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BRAF inhibition generates a host/tumor niche that mediates therapeutic escape

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Abstract

The current study defines a fibroblast-derived niche that facilitates the therapeutic escape of melanoma cells from BRAF inhibition. Vemurafenib treatment led to the release of TGF-β from the melanoma cells that increased the differentiation state of the fibroblasts; an affect associated with fibronectin deposition, increase in α-smooth muscle actin (α–SMA) expression and the release of neuregulin (NRG). At the same time, vemurafenib directly activated the fibroblasts through paradoxical stimulation of the MAPK pathway, causing them to secrete hepatocyte growth factor (HGF). Treatment with the BRAF/MEK inhibitor combination reversed the release of HGF. Adhesion of melanoma cells to fibronectin was critical in amplifying the fibroblast-derived NRG and HGF-mediated PI3K/AKT survival signaling in the melanoma cells following BRAF inhibition. In co-culture studies, combination treatment with inhibitors of BRAF/MET/HER kinase was ineffective at reversing the fibroblast-mediated therapeutic escape from BRAF inhibition. Instead, it was noted that combined BRAF/PI3K inhibition overcame fibroblast-mediated drug resistance *in vitro* and was associated with enhanced anti-tumor effects in an *in vivo* xenograft model. Thus, we show melanoma cells and fibroblasts remodel their microenvironment in response to BRAF inhibition and that these adaptations allow tumor cells to evade therapy through increased PI3K/AKT survival signaling.

Keywords

melanoma; fibroblasts; BRAF; resistance

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Introduction

Our emerging understanding of therapeutic resistance suggests a role for both tumor autonomous mechanisms as well as adaptive pro-survival signals from the host microenvironment (Abel *et al.,* 2013; Lito *et al.,* 2012; Straussman *et al.,* 2012; Wilson *et al.,* 2012). During progression, melanoma cells lose contact with their natural binding partners, the keratinocytes, and instead interact with host endothelial cells and fibroblasts (Hsu *et al.,* 2000; Li *et al.,* 2001). Fibroblasts also contribute to the escape of melanoma cells from vemurafenib therapy, in part through hepatocyte growth factor (HGF) signaling (Straussman *et al.,* 2012). Additionally, tumor-derived growth factors, such as epithelial growth factor (EGF), neuregulin (NRG) and insulin-like growth factor (IGF)-I, impact responsiveness to BRAF inhibition and may even drive BRAF inhibitor resistance (Abel *et al.,* 2013; Villanueva *et al.,* 2010; Wilson *et al.,* 2012). Other studies have suggested that adhesion to the extracellular matrix (ECM) protects cancer cells from apoptosis following treatment with chemotherapeutics. This phenomenon, known as cell adhesion-mediated drug resistance (CAM-DR), was first described for multiple myeloma, with adhesion to fibronectin (FN) decreasing the sensitivity to melphalan (Damiano *et al.,* 1999; Hazlehurst and Dalton, 2001; Hazlehurst *et al.,* 2000). The extent to which melanoma cells and their microenvironment interact to provide a “protective sanctuary” that allow the cancer cells to evade therapy is not well understood. In the present study, we uncovered a previously uncharacterized ECM-derived protective niche that drives therapeutic escape through the amplification of host-derived survival signals. Unexpectedly, inhibition of BRAF also led to paradoxical MAPK signaling-mediated differentiation and ECM deposition in normal skin fibroblasts (*BRAF* wild-type), suggesting that off-target effects of kinase inhibitors remodel the host environment. We propose a role for bi-directional signaling between the tumor and host in the adaptive responses to therapy and demonstrate that host cells are an active player in the escape process. Our data suggest that future therapeutic strategies will require the targeting of both the tumor and host responses.

Results

Plating of GFP-tagged melanoma cells onto a confluent fibroblast monolayer conveyed near-total protection to the growth inhibitory effects of vemurafenib treatment (3 μM, 72h) (Figure 1A). Mechanistically, it was found that treatment of the fibroblasts using transforming growth factor (TGF)-β, conditioned media (CM+vemu) from melanoma cells treated with vemurafenib (3 μM, 48h) or vemurafenib alone (3 μM, 48h) increased their differentiation as shown by the increased expression of fibronectin (FN) and α-smooth muscle actin expression (α-SMA) (Figures 1B,C). Although vemurafenib alone induced fibroblast differentiation, the extent of this was less than either CM+vemu or TGF-β alone. The stimulatory effects of the melanoma CM on the fibroblasts was TGF-β-dependent, with the addition of the TGF-kinase inhibitor SB505124 found to partially inhibit fibroblast activation (Supplemental Figure 1). The increased expression of FN was required for fibroblast survival, with its siRNA-mediated knockdown associated with increased fibroblast cell death under serum-free conditions (Supplemental Figure 2).

Previous work from our lab has demonstrated BRAF inhibitor treatment to induce an EMT-like state in melanoma cells, a phenotype often driven through TGF-β signaling (Fedorenko *et al.,* 2015). As exogenous TGF-β, vemurafenib and CM from vemurafenib-treated melanoma cells induced the differentiation of fibroblasts, we next asked whether BRAF inhibition led to the release of TGF-β from the melanoma cells. Treatment with vemurafenib increased the protein expression of TGF-β in 3 out of 6 *BRAF*-mutant melanoma cell lines (Figure 2A). Also noted was an increase in mRNA levels as well as the release of TGF-β - as measured by qRT-PCR and ELISA assays (Figures 2B,C). The potential clinical relevance of these findings were suggested by the analysis of pre- and post-treatment specimens from melanoma patients on BRAF inhibitor therapy, with increased post-treatment levels of TGF-β mRNA observed in 2 out of 4 of patients analyzed (Figure 2D). While the origin of the TGF-β could not be attributed exclusively to melanoma cells (due to the nature of patient tissue, in which many cell types interact intimately), these clinical results are consistent with our in vitro findings.

