crossover trial in healthy volunteers. *Acta Derm Venereol* 70:429–31

Korting HC, Lukacs A, Vogt N *et al.* (1992) Influence of the pH-value on the growth of *Staphylococcus epidermidis, Staphylococcus aureus* and *Propionibacterium acnes* in continuous culture. *Zentralbl Hyg Umweltmed* 193:78–90

Krien PM, Kermici M (2000) Evidence for the existence of a self-regulated enzymatic process within the human stratum corneum—an unexpected role for urocanic acid. *J Invest Dermatol* 115:414–20

Mauro T, Holleran WM, Grayson S *et al.* (1998) Barrier recovery is impeded at neutral pH, independent of ionic effects: implications for extracellular lipid processing. *Arch Dermatol Res* 290:215–22

Presland RB, Boggess D, Lewis SP *et al.* (2000) Loss of normal profilaggrin and filaggrin in flaky tail (ft/ft) mice: an animal model for the filaggrin-deficient skin disease ichthyosis vulgaris. *J Invest Dermatol* 115: 1072–81

Puhvel SM, Reisner RM, Sakamoto M (1975) Analysis of lipid composition of isolated human sebaceous gland homogenates after incubation with cutaneous bacteria. Thin-layer chromatography. *J Invest Dermatol* 64:406–11

Rawlings AV, Matts PJ (2005) Stratum corneum moisturization at the molecular level: an update in relation to the dry skin cycle. *J Invest Dermatol* 124:1099–110

Schmid-Wendtner MH, Korting HC (2006) The pH of the skin surface and its impact on the barrier function. *Skin Pharmacol Physiol* 19:296–302

Selden C, Calnan D, Morgan N *et al.* (1995) Histidinemia in mice: a metabolic defect treated using a novel approach to hepatocellular transplantation. *Hepatology* 21:1405–12

Thueson DO, Chan EK, Oechsli LM *et al.* (1998) The roles of pH and concentration in lactic acid-induced stimulation of epidermal turnover. *Dermatol Surg* 24: 641–5

Oncogenic B-RAF^{V600E} Promotes Anchorage-Independent Survival of Human Melanocytes

Journal of Investigative Dermatology (2010) **130,** 2144–2147; doi:10.1038/jid.2010.117; published online 6 May 2010

TO THE EDITOR

Activation of the extracellular signal-regulated kinase (ERK) pathway has an undisputed role in melanoma development. The majority of human melanomas harbor activating mutations in N-RAS (4–50% of primary melanoma) or B-RAF (20–80% of primary melanoma) (reviewed by Platz *et al.*, 2008). These mutations are mutually exclusive, appear early, and are detectable in up to 80% of benign nevi (reviewed by Reifenberger *et al.*, 2002; Thomas, 2006). Thus, aberrant ERK signaling is not sufficient to induce melanoma but triggers an initial cycle of melanocyte proliferation that is subsequently limited by the onset of cellular senescence (Michaloglou *et al.*, 2005).

There is also accumulating evidence that oncogenic activity differences may exist between activated N-RAS and B-RAF, as demonstrated by the unique transcriptional signatures resulting from mutations in these oncogenes (Pavey *et al.*, 2004) and the distinct histology of B-RAF versus N-RAS mutation-positive melanomas (Viros *et al.*, 2008). To evaluate the oncogenic activity of activated N-RAS and B-RAF, the melanoma-associated N-RAS^{Q61K} or B-RAF^{V600E} mutants were transduced into two primary human melanocytes (HEM1455 and HEM1259). As expected, the accumulation of wild-type B-RAF or the coexpressed copepod green fluorescent protein (copGFP) did not induce ERK activation, whereas N-RAS^{Q61K} and B-RAF^{V600E} induced increased levels of phosphorylated ERK (Figure 1a). Importantly, the level of ERK phosphorylation in oncogene-transduced melanocytes was comparable with endogenous ERK phosphorylation in melanoma cells with activated mitogen-activated protein kinase signaling (Supplementary Figure S1 online).

The accumulation of B-RAF^{V600E}, but not wild-type B-RAF, oncogenic N-RAS, or copepod green fluorescent protein, also induced dramatic morphological changes; melanocytes displayed fewer dendrites, appeared rounded, and the majority detached from the culture flask (Figure 1b and c). Importantly, V600E-induced loss of substrate adhesion continued for the length of our experiments, up to 7 days post-transduction (data not shown). The resulting suspension melanocytes appeared rounded, viable, formed loose clusters, and continued to express B-RAF^{V600E} and activated ERK (Figure 1a and b). Trypan blue exclusion assays demonstrated that approximately 90% of B-RAF^{V600E} expressing suspension melanocytes remained viable, whereas a substantial proportion of the suspension melanocytes expressing wild-type B-RAF, N-RAS^{Q61K}, or copepod green fluorescent protein (suspension cells accounted for only 2–10% of these populations) were not viable (Figure 1c).

