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$BRAF^{V600E}$ melanoma cells secrete factors that activate stromal fibroblasts and enhance tumourigenicity

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Background: Melanoma, the most lethal form of skin cancer, is responsible for over 80% of all skin cancer deaths and is highly metastatic, readily spreading to the lymph nodes or metastasising to other organs. The frequent genetic mutation found in metastatic melanoma, $BRAF^{V600E}$, results in constitutive activation of the mitogen-activated protein kinase pathway.

Methods: In this study, we utilised genetically engineered melanoma cell lines and xenograft mouse models to investigate how $BRAF^{V600E}$ affected cytokine (IL-1β, IL-6, and IL-8) and matrix metalloproteinase-1 (MMP-1) expression in tumour cells and in human dermal fibroblasts.

Results: We found that $BRAF^{V600E}$ melanoma cells expressed higher levels of these cytokines and of MMP-1 than wild-type counterparts. Further, conditioned medium from the $BRAF^{V600E}$ melanoma cells promoted the activation of stromal fibroblasts, inducing expression of SDF-1 and its receptor CXCR4. This increase was mitigated when the conditioned medium was taken from melanoma cells treated with the $BRAF^{V600E}$ specific inhibitor, vemurafenib.

Conclusions: Our findings highlight the role of $BRAF^{V600E}$ in activating the stroma and suggest a mechanistic link between $BRAF^{V600E}$ and MMP-1 in mediating melanoma progression and in activating adjacent fibroblasts in the tumour microenvironment.

Metastatic melanoma is an aggressive cancer, with an increasing incidence worldwide, few durable and effective therapies, and an overall survival rate of $<10\%$ (Villares *et al*, 2011). Early stage melanoma, classified as radial growth phase (RGP), is curable by surgical excision. However, later stage vertical growth phase (VGP) is characterised by invasion into the dermal layer and is frequently metastatic with median survival times of less than 9 months.

Melanoma progression is often associated with a mutation in the BRAF oncogene. The most common mutation, $BRAF^{V600E}$, is found in $\sim 50\%$ of melanomas and promotes progression through constitutive activation of the mitogen-activated protein kinase (MAPK) signalling cascade (Hingorani *et al*, 2003; Sharma *et al*, 2006; Vultur *et al*, 2011) and target genes. Among these genes, matrix metalloproteinase-1 (MMP-1; Rutter *et al*, 1998; Huntington *et al*, 2004) and several cytokines and chemokines affect stromal cells in the tumour microenvironment (TME; Nicholas and Lesinski, 2011; Domanska *et al*, 2013). Importantly, the small molecule inhibitor, vemurafenib, specifically blocks $BRAF^{V600E}$ signalling and expression of these downstream genes, inducing tumour regression (Sullivan and Flaherty, 2013). Accumulating evidence highlights the critical role of the TME in enhancing the aggressive behaviour of melanomas, since products secreted by the tumour cells affect gene expression in adjacent stromal cells, causing them to adopt a carcinoma-activated fibroblast-like (CAF-like) phenotype (Eck *et al*, 2009).

The mechanisms that promote progression from less invasive RGP melanoma to aggressive VGP melanoma are unclear. However, this transition is associated with the destruction of the extracellular matrix, and an upregulation of proteolytic enzymes, particularly MMP-1 (Zigler *et al*, 2011; Austin *et al*, 2012).

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Matrix metalloproteinase-1 plays a prominent role in melanoma progression. It is secreted by melanoma and activated stromal cells and it degrades interstitial collagens (types I and III), which is an essential step in invasion and metastasis (Boire *et al*, 2005). Considering that melanomas are notorious for their capacity to invade, and that an activated TME facilitates tumour progression (Villanueva and Herlyn, 2008), a better understanding of how vemurafenib alters gene expression within the tumour and adjacent stromal cells is essential for determining the full impact of vemurafenib as a therapeutic agent.

Therefore, we investigated the effect of BRAF^{V600E} expression on the transition from RGP to VGP and its influence on dermal fibroblasts in the TME. We developed a genetically controlled model where RGP Bowes cells, which are BRAF wild-type (WT), ectopically express BRAF^{V600E}. By using Bowes cell lines containing an empty vector or BRAF^{V600E}, we measured expression of MMP-1 and several secreted chemokines and cytokines, and we analysed activation of stromal fibroblasts. We found that PLX4032 (the experimental version of vemurafenib) decreased expression of these proteins and subdued stromal activation. We also found that ectopic expression of BRAF^{V600E} can drive endogenous MMP-1 expression in the melanoma cells, resulting in enhanced tumourigenicity.

Our *in vitro* data confirm the findings of others (Khalili *et al*, 2012) on the induction of MMP-1, IL-1β, IL-6, and IL-8 by BRAF^{V600E} in melanoma cells (Sumimoto *et al*, 2006; Ryu *et al*, 2011). Importantly, our work extends these previous findings by demonstrating a central role for IL-1β (produced by melanoma cells) in activating stromal fibroblasts. In addition, we show that PLX4032 reduces expression of these cytokines and of MMP-1, and subsequent stromal cell activation. Finally, *in vivo* studies investigating the tumourigenicity of engineered Bowes cells in nude mice demonstrate cooperativity between BRAF^{V600E} and MMP-1 in mediating melanoma growth.

