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Global Analysis of BRAF^{V600E} Target Genes in Human Melanocytes Identifies Matrix Metalloproteinase-1 as a Critical Mediator of Melanoma Growth

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TO THE EDITOR

BRAF kinase has been found to be mutationally activated in up to 70% of benign nevi and melanomas (Davies *et al.*, 2002). It has been implicated as a critical mediator of melanoma development, with the V600E activating mutation representing the most commonly mutated form of BRAF in nevi and melanomas (Pollock *et al.*, 2003). Despite strong evidence implicating BRAF kinase as a bona-fide oncogene in melanoma, its precise downstream targets in melanocytes have not been defined to date, and a BRAF-specific gene signature in melanomas remains uncertain (Hoek *et al.*, 2006).

We have introduced activated BRAF^{V600E} into human primary melanocytes (HPMs) in order to assess its specific functions (Figure 1a and also see the supplemental information for details of experimental methods). The gene expression signature of HPMs induced by acute expression of the BRAF^{V600E} was assessed in comparison to HPMs expressing GFP (Figure 1c). The complete dataset is accessible as GSE13827[a]. We found that the BRAF^{V600E} signature of HPMs was characterized by upregulation of several growth promoting genes and cellular motility and inflammation associated genes (Figure 1b) with a common network activation of cellular growth/proliferation and apoptosis (Figure S1). This

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Conflict of Interest

The authors declare no conflicts of interest.

[a]http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13827

finding suggests that $BRAF^{V600E}$ may induce gene signatures of biphasic cellular responses, proliferation as observed in melanomas and oncogene-induced growth arrest observed in nevi. Detection of genes involved in proliferation such as *MMP1*, *AREG*, *CXCL5*, *IL-8*, and *EREG* (Figure 1c and Table S1) implies that short-term cellular response by acute $BRAF^{V600E}$ expression may be HPM proliferation rather than growth arrest which appears to be a long-term sustained effect of $BRAF^{V600E}$ (Michaloglou *et al.*, 2005; Woods *et al.*, 1997). Therefore, we reasoned that the cellular proliferation signal induced by the $BRAF^{V600E}$ in HPM may be reactivated in melanoma for tumor growth. In order to test this hypothesis, we sought to further examine functional roles of MMP-1 as a BRAF effector in melanoma because MMP-1 is most strongly induced by BRAF (Table S1) and reported to be involved in melanoma progression and metastasis (Blackburn *et al.*, 2007).

In order to determine whether MMP-1 expression correlated with $BRAF^{V600E}$ expression in melanomas, melanoma cell lines and HPMs were examined for MMP-1 mRNA expression using gene expression profiling as previously described (Ryu *et al.*, 2007). We identified 25-fold increased expression of MMP-1 mRNA in melanoma cells possessing $BRAF^{V600E}$ compared to wildtype while HPMs expressed similar transcript levels with BRAF wildtype melanoma cell (Figure 2a), suggesting that increased BRAF kinase activity may be associated with elevated MMP-1 expression in melanomas. We also found that melanocytes expressing $BRAF^{V600E}$ have increased levels of secreted MMP-1 protein (Figure 2b) and collagenase activity (Figure 2c) versus HPM controls, suggesting that activated BRAF can induce both MMP-1 protein expression and activity. In order to determine the functional significance of BRAF kinase induction of MMP-1 in human melanomas, we assessed the effect of MMP-1 gene silencing on the proliferative functions of BRAF kinase. MMP-1 mRNA and protein levels were efficiently reduced in melanomas possessing either wildtype BRAF (WM852) or mutant $BRAF^{V600E}$ (WM793) using targeted MMP-1 siRNA (Figure 2d and 2e). Cellular proliferation was assessed in both BRAF wildtype and mutant melanomas following MMP-1 silencing by siRNA (Figure 2f) and a neutralizing MMP-1 antibody (data not shown). Significant inhibition of proliferation was seen in both BRAF wildtype and mutant melanoma cells following MMP-1 knockdowns; however, while cell growth was inhibited by 17% with MMP-1 siRNA versus control siRNA in BRAF wildtype melanomas, growth inhibition by MMP-1 siRNA in the BRAF mutant melanoma cells was significantly more effective at 80% inhibition despite comparable gene silencing (Figure 2f). We therefore conclude that $BRAF^{V600E}$ may promote cellular growth in melanomas through activated expression of MMP-1. It should be noted that MMP-1 silencing by RNAi was previously shown to affect only metastasis but not tumor growth in a melanoma cell line (VMM12) (Blackburn *et al.*, 2007). However, Blackburn *et al.* also reported that stable overexpression of MMP-1 in Bowers melanoma cells promotes xenograft tumor growth in a recent study (Blackburn *et al.*, 2009). These data suggest variable effects of MMP-1 in melanoma cell lines likely attributable to the molecular heterogeneity of melanomas. It is likely that WM792 and Bowers melanoma cells depend on BRAF/MMP-1 mediated cellular pathways for tumor growth which may not be the case for VMM12 melanoma cells. As we were able to show that MMP-1 promotes growth in melanoma cells expressing $BRAF^{V600E}$, we sought to clarify functional targets for MMP-1 in this setting. Since amphiregulin (AREG), a ligand for the epidermal growth factor receptor (EGFR), was also found to be

