhibited NRG1-stimulated ERBB3 and AKT phosphorylation in UM001 cells (Fig. 2D). These data indicate that NRG1–ERBB3/ERBB2 signaling to AKT is elevated in MEK-inhibited, metastatic uveal melanoma cells.

Resistance to Targeted Therapies

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in trametinib-treated UM001 (Fig. 3D and E) and UM003 cells (Fig. 3F). Together, these data demonstrate that targeting NRG1 signaling with ERBB3 antibodies and EGFR/ERBB2 inhibitors overcomes NRG1-mediated resistance to trametinib in metastatic uveal melanoma cells.

HGF induces sustained activation of AKT in trametinib-treated uveal melanoma cells

Our data show that HGF also effectively protects against MEK inhibitor effects on uveal melanoma cell growth. To investigate these effects further, UM001 cells were pretreated with trametinib...
overnight following by stimulation with increasing doses of HGF. In contrast to the effect of NRG1, HGF promoted the initial phosphorylation of cMET and downstream activation of AKT equivalently in trametinib-treated versus vehicle-treated UM001 and UM003 cells (Fig. 4A and B). However, HGF-induced phosphorylation of cMET and AKT was maintained at a higher level in trametinib-treated cells at later time points (Fig. 4C). These effects were associated with upregulated cell surface expression of cMET following MEK inhibition in both cell lines (Supplementary Fig. S5A). In contrast, IGF1 induced a transient activation of IGF1R and AKT, irrespective of trametinib treatment, with phosphorylation returning to basal levels at later (>8 hours) time points (Supplementary Fig. S5B). Our data suggest that the sustained activation of AKT by HGF may compensate for the loss of ERK1/2 activation in trametinib-treated cells and contributes to resistance to trametinib in uveal melanoma cells.

cMET inhibition overcomes HGF-mediated resistance to trametinib in uveal melanoma cells

To test whether targeting cMET could abrogate HGF-mediated resistance to trametinib in uveal melanoma cells, we first used crizotinib, a cMET/anaplastic lymphoma kinase (ALK) inhibitor (30, 31). In UM001 and UM003 cells, crizotinib blocked HGF-induced cMET phosphorylation in a dose-dependent manner (Fig. 5A). Uveal melanoma cells were treated with trametinib alone, in combination with HGF and/or crizotinib. At the two doses tested, crizotinib alone did not affect viability (Fig. 5B) or proliferation (Supplementary Fig. S6A) of UM001 and UM003 cells. As above, HGF partially restored the viability and cell proliferation of trametinib-treated cells; an effect that was reversed by crizotinib (Fig. 5B and C). As a second approach, we tested the effect of silencing cMET expression on sensitivity to trametinib in uveal melanoma cells. cMET knockdown alone neither altered ERBB3 and ERBB2 levels nor affected cell growth in UM001 and UM004 cells (Supplementary Fig. S6B). Whereas control cells were protected from trametinib-induced inhibition of colony growth by HGF, cMET knockdown cells were sensitive to trametinib despite the presence of HGF (Supplementary Fig. S6C). Thus, cMET is required for HGF-mediated resistance to trametinib.

AKT inhibition reverses NRG1- and HGF-mediated resistance to trametinib in uveal melanoma cells

Both NRG1 and HGF promote AKT signaling in trametinib-treated cells. To test whether NRG1- and HGF-driven resistance to MEK inhibitors is mediated by AKT, we used the inhibitor MK2206. Addition of MK2206 completely abrogated the protective effect of NRG1 and HGF in trametinib-treated UM001 (Fig. 5D) and UM003 cells (Supplementary Fig. S7). Notably, MK2206 alone slightly inhibited growth of UM001 cells, while it did not affect the growth of UM003 cells. MK2206 effectively blocked NRG1- and HGF-initiated signaling, leading to AKT phosphorylation and downstream AKT targets in trametinib-treated UM001 cells (Fig. 5E). Thus, AKT contributes, at least in part, to the NRG1- and HGF-mediated protection from MEK inhibitors.