MATERIALS AND METHODS

Cell culture and conditioned media. Bowes cells and normal human neonatal dermal fibroblasts (HDFs) were obtained from American Type Cell Culture (ATCC, Manassas, VA, USA; Blackburn *et al*, 2009) and cultured according to manufacturer's directions. Complete medium, EMEM (ATCC), contained 5% FBS, 5% HEPES (Fisher Bioreagents, Pittsburg, PA, USA), and penicillin/streptomycin (100 U ml^{-1} and 100 μg ml^{-1}, respectively; Corning Inc., Corning, NY, USA). For serum-free (SF) conditions, cells were placed in EMEM (ATCC) with 0.2% lactalbumin hydrolysate (Sigma, St Louis, MO, USA), 5% HEPES, and penicillin/streptomycin. For conditioned medium (CM), 10^6 tumour cells were seeded in a 10-cm dish, incubated 24 h, washed with HBSS (Cellgro Mediatech Inc., Manassas, VA, USA), and 4 ml of SF medium was added to each dish. After 48 h, the CM was collected, spun at 1500 r.p.m. for 2 min, and used immediately or stored at $-80\,^{\circ}$C. All cells were cultured at 37 °C in humidified air with 5% CO_2.

Construction and selection of expression clones with Bowes cells. Bowes cells were stably transfected with a BRAF^{V600E} overexpression construct (under control of the 5′ LTR of the MoMuLV retrovirus in the pBABE vector (Addgene, Cambridge, MA, USA)) utilising Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer's directions. A panel of engineered clones: WT for BRAF (vector only) or mutant BRAF^{V600E} was isolated using 2 μg ml^{-1} of puromycin (Invitrogen).

cDNA synthesis and BRAF^{V600E} sequencing. Clones were seeded in a 6-cm dish (3.5×10^5), incubated overnight, and RNA was isolated utilising the Qiagen RNeasy kit (Qiagen, Venlo, Limburg, Netherlands) following manual instructions. After cDNA synthesis utilising the iScript cDNA Synthesis kit (Bio-Rad, Hercule, CA, USA), the BRAF template was amplified via standard PCR profile. To confirm the presence of BRAF^{V600E}, DNA was sequenced and compared to normal BRAF (nm_004333), with a T > A transversion at nucleotide 1799 indicating the presence of the mutant BRAF allele.

Real-time RT–PCR. cDNA was subjected to RT–PCR using Bio-Rads iQSybr Green Supermix and CFX96 Real Time System C1000 Thermal Cycler (Bio-Rad). All samples were normalised to B_2M and relative fold change was calculated as $2^{-\Delta\Delta Ct}$. Primer sequences are listed in Supplementary Table 1.

Immunoblotting. Bowes cells, 2.5×10^5 or 5×10^5, were plated in 6- or 10-cm dishes, respectively, and incubated overnight. Cells were pelleted and lysed via sonication in cold cell extraction buffer (Invitrogen) 1X proteinase inhibitors (Calbiochem, Darmstadt, Germany) and 0.1 mM PMSF. Standard immunoblotting techniques were used (Huntington *et al*, 2004; Croteau *et al*, 2013): 2–5 μg total protein was visualised with anti-pMEK (1 : 1000, Cell Signaling, Beverly, MA, USA), anti-Total MEK (1 : 1000, Cell Signaling), or anti-MMP-1 (1 : 5000, Millipore, Billerica, MA, USA), followed by the donkey anti-rabbit HRP secondary antibody at 1 : 5000 (Millipore). Signal was detected with Western Lightning Plus—ECL (PerkinElmer, Waltham, MA, USA).

Trichloroacetic acid precipitation. Conditioned medium was spun at 1500 r.p.m. for 2 min, 500 μl cold 10% trichloroacetic acid (Fisher Scientific, Pittsburg, PA, USA) was added to 1 ml CM for 60 min on ice. Samples were spun for 15 min at 13 000 r.p.m., pellets were washed with cold 95% ETOH (containing 0.1 M potassium acetate), and centrifuged at 13 000 r.p.m. at 4 °C for 5 min. Pellets were air-dried; 30 μl of Laemmli buffer (Bio-Rad) was added; samples were boiled for 5 min, and 20 μl were loaded in a 12% Tris-glycine SDS–PAGE gel.

PLX4032 inhibition/cytokine production in tumour cells. One million cells were plated in a 10-cm dish, incubated 24 h in complete EMEM medium (ATCC), washed with HBSS (Cellgro Mediatech Inc.), and placed in 4 ml of SF medium with/without 3 μM PLX4032 (ChemieTek, Indianapolis, IN, USA). After 48 h, the medium was spun 2 min at 1500 r.p.m., and stored at $-80\,^{\circ}$C. Total protein and mRNA were isolated.

PLX4032 inhibition/cytokine production in HDFs. Bowes cells, 1×10^6, were plated in a 10-cm dish, incubated overnight. Approximately, 4 ml of fresh serum-containing EMEM medium (ATCC), with/without 3 μM PLX4032, was added to each plate. After 48 h, CM was collected. Human dermal fibroblasts, 2.5×10^5, were plated per well of a six-well dish for 24 h and 1 ml of CM was added to each well. After 24 h, the CM was removed; the cells were pelleted for subsequent RNA isolation and RT–PCR analysis of cytokine expression.

IL-1β rescue. Human dermal fibroblasts were treated as described above, except 10 ng ml^{-1} of IL-1β was added to the tumour cell CM before adding it to the HDFs. As a negative control, SF medium with/without 3 μM of PLX4032 (ChemieTek) was placed in an empty 10-cm dish and incubated for 48 h alongside the tumour cells producing CM. This SF medium (with and without PLX) and the CM removed from the tumour cells were placed on the HDFs for 24 h as described above.

Tumour growth and analysis. Bowes cells were grown in culture to 80–90% confluency, washed, counted, and resuspended 1×10^6 cells per 50 μl of PBS. Six- to eight-week-old female nude mice (strain nu/nu, Charles River, Wilmington, MA, USA) were injected intradermally (1×10^6 cells) into the right flank. For statistical

significance, there were eight mice per treatment group (Blackburn *et al*, 2009). Tumours were measured weekly with calipers. When the tumours were ~10–12 mm in diameter, the mice were killed and tumours were excised.