To determine whether the impact of oncogenic B-RAF on cell adhesion was specific to melanocytes, we repeated the transduction experiments in two primary human dermal fibroblasts (HDF1314 and WS-1). Oncogenic B-RAF and N-RAS activated the ERK pathway. Occasionally, wild-type B-RAF also induced ERK phosphorylation in these cells (Figure 1a), and although this has been observed previously (Gray-Schopfer *et al.*, 2006) it was not a consistent finding in our studies. Expression of oncogenic B-RAF also altered the morphology of fibroblasts (Figure 1d), but these cells remained adhered (Figure 1e). Moreover, the small proportion of B-RAF^{V600E} fibro-

Abbreviation: ERK, extracellular signal-regulated kinase

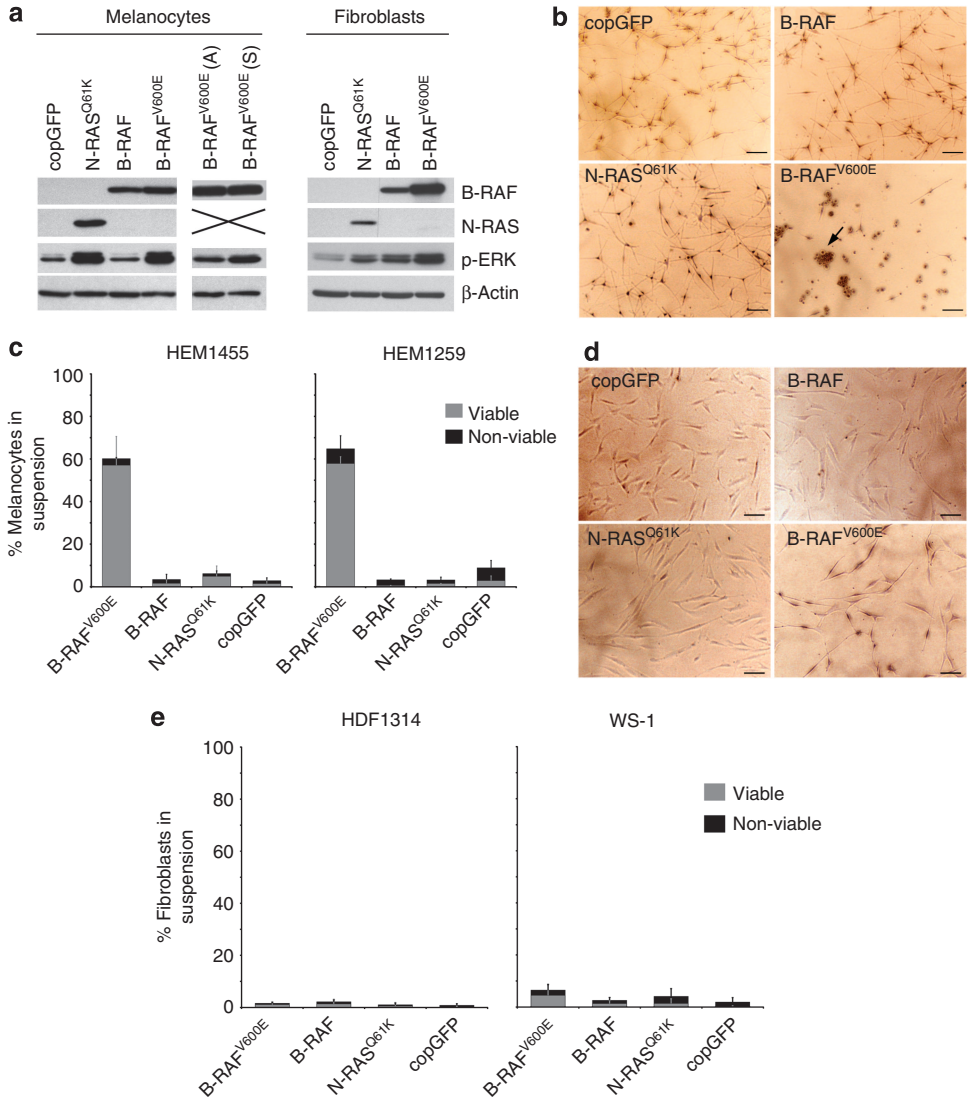

Figure 1. B-RAF^{V600E} promotes loss of substrate adhesion in human melanocytes. (**a**) Melanocytes and fibroblasts were infected with the indicated lentivirus constructs (Haferkamp *et al.*, 2009). The levels of phosphorylated extracellular signal-regulated kinase (p-ERK) were compared 3 days post-infection in adhered and suspension cells, suspension cells only (S), or adherent cells only (A). Adequate numbers of suspension cells could only be obtained from B-RAF^{V600E}-transduced melanocytes, and no N-RAS samples are included in the suspension-only blot. (**b**) Morphological changes induced in melanocytes transduced for 3 days with the indicated constructs. A cluster of suspension melanocytes expressing B-RAF^{V600E} is indicated with an arrow (bar = 100 μm). (**c**) Melanocytes were transduced with the indicated constructs for 3 days and the percentage of viable and non-viable suspension cells was determined by trypan blue exclusion. (**d**) Morphological changes in fibroblasts transduced with the indicated expression constructs (bar = 100 μm). (**e**) Fibroblasts were transduced with the indicated lentiviral constructs for 3 days and the percentage of viable and non-viable suspension cells was determined.

