Fibroblast-derived Neuregulin 1 Promotes Compensatory ErbB3 Receptor Signaling in Mutant BRAF Melanoma*

Claudia Capparelli†, Sheera Rosenbaum‡, Adam C. Berger§, and Andrew E. Aplin††

From the ††Department of Cancer Biology, Sidney Kimmel Cancer Center, and ‡§Department of Surgery, Division of General Surgery, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Background: Mutant BRAF melanomas respond to RAF inhibitors by up-regulating ErbB3 signaling.
Results: NRG1 derived from fibroblasts activates ErbB3/ErbB2 signaling in vemurafenib-treated mutant BRAF melanoma cells, and NRG1 signaling is inhibited by ErbB3-targeting antibodies.
Conclusion: Targeting ErbB3/ErbB2 enhances vemurafenib effects in mutant BRAF melanoma.
Significance: Our data provide the preclinical basis to improve targeted therapies for mutant BRAF melanoma.

Rapidly accelerated fibrosarcoma (RAF) inhibitors are first-line treatments for patients harboring V600E/K mutant BRAF melanoma. Although RAF inhibitors produce high response rates, the degree of tumor regression is heterogeneous. Compensatory/adaptive responses to targeted inhibitors are frequently initiated by the activation of growth factor receptor tyrosine kinases, including ErbB3, and factors from the tumor microenvironment may play an important role. We have shown previously that mutant v-raf murine sarcoma viral oncogene homolog B1 (BRAF) melanoma cells have enhanced activation of ErbB3 following RAF inhibition. However, the source of neuregulin 1 (NRG1), the ligand for ErbB3, is unknown. In this study, we demonstrate that NRG1 is highly expressed by dermal fibroblasts and cancer-associated fibroblasts (CAFs) isolated from mutant BRAF melanomas. Conditioned medium from fibroblasts and CAFs enhanced ErbB3 pathway activation and limited RAF inhibitor cytotoxicity in V600 mutant BRAF-harboring melanomas. Targeting the ErbB3/ErbB2 pathway partially reversed the protective effects of fibroblast/CAF-derived NRG1 on cell growth properties of RAF inhibitor-treated melanoma cells. These findings support the idea that NRG1, acting in a paracrine manner, promotes resistance to RAF inhibitors and emphasize that targeting the ErbB3/ErbB2 pathway will likely improve the efficacy of RAF inhibitors for mutant BRAF melanoma patients.

The RAF inhibitors vemurafenib and dabrafenib and the RAF/MEK inhibitor combination dabrafenib plus trametinib are first-line treatments for melanomas that harbor BRAF V600E/K mutations (1–3). These targeted therapies are limited because patients nearly always progress because of drug resistance (4). Durable response is frequently mediated by stable alternations intrinsic to the tumor cells that lead to ERK1/2 pathway reactivation (5–8). However, the initial response is likely determined by the degree of compensatory/adaptive activation of growth factor receptor tyrosine kinases (9–12). The tumor microenvironment plays a key role in these adaptive responses to targeted inhibitors. For example, stromal fibroblasts may be subverted to phenotypically distinct CAFs, which promote supportive conditions for cancer cell growth through the production of soluble growth factors and the extracellular matrix (13–16), induction of angiogenesis, and recruitment of inflammatory cells (14). The extent to which stromally derived factors are required for resistance to targeted inhibitors remains to be fully understood. Defining the molecular signals that control the cross-talk between malignant cells and the surrounding stroma may open avenues for new targeted therapy strategies to enhance the cytotoxic effects of RAF and MEK inhibitors.

Compensatory resistance mechanisms to mutant BRAF inhibition are rapid and reversible responses that counteract the actions of RAF inhibitors. We have demonstrated previously that up-regulation of ErbB3 signaling in V600E/D BRAF melanoma cells promotes survival of cells targeted with RAF inhibitors (10). Levels of phospho-ErbB3 were elevated following RAF inhibitor treatment of melanomas cells in culture, melanoma xenografts, and patient samples (10). Furthermore, application of an ErbB3-neutralizing antibody enhanced the efficacy of the RAF inhibitor PLX4720 in vivo (15). ErbB3 is a member of the EGF receptor family of receptor tyrosine kinases. Unlike the other members, ErbB3 shows low intrinsic kinase activity (17). Nevertheless, it still plays a role in the progression of several cancer types and is implicated in driving resistance to targeted therapies (10, 18–21). Following the binding of its ligand, NRG1, ErbB3 heterodimerizes with ErbB2 and ErbB4, activating downstream signaling pathways that support cell proliferation, survival, and invasion.

**This work was supported, in whole or in part, by National Institutes of Health Grant R01 CA160495 (to A. E. A.). The Thomas Jefferson University, Sidney Kimmel Cancer Center core facilities are funded by NCI/National Institutes of Health Support Grant P30CA6056. This work was also supported by grants from OutRun the Sun (to C. C.), an American-Italian Cancer Foundation postdoctoral research fellowship (to C. C.), and the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation (to A. E. A.). The authors declare that they have no conflicts of interest with the content of this article.