Tumour pieces were flash frozen for mRNA isolation. Another portion of the tumour was cultured as explant in SF medium. For the tumour explant, tumour pieces were weighed, minced into 2–3 mm^3 pieces, and divided between several wells of a 24-well dish, each well with 1 ml SF medium containing gentamicin (1 : 200) and Fungizone (1 : 250). After 48 h, CM was collected, spun at 1500 r.p.m. for 3 min, and stored at −80 °C. Cytokines in CM were measured by testing the CM in a MSD Multiplex Array per kit instructions (Meso Scale Diagnostics, Rockville, MD, USA). Matrix metalloproteinase-1 was measured with R&D Systems Fluorokine E Enzyme Activity Assay for Human Active MMP-1 per kit instructions (Minneapolis, MN, USA). All Animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the Geisel School of Medicine at Dartmouth College.

Statistical analysis. Unless otherwise noted, Student's *t*-test was used for statistical analysis, with $P<0.05$ defined as significant. All experiments were done in triplicate, at least three separate times. All numerical values represent the mean ± s.e.

RESULTS

Isolation and characterisation of Bowes melanoma clones. We transfected RGP Bowes cells with empty vector or vector expressing BRAF^{V600E}. We isolated 10 clones harbouring the empty vector and 32 clones with BRAF^{V600E} (data not shown). From these, we selected three for further investigation. Figure 1A shows total BRAF mRNA levels in WT (empty vector; clone 8) and in two clones expressing ectopic BRAFV600E (clones 7 and 21). Initial experiments in medium with 10% serum showed a significant increase (five-fold, $P=0.0015$) in total BRAF mRNA in clone 21 compared to clone 7 (Figure 1A). In WT Bowes cells (clone 8), BRAF mRNA levels are low, whereas ectopic expression of BRAF^{V600E} increases total BRAF levels in clones 7 and 21. Next, we compared expression of mRNA for BRAF^{V600E} in cells after 48 h culture in SF medium, conditions that we used to prepare the medium from melanoma cells to be tested on stromal fibroblasts (see below). Figure 1B shows that clone 21 displays higher BRAF mRNA than clone 7, which although not significantly higher than clone 7, was associated with high constitutive levels of MMP-1 and cytokines (see below). We attribute the difference to nutrients in serum-containing *vs* SF medium; SF medium may be limiting in allowing clone 21 to maintain higher levels of BRAF mRNA.

Effect of PLX4032 on pMEK. To confirm that BRAF^{V600E} conferred increased constitutive signalling through the MAPK pathway and that PLX4032 could reduce this signalling, we measured pMEK after 48 h in SF medium. Wild-type Bowes (clone 8) displayed low pMEK, which was not affected by PLX4032 treatment (Figure 1C). In contrast, clones with BRAF^{V600E} (clones 7 and 21) expressed higher pMEK and this was antagonised by PLX4032. Despite the slightly higher level of BRAF mRNA in clone 21, compared to clone 7 (Figure 1B), pMEK levels were not higher, suggesting that differences in BRAF mRNA levels (under SF conditions; Figure 1B) were not sufficient to alter levels of pMEK.

Effect of BRAF^{V600E} and PLX4032 on proteins secreted by melanoma cells. Next, we compared gene expression in cells with WT *vs* mutant BRAF^{V600E}. We focused on secreted proteins: IL-1β (Khalili *et al*, 2012), IL-6, and IL-8 (Frederick *et al*, 2013; cytokines known to contribute to melanoma progression), and MMP-1 (Ryu *et al*, 2011). Cells were cultured in SF medium for 48 h with or without 3 μM PLX4032 (ChemieTek) and mRNA and total protein were isolated. Wild-type BRAF cells (clone 8) displayed low levels of IL-1β, IL-8, IL-6, and MMP-1, which were not affected by PLX4032 (Figure 2A-D, respectively). Similar to Khalili *et al* (2012), who found that introducing BRAF^{V600E} into melanocytes increased expression of IL-1β, clones 7 and 21 displayed increased IL-1β mRNA and this expression was antagonised by PLX4032 (Figure 2A).

However, we also found increased expression of IL-8, IL-6, and MMP-1 in BRAF^{V600E} cells (Figure 2B–D, respectively), and PLX4032 antagonised this expression. There were differences in the induction of cytokines and MMP-1 in clone 7 (lower BRAF mRNA expression) compared to clone 21 (higher BRAF mRNA). For example, IL-1β and IL-6 levels were higher in clone 7, while IL-8 and MMP-1 levels were higher in clone 21. These differences may reflect downstream mechanisms differentially regulating expression of these genes in response to BRAF^{V600E}. Lastly, to confirm the mRNA data with protein analysis, we performed a human multiplex assay on CM collected from the tumour cells. In keeping with the results of mRNA, protein levels of cytokines, IL-1β and IL-8, increased with BRAF^{V600E} (clones 7 and 21) and PLX4032 abrogated this increase (Table 1). Similarly, MMP-1 levels were increased with BRAF^{V600E} clone 21 (although not detected, with clones 7 and 8). Thus, the changes in protein levels with PLX4032 mirrored those seen with mRNA.