Increased receptor tyrosine kinase (RTK) signaling is known to mediate the escape of melanoma cells from BRAF inhibition (Abel *et al.,* 2013; Nazarian *et al.,* 2010; Straussman *et al.,* 2012). A role for melanoma- and vemurafenib-activated fibroblasts in the release of pro-survival melanoma growth factors was demonstrated by ELISA assays in which TGF-β (0.1 and 1 ng/ml) or vemurafenib (3 μM) treatment increased the fibroblast-mediated release of NRG and HGF, respectively (Figures 3A,B). Interestingly, maximal fibroblast activation seemed to be dependent upon dual TGF-β/vemurafenib treatment, with studies showing that vemurafenib alone failed to induce NRG release from fibroblasts, TGF-β1 alone failed to induce HGF release from fibroblasts and the observation that TGF-β inhibition did not fully suppress fibroblast activation (Supplemental Figures 1, 3). Previous work has shown that BRAF inhibition activates MAPK signaling in systems with either *Ras* mutations or upstream RTK signaling, as a result of CRAF transactivation (Hatzivassiliou *et al.,* 2010; Poulikakos *et al.,* 2010). To date, the ability of BRAF inhibitors to activate ERK signaling in normal, primary cells has not been reported. We next determined the requirement for paradoxical ERK activation in the vemurafenib-mediated release of fibroblast HGF. Western blot analyses showed single-agent vemurafenib to induce paradoxical MAPK signaling in primary human skin fibroblasts and that this was reversed through combination with the MEK inhibitor trametinib (Figure 3C). A role for paradoxical ERK activation in fibroblast-mediated melanoma therapeutic escape was demonstrated in ELISA assays that showed the combination of trametinib with vemurafenib to completely suppress the vemurafenib-mediated increase in HGF expression (Figure 3D). Adhesion to ECM proteins, such as FN, can increase cell survival by amplifying RTK signals. To determine the role of FN expression in amplifying fibroblast-derived growth signals we identified three melanoma cell lines whose FN expression increased following vemurafenib treatment (Supplemental Figure 4). Knockdown of FN using siRNA limited EGFR, c-MET and ERBB3 receptor phosphorylation following ligand challenge, an effect associated with impaired PI3K/AKT signaling (Figures 4A,B).

An IHC analysis of specimens from melanoma patients treated with vemurafenib (n=9) was then performed to validate that melanoma cells and fibroblasts co-exist in close proximity, and that FN expression was increased at these sites of interaction. Examination by two

independent pathologists confirmed areas in the post-treatment specimens with high levels of FN staining, with infiltrating spindlelike cells, characteristic of fibroblasts noted in 3/9 samples (Figure 4C). Areas of strong FN staining were also seen at the tumor/stroma interface, at the sites of melanoma cells and fibroblast interaction (Figure 4C).

Our studies thus far demonstrated that activated fibroblasts increased the cooperative effects of RTKs and FN upon PI3K signaling in melanoma cells. We next asked whether inhibition of PI3K/AKT signaling was sufficient to reverse the protection conferred by the fibroblasts to the melanoma cells. Quantification of pAKT staining in GFP-tagged WM9 melanoma cells revealed higher basal signaling levels following plating upon fibroblasts compared to plastic (Figure 5A). Upon treatment with vemurafenib (3 μM, 24h), significant increases in pAKT staining were observed in the melanoma cells following adhesion to three independent fibroblast cell lines (Figure 5A,B). Vemurafenib was not noted to induce AKT signaling in the fibroblasts (Supplemental Figure 5). In line with the observation that FN amplifies AKT signaling through multiple RTKs (Figure 4A-B), only limited inhibition of AKT signaling was seen when the co-cultures were treated with the combination of crizotinib and lapatinib with vemurafenib (Figure 5C,D). As expected, the inhibitory effects of the RTK inhibitors were stronger when the melanoma cells were plated on fibroblasts as opposed to on plastic, however these effects were still quite limited (Figure 5C,D). Interestingly, while the combination of the BRAF inhibitor with lapatinib and crizotinib induced a relatively small decrease in cell proliferation, this triple combination was ineffective at inducing apoptosis as measured by PARP cleavage – a possible reflection of the incomplete inhibition of PI3K/AKT signaling (Supplemental Figure 6; Figure 5C,D). The combination of vemurafenib with the PI3K inhibitor GDC-0941 was associated with a near-complete inhibition of the fibroblast-mediated AKT signaling in the melanoma cells, an effect associated with a dramatic increase in PARP cleavage (Figures 5C,D). Evidence for the role of PI3K in facilitating fibroblast-mediated therapeutic escape was demonstrated by the ability of the BRAF+PI3K inhibitor combination to enhance vemurafenib-mediated apoptosis in two additional melanoma cells lines, each plated on three individual primary human skin fibroblast cultures (Figure 6A). The extent of apoptosis induction following BRAF+PI3K inhibitor treatment was slightly increased on fibroblasts compared to plastic in the WM793 cell line but not the 1205Lu (Supplemental Figure 7). The *in vivo* relevance of microenvironment-mediated PI3K/AKT signaling in the escape response of melanoma cells was demonstrated in a human melanoma mouse xenograft model, where dosing with the combination of the BRAF inhibitor PLX4720 and the PI3K inhibitor GDC-0941 caused significant levels of tumor regression compared to either PLX4720 or GDC-0941 alone (Figure 6B). A model showing the proposed interaction of the host/melanoma cells under vemurafenib treatment is shown in Figure 6C.