1 To whom correspondence should be addressed: Dept. of Cancer Biology, Thomas Jefferson University, 233 S. 10th St, Philadelphia, PA 19107. Tel.: 215-503-7296; Fax: 215-923-9248; E-mail: aea004@jefferson.edu.

2 The abbreviations used are: RAF, rapidly accelerated fibrosarcoma; BRAF, v-raf murine sarcoma viral oncogene homolog B1; HFF, human foreskin fibroblasts; HTERT BJ1, human foreskin fibroblast immortalized with human telomerase reverse transcriptase; CAF, cancer-associated fibroblast; DMSO, dimethyl sulfoxide.
erodimerizes with other ErbB family receptors, including ErbB2 and EGF receptor, to promote the activation of the AKT and ERK1/2 pathways (22). However, the cellular source of NRG1 remains unidentified.

In this study, we demonstrate that fibroblasts express high levels of NRG1 compared with mutant BRAF melanoma cells and that conditioned medium from fibroblasts and CAFs limits RAF inhibitor cytotoxicity. Additionally, ErbB3- and ErbB2-targeting antibodies partially reverse the protective effects of fibroblast- and CAF-derived medium. Together, these data suggest a functional role for fibroblast-derived NRG1 in promoting resistance to RAF inhibitors in mutant BRAF melanoma.
Experimental Procedures

**Growth Factors and Inhibitors**—Recombinant human NRG1, insulin, and vemurafenib (PLX4032) were purchased from Cell Signaling Technology (Danvers, MA), Sigma-Aldrich (St. Louis, MO) and Selleck Chemicals LLC (Houston, TX), respectively. Seribatum MMA/M121 was a gift from Merrimack Pharmaceuticals, and pertuzumab was obtained from the pharmacy at Thomas Jefferson University.

**Cell Culture**—WM115, WM239-A, and WM266-4 cells were cultured in MCDB153 with 2% FBS, 20% Leibovitz L-15 medium, and 5 μg/ml insulin. M238 cells were cultured in RPMI medium enriched with 10% FBS and 2 mM L-glutamine. A375, human foreskin fibroblasts (HFF), and human foreskin fibroblast immortalized with human telomerase reverse transcriptase (HTERT BJ1) cells were cultured in DMEM supplemented with 10% FBS. All media contained 1% penicillin/streptomycin. Cells were cultured at 37 °C and 5% CO2 in a humidified chamber.

**Isolation of CAFs**—Human melanoma cancer biopsies (TJUMEL25 and TJUMEL41) were obtained from Thomas Jefferson Hospital with patient consent. Following tumor excision, small pieces were digested with collagenase (Sigma) in complete medium at 37 °C for 2–4 h. For the TJUMEL41 sample, pieces derived from different sections of the tumor were digested to generate CAF41A and CAF41B. Samples were then centrifuged at 4000 rpm for 4 min, the pellet was washed with complete medium, and then a second centrifugation was performed. The subsequent pellet was resuspended, and cells were cultured in DMEM supplemented with 10% FBS containing 5 μg/ml insulin. CAFs were maintained in culture until passage 10. Cells were authenticated by morphology and by the expression level of α-smooth muscle actin and fibroblast activation protein.

**Genomic DNA Sequencing**—DNA was extracted from a portion of tumor samples and sequenced at the BRAF V600 loci.

**Hematoxylin and Eosin Staining**—The human melanoma sample TJUMEL25 was formalin-fixed, embedded in paraffin, and stained with hematoxylin and eosin.

**ELISA**—HFF, HTERT BJ1, and CAF25 cells were cultured in serum-free DMEM with or without 1 μM vemurafenib for 24 h. Medium was collected and spun down to remove floating cells. Collected medium samples were analyzed using the NRG1-β1 human ELISA kit (Abcam, Cambridge, MA) according to the instructions of the manufacturer. NRG1-β1 concentrations were calculated from standard curves completed at the time of each assay. Data are representative of three independent experiments.

**siRNA Transfections**—HFF and HTERT BJ1 cells were transfected with chemically synthesized siRNAs that target multiple different isoforms of NRG1 (Dharmacon Inc., Lafayette, CO) at a final concentration of 25 nM using Lipofectamine RNAiMAX (Invitrogen). The sequences used were as follows: control, UGG-UUAACAUUGCUAGUAA; NRG1 SMARTpool, ACAUCCAC-CACUGGGACAA, UUUGAAUUGGGCGGAGAA, GGGGA-GGGCUUCUAGUGGA, and UUUAAACCCUCUCGAGAU.