significantly induced by $BRAF^{V600E}$ in HPMs (Figure 1c and Table S1) and is synthesized as a precursor protein that is released from the plasma membrane by metalloproteinases (Lu *et al.*, 2009; Zhang *et al.*, 2004), we sought to evaluate whether HPMs expressing $BRAF^{V600E}$ expressed elevated levels of activated AREG. We found >100-fold expression of activated AREG in HPMs expressing $BRAF^{V600E}$ versus controls (Figure 2g). In order to determine whether induction of activated AREG in HPMs expressing $BRAF^{V600E}$ was due to cleavage by activated MMP-1, we evaluated the effect of silencing MMP-1 on expression of activated AREG in melanoma cells expressing $BRAF^{V600E}$ versus wildtype BRAF-expressing melanoma cells. We found that silencing of MMP-1 led to a significant reduction in levels of cleaved AREG in $BRAF^{V600E}$ melanoma cells, but no significant change in expression in the BRAF-wildtype melanoma cells (Figure 2h).

Although previous studies suggested that BRAF kinase activity promotes expression of MMP-1 in melanoma (Huntington *et al.*, 2004) and that MMP-1 promotes melanoma progression (Blackburn *et al.*, 2007), these authors conclude that induction of MMP-1 in melanoma is specifically important for melanoma progression and metastasis through degradation functions on interstitial collagens. Here we show that MMP-1 is a critical mediator of the growth promoting functions of BRAF kinase in melanoma cells which is consistent with a proliferative role for $BRAF^{V600E}$ in the development of melanomas. Indeed, recent studies have suggested an additional important role for MMPs in activating latent growth factors which may be critical to the effects of MMP-1 seen in our studies. Notably, MMP-1 has been implicated in activating breast cancer and melanoma cell growth through proteolytic activation of the cell surface receptor PAR1 (Blackburn *et al.*, 2009; Boire *et al.*, 2005). Together with the report that EGFR is highly expressed in vertical growth phase primary (89%) and metastatic melanoma (80%) (Rodeck *et al.*, 1991), data presented in this study demonstrating the growth promoting function of MMP-1 in human melanomas, suggests that $BRAF^{V600E}$ induced activation of an autocrine feedback loop (MMP-1/AREG/EGFR/RAS/BRAF) may play a critical role in melanoma growth and metastasis. It is also possible that tumor cell-induced AREG expression and activation may affect the growth of neighboring endothelial and stromal cells. This may further promote tumor cell metastasis by modulating the tumor-specific microenvironment. Taken together, this feedback loop could be an important player for melanoma progression and a molecular target for melanoma therapy. Further studies are currently ongoing to test this hypothesis using EGFR inhibitors in our experimental model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HPM Human Primary Melanocyte