blasts in suspension (between 0.5 and 8%) showed low viability (Figure 1e). These data confirm that cell rounding and loss of substrate adhesion are specific to oncogenic B-RAF in melanocytes.

When anchorage-dependent cells detach from the extracellular matrix, they usually undergo apoptotic cell death known as anoikis, and acquired resistance to anoikis is associated with transformation and immortalization (reviewed by Gilmore, 2005). To examine whether oncogenic B-RAF^{V600E} prevented anoikis in human melanocytes, the proportion of cells undergoing apoptosis was determined using annexin V and propidium iodide staining. The loss of adhesion induced by B-RAF^{V600E} did not induce anoikis; 80–90% of suspension melanocytes remained viable (Figure 2a and b). In contrast, the minor proportion of melanocytes in suspension post-transduction with copepod green fluorescent protein or wild-type B-RAF underwent apoptosis (Figure 2a and b). Intriguingly, although mutant N-RAS did not induce a significant loss of adhesion (see Figure 1c), a considerable proportion of N-RAS^{Q61K}-positive suspension melanocytes remained viable, suggesting that anoikis prevention may involve the common downstream ERK pathway (Figure 2a and b). Indeed, N-RAS^{Q61K} has been shown to prevent anoikis efficiently by Bim repression in immortalized PIG1 melanocytes (Goldstein *et al.*, 2009).

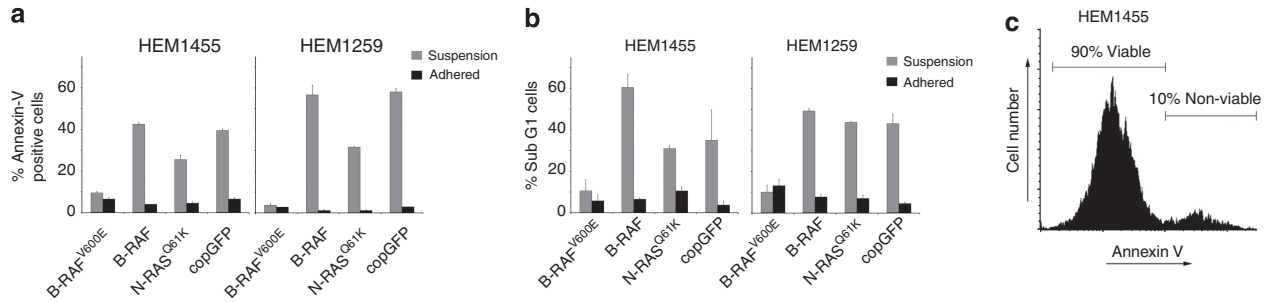

Figure 2. B-RAF^{V600E} prevents anoikis. (**a**) Adhered and suspension melanocytes transduced with the indicated lentiviral constructs for 3 days were analyzed for the proportion of apoptotic cells using annexin V staining by flow cytometry analysis. (**b**) The same cell populations were tested for their proportion of apoptotic, sub-G1 cells with propidium iodide staining and flow cytometry analysis. (**c**) B-RAF^{V600E}-transduced melanocytes were maintained in suspension culture for 3 weeks and tested for the proportion of apoptotic cells with annexin V staining by flow cytometry analysis.