**Western Blotting**—Cells were washed twice in cold PBS and lysed with Laemmli sample buffer. For secreted NRG1 detection, medium was collected and centrifuged at 4000 rpm for 5 min to eliminate cellular debris and concentrated by centrifugation for 30 min at 4000 rpm using Amicon ultraconical tubes. Proteins were resolved by SDS-PAGE, and proteins were transferred to PVDF membranes. After blocking in 5% BSA, membranes were incubated with the indicated primary antibodies overnight at 4 °C, followed by incubation with peroxidase-coupled secondary antibodies. Immunoreactivity was detected using HRP-conjugated secondary antibodies (CalBioTech, Spring Valley, CA) and chemiluminescence substrate (ThermoScientific, Rockford, IL) on the Versadoc imaging system (Bio-Rad). The primary antibodies used were as follows: secreted NRG1 (catalog no. MAB377) from R&D Systems (Minneapolis, MN); NRG1 (catalog no. 2573), phospho-ErbB3 (Tyr-1197, catalog no. 4561), ErbB3 (catalog no. 4754), phospho-ErbB2 (Tyr-1196, catalog no. 6942), ErbB2 (catalog no. 4290), phospho-AKT (Thr-308, catalog no. 2965; Ser-473, catalog no. 6942), AKT (catalog no. 9272), and phospho-ERK1/2 (Thr-202/Tyr-204, catalog no. 9101) from Cell Signaling Technology; ERK2 (catalog no. sc-154) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); α-smooth muscle actin (catalog no. 5694) and fibroblast-activation protein (catalog no. 54651) from Abcam; mutationally selective BRAFV600E (catalog no. E19290) from Spring Bioscience (Atlanta, GA); and actin (catalog no. A2066) antibody from Sigma.

**Coculture System**—Cancer cells were plated in complete medium in individual wells of a 6-well plate. Following the indicated pretreatments, fibroblasts were plated in the transwell insert set on top of the cancer cells. The medium was replaced with fibroblast complete medium, and cells were cocultured for 3 days.

**Conditioned Medium Preparation**—For Western blot analysis, fibroblasts were plated at 60–70% confluence in single wells of a 6-well plate. Fibroblasts and cancer cells were treated similarly in DMEM containing 10% FBS. Conditioned medium from fibroblasts was then collected and transferred onto the melanoma cells. For longer-term growth and apoptosis assays, fibroblasts were plated in 15-cm plates at 50% confluence in 18 ml of complete medium. After 48 h, the medium was collected and concentrated to 450 μl. 150 μl of concentrated medium was added to 2 ml of conditioned medium.
melanoma cell medium in 6-well plates on the basis of calculations to give equivalent NRG1 levels to unconcentrated conditioned medium used for Western blot experiments. To promote reproducibility, after conditioned medium collection, fibroblasts were counted with the Scepter handheld automated cell counter (EMD Millipore, Billerica, MA). Counted cells per plate were $3.63 \pm \ldots$
0.21 \times 10^6 \text{ for HFF cells, } 3.23 \pm 0.21 \times 10^6 \text{ for HTERT cells, and } 1.53 \pm 0.11 \times 10^7 \text{ for CAFs.}

**Alamar Blue Assay**—Cells were plated in complete medium in individual wells of 6-well plates. The next day, cells were treated with vemurafenib (1 \mu M) for 24 h or left untreated. Cells were either cocultured with fibroblasts (HFF or HTERT BJ1) using transwell inserts for 3 days or treated for 6 days with fibroblast conditioned medium with fresh vemurafenib. Treatments were renewed every 72 h. After 3 and 6 days, the medium was replaced with complete medium containing Alamar Blue (Invitrogen), and cells were allowed to reduce Alamar Blue for 2–3 h. The medium was collected in triplicate from each condition, and the absorbance of oxidized and reduced Alamar Blue was measured at wavelengths of 600 and 570 nm, respectively, in a Multiskan Spectrum spectrophotometer (Thermo Fisher Scientific). Changes in viability were calculated from the resulting absorbance reading using the guidelines of the manufacturer. All conditions were normalized to the DMSO control that was set to a value of 100.

**Annexin V Staining**—Cells were plated in complete medium in individual wells of 6-well plates. The next day, cells were treated with vemurafenib (1 \mu M) or left untreated. After 24 h, vemurafenib treatment was renewed, and cells were treated for 72 h with fibroblast conditioned medium concentrated previously as described above. Cells were collected and washed twice with PBS and incubated with annexin V-allophycocyanin (BD Biosciences) for 15 min at room temperature protected from light. Samples were analyzed using the FACSCalibur flow cytometer.

**Cell Growth Assay**—Cells were treated for 6 days with fibroblast Conditioned medium containing vemurafenib and seribantumab/MM121 or pertuzumab, as indicated. Cells were then washed with PBS and stained with 0.2% crystal violet in 10% buffered formalin for 20 min. Subsequently, wells were washed and air-dried. Plates were scanned, and a quantitative analysis was performed using ImageJ software. The intensity mean was calculated for each well. Pictures were taken with a Nikon Eclipse Ti inverted microscope with NIS-Elements AR 3.00 software (Nikon, Melville, NY).

**Statistical Analysis**—Statistical analyses for 5-ethynyl-2'-deoxyuridine staining, annexin V, Alamar Blue, and crystal violet assays were performed using Student’s *t* test (two-tailed, unpaired, and assuming unequal variance).