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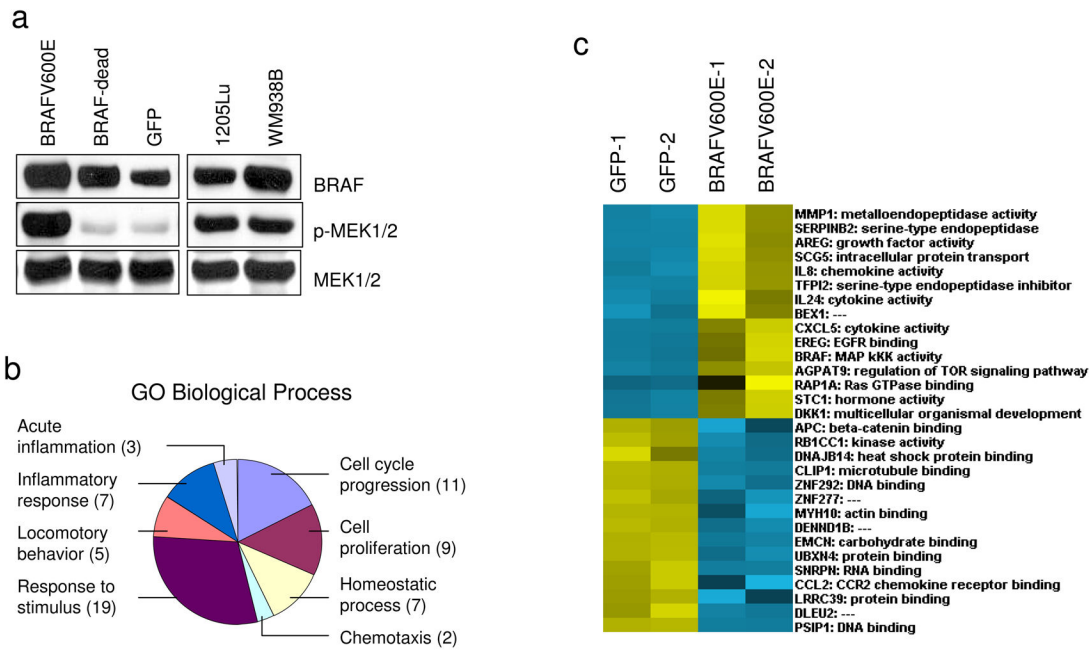

Figure 1. A proliferative gene signature induced by oncogenic BRAF in human primary melanocyte (HPM) and gene ontology (GO) annotation analysis

(a) Activation of MAPK signal transduction pathway by acute expression of $BRAF^{V600E}$ in HPM. (b) A pie chart of the GO annotation analysis. Eighty two annotated genes from 137 probe sets which are identified as greater than 3-fold differentially expressed genes were analyzed. p-value <0.005 was used for identification of the biological processes that may be regulated by BRAF downstream effectors. Numbers shown in parenthesis indicate number of genes classified as the suggested categories. (c) Heat map presentation of the downstream effector gene signature induced by $BRAF^{V600E}$. Top 15 up- and down-regulated genes were shown. GFP; HPM expressing GFP, $BRAF^{V600E}$; HPM expressing mutant $BRAF^{V600E}$.

