Expression of Betapapillomavirus Oncogenes Increases the Number of Keratinocytes with Stem Cell-Like Properties

Martin Hufbauer,^a Adrian Biddle,^b Cinzia Borgogna,^c Marisa Gariglio,^c John Doorbar,^d Alan Storey,^e Herbert Pfister,^a Ian Mackenzie,^b Baki Akgül^a

Institute of Virology, University of Cologne, Cologne, Germany^a; Blizard Institute of Cell and Molecular Science, Barts and The London School of Medicine and Dentistry, London, United Kingdom^b; Department of Clinical and Experimental Medicine, Medical School of Novara, Novara, Italy^c; National Institute for Medical Research, London, United Kingdom^d; Department of Oncology, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, United Kingdom^e

Human papillomaviruses (HPV) of genus *Betapapillomavirus* (betaPV) are associated with nonmelanoma skin cancer development in epidermodysplasia verruciformis (EV) and immunosuppressed patients. Epidemiological and molecular studies suggest a carcinogenic activity of betaPV during early stages of cancer development. Since viral oncoproteins delay and perturb keratinocyte differentiation, they may have the capacity to either retain or confer a “stem cell-like” state on oncogene-expressing cells. The aim of this study was to determine (i) whether betaPV alters the expression of cell surface markers, such as CD44 and epithelial cell adhesion molecule (EpCAM), that have been associated with epithelial stemness, and (ii) whether this confers functional stem cell-like properties to human cutaneous keratinocytes. Fluorescence-activated cell sorter (FACS) analysis revealed an increase in the number of cells with high CD44 and EpCAM expression in keratinocyte cultures expressing HPV type 8 (HPV8) oncogenes E2, E6, and E7. Particularly through E7 expression, a distinct increase in clonogenicity and in the formation and size of tumor spheres was observed, accompanied by reduction of the epithelial differentiation marker Calgranulin B. These stem cell-like properties could be attributed to the pool of CD44^{high} EpCAM^{high} cells, which was increased within the E7 cultures of HPV5, -8, and -20. Enhanced EpCAM levels were present in organotypic skin cultures of primary keratinocytes expressing E7 of the oncogenic HPV types HPV5, -8, and -16 and in clinical samples from EV patients. In conclusion, our data show that betaPV may increase the number of stem cell-like cells present during early carcinogenesis and thus enable the persistence and accumulation of DNA damage necessary to generate malignant stem cells.

Papillomaviruses (PV) are highly species-specific DNA tumor viruses with a life cycle inseparably linked to differentiation processes in stratified epithelia. An infection of the skin with human papillomaviruses (HPV) may result in benign tumors with limited growth, which tend to regress spontaneously. However, some HPV types, called high-risk or oncogenic types, cause lesions which have a high risk for conversion to malignancy (1). Among mucosal HPV of genus *Alphapapillomavirus* (alphaPV), the DNAs of HPV16 and HPV18 are frequently found integrated into the host genome in high-grade intraepithelial neoplasia and in carcinomas of the anogenital tract (2–4). Members of the genus *Betapapillomavirus* (betaPVs) are part of the normal microbiological flora of the cutaneous skin, and viral infection *per se* does not represent the major event in skin carcinogenesis (5). In immunosuppressed individuals and those suffering from the rare inherited disease epidermodysplasia verruciformis (EV), betaPVs (e.g., HPV5 and HPV8) are found with high viral loads and have been implicated in the development of squamous cell carcinomas (SCC) (6, 7). Based on the observation that higher viral loads of betaPV have been found in precancerous actinic keratoses than in SCC in the general population, it has been suggested that betaPV act early in carcinogenesis and are not necessary for the maintenance of the malignant phenotype (8, 56).

The bulge region of the hair follicle represents the natural reservoir of cutaneous PV (9, 10), and it is thought that PV also reach stem cells of the interfollicular epidermis through microtrauma of the skin. Both E6 and E7 oncoproteins of PV have the ability to delay keratinocyte differentiation, and it is tempting to speculate that E6 and/or E7 may have the capacity to retain some infected

epithelial cells in a stem cell-like state (11), leading to disturbance of their normal proliferation and differentiation.

Human malignancies are propagated and reinitiated by a small population of cells, designated “cancer stem cells” or “tumor-initiating cells,” that have the ability both to self-renew and to generate daughter cells which differentiate into the heterotypic cell populations that comprise the tumor (12–14). Cancer stem cells share several features of embryonic and adult stem cells, including signaling pathways that guide their fate (15), a slow progression through the cell cycle (16), a requirement for a niche that supports their homing and survival (17), and a high level of radiation and drug resistance (18, 19). Subpopulations of cells with stem cell-like properties persist in cell lines derived from a range of cancers (20–24). Two hypotheses have been proposed concerning the origin of cancer stem cells. One is that adult stem cells transform into malignant cells due to the accumulation of multiple mutations, and the other that differentiated cells can dedifferentiate to produce malignant stem cells (14, 25, 26).