**Results**

**NRG1 Is Highly Expressed in Dermal Fibroblasts and Cancer-associated Fibroblasts**—To investigate stromally versus tumor-derived NRG1, we first analyzed NRG1 expression across a panel of mutant BRAF melanoma cells compared with primary human dermal fibroblasts (HFF) and immortalized dermal fibroblasts (HTERT BJ1). Expression of NRG1 was low or undetectable in mutant BRAF melanoma cells but was readily detected in HFF and HTERT BJ1 cells (Fig. 1A). Because RAF inhibitors elicit paradoxical signaling effects in wild-type BRAF cells (23–26), we tested the effect of the RAF inhibitor vemurafenib on NRG1 expression in fibroblasts. In contrast to keratinocytes harboring Harvey rat sarcoma viral oncogene homolog mutations (27), we observed neither paradoxical activation of ERK1/2 nor NRG1 up-regulation following vemurafenib treatment throughout a 24–72 h time course in either HFF or HTERT cells (Fig. 1B). To further focus our studies on the tumor-associated stroma, we isolated CAFs from two independent BRAF V600E melanoma biopsy samples (Fig. 1, C and D). CAFs were characterized by their elevated expression of fibroblast activation protein and α-smooth muscle actin compared with normal fibroblasts (28, 29) and by their lack of BRAF mutation (Fig. 1, E and F). NRG1 was poorly detectable in tumor lysates but was expressed readily in CAFs generated from the corresponding tumor, supporting our view that NRG1 is provided to mutant BRAF melanomas by the stroma (Fig. 1, E and F). Furthermore, vemurafenib treatment did not elicit paradoxical activation of ERK1/2 or enhance NRG1 expression in CAFs (Fig. 1G). To analyze the effects on secreted levels of NRG1, ELISAs were performed and showed that NRG1β1 secretion from fibroblasts and CAFs was not affected dramatically following vemurafenib treatment (Fig. 1H). Of note, the ELISA detects NRG1β1, but multiple isoforms of NRG1 may be generated through alternative promoter use and splicing (30). Therefore, the total NRG1 concentration in the fibroblast conditioned medium is likely higher than the 0.5–1.2 ng/ml detected in this assay.

Because tumor cells can influence the expression profile of proximal cells (29), we also tested whether mutant BRAF melanoma-derived factors alter NRG1 expression in fibroblasts. Conditioned medium from untreated and vemurafenib-treated melanoma cells, WM115 and M238, did not alter NRG1 expression in fibroblasts (Fig. 1I). Also, in coculture transwell assays, vemurafenib-treated A375 and WM266-4 melanoma cells did not alter NRG1 expression in fibroblasts (Fig. 1I). Together, these data suggest that NRG1 is highly expressed in fibroblasts but that its expression is not enhanced by the paradoxical actions of RAF inhibitors.

**Fibroblast-derived NRG1 Promotes ErbB3 Signaling and Increases AKT Phosphorylation in Melanoma Cells**—Next we investigated whether fibroblast-derived NRG1 enhanced signaling in RAF-inhibited melanoma cells. To this end, we treated RAF-inhibited BRAF V600E/D melanoma cells (WM115, WM239-A, WM266-4, A375, and M238) with conditioned medium from vemurafenib-treated dermal fibroblasts. Exogenous NRG1 was added to cultures as a positive control (10).
Consistently, conditioned medium from HFF and HTERT BJ-1 cells induced phosphorylation of ErbB3 and downstream signaling to AKT and ERK1/2 to a level comparable with activation by exogenous NRG1 (Fig. 2A). Similar effects were obtained with CAF-derived conditioned medium (Fig. 2B). To test whether the protective effects of conditioned medium were dependent on NRG1 expression, we depleted NRG1 from fibroblasts using siRNAs (Fig. 2C). Notably, conditioned medium from NRG1 knockdown fibroblasts only weakly induced ErbB3 and AKT phosphorylation in all three RAF-inhibited mutant BRAF melanoma cell lines analyzed (Fig. 2D). These data show that the ErbB3 pathway in RAF-inhibited melanoma cells is activated by fibroblast-derived NRG1.

**Fibroblast Conditioned Medium Drives Resistance in RAF-inhibited Mutant BRAF Melanoma Cells—Exogenous NRG1 enhances growth and viability in RAF-inhibited melanoma cells (10). Therefore, we evaluated whether fibroblast-derived conditioned medium elicited similar effects. Initially, we analyzed the ability of fibroblast conditioned medium to rescue the effects of a range of vemurafenib doses on the BRAF V600D melanoma cell line WM115. Alamar Blue analysis showed that cell viability was effectively inhibited between dosages of 0.5–1 μM of vemurafenib and that fibroblast-derived conditioned medium enhanced cell viability (Fig. 3A). On the basis of these findings, we continued our studies utilizing 1 μM vemurafenib. Next we explored the effects of fibroblast conditioned medium on a panel of RAF-inhibited BRAF mutant melanoma cell lines. Vemurafenib-treated BRAF V600E/D melanoma cells were either cocultured with fibroblasts for 3 days in transwell chambers or treated with fibroblast-conditioned medium for 6 days. In both cases, fibroblast conditioned medium was able to partially prevent vemurafenib-mediated inhibition of melanoma cell viability (Fig. 3, B and C). Furthermore, fibroblast conditioned medium reduced the percentage of vemurafenib-induced apoptotic cells (Fig. 3D) and promoted cell growth in vemurafenib-treated WM115, W266-4, WM239-A, and M238 cells (Fig. 3E). In a similar way, conditioned medium from CAFs partially rescued cell growth in RAF-inhibited WM115 melanoma cells (Fig. 3F). These data show that fibroblast and CAF conditioned medium counteracts RAF inhibitor effects on the growth of mutant BRAF melanoma cells.