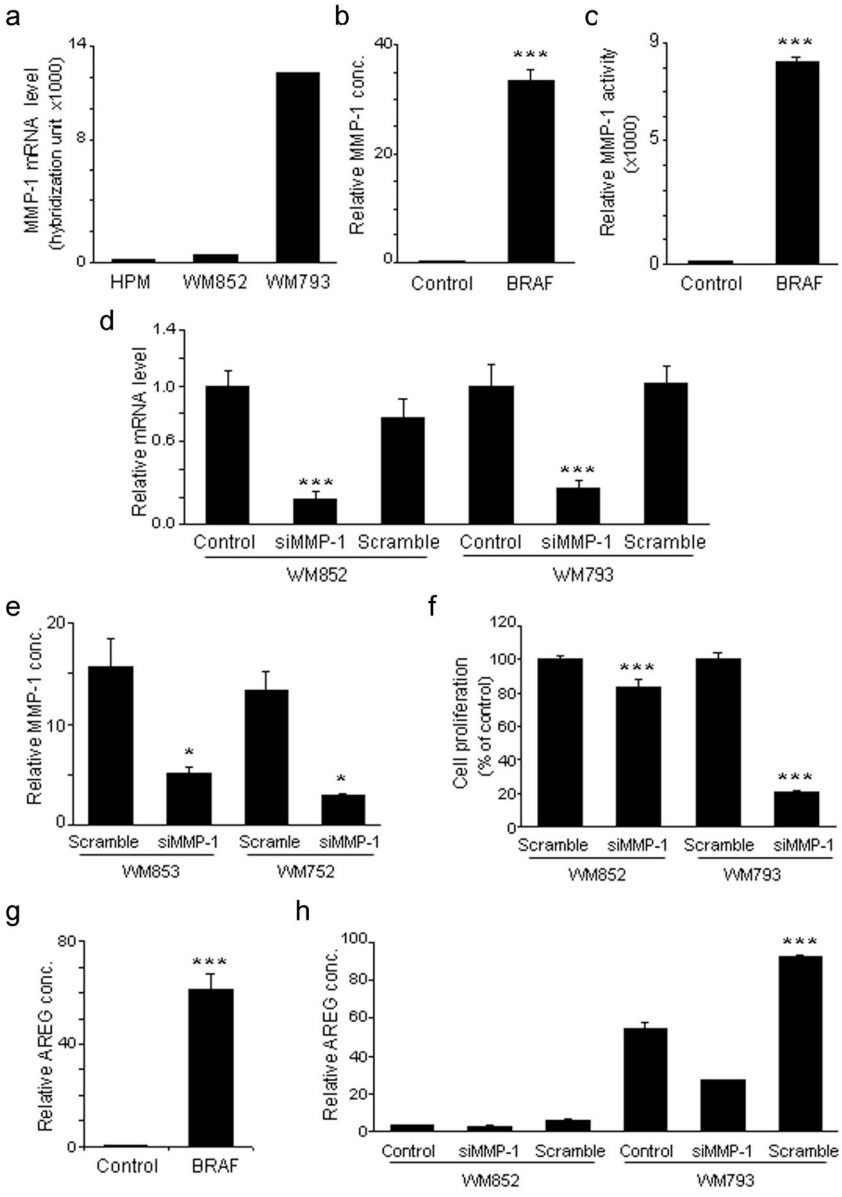

Figure 2. Activated BRAF promotes melanoma cell growth by MMP-1

(a) Relative MMP-1 mRNA levels in HPMs and melanoma cells expressing wildtype (WM852), or mutant BRAF (WM793). (b) Relative levels of secreted MMP-1 in conditioned media obtained from HPMs expressing GFP or $BRAF^{V600E}$ at 72 hours following lentiviral infection. (c) Relative MMP-1 collagenase activity in conditioned media obtained from HPMs expressing GFP or $BRAF^{V600E}$ at 72 hours following lentiviral infection. (d) qRT-PCR analysis of MMP-1 expression following gene silencing by siRNA in melanomas possessing either wildtype (WM852) or mutant $BRAF^{V600E}$ (WM793). (e) Relative MMP-1 concentration in cell culture media following MMP-1 gene silencing in melanomas possessing wildtype (WM852) and $BRAF^{V600E}$ (WM793) cells. (f). ^{3}H-thymidine cell proliferation assay of melanomas possessing wildtype (WM852) and $BRAF^{V600E}$ (WM793) following MMP-1 gene silencing. (g) Relative expression of

activated AREG in conditioned media from HPMs expressing GFP or $BRAF^{V600E}$. (h) Relative expression of activated AREG in melanomas expressing wildtype (WM852), or mutant BRAF (WM793) following MMP-1 gene silencing. *Columns*, mean of three individual experiments done in triplicate; *bars*, SD. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, compared with GFP control in the Figure 2b, c, g, and compared with siRNA control (Scramble) in the Figure 2d, e, f, h.