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SUPPLEMENTARY MATERIAL

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

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Human Basal Cell Carcinoma Tumor-Initiating Cells Are Resistant to Etoposide

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TO THE EDITOR

Human basal cell carcinoma (BCC), the most common cancer prevailing in the United States of America, is resistant to conventional chemotherapy, although early surgical excision typically results in cure (Pfeiffer *et al.*, 1990). An *in vivo* human BCC xenograft model led to the recent identification of CD200+ CD45− BCC tumor-initiating cells (TICs) (Colmont *et al.*, 2013), one of only 16 human cancer TIC populations characterized by an *in vivo* assay (Colmont *et al.*, 2012). The reproducible propagation of human BCC in a xenograft model was dependent upon the generation of a ''humanized'' fibrovascular stromal bed in athymic nude mice, similar to the human squamous cell carcinoma *in vivo* assays (Patel *et al.*, 2012), but additionally required administration of intraperitoneal etoposide 1 day before BCC grafting. We hypothesized that etoposide in this assay might (1) kill BCC TICs leading to an underestimation of the true TIC frequency and/or (2) might result in an *in vivo* selection bias favoring resistant BCC cells including non-TICs.

BCC cells in tissue culture formed spheroidal colonies that when enumerated correlated with the *in vivo* TIC frequency, allowing us to assess the effect of etoposide on BCC TICs (Supplementary Material online). Colony numbers and average sizes were measured before and after etoposide treatment for 1 hour, 24 hours, and 3 days ($n=6$ per condition; Figure 1). Etoposide exposure for 24 and 72 hours led to reductions in colony numbers at 1 mM etoposide ($8.5+5.2$, $P=0.01$, $t=4$, d.f. $=5$, $r=0.8$ and $4.3+5.7$, $P<0.01$, $t=4.6$, d.f. $=5$, $r=0.4$) but not at 60 μM ($P=0.45$ and $P=0.12$) or 100 μM ($P=0.05$ and $P=0.12$). There was no overall difference in mean colony sizes before, $0.029+0.007\,mm^2$ (BCC1 $0.0295+0.0097\,mm^2$, BCC2 $0.0295+0.0052\,mm^2$, BCC3 $0.028+0.0066\,mm^2$), and after etoposide treatment, $0.028+0.006\,mm^2$ (BCC1 $0.031+0.0083\,mm^2$, BCC2 $0.030+0.0030\,mm^2$, BCC3 $0.024+0.0040\,mm^2$) ($P=0.65$). Hence, etoposide at clinically relevant