**Targeting ErbB3/ErbB2 Combines with RAF Inhibitors to Inhibit V600E/D BRAF Melanoma Cell Growth—**To address the translational potential of our studies, we tested the effect of ErbB3- and ErbB2-neutralizing antibodies in blocking the protective effects of stromally derived NRG1. We utilized seribantumab/MM121, an ErbB3-neutralizing antibody that binds to the ectodomain 1 of ErbB3 (31), and pertuzumab, a clinical-grade antibody that binds to ErbB2 subdomain II to block the ligand-dependent dimerization of ErbB2-ErbB3 (32). Administration of either seribantumab or pertuzumab strongly reduced the phosphorylation of ErbB3 and AKT in RAF-inhibited melanoma cells induced by normal fibroblast-derived or CAF-derived conditioned medium (Fig. 4, A and B). Consistent with previous findings (10), pertuzumab-mediated inhibition of ErbB3 phosphorylation indicates that ErbB3 utilizes ErbB2 as its main heterodimer partner in melanoma cells (Fig. 4A). Consistently, seribantumab and pertuzumab partially blocked the protective effects induced by fibroblast-derived conditioned medium on melanoma cell viability (Fig. 4C), apoptosis (Fig. 4D), and growth (Fig. 4E). Seribantumab and pertuzumab also inhibited the increase in melanoma cell growth induced by CAF conditioned medium (Fig. 4F). These data demonstrate that the protective effect of fibroblast-derived factors is targetable by ErbB3- and ErbB2-neutralizing agents.

**Discussion**

The NRG1-ErbB3 pathway is implicated in melanocyte homeostasis (33) and is related to the progression of several cancer types (18). Additionally, NRG1-ErbB3 signaling is implicated in driving resistance to targeted inhibitors (10, 19–21). This knowledge prompted us to investigate the role and source of NRG1 in melanoma tumors and to better understand the ability of NRG1 to drive resistance to targeted therapies. We demonstrate that NRG1 secreted by fibroblasts is likely to provide the initiating signal for the compensatory/adaptive ErbB3 response in mutant BRAF melanomas treated with vemurafenib. These studies demonstrate the role of paracrine NRG1 signaling in regulating the response to targeted therapies in V600 mutant BRAF-harboring melanoma cells (Fig. 5). Additionally, our findings highlight the need to understand the mechanisms of NRG1 expression in normal and cancer-associ-
The Tumor Microenvironment and Targeted Inhibitor Resistance

A

Cond. medium
DMSO CTL Serban Pertuz Serban Pertuz DMSO CTL Serban Pertuz DMSO CTL Serban Pertuz
Vemurafenib
pErbB3 Y1197
ErbB3
pErbB2 Y1196
ErbB2
pAKT S473
pAKT T308
AKT
pERK1/2
ERK2
Actin

B

Cond. medium
DMSO CTL Serban Pertuz
Vemurafenib
pErbB3 Y1197
ErbB3
pAKT S473
AKT
pERK1/2
ERK2
Actin