Modulation of stromal cell gene expression by tumour-derived proteins. As tumour cells interact with adjacent fibroblasts, the

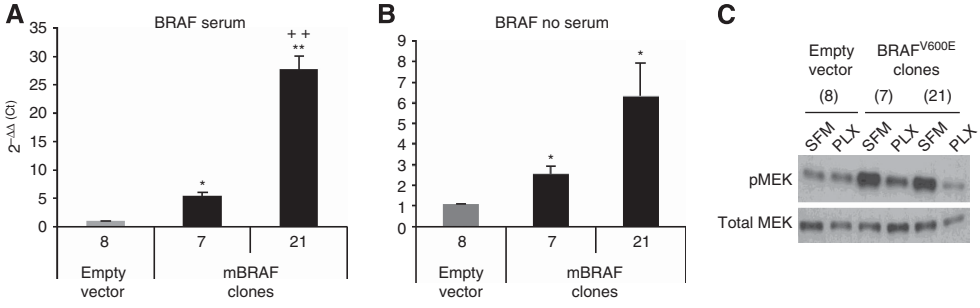

Figure 1. BRAF mRNA and pMEK levels in Bowes clones. Bowes cells stably transfected with BRAF^{V600E} (clones 7 and 21), or with empty vector (clone 8) were grown in either 10% serum or in serum-free medium for 48 h and real-time RT–PCR was used to measure BRAF gene expression. (**A**) Bowes clones with serum. (**B**) Bowes cells in serum-free medium (SFM). (**C**) Bowes cells were grown in SFM either with or without 3 μM PLX4032 (PLX) for 48 h. Total protein was isolated and probed for pMEK and total MEK via western blot analysis. RT–PCR data were normalised to B2M, analysed by the $2^{\Delta\Delta(Ct)}$ method (values are relative to clone 8) and are representative of three experiments. *$P\leqslant0.05$; **$P\leqslant0.005$ when compared to clone 8. $^{++}P\leqslant0.005$ when compared to clone 7.

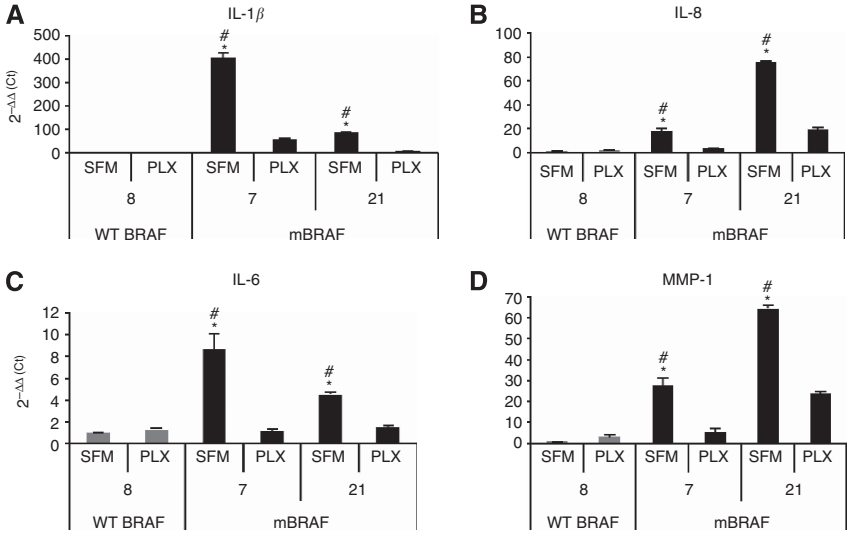

Figure 2. mBRAF increases MMP-1 and inflammatory cytokine levels and PLX4032 abrogates this increase. Bowes cells with empty vector (WT BRAF, clone 8) or BRAF^{V600E} (mBRAF, clones 7 and 21) were incubated in serum-free medium (SFM) or in SFM containing 3 μM PLX4032 (PLX) for 48 h. Real-time RT–PCR was used to measure the mRNA levels of (**A**) IL-1β, (**B**) IL-8, (**C**) IL-6, and (**D**) MMP-1. RT–PCR data were normalised to B_2M, analysed by the $2^{\Delta\Delta(Ct)}$ method (values are relative to clone 8 SFM) and are representative of three experiments. *$P \leqslant 0.05$ compared to clone 8. $^{\#}P \leqslant 0.05$ compared to same clone but with PLX4032 treatment.

Table 1. Conditioned medium from BRAF^{V600E} tumour cells exhibits increased cytokine and MMP-1 levels

Human multiplex cytokine and MMP-1 levels in Bowes cells				
	IL-1β (pg ml^{-1})	**IL-6 (pg ml^{-1})**	**IL-8 (pg ml^{-1})**	**MMP-1 (ng ml^{-1})**
Empty vector clone 8				
CM	0.12	239.42	0.51	ND
CM & PLX	0.08	171.12	0.08	ND
mBRAF clone 7				
CM	5.31	329.83	9.76	ND
CM & PLX	3.28	98.4	2.83	ND
mBRAF clone 21				
CM	1.98	400.72	51.73	19.09
CM & PLX	1.01	194.22	21.83	10.32

Abbreviations: CM = conditioned medium; IL = interleukin; MMP-1 = matrix metalloproteinase-1. Conditioned medium from Bowes clones 7, 8, and 21 was collected and subjected to a human multiplex cytokine assay. Matrix metalloproteinase-1 levels were not detected (ND) for clones 7 and 8.

TME becomes activated (Blackburn *et al*, 2007; Gogas *et al*, 2007; Blackburn and Brinckerhoff, 2008; Melnikova *et al*, 2009; Boni *et al*, 2010; Villares *et al*, 2011; Zigler *et al*, 2011; Khalili *et al*, 2012; Koya *et al*, 2012; Straussman *et al*, 2012; Wang *et al*, 2012). Gene expression of these activated stromal cells is altered as they secrete their own profile of growth factors, cytokines and MMPs, which can disrupt normal stromal architecture (Blackburn *et al*, 2007; Gogas *et al*, 2007; Blackburn and Brinckerhoff, 2008; Blackburn *et al*, 2009; Eck *et al*, 2009; Zigler *et al*, 2011; Lu *et al*, 2012; Straussman *et al*, 2012; Wang *et al*, 2012). The result is the emergence of carcinoma-associated fibroblasts (Eck *et al*, 2009), which express a variety of markers such as alpha-smooth muscle actin, MMPs, stromal-derived factor-1 (SDF-1; CXCL12), and its receptor, CXCR4 (Eck *et al*, 2009; Lu *et al*, 2012). CXCR4 and its ligand SDF-1 are two factors mediating communication between the tumour cells and the TME, and increased expression of CXCR4 and SDF-1 correlates with enhanced tumour progression (Scala *et al*, 2005; Mantovani *et al*, 2010; Teicher and Fricker, 2010; Domanska *et al*, 2013; Portella *et al*, 2013).