Abbreviations: BCC, basal cell carcinoma; TIC, tumor-initiating cell

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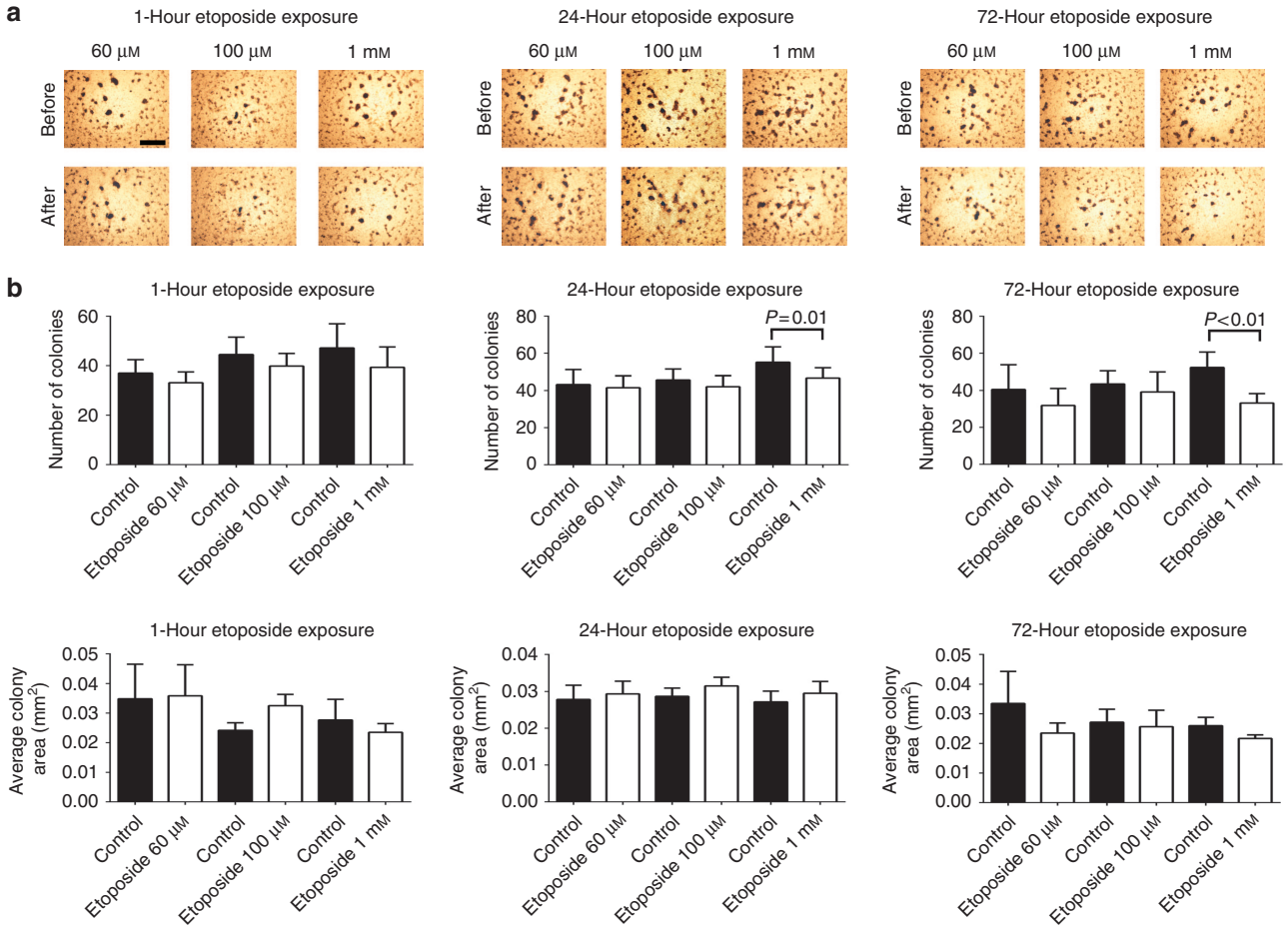

Figure 1. Etoposide treatment of established basal cell carcinoma (BCC) colonies. Freshly dissociated BCC samples ($n = 3$) were plated and colonies establish after 2 weeks in culture were photographed using an inverted microscope with a ×2 lens immediately before and after exposure to etoposide for 1, 24, and 72 hours. Only colonies adherent to the tissue culture plate were photographed. (**a**) Representative images are shown of one of six replicates for etoposide concentrations 60 μM, 100 μM, and 1 mM. Scale bars = 1 mm. (**b**) Colony numbers and sizes (average area) were enumerated using ImageJ software (NIH, Bethesda, MD) for each well (18 wells per BCC sample) before and after treatment and compared by a paired *t*-test using GraphPad Prism software (GraphPad, San Diego, CA) for each BCC sample.

concentrations (60 μM) had little impact on BCC colonies, although prolonged exposure at 1 mM led to the detachment of fibroblasts and loss of some BCC colonies.

In response to etoposide, BCC colonies mounted a DNA-damage response, with serine 139 histone H2A variant H2AX phosphorylation (Supplementary Figure S1a and S1b online) and p53 activation (Supplementary Figure S1c and S1d online). This was associated with a rapid induction of PIG8, a P53-regulated early response gene, and a transient expression of genes involved in G1 cell cycle arrest (p21) and DNA repair (p48) (Supplementary Figure S2a online). In contrast to U2OS control cells, BCC cells failed to express p53-regulated proapoptotic genes (Fas, Bax, and GADD45α), genes involved in G2 and S-phase cell cycle arrest (14-3-3-σ and GADD45α), or the p53-negative feedback gene MDM2 (Supplementary Figure 2a online). Thus, exposure to etoposide led to a transient DNA-damage response that favored cell survival over apoptosis or senescence.

Cellular resistance to etoposide is typically mediated by ABC transporters, in particular ABCB1 (p-glycoprotein, multidrug-resistance protein 1), ABCC1 (multidrug resistance–associated protein-1), and ABCG2 (breast cancer–resistance protein) (Gillet and Gottesman, 2012). Primary human renal cells (HEPTC, positive control), human BCC tissue, and BCC cultured cells were analyzed by reverse transcriptase–PCR, revealing constitutive expression of only ABCB1 in both BCC tumor tissue and cultured cells (Supplementary Figure S2b online), with an increased expression after etoposide treatment (Supplementary Figure S2c online). As ABCB1 is not expressed by normal human keratinocytes (Pfützner *et al.*, 1999; Baron *et al.*, 2001; Therrien *et al.*, 2010) and has not been described in human BCC, we confirmed protein expression by FACS analysis (Figure 2a) and functional activity by the rhodamine dye extrusion assay (Supplementary Figure S3 online).