Discussion

Although there is some evidence that host fibroblasts also mediate resistance to BRAF inhibition through increased HGF release, the mechanisms underlying the melanoma cell/fibroblast interaction remain poorly described (Straussman *et al.*, 2012). Fibroblast survival is dependent upon attachment to an appropriate ECM, with adhesion to FN constituting a major survival signal (Almeida *et al.*, 2000; Ilic *et al.*, 1998; Lin *et al.*, 2011; Zhang *et al.*,

1995). FN is also a potent chemo-attractant for fibroblasts that stimulates their motility (Postlethwaite *et al.,* 1981). Our data suggests that the release of TGF-β from melanoma cells treated with a BRAF inhibitor, and the effects of the BRAF inhibitor itself play critical roles in the activation of host fibroblasts. Treatment with TGF-β, vemurafenib or CM from vemurafenib-treated melanoma cells increased fibroblast differentiation. At the same time, either exogenous TGF-β or vemurafenib enhanced NRG and HGF release from fibroblasts, respectively. Recent reports have suggested that TGF-β released from melanoma cells upon BRAF inhibition may also increase RTK expression in melanoma cells (Sun *et al.,* 2014). This, along with the data contained herein, suggests TGF-β release to set the stage for complex growth factor-mediated crosstalk between melanoma cells and fibroblasts. Analysis of tumor specimens from melanoma patients on BRAF inhibitor therapy suggest that melanoma cells and fibroblasts exist in close proximity *in vivo*, suggesting the likelihood of this cross-talk occurring.

The ability of vemurafenib to stimulate HGF release from normal primary skin fibroblasts was unexpected, and was linked to the ability of vemurafenib to induce paradoxical MAPK signaling in normal human fibroblasts. Paradoxical MAPK signaling occurs when BRAF inhibitors transactivate CRAF as result of upstream signals emanating from either *Ras* mutations, or increased levels of growth factor signaling (Gibney *et al.,* 2013; Hatzivassiliou *et al.,* 2010; Joseph *et al.,* 2010; Poulikakos *et al.,* 2010). Although the BRAF/MEK inhibitor combination was noted to suppress the release of HGF from the fibroblasts, this would not be expected to fully reverse host-mediated resistance. Other signals, such as melanoma-derived TGF-β, would still be able to activate the host fibroblasts, perhaps partly explaining why the dabrafenib/trametinib combination can delay, but not prevent the onset of acquired resistance in melanoma patients (Flaherty *et al.,* 2012; Larkin *et al.,* 2014). Although TGF-β released locally from BRAF-inhibitor treated melanoma cells appeared to constitute an important mechanism of fibroblast activation, it is worth noting that melanoma cells release other factors known to stimulate fibroblasts including PDGF and stromal derived factor (SDF) (Orimo *et al.,* 2005; Whipple and Brinckerhoff, 2014; Willenberg *et al.,* 2012). There is also evidence that the introduction of mutant *BRAF* into melanoma cells increases their secretion of interleukin (IL)-1α that causes tumor-associated fibroblasts to induce immune suppression (Khalili *et al.,* 2012).

The observation that drug-treated melanoma cells activated fibroblasts, increasing NRG and HGF release, was suggestive of a role for host cells in mediating therapeutic escape. Under baseline conditions, *BRAF*-mutant melanoma cells exhibit high levels of feedback inhibition in the MAPK signaling pathway that suppresses the ability of RTKs to activate ERK (Lito *et al.,* 2012). Following vemurafenib treatment, the feedback inhibition of the MAPK pathway becomes deregulated, increasing the responsiveness to growth factors such as EGF, NRG, HGF and fibroblast growth factor (FGF) (Lito *et al.,* 2012). There is already evidence that both HGF and NRG limit responses to vemurafenib and its analogue PLX4720 (Abel *et al.,* 2013; Sharma *et al.,* 2010; Straussman *et al.,* 2012). For all growth factors evaluated, the expression of FN was required for the maximal activation of PI3K/AKT signaling, suggesting a critical role for environmental remodeling in the amplification of these escape signals. In this instance the FN seemed to be derived from the activated fibroblasts and from the melanoma cells themselves. It is already known in lung cancer that co-operation between

integrin and RTK signaling is required for the optimal activation of downstream PI3K/AKT pathway (Morello *et al.*, 2011). Similar findings have also been reported for $\alpha_5\beta_1$ integrin, where a functional association between the integrin with the VEGFR3 receptor in lymphatic vessels and EGFR and ERBB3 in intestinal epithelial cells being required for efficient signaling (Lee and Juliano, 2002; Zhang *et al.*, 2005). In some experimental systems, integrin $\alpha_5\beta_1$ also complexes with c-MET, with the presence of either vitronectin or FN found to significantly increase the level of MET receptor phosphorylation (Rahman *et al.*, 2005).

When co-cultured with fibroblasts, vemurafenib markedly enhanced increased AKT signaling in the melanoma cells. The activation of AKT was mediated through multiple RTKs and by direct melanoma/fibroblast adhesion, with the BRAF/PI3K inhibitor combination found to be significantly more effective at reversing the adaptive survival than a BRAF inhibitor combined with multiple RTK inhibitors. It was also found that combined BRAF/PI3K inhibition was significantly more effective at reducing the growth of melanoma xenografts than either BRAF or PI3K inhibitor alone. These data are in agreement with recent preclinical studies demonstrating that the combination of a BRAF and PI3K inhibitor induces a more rapid regression of tumors in *BRAF* V600E/PTEN-null GEMMs than BRAF inhibitor alone (Marsh Durban *et al.*, 2013). In our xenograft model, PLX4720 was relatively weak as a single agent. This is likely a consequence of the 1205Lu melanoma cell line being both *BRAF* mutant and null for PTEN. There is already evidence from our lab and others that PTEN loss can be a mediator of intrinsic BRAF inhibitor resistance and there is evidence that aberrant PTEN function is associated with a shorter-PFS in melanoma patients receiving BRAF inhibitor therapy (Nathanson *et al.*, 2013; Paraiso *et al.*, 2011; Xing *et al.*, 2011). The observed heterogeneity in TGF-β secretion (highest in PTEN null cell lines) supports these findings.