Fibroblast-derived growth factors elicit uveal melanoma cell resistance to trametinib

Stromal fibroblasts in the tumor microenvironment may promote tumor growth and regulate drug response in a variety of cancer types (32, 33). To determine whether trametinib resistance...
may be mediated through a paracrine effect from fibroblasts, we examined the activation of ERBB3 and cMET in uveal melanoma cells by conditioned medium from two fibroblast cell lines: hTERT immortalized BJ1 (HT-BJ1) and Wi38, which produce NRG1 and HGF, respectively (34). Vehicle- or trametinib-treated UM001 and UM003 cells were cultured in conditioned medium for 1 hour. Conditioned medium from HT-BJ1 cells induced phosphorylation of ERBB3 and AKT, whereas conditioned medium from Wi38 cells induced phosphorylation of cMET, AKT, and ERK1/2 (Fig. 6A). UM001 cells cultured with HT-BJ1 conditioned medium were partially protected from MEK inhibition as assessed by growth (Fig. 6B, left) and viability (Fig. 6B, right). Addition of lapatinib restored the sensitivity to trametinib in these cells (Fig. 6B), consistent with the involvement of ERBB3–ERBB2 signaling. In vivo, we first examined uveal melanoma xenografts. UM001 cells were injected directly into the liver of NOD/SCID gamma (NSG, NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice and were treated with crizotinib for 4 hours, followed by 10 ng/mL of HGF stimulation for 15 minutes. Phosphorylation of cMET was evaluated by Western blotting of cell lysates with p-cMET antibody. Actin was used for loading. B, exponentially growing UM001 and UM003 cells were treated with DMSO or 100 nmol/L trametinib, in combination with 10 ng/mL of HGF and/or crizotinib for 3 days (UM001) and 5 days (UM003). Cell viability was determined by AlamarBlue staining. **, P < 0.01, based on the two-tailed Student t test assuming unequal variance. C, exponentially growing UM001 and UM003 cells were treated with 100 nmol/L trametinib, in combination with HGF and/or crizotinib for 3 days (UM001) and 5 days (UM003). Culture medium was changed and new drug and growth factors were added on day 3. Cells were washed and stained with crystal violet. Magnification, ×200. D, UM001 and UM003 cells were treated with DMSO or 100 nmol/L of trametinib, in combination with 10 ng/mL of NRG1 (left) or 10 ng/mL of HGF (right) for a total of 3 days. In some conditions, 2 μg/mL of MK2206 was also added. Cell growth was determined by crystal violet staining. Images were taken at ×200 magnification. E, UM001 cells were pretreated with vehicle, 100 nmol/L of trametinib, or 2 μmol/L of MK2206 overnight. Cells were then stimulated with 10 ng/mL NRG1 (left) or HGF (right) for 1 hour, as indicated. Activation of AKT, ERK1/2, and TSC2 was analyzed by Western blotting.
allowed to grow for 4 to 5 weeks. Inoculated UM001 cells grew and developed intrahepatic metastases with a high (18 of 19) success rate. UM001 xenografts growing in NSG mice stained positive for phospho ERBB2 (Fig. 7A, left). Because of the incompatibility of HGF across species (35), we implanted UM001 cells into hHGFki mice, in which the cDNA of human HGF was knocked into the mouse HGF locus by homologous recombination for analysis of phospho cMET. UM001 xenografts growing in hHGFki mice stained positive for phospho cMET (Fig. 7A, right).

We next extended our study to analyze liver metastases of uveal melanoma patients (Supplementary Table S1). Biopsies from 7 uveal melanoma patients with liver metastasis were stained with anti-phospho ERBB2 and anti-phospho cMET antibodies. Staining intensity was scored 0 (negative staining), 1 (weak to medium positive staining), and 2 (strong positive). Percentage of tumor cells was semi-quantitated. We observed positive staining of phospho ERBB2 in all seven samples and positive staining of phospho cMET in five out of seven samples (Fig. 7B). Representative images with various staining intensity were shown in Fig. 7C. These data suggest that cMET and ERBB2 are activated in uveal melanoma in the liver metastatic microenvironment.