We hypothesized that BCC cell suspensions, similar to those used in xenografts (Colmont *et al.*, 2013), would also be resistant to etoposide. Dissociated BCC cells (10^5) had similar colony-forming efficiencies, with and

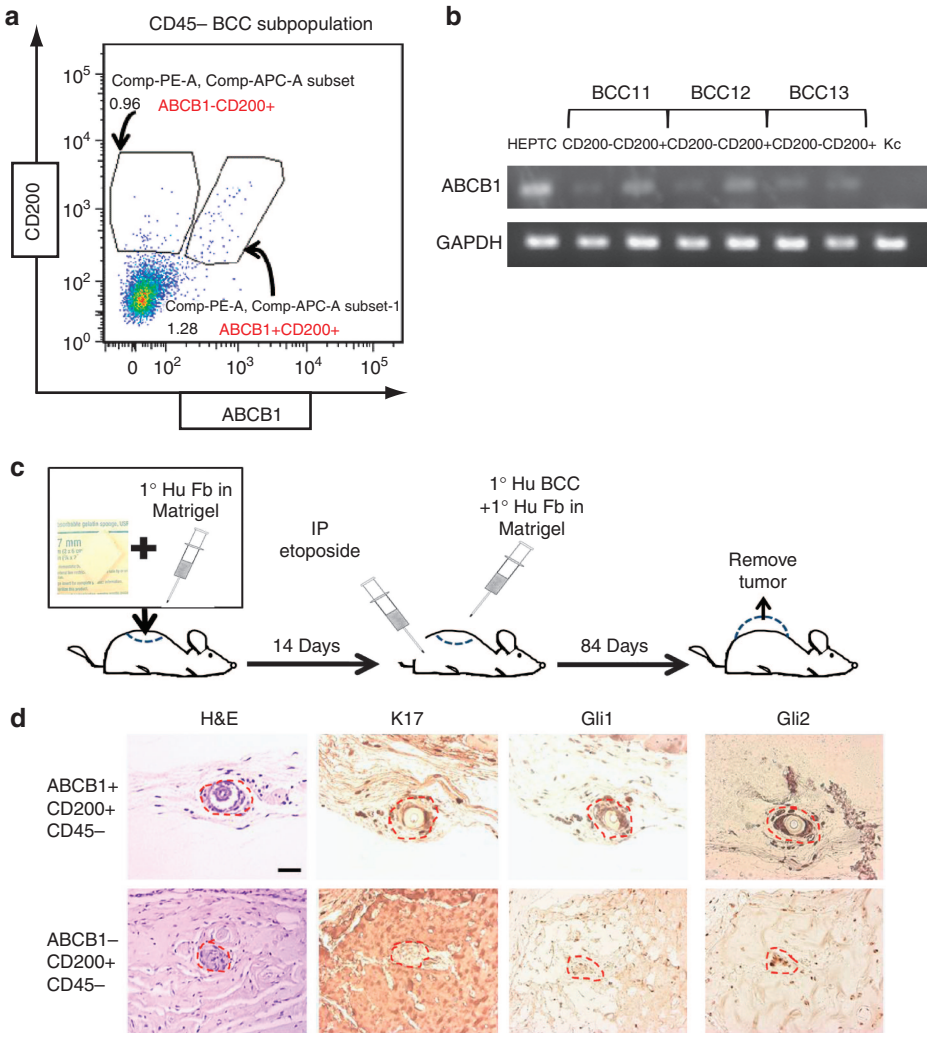

Figure 2. *In vivo* basal cell carcinoma (BCC) xenografts growth is not dependent upon ABCB1 expression. (**a**) Both CD200 + CD45 – and CD200 – CD45 – BCC flow-sorted subpopulations express ABCB1 by reverse transcriptase–PCR of equal cDNA amounts. (**b**) FACS analysis of freshly dissociated BCC cells showing both ABCB1 + CD200 + CD45 – and ABCB1 – CD200 + CD45 – subpopulations, representing 1.28 and 0.96%, respectively, of the CD45 – BCC tumor sample cell populations. (**c**) Schematic of the modified BCC xenografts model with concomitant administration of intraperitoneal etoposide and implantation of BCC subpopulations. (**d**) H&E-stained and immunohistochemically stained sections of xenografts tumors from ABCB1 + CD200 + CD45 – and ABCB1 – CD200 + CD45 – subpopulation implantations using the modified BCC xenografts model. Similar to human BCCs, xenografts from both subpopulations express K17, Gli1, and Gli2. All scale bars = 100 μm. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, hematoxylin and eosin; IP, immunoprecipitation.