Thus, we examined the ability of SF CM from the Bowes melanoma clones to activate HDFs, and whether PLX4032 could attenuate this activation. Figure 3 shows that CM from WT Bowes cells (clone 8) failed to increase expression of CXCR4, and its ligand SDF-1. However, CM from clone 7 mediated a small but significant increase in SDF-1 and CXCR4, while clone 21 gave a greater increase. For both clones, CM from PLX4032-treated tumour cells significantly reduced CXCR4 levels (Figure 3A); SDF-1 levels were also reduced with PLX4032 (Figure 3B). Perhaps the modest increase in SDF-1 with CM from clone 7 is due to the lower level of BRAF mRNA. Indeed, CM from clone 21, with higher expression of BRAF, substantially increased expression of CXCR4 and SDF-1, and CM from PLX4032-treated melanoma cells reduced this expression (Figure 3B). Importantly, non-conditioned SF medium with or without PLX4032 failed to modulate HDF gene expression (light grey bars, Figure 3). These findings suggest that cells with ectopic BRAF^{V600E} secrete factors that activate a CAF-like phenotype in HDFs and BRAF^{V600E} inhibition can mitigate these changes.

We examined the ability of CM from melanoma cells to influence expression of IL-1β, -8 and -6, and of MMP-1 in activated HDFs (Figure 3C–F, respectively). Serum-free CM from the melanoma cells was placed on cultures of HDFs and mRNA was measured at 24 h. CM from WT Bowes slightly increased gene expression in the HDFs, whereas CM from PLX4032-treated melanoma cells did not decrease this expression (Figure 3C–F). In contrast, CM from BRAF^{V600E} clones increased expression of all four-marker genes, which are expressed by cells in the TME (Hwang *et al*, 2004; Goldstein *et al*, 2005; Crawford *et al*, 2008; van Kempen *et al*, 2008; Waugh and Wilson, 2008; Halaban *et al*, 2010; Lu *et al*, 2012; Yin *et al*, 2012). Further, CM from PLX4032-treated tumour cells decreased this expression. Therefore, it is likely that in the tumour cells, PLX4032 decreased production of factors that induce these proteins in the HDFs.