C

% Cell viability
DMSO
Vemurafenib
HFF CM HTERT CM
WM115

**
**

% Annexin V positive cells
DMSO
Vemurafenib
HFF CM HTERT CM
WM115

**
**

E

HFF CM
HTERT CM
Vemurafenib
Serban Pertuz

F

WM115
CAF25 CM
Vemurafenib
Serban Pertuz

High mag
Low mag

High mag
Low mag

201 ± 26.3 54 ± 10** 120 ± 18 57 ± 11** 62 ± 17** 114 ± 14** 58 ± 13 66 ± 14**

176 ± 12 41 ± 12** 79 ± 1.5** 51 ± 9.0** 53.5 ± 1**

24274 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 290 • NUMBER 40 • OCTOBER 2, 2015
First, we show that NRG1 expression is low/undetectable in mutant BRAF melanoma cell lines and melanoma patient samples but highly expressed in fibroblasts and CAFs isolated from mutant BRAF melanomas. Our data are consistent with previous reports of low NRG1 expression in melanoma cells (20) and with studies demonstrating the presence of NRG1 in skin fibroblasts (34) and CAFs from pancreatic ductal adenocarcinoma (35). However, concurrent expression of ErbB3 and NRG1 may exist in a subset of human metastatic melanomas (36), and, in these tumors, direct targeting of ErbB3/ErbB2 may be efficacious (37). In other studies, we have identified a subset of wild-type BRAF/wild-type NRAS (neuroblastoma rat sarcoma viral oncogene homolog) melanomas that express both NRG1 and ErbB3 and are dependent on NRG1-ErbB3 signaling for growth (38). Despite the well characterized paradoxical signaling effects of first-generation RAF inhibitors (25, 39), RAF inhibitors do not further promote NRG1 expression in fibroblasts. This contrasts with paradoxical effects of vemurafenib enhancing the production of VEGF from macrophages (40). Next-generation paradox breaker RAF inhibitors have been generated in an attempt to lessen these side effects (27, 41). Additionally, we demonstrate the ability of fibroblast- and CAF-derived NRG1 to mediate the activation of the ErbB3 pathway in RAF-inhibited mutant BRAF melanoma cells. We show that fibroblast-derived NRG1 partially blocks the effects of vemurafenib on cell growth and death in V600 mutant BRAF-harboring melanomas. Previous studies have reported the role of growth factors such as HGF (hepatocyte growth factor) produced from fibroblasts and exogenous NRG1 in driving resistance to vemurafenib (10, 21, 42, 43). Importantly, our data demonstrate, through depletion experiments, the requirement for a fibroblast-derived growth factor, NRG1, in mediating resistance to RAF inhibitors. NRG1 does not promote the growth of mutant BRAF melanoma cells under basal conditions (10). Therefore, it
The Tumor Microenvironment and Targeted Inhibitor Resistance

is likely that the presence of NRG1 in the tumor microenvironment becomes critical following treatment with RAF inhibitors.

To support the translational potential of our studies, we tested the role of ErbB3- and ErbB2-neutralizing antibodies in counteracting the effects of stromal NRG1. We used two different antibodies: seribantumab, which is currently being tested in several clinical trials (ClinicalTrials.gov identifier NCT02387216), and pertuzumab, a Food and Drug Administration-approved agent for combination therapy with trastuzumab and docetaxel for metastatic breast cancer (44). Both antibodies were able to partially reverse the effect of fibroblast-derived conditioned medium on ErbB3 pathway activation and mutant BRAF melanoma cell growth properties. These data are consistent with the ability of ErbB3-targeting agents to increase the efficacy and duration of response of RAF inhibitors in vivo (15) and underscore the use of ErbB3-targeting agents to reduce the protective effects of fibroblast-derived NRG1 on RAF-inhibited melanoma cells. Although pertuzumab and seribantumab target ErbB3/ErbB2 signaling by preventing ErbB3/ErbB2 dimerization or the binding of NRG1 to ErbB3, respectively, other agents may be developed to directly sequester NRG1 (45) or to directly target CAFs (46). Our findings also have relevance to other tumor types in which ErbB3 has been implicated in driving resistance to targeted inhibitors (19, 20). ErbB2-targeting antibodies are already Food and Drug Administration-approved for breast cancer, and there is considerable research activity with ErbB3-neutralizing antibodies for several cancer types (47, 48).

ErbB3 is overexpressed in a subset of melanoma patients and associated with a poor prognosis (49). However, our findings emphasize the importance of stratification on the basis of NRG1 and ErbB3 levels as well as genetic status. Recent studies report that NRG1 expression is a predictor of response to seribantumab (31), providing the basis for using NRG1 levels as a biomarker. Therefore, further studies investigating NRG1 and ErbB3 as biomarkers for the combined therapy with RAF or MEK inhibitors and ErbB3/ErbB2-targeting agents in melanoma could prove to be beneficial. Such studies may identify BRAF V600 melanoma patients in whom stromal NRG1 contributes to compensatory/adaptive ErbB3 signaling and in whom targeting the ErbB3/ErbB2 signaling axis may improve the efficacy of RAF inhibitors.

Author Contributions—C. C. and A. E. A. conceived and coordinated the study and wrote the paper. C. C. performed most of the experiments. S. R. performed and analyzed the experiments shown in Fig. 1, A, B, H, and L, and aided in the writing of the manuscript. A. C. B. provided patient tissue samples. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgments—We thank Dr. Meenhard Herlyn for the WM cell lines, Dr. Antoni Ribas for M238 cells, Dr. Gabriela Garcia (Merck–mack Pharmaceuticals) for MM121/seribantumab, Neda Dadpey for performing the DNA sequencing, Tim Purwin for help with database analysis, and Curtis H. Kugel, III, for isolating CAF25 from patient tumors.

References

1. Flaherty, K. T., Infante, J. R., Daud, A., Gonzalez, R., Kefford, R. F., Sosman, J., Hamid, O., Schuchter, L., Cebon, I., Ibrahim, N., Kudchadkar, R., Burris, H. A., 3rd., Falchuck, G., Alzagi, A., Lewis, K., Long, G. V., Puzanov, I., Lebowitz, P., Singh, A., Little, S., Sun, P., Allred, A., Ouellet, D., Kim, K. B., Patel, K., and Weber, J. I. (2012) Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. N. Engl. J. Med. 367, 1694–1703

2. Sosman, J. A., Kim, K. B., Schuchter, L., Gonzalez, R., Pavlick, A. C., Weber, J. S., McArthur, G. A., Hutson, T. E., Moschos, S. J., Flaherty, K. T., Hersey, P., Kefford, R., Lawrence, D., Puzanov, I., Lewis, K. D., Amaravadi, R. K., Chmielewski, B., Lawrence, H. J., Shyr, Y., Ye, F., Li, J., Nolop, K. B., Lee, R. J., Joe, A. K., and Ribas, A. (2012) Survival in BRAF V600-mutant advanced melanoma treated with vemurafenib. N. Engl. J. Med. 366, 707–714