It is worth noting that protection from anoikis has been associated with cell cycle arrest (Collins *et al.*, 2005) and B-RAF^{V600E} rapidly arrests melanocytes (Supplementary Figure S2a online). It is unlikely that arrest is the only requirement for protection from anoikis, however, as N-RAS^{Q61K} also promotes arrest in melanocytes, and we have not detected a significant increase in suspension melanocytes up to 15 days post-N-RAS^{Q61K} transduction (Haferkamp *et al.*, 2009). Furthermore, the B-RAF^{V600E}-positve NM182 melanoma cells have 10–15% of cells in suspension and these cells show no signs of cell cycle arrest and depend on B-RAF^{V600E} signaling for their survival (Supplementary Figure S2b, S2c online). These data suggest that B-RAF^{V600E} can protect melanocytes from anoikis independently of cell cycle inhibition.

To ensure that anoikis was not simply delayed in response to B-RAF^{V600E} expression, the suspension melanocytes were maintained for up to 3 weeks post-transduction. These cells remained rounded, in loose clusters and most were viable (Figure 2c). These findings demonstrate that oncogenic B-RAF is distinct from oncogenic N-RAS in inducing cell detachment and preventing anoikis of human melanocytes. This is supported by recent reports that confirmed B-RAF^{V600E} inhibited anoikis in melanoma cell lines by repressing the proapoptotic Bim and Bad proteins and inducing the prosurvival Mcl-1 protein (Boisvert-Adamo and Aplin, 2006; Boisvert-Adamo *et al.*, 2009).

The impact of B-RAF^{V600E} on melanocyte adhesion and survival could conceivably increase cell motility and may account for the unique histopathological characteristics of B-RAF^{V600E} melanomas, including upward migration of cells into the epidermal layer and intraepidermal ''nest'' formation (Viros *et al.*, 2008). Our data are also in line with evidence demonstrating that oncogenic B-RAF is more potent than N-RAS in supporting anchorage independency. In particular, melanoma cells derived from a single tumor carrying cells with the B-RAF^{V600E} or N-RAS^{Q61R} mutation formed significantly more soft agar colonies and exhibited greater viability in suspension if they were B-RAF^{V600E} positive (Sensi *et al.*, 2006). Taken together, these data highlight that activated B-RAF may not only signal through the mitogen-activated protein kinase cascade but may also signal with different intensity or through alterative pathways that regulate the adhesion and survival of melanocytes. These B-RAF-specific pathways deserve careful exploration as they may confer distinct proliferative and survival characteristics on B-RAF^{V600E} melanomas.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work is supported by the National Health and Medical Research Council of Australia (NHMRC), the Cancer Council of New South Wales, and an infrastructure grant to Westmead Millennium Institute by the Health Department of NSW through Sydney West Area Health Service. Westmead Institute for Cancer Research is the recipient of capital grant funding from the Australian Cancer Research Foundation. LS is a Cameron Melanoma Research Fellow, supported by the Melanoma and Skin Cancer Research Institute, University of Sydney. HR is a Cancer Institute New South Wales Research Fellow.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Boisvert-Adamo K, Aplin AE (2006) B-RAF and PI-3 kinase signaling protect melanoma cells from anoikis. *Oncogene* 25:4848–56

Boisvert-Adamo K, Longmate W, Abel EV *et al.* (2009) Mcl-1 is required for melanoma cell resistance to anoikis. *Mol Cancer Res* 7:549–56

Collins NL, Reginato MJ, Paulus JK *et al.* (2005) G1/S cell cycle arrest provides anoikis resistance through Erk-mediated Bim suppression. *Mol Cell Biol* 25:5282–91

Gilmore AP (2005) Anoikis. *Cell Death Differ* 12(Suppl 2):1473–7

Goldstein NB, Johannes WU, Gadeliya AV *et al.* (2009) Active N-Ras and B-Raf inhibit anoikis by downregulating Bim expression in melanocytic cells. *J Invest Dermatol* 129:432–7

Gray-Schopfer VC, Cheong SC, Chong H *et al.* (2006) Cellular senescence in naevi and immortalisation in melanoma: a role for p16? *Br J Cancer* 95:496–505

Haferkamp S, Scurr LL, Becker TM *et al.* (2009) Oncogene-induced senescence does not require the p16(INK4a) or p14ARF melanoma tumor suppressors. *J Invest Dermatol* 128:1983–91

Michaloglou C, Vredeveld LC, Soengas MS *et al.* (2005) BRAFE600-associated senescence-like

cell cycle arrest of human naevi. *Nature* 436:720–4

Pavey S, Johansson P, Packer L *et al.* (2004) Microarray expression profiling in melanoma reveals a BRAF mutation signature. *Oncogene* 23:4060–7

Platz A, Egyhazi S, Ringborg U *et al.* (2008) Human cutaneous melanoma; a review of NRAS and BRAF mutation frequencies in relation to histogenetic subclass and body site. *Mol Oncol* 1:395–405