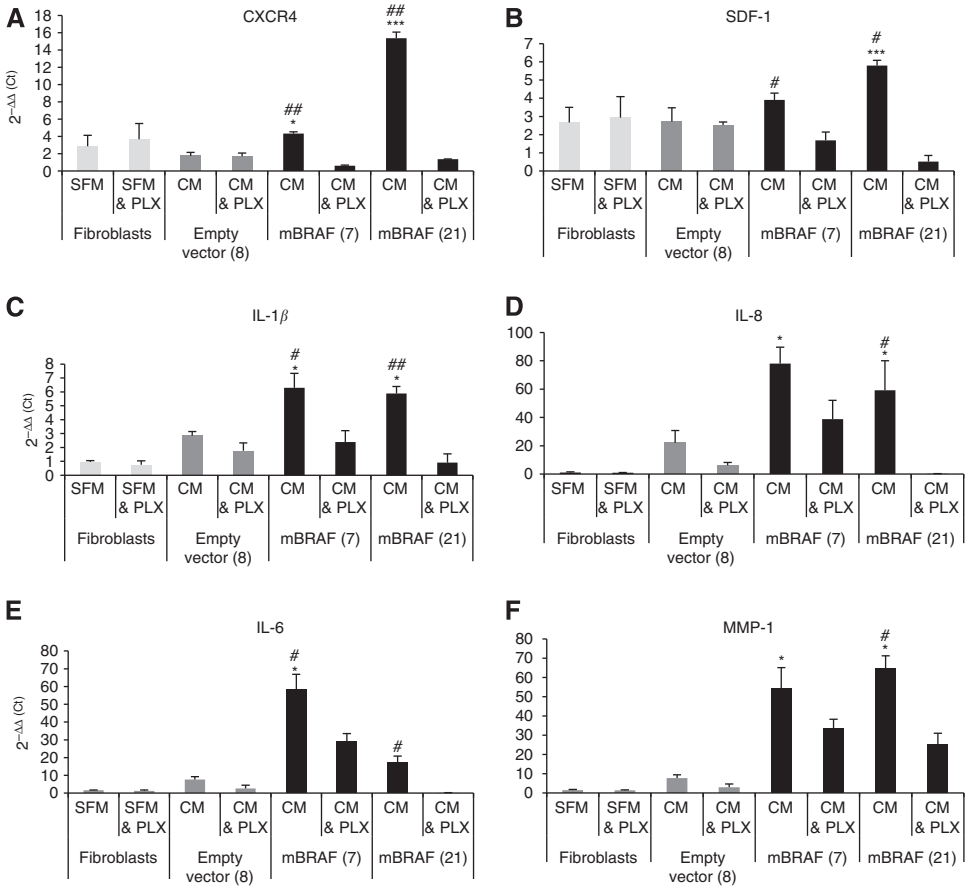

Figure 3. Conditioned medium (CM) from BRAF^{V600E} cells induces MMP-1, cytokine expression, and a CAF-like phenotype. Conditioned medium with or without 3 μM PLX4032 ('CM' and 'CM & PLX', respectively) was isolated from Bowes cells (clones 7, 8, and 21). As a negative control, serum-free medium with or without 3 μM PLX4032 ('SFM' and 'SFM & PLX', respectively) was placed on the human dermal fibroblasts ('Fibroblasts') and incubated for 24 h. Real-time RT–PCR was used to analyse mRNA levels of (**A**) CXCR4, (**B**) SDF-1, (**C**) IL-1β, (**D**) IL-8, (**E**) IL-6, and (**F**) MMP-1. RT–PCR data were normalised to B_2M, analysed by the $2^{\Delta\Delta(Ct)}$ method (values are relative to clone 8) and are representative of three experiments. $^{*}P \leqslant 0.05$, $^{***}P \leqslant 0.0005$ compared to clone 8. $^{\#}P \leqslant 0.05$, $^{\#\#}P \leqslant 0.005$ compared to same clone but with PLX4032 treatment.

Ability of IL-1β to rescue cytokine expression in HDFs. PLX4032 treatment of melanoma cells suppresses the ability of CM from these cells to increased expression of IL-1β, IL-6, IL-8, and MMP-1 in HDFs (Figure 3). Since induction of these proteins occurs in response to IL-1β (Apte *et al*, 2006; Apte and Voronov, 2008; Weber *et al*, 2010a,b), we asked whether adding exogenous IL-1β to the CM from melanoma cells treated with PLX4032 could reverse this reduction. Indeed, exogenous IL-1β significantly increased mRNA levels of IL-8, IL-6, and MMP-1 (even in clone 8; empty vector) over medium alone (Figure 4A–D, respectively). However, exogenous IL-1β could not rescue IL-1β expression (Figure 4A; see Discussion section). We also investigated whether exogenous IL-1β could reverse the reduction in SDF-1 and CXCR4 in the HDFs when these cells were exposed to CM from PLX4032-treated melanoma cells (Figure 3A and B). We found a partial rescue of CXCR4 in clone 21 and a full rescue in clone 7 (Figure 5A). However, IL-1β did not rescue SDF-1 in clone 7 or 21 (Figure 5B).

Tumourigenicity of Bowes cells, WT, and ectopic expression of BRAF^{V600E}. Ectopic expression of MMP-1 can drive tumourigenicity in RGP Bowes cells (Blackburn *et al*, 2009). Therefore, we investigated whether ectopic expression of BRAF^{V600E}, which increases endogenous MMP-1 (Figure 2D), might increase tumourigenicity of these cells when these cells were injected intradermally into nude mice. Figure 6A shows that tumour incidence varied, with 100% (8 of 8) tumour take for clone 21 and 3 of 8 for clones 7 and 8. The tumour incidence and growth rate for clone 8 were similar to that of WT Bowes cells (Blackburn *et al*, 2009), while clone 21 resembled Bowes cells ectopically expressing MMP-1 (Blackburn *et al*, 2009). Thus, our data suggest that while BRAF^{V600E} confers some growth advantage to melanomas, MMP-1 is a mediator of tumourigenicity (see Discussion section).

The time at which mice were killed, tumours were excised and mRNAs for MMP-1, BRAF, and IL-1β were measured. Figure 6B–D demonstrates that clone 21 had higher expression of MMP-1 and BRAF, while clone 7 had the highest level of IL-1β. To confirm that protein levels mirrored mRNA expression in tumours, excised tumours were cultured as explants in SF medium for 48 h and CM subjected to a human multiplex assay for protein levels of selected cytokines and MMP-1. Results reflected those observed with mRNA: clone 7 showed significantly elevated IL-1β levels, whereas clone 21 showed significantly high IL-6, IL-8, and MMP-1 (compared to clone 8; Table 2).

DISCUSSION

In this study, we demonstrate that ectopic expression of BRAF^{V600E} in RGP BRAF WT Bowes melanoma cells confers increased expression of BRAF mRNA and a cohort of genes that are targets of the Ras-Raf-MEK-ERK pathway: cytokines, IL-1β, IL-6 and IL-8, and MMP-1 (Huntington *et al*, 2004; Ryu *et al*, 2011;