3. Hauschild, A., Grob, J. J., Demidov, L. V., Jouary, T., Gutzmer, R., Millward, M., Rutkowski, P., Blank, C. U., Miller, W. H., Jr., Kaempgen, E., Martin-Algarra, S., Karasewszka, B., Mauch, C., Chiarion-Sileni, V., Martin, A. M., Swann, S., Haney, P., Mirakhur, B., Guckert, M. E., Goodman, V., and Chapman, P. B. (2012) Dabrafenib in BRAF-mutated metastatic melanoma: a multicentre, open-label, phase 3 randomised controlled trial. Lancet 380, 358–365

4. Hartsough, E., Shao, Y., and Aplin, A. E. (2014) Resistance to RAF inhibitors revisited. J. Invest. Dermatol. 134, 319–325

5. Nazarian, R., Shi, H., Wang, Q., Kong, X., Koya, R. C., Lee, H., Chen, Z., Lee, M. K., Attar, N., Szegaz, H., Chodon, T., Nelson, S. F., McArthur, G., Sosman, J. A., Ribas, A., and Lo, R. S. (2010) Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. Nature 468, 973–977

6. Poulikakos, P. I., Persaud, Y., Janakiraman, M., Kong, X., Ng, C., Moriceau, G., Shi, H., Atefi, M., Titz, B., Gabay, M. T., Salton, M., Dahlman, K. B., Tadi, M., Wargo, J. A., Flaherty, K. T., Kelley, M. C., Misteli, T., Chapman, P. B., Sosman, J. A., Greaber, T. G., Ribas, A., Lo, R. S., Rosen, N., and Solit, D. B. (2011) BRAF inhibitor resistance is mediated by dimerization of aberrantly spliced BRAF(V600E). Nature 480, 387–390

7. Hartsough, E. J., Basile, K. J., and Aplin, A. E. (2014) Beneficial effects of RAF inhibitor in mutant BRAF splice variant-expressing melanoma. Mol. Cancer Res. 12, 795–802

8. Basile, K. J., Abel, E. V., Dadpey, N., Hartsough, E. J., Fortina, P., and Aplin, A. E. (2013) In vivo MAPK reporting reveals the heterogeneity in tumoral selection of resistance to RAF inhibitors. Cancer Res. 73, 7101–7110

9. Villanueva, J., Vultur, A., and Herlyn, M. (2011) Resistance to BRAF inhibitors: unraveling mechanisms and future treatment options. Cancer Res. 71, 7137–7140

10. Abel, E. V., Basile, K. J., Kugel, C. H., 3rd., Witkiewicz, A. K., Le, K., Amaravadi, R. K., Karakousis, G. C., Xu, X., Xu, W., Schuchter, L. M., Lee, J. B., Bertin, A., Fortina, P., and Aplin, A. E. (2013) Melanoma adapts to RAF/MEK inhibitors through FOXD3-mediated upregulation of ERBB3. J. Clin. Invest. 123, 2155–2168

11. Girotti, M. R., Pedersen, M., Sanchez-Laorden, B., Viros, A., Turajlic, S., Niculescu-Duvaz, D., Zambon, A., Sinclair, J., Hayes, A., Gore, M., Lorigan, P., Springer, C., Larkin, J., Jorgensen, C., and Marais, R. (2013) Inhibiting EGF receptor or SRC family kinase signaling overcomes BRAF inhibitor resistance in melanoma. Cancer Discov. 3, 158–167

12. Kugel, C. H., 3rd., and Aplin, A. E. (2014) Adaptive resistance to RAF inhibitors in melanoma. Pigment Cell Melanoma Res. 27, 1032–1038

13. Mueller, M. M., and Fusseneg, N. E. (2004) Friends or foes: bipolar effects of the tumour stroma in cancer. Nat. Rev. Cancer 4, 839–849

14. Lee, K. W., Yeo, S. Y., Sung, C. O., and Kim, S. H. (2015) Twist1 is a key regulator of cancer-associated fibroblasts. Cancer Res. 75, 73–85

15. Kugel, C. H., 3rd., Hartsough, E. J., Davies, M. A., Setiyadi, Y. Y., and Aplin, A. E. (2014) Function-blocking ERBB3 antibody inhibits the adaptive response to RAF inhibitor. Cancer Res. 74, 4122–4132

16. Fedorenko, I. V., Abel, E. V., Koomen, J. M., Fang, B., Wood, E. R., Chen, Y. A., Fisher, K. J., Iyengar, S., Dahlman, K. B., Wargo, J. A., Flaherty, K. T., Sosman, J. A., Sondak, V. K., Messina, J. L., Giblyn, G. T., and Smalley, K. S. (2015) Fibronectin induction abrogates the BRAF inhibitor response of BRAF V600E/PTEN-null melanoma cells. Oncogene 10.1038/onc.2015.188

17. Guy, P. M., Platko, J. V., Cantley, L. C., Cerione, R. A., and Carraway, K. L., 2015.
3rd (1994) Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. Proc. Natl. Acad. Sci. U.S.A. 91, 8132–8136.