The requirement for PI3K signaling in microenvironment-mediated therapeutic escape was demonstrated by the ability of combined BRAF/PI3K inhibition to overcome this protection. Together these data suggest the PI3K/AKT signaling pathway integrates multiple host-derived signals required for therapeutic adaption. This idea is also supported by recent work suggesting that mutations in *AKT1* are involved in tumor-intrinsic therapeutic adaptation (Shi *et al.*, 2014). Loss of PTEN expression and/or function on BRAF inhibitor therapy has been suggested as a mechanism of therapeutic escape (Van Allen *et al.*, 2014).

While previous studies showed variable cytokine production in established fibroblast lines, the current study used early-passage primary cultures from three healthy human donors and is likely to better represent fibroblast biology. A more extensive characterization of clinical samples may improve our understanding of the cytokines produced by the stroma in patients treated with BRAF inhibitors (Khalili *et al.*, 2012; Straussman *et al.*, 2012).

Our current understanding of BRAF inhibitor escape suggests a role for short-term adaptation in which cells evade the immediate effects of the drug. The data contained herein suggest a role for host-tumor cross talk in the earliest phases of adaptation, however it is also likely that pressure from the host may also help to select for escaping clones or mutations. Long-term treatment of melanoma patients with small molecule inhibitors such as

dabrafenib and vemurafenib will depend upon their ability to suppress the escaping population of cells that ultimately repopulate the tumor. This study presents evidence that adaptive changes in normal host cells facilitate the escape of melanoma cells from BRAF inhibition. It is likely that combination therapies such as BRAF+PI3K inhibition may be one strategy to limit the protection conveyed by the host.

Materials and methods

Cell culture and reagents

The 1205Lu, WM9, WM793, WM164, WM983A and 451Lu melanoma cells lines and FF2504, FF2507 and FF2447 human primary skin fibroblasts were a gift from Dr. Meenhard Herlyn (The Wistar Institute, Philadelphia, PA). WM9-GFP was from Dr. Peter Forsyth (Moffitt Cancer Center, Tampa, FL). The identities of all cell lines were confirmed by Biosynthesis Inc (Lewisville, Tx) through STR validation analysis. Cell lines were maintained in 5% FBS/RPMI-1640. Conditioned media was prepared by adding fresh media to 1205Lu cells for 48 hrs in the presence of vehicle or 3 μM vemurafenib. Then the media was collected and diluted 1:1 with fresh media, matching the concentrations of drugs/vehicle. SB505124 was obtained from Selleck Chemicals (Houston, TX).

Western blotting

Proteins were extracted and western blotting was performed as previously described (Fedorenko *et al.*, 2015). Uniform protein loading was confirmed by blotting for GAPDH. The antibodies to pAKT S473, total AKT, pERK, total ERK, pMet (Tyr1234/1235), pEGFR (Y1172), pHER3 (Y1289) and TGFβ were from Cell Signaling Technology (Beverly, MA). The α-SMA antibodies were from Abcam (Cambridge, MA) while the antibody against Fibronectin antibody was from BD (San Jose, CA), GAPDH was from Sigma (St. Luis, MO), and Phalloidin was from Invitrogen (Life Technologies, Carlsband, CA).

Immunofluorescent staining

Melanoma and primary skin fibroblast cells were plated on glass coverslips overnight prior to treatment. Cells were then fixed, stained and imaged as previously described (Fedorenko *et al.*, 2015). Images were analyzed using Definiens® Developer v2.0 (Definiens AG, Munich, Germany) software suite. The total fluorescence intensity was normalized to the number of nuclei (stained by DAPI) for monocultures or to the number of GFP-positive cells in co-cultures. For co-culture experiments, melanoma cells were green-fluorescent protein (GFP) labeled, plated on un-labeled fibroblasts and imaged using fluorescent microscopy. Melanoma and fibroblast cells were differentiated by the GFP label. In rescue experiments, the number of GFP+ cells were counted for three 5× field of view images per treatment (N=3). Fibroblast differentiation was measured by level of FN and α-SMA expression.

RNA interference

Cells were plated and left to grow overnight. The 5%FBS/RPMI media was replaced with Opti-MEM (Invitrogen). Fibronecin pool siRNA's in complex with Lipofectamine 2000(Invitrogen) were added. Scrambled, non-targeting siRNA's were used as controls. Cells were transfected for 24-72 hours prior to treatment.

ELISA Assays

The TGFβ1 and HGF ELISA kits were purchased from R&D Systems (Minneapolis, MN). The neuregulin ELISA Kit was from Abcam (Cambridge, MA).

qRT PCR for cell lines and patient specimens

Cells were treated for 72 hours then total RNA was isolated using Qiagen's RNeasy mini kit. cDNA from a cohort of patient specimens was generously shared by Keith Flaherty from Massachussetts General Hospital. Patient specimens were obtained with written, informed patient consent according to approved protocols by the Institutional Review Board at the Massachussetts General Hospital. The pretreatment biopsies were performed between 0 and 30 days before initiating therapy and on-treatment biopsies were collected between 7 and 24 days following the initiation of therapy. The following TaqMan® Gene Expression Assays primer/probes were used: Hs00365052_m1 (Fibronectin), P/N 4319413E (18S) and Hs99999905_m1 (GAPDH). The 18S and GAPDH data were utilized to normalize TGFβ1, accounting for cellularity. qRT-PCR reactions were performed as previously described (Paraiso et al 2011).