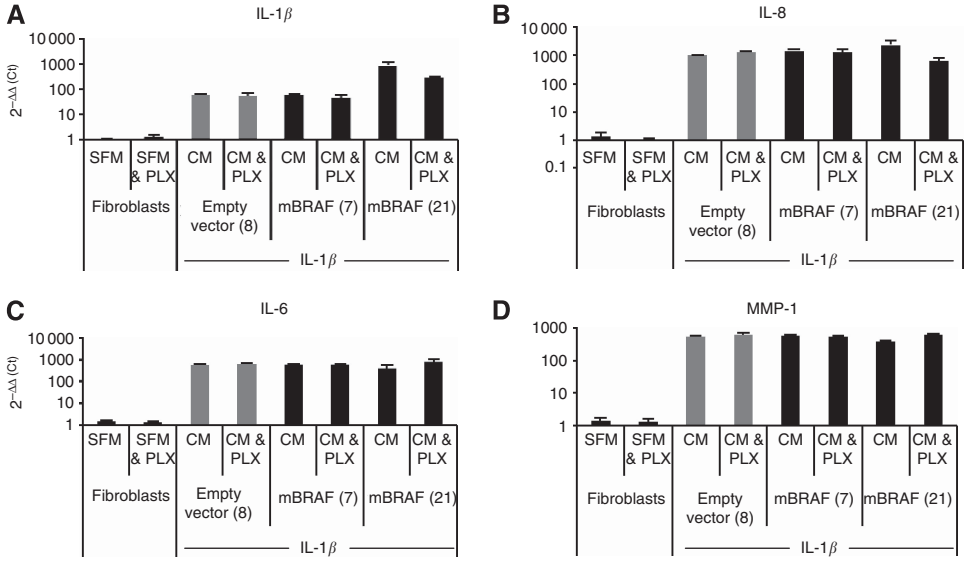

Figure 4. Exogenous addition of IL-1β restores cytokine and MMP-1 levels. Conditioned medium (CM) with or without 3 μM PLX4032 ('CM' and 'CM & PLX', respectively) was isolated from Bowes cells (clones 7, 8, and 21). Next, 10 ng ml^{-1} of IL-1β was added to the CM isolated from the Bowes cells and incubated on the HDFs for 24 h. As a negative control, serum-free medium with or without 3 μM PLX4032 ('SFM' and 'SFM & PLX', respectively) was placed on the human dermal fibroblasts ('Fibroblasts') and incubated for 24 h. Total RNA was isolated from HDFs and real-time RT–PCR analysis was used to measure (**A**) IL-1β, (**B**) IL-8, (**C**) IL-6, and (**D**) MMP-1 mRNA levels. No significant difference in expression was observed in the HDFs with or without the presence of PLX4032. Data are shown on a log10 scale.

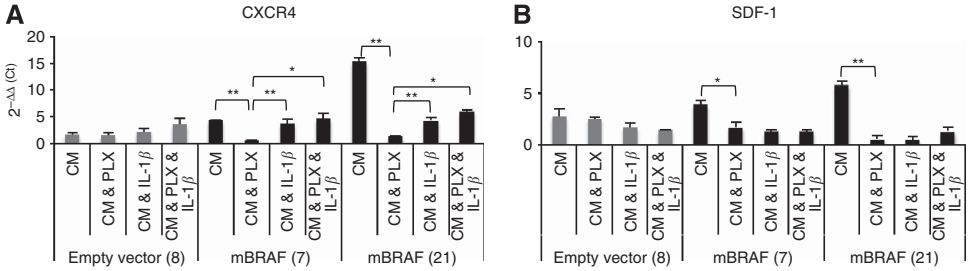

Figure 5. Conditioned medium (CM) from BRAF^{V600E} cells increases the production of CXCR4 and SDF-1 by human dermal fibroblasts. Conditioned medium was collected from Bowes cells (clones 7, 8, and 21) that had been incubated for 48 h in serum-free medium either with or without 3 μM PLX4032. This CM was placed on human dermal fibroblasts ('Fibroblasts') and allowed to incubate on these cells for 24 h ('CM' and 'CM & PLX'). Simultaneously, but separately, 10 ng ml^{-1} of IL-1β was added to a portion of the CM collected from the Bowes cells, placed on the HDFs, and incubated for 24 h ('CM and IL-1β' and 'CM and PLX and IL-1β'). RNA was then isolated from each HDF sample and real-time RT–PCR was utilised to analyse (**A**) CXCR4 and (**B**) SDF-1 mRNA. *$P \leqslant 0.05$ and **$P \leqslant 0.005$.

Frederick *et al*, 2013; Sullivan and Flaherty, 2013), a major mediator of melanoma progression (Hofmann *et al*, 2005; Blackburn *et al*, 2009; Hua *et al*, 2011). BRAF^{V600E} also increased expression of pMEK, which is abrogated by the small molecule inhibitor, PLX4032 (Ascierto *et al*, 2012). We show that CM from these BRAF^{V600E} Bowes cells contains secreted factors that upregulate this same panel of target genes in human dermal fibroblasts. In addition, these fibroblasts express a CAF-like phenotype, with increases in SDF-1 and its receptor CXCR4, suggesting that BRAF^{V600E} melanoma cells co-opt adjacent stromal cells in the TME and facilitate melanoma progression. Finally, we note that tumours from Bowes cells expressing BRAF^{V600E} are more tumourigenic and grow more rapidly than their WT counterparts.

In vitro, BRAF mRNA levels fluctuate depending on whether cells are cultured in serum-containing or SF medium (Figure 1A and B). *In vivo*, the rate of tumour growth is associated with the level of BRAF mRNA, with the higher expressing clone (clone 21) more tumourigenic than the lower expresser (clone 7). Interestingly, high levels of BRAF mRNA expression correlated with high levels of MMP-1, confirming our previous studies showing that MMP-1 is a target of the Ras-Raf-MEK-ERK pathway in melanoma (Huntington *et al*, 2004; Blackburn and Brinckerhoff, 2008; Blackburn *et al*, 2009). Here we show that PLX4032 abrogates MMP-1 expression, thereby demonstrating that MMP-1 is a target of BRAF^{V600E}.

We attribute differences in BRAF expression in clones 7 and 21 to a variation in the insertion site within genomic DNA (Kay *et al*, 2001; Schroder *et al*, 2002), which influences mRNA expression. Of potential importance, the higher level of BRAF mRNA seen in clone 21 may mimic a recent report describing BRAF^{V600E} amplification in melanoma specimens (Koya *et al*, 2012; Shi *et al*, 2012), which appears to be necessary and sufficient for acquired resistance to PLX4032 (Koya *et al*, 2012; Shi *et al*, 2012). Thus, this clone may be a valuable experimental tool for investigating the molecular and biological effects of amplified BRAF on proteins secreted by the melanoma cells that affect the TME.