without etoposide exposure for 1 hour at increasing concentrations, when plated in fresh media (Supplementary Figure S4 online). The mean colony number in the absence of pre-treatment was 66 + 8.9 (BCC4 69 + 2.9, BCC5 72 + 8, BCC6 57 + 6.5) compared with that after etoposide pre-treatment (64.7 + 12.0 (BCC4 74.4 + 8.4, BCC5 65.9 + 8.4, BCC6 53.7 + 8.9)) ($P = 0.67$). Similarly, the mean colony sizes were also similar, 0.034 + 0.011 mm^2 (BCC4 0.022 + 0.0012, BCC5 0.045 + 0.0063, BCC6 0.038 + 0.0062) and 0.034 + 0.01 mm^2 (BCC4 0.023 + 0.003, BCC5 0.038 + 0.0066, BCC6 0.042 + 0.0079), respectively ($P = 0.85$). There was also no reduction in colony number when freshly dissociated BCC cells were continuously treated with a 20–100 μM etoposide concentration over a period of 2 weeks (Supplementary Figure S5 online). In aggregate, our *in vitro* data suggest that BCC cells resist etoposide killing at concentrations relevant for (1) clinical practice where the standard chemotherapy dose is 100 mg m^{-2} body surface area resulted in an estimated peak plasma etoposide concentration of 64 μM and (2) in our *in vivo* model using a dose of 30 mg kg^{-1} body weight that has a maximum peak plasma concentration of 0.5 mM.

Both CD200 + CD45 – and CD200 – CD45 – flow-sorted human BCC populations were found to constitutively express ABCB1 (Figure 2a and b), but only the CD200 + CD45 – population has tumor-initiating capacity. As all BCC samples ($n = 6$) contained an ABCB1 – CD200 + CD45 – population, representing 54–91% of CD200 + CD45 – BCC cells (Figure 2b), we next tested the tumor-initiating capacity of this subpopulation using an *in vivo*

assay in which intraperitoneal etoposide is simultaneously administered rather than 1 day before grafting (Figure 2c). Dissociated ABCB1 + CD200 + CD45 − and ABCB1 − CD200 + CD45 − BCC cells both gave rise to *in vivo* BCC xenograft growth ($n = 3$), but ABCB1 − cells resulted in smaller tumors (Figure 2d and Supplementary Figure S6 online). Both ABCB1 + CD200 + CD45− and ABCB1 − CD200 + CD45 − derived xenograft tumors exhibited ABCB1 labeling (Supplementary Figure S7 online) (Pfützner *et al.*, 1999, Georges *et al.*, 1992), suggesting that ABCB1 − CD200 + CD45 − BCC cells upregulated ABCB1 acquiring resistance to etoposide *in vivo*.

ABCB1 is both constitutively expressed by a subpopulation of BCC TICs and can be induced in ABCB1 − cells upon etoposide exposure, potentially explaining why BCC does not respond to etoposide (Coker *et al.*, 1983; Pfeiffer *et al.*, 1990). The ability of BCC cells to express ABCB1, a cell surface transporter protein known to extrude etoposide, may also explain the transient nature of the DNA-damage response observed after etoposide exposure, as well as the development of smaller tumors from the ABCB1 − subpopulation. We did not test metastatic BCC cells or samples from patients previously treated with etoposide, and therefore we cannot comment on drug-transporter expression or chemotherapy resistance of these tumors. The ability of the *in vivo* BCC xenograft model to faithfully recapitulate BCC tumor growth, within which etoposide has no effect on TIC frequency, may allow exploration of these and related questions in future studies.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

GKP and MCU designed research; CSC, ABK, and GKP performed research; SHR and RE contributed new reagents/analytic tools; CSC, ABK, and GKP analyzed data; and CSC, MCU, and GKP wrote the manuscript.

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SUPPLEMENTARY MATERIAL

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