Flow cytometry

Cells were grown overnight, then treated with vehicle (DMSO), 3 μM vemurafenib, 3 μM GDC-0941, or the two drugs in combination (72 hours). Cells were stained for Annexin V and TMRM as previously described (Fedorenko *et al.,* 2015). For analysis of pAKT and cleaved PARP, FF2504 human primary skin fibroblasts (3.0×10^5 cells) were plated in 6-well plates overnight. GFP-tagged WM9 melanoma cells (3.0×10^5 cells) were then plated either on plastic or on FF2504 fibroblasts and incubated overnight. Cells were then treated with 3μM vemurafenib (BRAFi), 3μM GDC-0941 (PI3Ki), 200nM crizotinib (METi), and/or 1μM lapatinib (HER2i) for 24 hours prior to being collected by scraping, fixed with 4% paraformaldehyde (10mins, RT) and permeabilized with 100% cold methanol (1hr, RT). Cells were stained with pAKT conjugated to AlexaFluor 647 (Ser473, Cell Signaling Technology, Beverly, MA) and cleaved PARP conjugated to PE (BD, San Jose, CA) in i0.5% bovine serum albumin in PBS (1hr, RT). Cells were analyzed by flow cytometry.

Animal Studies

All animal studies were carried out under approved protocols by the Institutional Animal Care and Use Committee at the University of South Florida. Female SHO mice (Charles River) were subcutaneously injected with 2.5×10^6 cells per mouse. Tumors were allowed to grow to approximately $100mm^3$. Mice were administered D10001 control chow, AIN-76A 417 mg/kg PLX4720-formulated chow (Research Diets, New Brunswick, NJ), vehicle (0.5% methylcellulose, 0.2% Tween-80) oral gavage, or GDC-0941 oral gavage (150mg/kg) daily for 8 days. Mouse tumor volumes (1/2 × L × W2) were measured.

Statistical Analysis

Results are reported as mean values, error bars indicating ± SEM. GraphPad Prism 6 software was used to calculate statistical significance of magnitude of changes between

different conditions was calculated using the parametric paired t-test with p-values depicted as follows: *p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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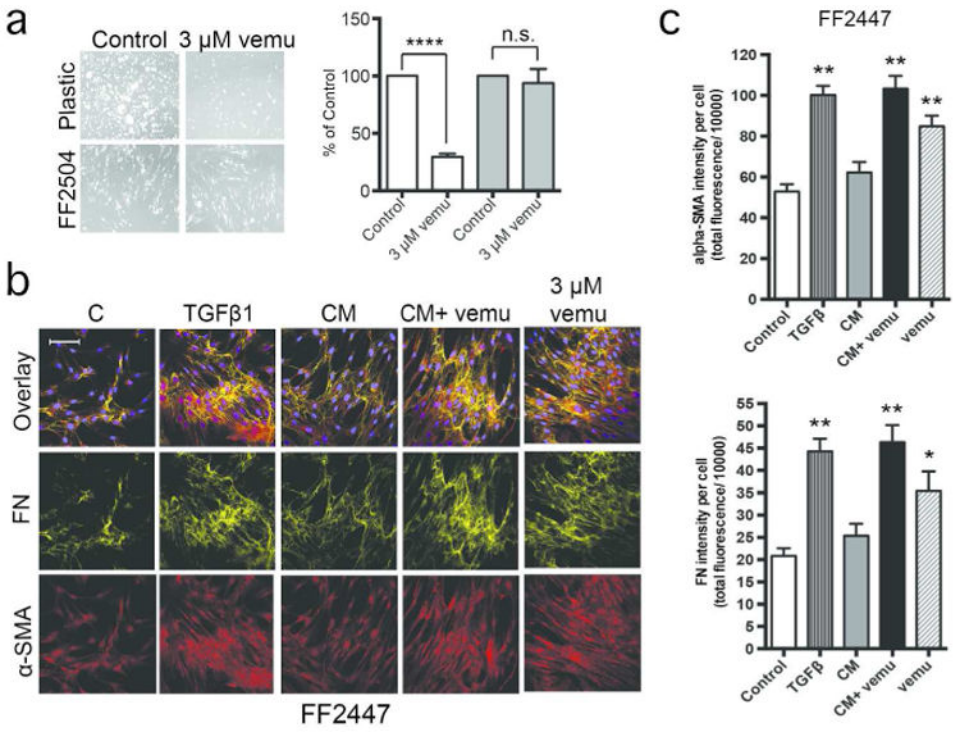

Figure 1.

***BRAF*^{V600E} melanoma cells and vemurafenib induce fibroblast differentiation. A:** (Left) GFP-tagged 1205Lu melanoma cells were plated on either tissue culture plastic or confluent monolayers of unlabeled FF2504 fibroblasts and treated with vemurafenib (3μM, 72 hrs). (Right) Quantification of GFP+ melanoma cells, N=3. **B:** FF2447 fibroblasts were treated with either conditioned media from 1205Lu cells (CM), conditioned media from 1205Lu cells treated with vemurafenib, 3 μM 48h (CM+vemu), 12.5pg/ml TGF-β1 (TGFβ1), or vemurafenib (vemu) before being stained for FN (FN, yellow) and α-smooth muscle actin (α-SMA, red). scale bar = 100 μm. **C:** Fibroblast differentiation was measured by level of FN and α-SMA expression. FN and α-SMA expression was analyzed using Definiens® Developer v2.0 software suite, total fluorescence intensity per nuclei was quantified.