Khalili *et al* (2012) reported that ectopic expression of BRAF^{V600E} in human primary melanocytes induces IL-1α and β as well as IL-8, which along with IL-6 constitute a cohort of target genes that can promote tumour growth by regulating a network of cytokines (Walker and Woolley, 1999; Lederle *et al*, 2011). In addition, they showed that increased IL-1β levels were associated

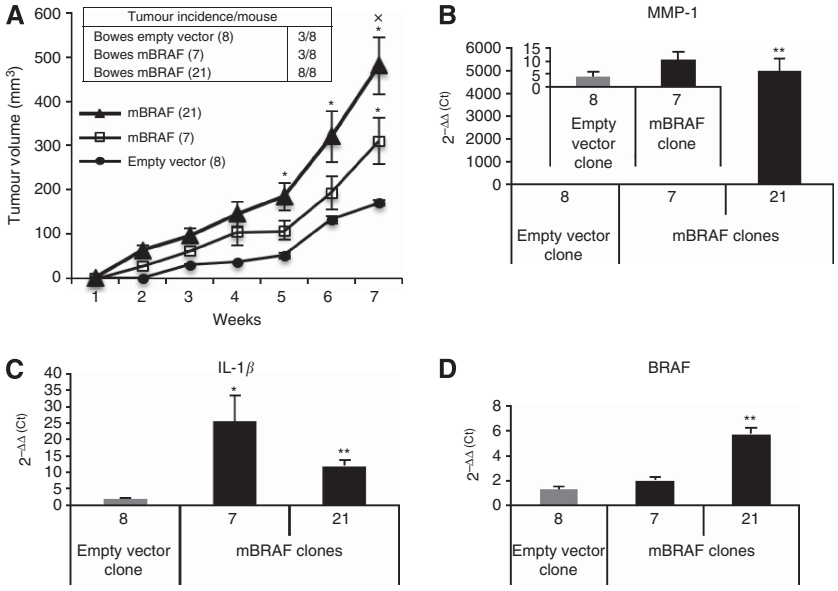

Figure 6. MMP-1 and BRAF^{V600E} drive tumour growth in nude mice. (**A**) Bowes cells with empty vector (clone 8), mBRAF (clone 7), and mBRAF (clone 21) were injected intradermally into nude mice (10^6 cells per injection). Tumours were measured weekly with calipers and tumour incidence is shown on graph. When tumour burden became large, the mice were killed, tumour pieces were flash frozen, and total RNA was subsequently isolated. Real-time RT–PCR analysis was utilised to measure (**B**) MMP-1, (**C**) IL-1β, and (**D**) BRAF mRNA levels. *$P \leqslant 0.05$ compared to clone 8; **$P \leqslant 0.005$ compared to clone 8; and $^{\times}P \leqslant 0.05$ compared to clone 7.

Table 2. Tumour explant medium collected from BRAF^{V600E} tumours shows heightened cytokine and MMP-1 levels

Human multiplex cytokine and MMP-1 levels in tumour explant media				
	IL-1β (pg ml^{-1})	**IL-6 (pg ml^{-1})**	**IL-8 (pg ml^{-1})**	**MMP-1 (ng ml^{-1} of tumour)**
Empty vector clone 8	0.42 ± 0.05	225.01 ± 50.06	9.52 ± 0.49	ND
mBRAF Clone 7	6.77 ± 0.07	137.58 ± 56.09	8.12 ± 0.73	26.5 ± 26.5
mBRAF Clone 21	1.02 ± 0.41	478.67 ± 116.09	14.79 ± 3.73	538.27 ± 108.88

Abbreviations: IL = interleukin; MMP-1 = matrix metalloproteinase-1; ND = not detected. Tumours were isolated from mice and a portion of each tumour was weighed and placed in serum-free culture medium for 48 h. Data are an average of three tumours for empty vector clone 8, three tumours for mBRAF clone 7, and seven tumours for mBRAF clone 21.

with stromal cell-mediated immunosuppression, and that vemurafenib decreased IL-1β, leading them to conclude that BRAF^{V600E} promoted stromal cell-mediated immunosuppression via induction of IL-1β (Khalili *et al*, 2012).

Our data suggest that IL-1β is a master regulator of gene expression in melanoma cells and stromal cells. We treated the Bowes cells with PLX4032 and found that expression of IL-1β, IL-8, IL-6, and MMP-1 were all reduced, as expected. However, we could rescue IL-8, IL-6, and MMP-1 expression with exogenous IL-1β, suggesting that this cytokine is an upstream regulator of these genes (Lazar-Molnar *et al*, 2000; Apte *et al*, 2006; Apte and Voronov, 2008). Further, tumour-derived IL-1β may increase gene expression in the stromal cells, since IL-1β is a known inducer of growth factors, chemokines (specifically SDF-1 and CXCR4), and MMP-1 (Apte *et al*, 2006; Apte and Voronov, 2008; Weber *et al*, 2010a,b). However, since exogenous IL-1β could not restore expression of IL-1β in the melanoma cells, IL-1β does not appear to be regulated by an autocrine feedback loop.

Finally, we note the correlation among increased BRAF, MMP-1, and tumourigenicity. We have shown that ectopic expression of MMP-1 in Bowes cells confers tumourigenicity and metastasis (Blackburn *et al*, 2009). In those studies, MMP-1 mediated invasion and metastasis by degradation of the interstitial collagens, and it cleaved protease activator receptor-1 to mediate signal transduction and the expression of genes associated with tumour invasion and angiogenesis (Blackburn *et al*, 2007; Blackburn and Brinckerhoff, 2008; Blackburn *et al*, 2009). It is likely that the levels of MMP-1 in clone 21 may have mediated the tumourigenicity by similar mechanisms. Although other genes probably contribute, our data suggest a mechanistic link between BRAF^{V600E} and MMP-1 in mediating melanoma progression. They also suggest that activation of similar genes in adjacent fibroblasts creates a 'spill-over' mechanism to enhance the tumourigenic behaviour of melanoma cells with BRAF^{V600E}.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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