Figure 6.
Paracrine effects of NRG1 and HGF from fibroblasts drive resistance to trametinib in uveal melanoma cells. A, UM001 and UM003 cells were cultured for 1 hour in unconditioned growth medium or fibroblast conditioned medium (CM) collected from either HT-BJ1 cells or Wi38 cells. Cells treated with 10 ng/mL of NRG1 and HGF were used as control. Activation of ERBB3, cMET, AKT, and ERK1/2 was analyzed by Western blotting. B, UM001 cells were cultured in conditioned medium collected from HT-BJ1 cells (CM) or unconditioned medium (non-CM). Cells were treated with 100 nmol/L of trametinib ± 1 μmol/L of lapatinib, as indicated. After 72 hours, cells were stained with crystal violet. Representative microscopic images are shown with a ×200 magnification. Scale bar, 50 μm (left). Cell viability was also assessed by AlamarBlue staining after 72 hours (right). **, P < 0.01; ***, P < 0.001, based on the two-tailed Student t test assuming unequal variance. C, UM001 cells were cultured in conditioned medium collected from Wi38 cells or unconditioned medium, as a control. Cells were treated with 100 nmol/L of trametinib and 0.5 μmol/L of crizotinib as indicated. After 72 hours, cells were stained with crystal violet. Representative microscopic images are shown with a ×200 magnification. Scale bar, 50 μm (left). AlamarBlue staining was also performed to determine viability (right). **, P < 0.01, based on the two-tailed Student t test assuming unequal variance.

Discussion
The response of genetically defined tumors to a targeted therapy is typically heterogeneous. Many patients display no tumor shrinkage and are regarded as exhibiting primary/intrinsic resistance mediated by either preexisting (innate) or rapid adaptive response mechanisms. Similar mechanisms may be present, albeit to a lesser extent, in tumors that effectively respond to targeted therapies and are likely to modulate the timing of acquired resistance. Here, we describe that both innate and adaptive mechanisms occur in mutant GNAQ metastatic uveal melanoma cells responding to clinical-grade MEK inhibitors.
Our studies use GNAQ-mutant human metastatic uveal melanoma cell lines. These represent an important resource to the field given the high percentage of uveal melanomas harboring GNAQ or GNA11 mutations, the noted lack of available cell lines for uveal melanoma (36), the concern that lines may be cutaneous melanoma (37), and the common use of lines derived from nonmetastatic lesions for drug response studies in the uveal melanoma literature. Using mutant GNAQ metastatic uveal melanoma lines, we show that clinical-grade MEK inhibitors (trametinib and selumetinib) block cell growth in vitro by either promoting cell death or inducing a proliferative arrest. This heterogeneous response is consistent with data from the Khalili and colleagues (19) who showed that trametinib promoted >10% increase in apoptosis in two out of six GNAQ/GNA11 uveal melanoma cell lines. We identify two growth factors, NRG1 and HGF, which are able to reverse the cytotoxic and growth-inhibitory effects of trametinib and selumetinib. However, the actions of NRG1 and HGF on their cognate receptors, ERBB3 and cMET, differ. NRG1 activation of ERBB3 is enhanced in MEK-inhibited cells in an adaptive manner. In contrast, initial HGF activation of cMET is comparable in untreated and MEK inhibitor–treated cells, although is more persistent in the MEK-inhibited cells. These data are similar to findings in cutaneous mutant BRAF melanoma cells, in which NRG1 adaptively upregulate ERBB3–AKT signaling in response to vemurafenib/PLX4720 (27) and in which HGF promotes resistance via adaptive and innate mechanisms depending on the cell line (34).