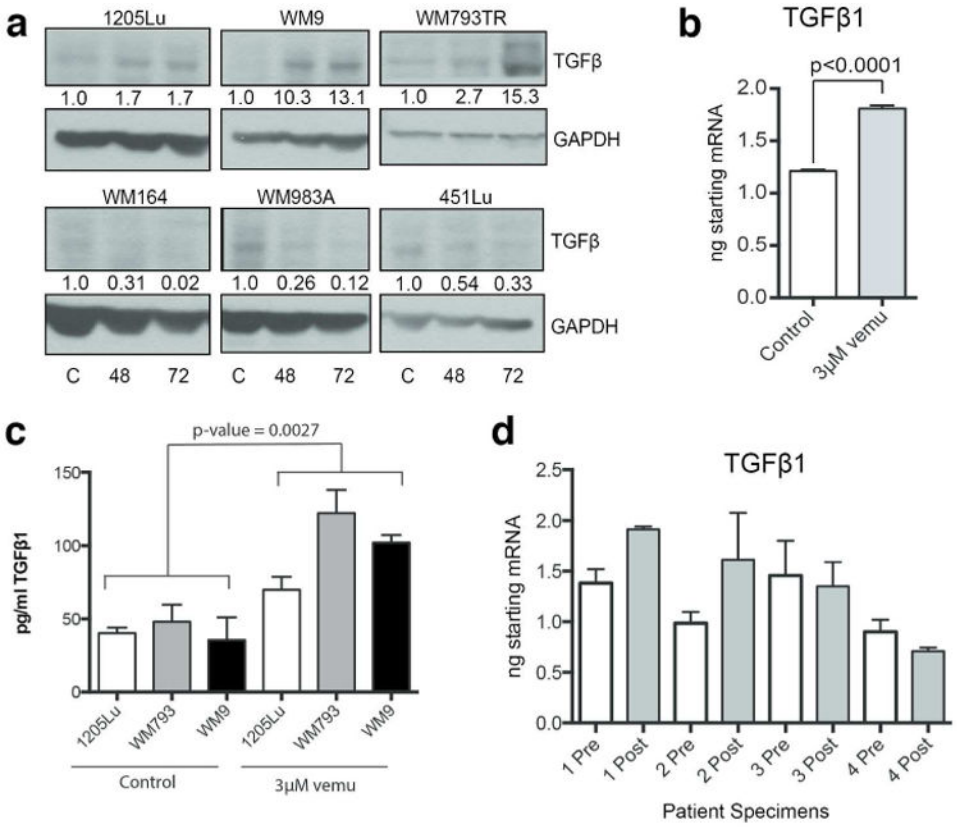

Figure 2. Vemurafenib induces the release and secretion of TGF-β from some *BRAF*-mutant melanoma cells

A Western blot analysis of 6 melanoma cell lines treated with vemurafenib (3 μM, 72Hrs). Densitometry for TGF-β is depicted in fold changes compared to each respective control. Scale bar = 50 μm. **B:** qRT-PCR for TGF-β1 shows vemurafenib-mediated induction of TGF-β1 mRNA expression in 1205Lu. Data was normalized to GAPDH and 18S endogenous controls. **C:** ELISA showing induction of TGF-β release from $BRAF^{V600E}$ melanoma cell lines following 3μM vemurafenib treatment (72 hours), expressed in pg/ml. **D:** Data shows q-RT-PCR experiments measuring levels of TGF-β1 mRNA in 4 matched (pre and post treatment) pairs of melanoma patient specimens receiving vemurafenib therapy (960 mg BID), error bars represent technical replicates of a single RNA extraction.

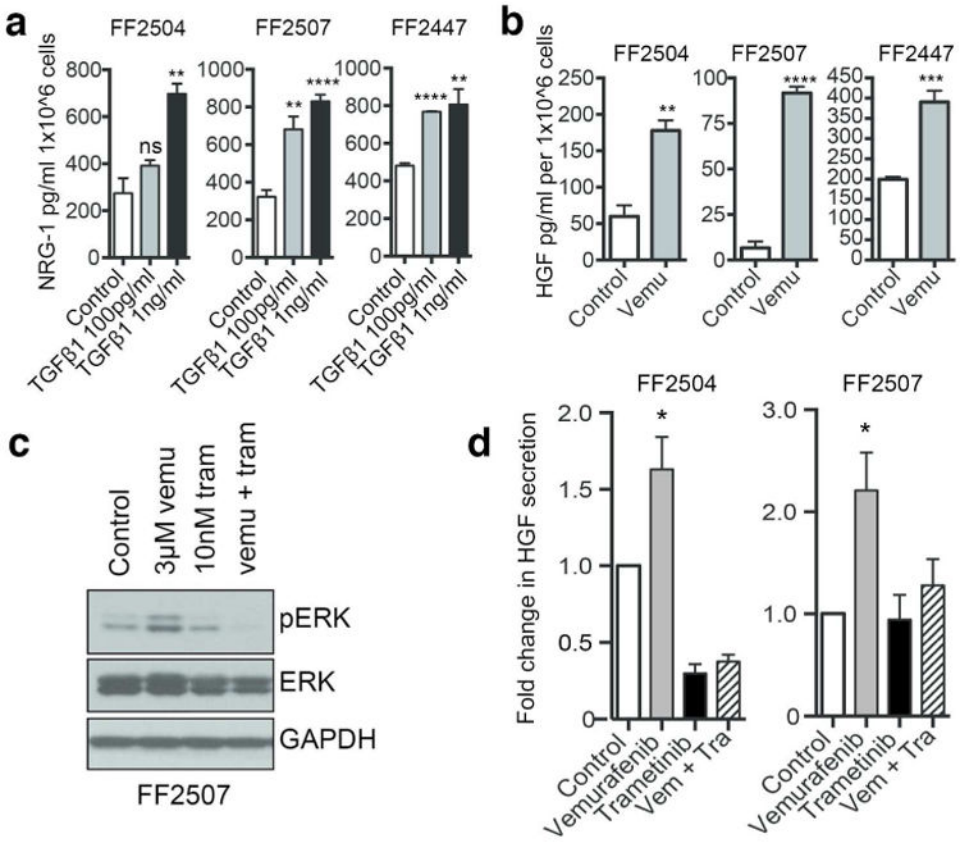

Figure 3. Vemurafenib and TGF-β co-operate to release growth factors from primary human fibroblasts