18. Yarden, Y., and Slivkovski, M. X. (2001) Untangling the ErbB signaling network. Nat. Rev. Mol. Cell Biol. 2, 127–137.

19. Sun, C., Hober, S., Bertotti, A., Zecchin, D., Huang, S., Galimi, F., Cottino, F., Prahallad, A., Gennarum, W., Tzani, A., Schlucker, A., Wessels, L. F., Smit, E. F., Thunnissen, E., Halonen, P., Liefk, C., Beijersbergen, R. L., Di Nicolantonio, F., Bardelli, A., Trusolino, L., and Bernards, R. (2014) Intrinsic resistance to MEK inhibition in KRAS mutant lung and colon cancer through transcriptional induction of ERBB3. Cell Rep. 7, 86–93.

20. Montero-Conde, C., Ruiz-Llorente, S., Dominguez, J. M., Knaf, I. A., Viale, A., Sherman, E. J., Ryder, M., Ghosein, R. A., Rosen, N., and Fagin, J. A. (2015) Relief of feedback inhibition of HER3 transition by RAF and MEK inhibitors attenuates their antitumor effects in BRAF-mutant thyroid carcinomas. Cancer Discov. 3, 520–533.

21. Wilson, T. R., Fridlyand, J., Yan, Y., Penuel, E., Burton, L., Chan, E., Peng, J., Lin, E., Wang, Y., Somman, J., Ribas, A., Li, J., Moffat, J., Sutherland, D. P., Koeppen, H., Merchant, M., Neve, R., and Settleman, J. (2012) Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors. Nature 487, 505–509.

22. Lin, M. C., Rojas, K. S., Cerione, R. A., and Wilson, K. F. (2014) Identification of mTORC2 as a necessary component of HRG/ErbB2-dependent cellular transformation. Mol. Cancer Res. 12, 940–952.

23. Hatzivassiliou, G., Song, K., Yen, I., Bruderhuber, B. J., Anderson, D. J., Marais, R. (2010) Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. J. Cell Sci. 123, 298–310.

24. Hadiorn, S. J., Milagre, C., Whittaker, S., Nourry, A., Niculescu-Duvaz, I., Dhomen, N., Reis-Filho, J. S., Springer, C. J., Pritchard, C., and Aplin, A. E. (2013) Selective RAF inhibitor impairs ERK1/2 phosphorylation and growth in mutant NRAS, vemurafenib-resistant melanoma cell lines. Pigment Cell Melanoma Res. 26, 523–533.

25. Garrett, J. T., Sutton, C. R., Kurupi, R., Bialucha, C. U., Ettenberg, S. A., Hutterer, M., Huang, A., Hambalkar, T., Wolchok, J. D., de Stanchina, E., Chandraharatap, S., Poulaki, P. I., Fagin, J. A., and Rosen, N. (2012) Relief of profound feedback inhibition of mitogenic signaling by RAF inhibitors attenuates their activity in BRAFV600E melanomas. Cancer Cell 22, 668–682.

26. Straussman, R., Morgaska, T., Sreek, K., Barzilay-Rokni, M., Qian, Z. R., Du, J., Davis, A., Szymanowski, M., Grund, J., Sutter, M., Cooper, A. Z., Chang, P. B., Solit, D. R., Ribas, A., Lo, R. S., Flaherty, K. T., Ogino, S., Wargo, J. A., and Golub, T. R. (2012) Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. Nature 487, 500–504.

27. Liles, J. S., Arnoletti, J. P., Kossenkov, A. V., Frost, A. R., Yarden, Y., and Aplin, A. E. (2015) ErbB3/ErbB2 complexes as a therapeutic target in a subset of wild-type BRAF/NRAS cutaneous melanomas. Cancer Res. 75, 10158–10157.

28. Setiady, Y. Y., Osman, I., Yarden, Y., and Aplin, A. E. (2015) ErbB3/ErbB2 oncogenic unit plays a key role in NRG1 signaling and melanoma cell growth and survival. Pigment Cell Melanoma Res. 28, 408–414.

29. Zhao, W., Cao, Y., Li, X., Zhang, J., Zhang, Z., Yang, Y., and Chen, W. (2014) Epidermal growth factor receptor (EGFR) signaling in esophageal adenocarcinoma. J. Gastrointest. Surg. 18, 1156–1164.

30. Ligon, L. K., Horsman, D. E., Shroff, R. M., Aguirre, A., Groom, C. R., Gruvberger, S., Wiklund, F., Greulich, H., Schmitt, L., Xu, H., Ng, W. Y., and Amler, L. R. (2014) ErbB3 as a therapeutic target in drug-resistant prostate cancer. Nature 509, 484–489.

31. Wolter, N., Akgul, A., Hepp, B., Bittner, S., Nauj, S., Schmitt, L., Duh, Y.-L., and Greulich, H. (2014) Targeting ErbB3 in colorectal cancer. J. Mol. Med. 92, 17–27.