Reifenberger J, Knobbe CB, Wolter M *et al.* (2002) Molecular genetic analysis of malignant melanomas for aberrations of the WNT signaling pathway genes CTNNB1, APC, ICAT and BTRC. *Int J Cancer* 100:549–56

Sensi M, Nicolini G, Petti C *et al.* (2006) Mutually exclusive NRASQ61R and BRAFV600E mutations at the single-cell level in the same human melanoma. *Oncogene* 25:3357–64

Thomas NE (2006) BRAF somatic mutations in malignant melanoma and melanocytic naevi. *Melanoma Res* 16:97–103

Viros A, Fridlyand J, Bauer J *et al.* (2008) Improving melanoma classification by integrating genetic and morphologic features. *PLoS Med* 5:e120

Observations of Skin Grafts Derived from Keratinocytes Expressing Selectively Engineered Mutant Laminin-332 Molecules

Journal of Investigative Dermatology (2010) **130,** 2147–2150; doi:10.1038/jid.2010.85; published online 15 April 2010

TO THE EDITOR

Laminin-332 is a large extracellular basement membrane zone protein that is critical for dermal–epidermal cohesion. The laminin-332 heterotrimer (α3/β3/γ2) is believed to link keratinocytes with the basement membrane zone by simultaneously binding epidermal integrin receptors via the C-terminal globular domain of its α3 chain, and type VII collagen through domains on the short arm of its β3 chain (Chen *et al.*, 1997; Rousselle *et al.*, 1997). Antibody-induced inhibition of laminin-332's integrin-binding domains produces extensive skin blistering (Rousselle *et al.*, 1991; Kirtschig *et al.*, 1995); however, the *in vivo* significance of the laminin β3 short arm in dermal–epidermal cohesion has not been tested directly.

To further study the laminin β3 short arm in dermal–epidermal cohesion, we produced two deletion mutants of the laminin β3 cDNA. One (ΔVI) contained a deletion of domain VI (LN) but left the type VII collagen-binding domain intact. The other contained a deletion of the entire β3 short arm I comprising domains VI and V-III (LN, LE, LF), which includes the collagen-binding region (ΔVI-III). Mutant and wild-type (WT) β3 chain cDNAs were retrovirally expressed in laminin β3 null junctional epidermolysis bullosa (JEB Null) primary keratinocytes (Waterman *et al.*, 2007).

ΔVI, ΔVI-III, and WT keratinocytes were cultured atop the devitalized dermis as described (Ortiz-Urda *et al.*, 2003) and the resulting skin equivalents were examined 3–4 weeks after grafting to severe combined immunodeficiency mice (Figure 1a). WT grafts showed no clinical or microscopic blistering, where as ΔVI and ΔVI-III grafts showed significant subepidermal blistering and erosions. Laminin-332 null grafts uniformly failed, with no evidence of overlying human epidermis (not shown).

Increased granulation tissue has long been recognized as a characteristic feature of patients with lethal and non-lethal JEB (Marinkovich and Bauer, 2008). Interestingly, ΔVI and ΔVI-III grafts also showed prominent granulation tissue (Figure 1b), confirming an association of granulation tissue with laminin-332 defects. Non-blistered areas of mutant grafts showed a linear deposition of laminin-332 and type VII collagen at the dermal–epidermal junction, similar to WT cells (Figure 1c), suggesting that blistering/granulation tissues in mutant skin equivalents were not due to reduced laminin-332 or type VII collagen deposition at the dermal–epidermal junction. As these studies were performed in immunodeficient mice, it is likely that laminin-332 defects promoted the development of granulation tissue without the involvement of the memory immune system.

Scanning and transmission electron microscopic analysis (Figure 2a) of JEB Null keratinocytes before grafting showed marked rounding and poor association with the culture surface, compared with the flat and well-attached WT keratinocytes. Both ΔVI and ΔVI-III cells showed degrees of flattening and culture substrate apposition intermediate between JEB Null and WT cells.

Mutant ΔVI and ΔVI-III skin analyzed by transmission electron microscopy 4 weeks after grafting (Figure 2b) showed rudimentary hemidesmosomes (HDs) compared with WT grafts. Anchoring fibrils in ΔVI grafts were hypoplastic compared with WT control, whereas little or no anchoring fibrils were seen in ΔVI-III skin equivalents. Separation of both ΔVI and ΔVI-III grafts occurred in the lamina lucida, with a continuous lamina densa (LD, arrows) on the dermal side of the split.

ΔVI-III skin grafts were noted to contain both HD defects associated

Abbreviations: HD, hemidesmosome; JEB Null, null junctional epidermolysis bullosa; WT, wild type