A ELISA data showing NRG release from 3 human skin fibroblast cell lines, following treatment with TGF-β (100 pg and 1 ng/ml) for 72 hours. **B:** ELISA data showing HGF release from 3 human skin fibroblast cell lines, following treatment with vemurafenib (3 μM, 72 hrs). **C:** Western blot analysis showing vemurafenib (3 μM) to induce paradoxical MAPK signaling in primary human skin fibroblasts that could be blocked through combination with trametinib (10 nM). **D:** ELISA data showing HGF release from 2 human skin fibroblast cell lines, following 72-hour treatment with 3 μM vemurafenib, 10nM Trametinib, or the combination.

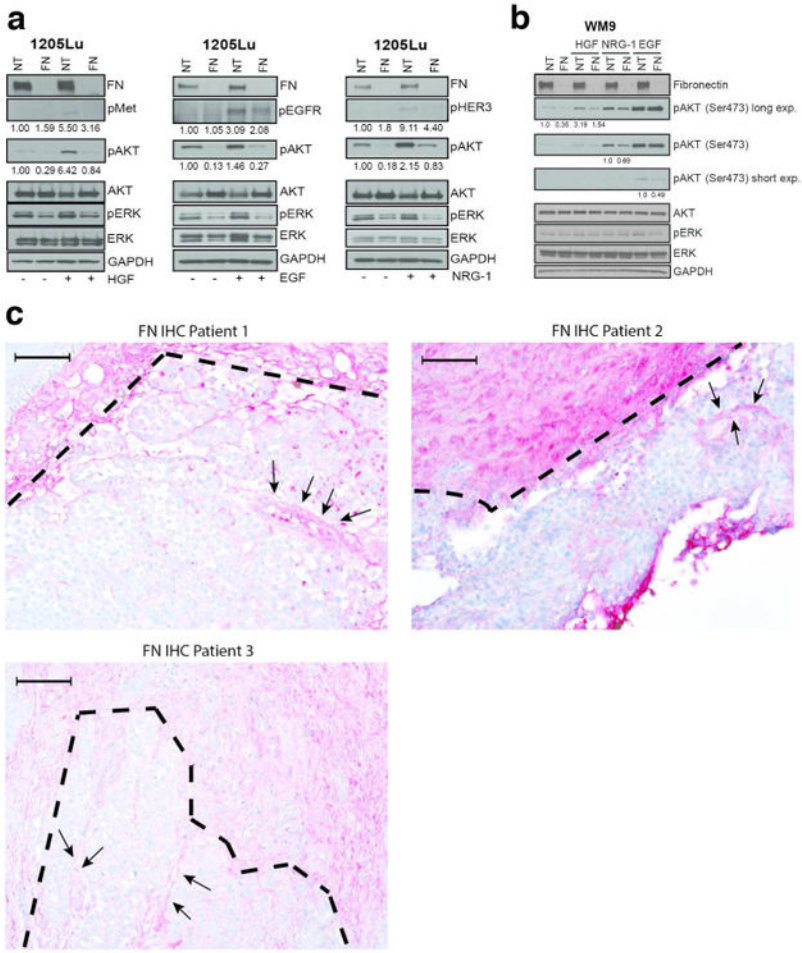

Figure 4. Adhesion to FN amplifies RTK signals in *BRAF*^{V600E} mutant melanoma cells

A 1205Lu cells were treated with either non-targeting (NT) or FN (FN) siRNA prior to stimulation with HGF, EGF or NRG (25ng/ml, 100ng/ml and 50ng/ml respectively). **B:** WM9 cells were treated with either NT or FN siRNA prior to stimulation with HGF, EGF or NRG (25ng/ml, 100ng/ml and 50ng/ml respectively). **C.** Representative IHC staining of post-vemurafenib failure melanoma patient specimens (N=9). The specimens were examined by two independent dermatopathologists who have indicated areas that are characterized by high FN staining (red) and spindle-like cells characteristic of fibroblasts infiltrating the tumor tissue (arrows). Areas of strong fibronectin staining are shown at the tumor/stroma interface where melanoma cells and fibroblasts also interact (dotted line). scale bar = 100 μm.