Both ERBB3 and cMET activate the PI3K–AKT pathway and the addition of a PI3K inhibitor to MEK treatment enhances apoptosis in mutant GNAQ cells in vitro (19). Furthermore, our data show that targeting AKT reverses growth factor–mediated resistance. It has long been recognized that the combination of ERK1/2 and PI3K pathway inhibitors will likely be beneficial in many tumor settings; however, the advancement of PI3K and AKT targeting agents is currently limited in the clinic by toxicity issues and poor target inhibition. An additional concern is that ERBB3 expression/
activity is frequently upregulated as a compensatory feedback mechanism to PI3K inhibitors (38, 39).

There is growing appreciation for the need for combinatorial targeted therapy studies. For example, recent preclinical studies show that combined inhibition of MEK and PKC improves efficacy compared with treatment with either single agent in GNAQ/11-mutant uveal melanoma (40, 41). We tested the effect of cotargeting the receptors, ERBB3 or cMET, in combination with MEK inhibitors. Targeting the ERBB3–ERBB2 complex with U3-1287/AMG88 or lapatinib effectively reversed the NRG1-mediated resistance to MEK inhibitors. U3-1287 is one example of a humanized ERBB3 antibody that has entered early-phase clinical trials (29, 42). Similarly, we targeted cMET either with crizotinib or by RNA interference. Crizotinib is an ATP-competitive inhibitor of cMET as well as ALK and is FDA-approved for non–small cell lung carcinoma patients harboring ALK gene fusions. Targeting cMET reversed HGF-mediated protection from MEK inhibitor–induced growth blockade. Others have used the cMET inhibitor, MK-8033, to inhibit growth in mutant GNAQ uveal melanoma cells (43). Overall, these data highlight that NRG1 and/or HGF-mediated resistance may underlie the modest response rate to MEK inhibitors in metastatic uveal melanoma. Furthermore, our findings suggest that targeting ERBB3 and/or cMET may enhance the effect of MEK inhibitor in advanced-stage, mutant GNAQ uveal melanoma patients.

Low levels of phosphorylated ERBB3 and cMET were detected in the absence of NRG1 and HGF, respectively, indicating that these ligands are poorly expressed by tumor cells. Uveal melanoma frequently metastasizes to the liver, a tissue in which both NRG1 and HGF are readily detected (44, 45), highlighting the possibility that these growth factors mediate resistance to MEK inhibitors via paracrine action. To this end, we tested the effect of stromal-produced growth factors on uveal melanoma cell resistance to MEK inhibitors. Wi38 and BJ1 fibroblasts produce high levels of NRG1 and HGF, respectively, and conditioned medium from these cells promoted AKT phosphorylation and growth in MEK-inhibited uveal melanoma cells in a manner dependent on the cognate receptor. These findings are similar to the notion that fibroblast-derived HGF protects against RAF inhibitors in cutaneous melanoma (34) and add to growing evidence for factors in the tumor microenvironment being able to modulate the response to targeted anticancer agents. Furthermore, our in vivo data from both uveal melanoma cell liver colonization model and liver metastatic patient samples show that the ERBB3–ERBB2 and cMET receptors are frequently phosphorylated.

In summary, we have identified that the growth factors, NRG1 and HGF, mediate resistance to MEK inhibitors in metastatic uveal melanoma cells. Targeting NRG1 or HGF signaling overcomes the resistance elicited by these growth factors. We have also provided evidence that paracrine effects of NRG1 and HGF from fibroblasts protect uveal melanoma cells from MEK inhibition. These data provide new insights into the mechanisms that regulate resistance to MEK inhibitors in metastatic uveal melanoma. Ongoing efforts are focused on using clinical-grade anti-ERBB3 and anti-cMET monoclonal antibodies in combination with MEK inhibitors in preclinical studies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: H. Cheng, T. Sato, A.E. Aplin
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Cheng, P.A. McCue, T. Sato, A.E. Aplin
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Other (supervised all experiment and data collection performed in Dr. Sato’s laboratory): T. Sato
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