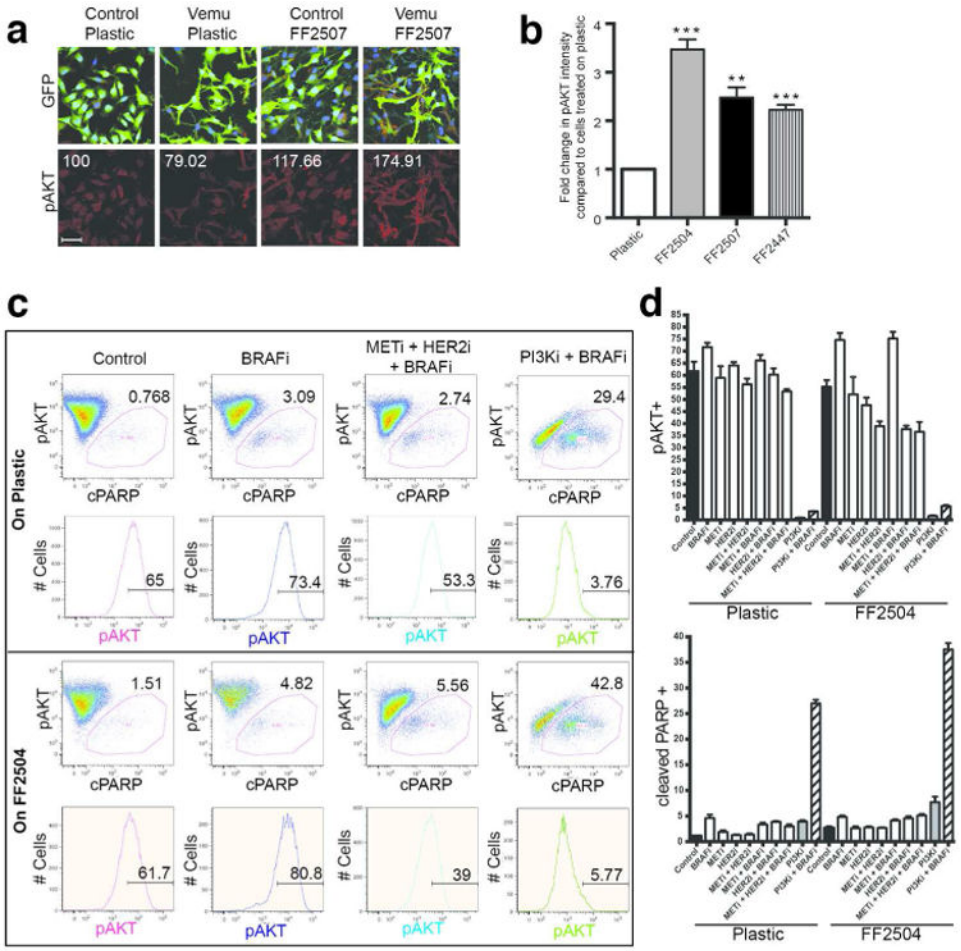

Figure 5. Fibroblasts protect melanoma cells from vemurafenib-mediated cytotoxicity through PI3K/AKT

A GFP-tagged WM9 melanoma cells were plated on plastic or fibroblast monolayers and treated with 3μM vemurafenib (24h) before being stained for pAKT (Ser473). Scale **B.** Fold changes in vemurafenib-induced pAKT from **A** were calculated. **C:** Melanoma cells treated with single agent or combinations of 3 μM vemurafenib (BRAFi), 3 μM GDC-0941 (PI3Ki), 200 nM crizotinib (METi), and 1 μM lapatinib (HER2i). Analysis of pAKT (Ser473) and cleaved PARP on individual GFP-tagged cells was performed using flow cytometry. Histograms show levels of pAKT, with an AKT+ gate drawn based on 3μM GDC-0941 treatment on plastic. **D.** Column graphs show the percentage of melanoma cells from **C** that are in the AKT+ and cleaved PARP+ populations.

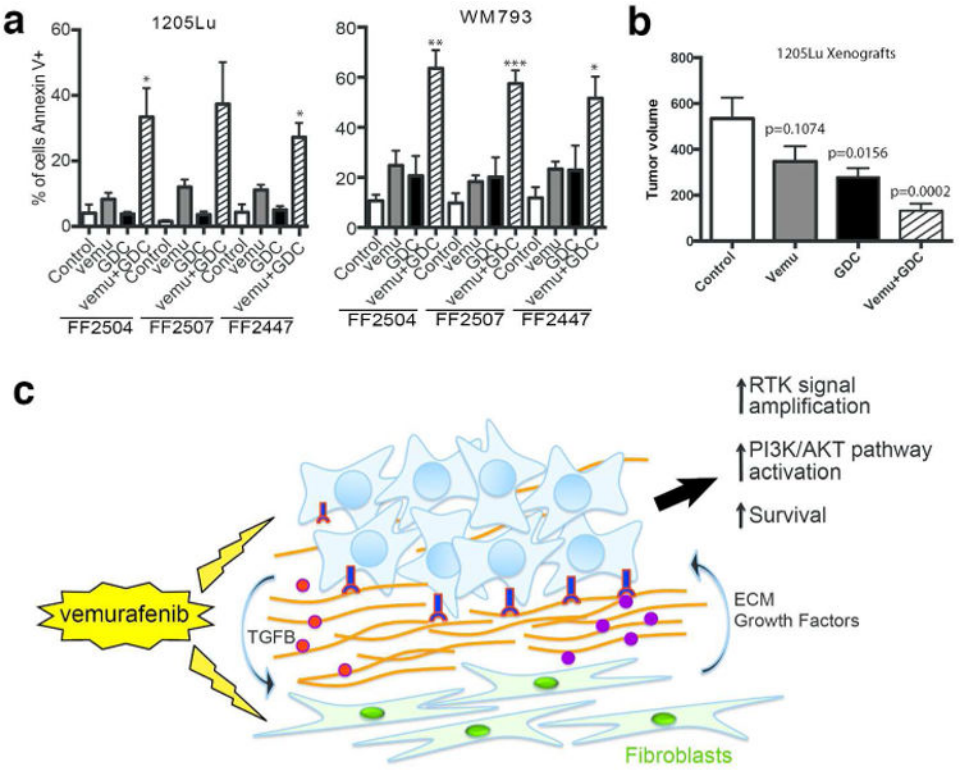

Figure 6. Combined BRAF/PI3K inhibition reverses fibroblast-mediated drug resistance and leads to tumor regression *in vivo*

A GFP-tagged melanoma cells (WM793, 1205Lu) were seeded on top of fibroblast cell lines (FF2504, FF2507, FF2447) overnight before being treated with either vehicle control or 3μM vemurafenib and 3μM GDC0941 for 72 hrs before being stained for annexin-V and analyzed by flow cytometry. P-values were calculated between vemurafenib only and vemurafenib/GDC0941 combination treatments. **B:** Xenograft tumor volume was calculated using the modified ellipsoid formula (tumor volume = ½ × L × W^2). P-values were calculated between control and treatment groups. **C:** Model showing the interaction of the host/melanoma cells under vemurafenib treatment.