Despite many attempts, a universal surface marker for epithelial stem cells has not been identified, but there are common patterns of expression of some markers. For example, the immunophenotype of cancer stem cells in breast cancer is CD44⁺ CD24[−]

Received 4 June 2013 Accepted 26 August 2013

Published ahead of print 4 September 2013

Address correspondence to Martin Hufbauer, martin.hufbauer@uk-koeln.de.

Copyright © 2013, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JVI.01510-13

EpCAM⁺ (epithelial cell adhesion molecule) (27), in colorectal cancer is CD44⁺ EpCAM⁺ (28), in pancreatic cancer is CD44⁺ EpCAM⁺ CD24⁺ (29), and in head and neck SCC is CD44⁺ (30) or CD44⁺ EpCAM⁺ (31, 32). In cutaneous SCC cell lines, CD44^{high} EpCAM^{high} cells also marked the epithelial cancer stem cell population (31). In these cell lines, differentiation, induced by GSK3 β inhibition, caused a reduction in the CD44^{high} EpCAM^{high} population, a shift toward CD44^{low} cell surface expression, and an increase in the expression of the differentiation marker Calgranulin B (32). EpCAM overexpression contributes to carcinogenesis by inducing anchorage-independent growth and by enhancing proliferation through upregulation of c-Myc and cyclins A and E (33, 34). EpCAM is an essential factor in the maintenance of embryonic stem cells, activating the promoters of the pluripotency-reprogramming genes Nanog, Oct4, c-Myc, Sox2, and KLF4 (35–37). CD44 increases cell proliferation and reprograms cancer cells to a cancer stem cell phenotype, and such cells have been identified *in vitro* by high cell surface expression of CD44 (CD44^{high}) (20, 21, 24, 31) and characterized as holoclones by colony morphology (consisting of small, tightly packed cells corresponding to stem cells), in contrast to meroclones (transient amplifying cells) and paraclones (terminally differentiating, CD44^{low} cells) (21, 22).

We hypothesized that the number of cells with stem cell properties present in the early stages of skin tumor development is increased by betaPV and that an increased number of stem cells predisposes to a greater frequency and permanency of transformed cells. To investigate this hypothesis, we assessed monolayer cell cultures for effects of HPV (i) on the functional stem cell properties of clonogenicity and sphere formation and (ii) on the expression of CD44 and EpCAM cell surface markers. EpCAM expression was also determined in organotypic skin cultures and in clinical samples from EV patients.

MATERIALS AND METHODS

Cell culture and patient material. The PM1 cell line (38) was maintained in RM+ medium (consisting of a 3:1 ratio of Dulbecco's modified Eagle's medium [DMEM]-F12 with 10% fetal calf serum [FCS], 1% glutamine, 0.4 μ g hydrocortisone, 10⁻¹⁰ M cholera toxin, 5 μ g/ml transferrin, 2 $\times$ 10⁻¹¹ M liothyronine, 5 μ g/ml insulin, 10 ng/ml epidermal growth factor, 1 $\times$ penicillin-streptomycin mixture). The cell line was cultivated at 37°C and 6% CO₂. Pooled primary human keratinocytes from several healthy donors were purchased from Lonza (Cologne, Germany) and cultured in KGM-Gold medium (Lonza). Organotypic skin cultures were generated as described elsewhere (39, 40). EV patients from whom skin lesions were collected (1 each of proliferative keratosis, seborrheic keratosis, and keratotic lesions and 4 SCC) have been described previously (6, 41, 42).

Retroviral expression vectors and infection of keratinocytes. We have previously described the generation of the pLXSN-based retroviral vectors coding for HPV8 E2 (43), E6 (44), or E7 (45). The construction of pLXSN-HPV4-E7, pLXSN-HPV5-E7, pLXSN-HPV16-E7, and pLXSN-HPV20-E7 is described in Westphal et al. (40). For retroviral infection, 1 $\times$ 10⁵ cells were seeded into 6-cm dishes and treated as described in Akgül et al. (45). Selection of infected cells was started 2 days later using G418. Positive clones were pooled and expanded.

FACS analysis and cell sorting. For fluorescence-activated cell sorter (FACS) analysis and cell sorting, cells were detached from cell culture flasks using enzyme-free, phosphate-buffered saline (PBS)-based cell dissociation buffer (Invitrogen, Darmstadt, Germany) and directly stained with anti-CD44 antibody (1:100 anti-CD44-phycoerythrin [PE]; BD Biosciences, Oxford, United Kingdom) and/or anti-EpCAM antibody (1:100 anti-EpCAM-allophycocyanin [APC]; Miltenyi Biotec, Bergisch Gladbach, Germany) and DAPI (4',6'-diamidino-2-phenylindole) or

7-AAD (7-amino-actinomycin D; BioLegend, San Diego, CA) for 30 min in the dark. Prior to staining with the Calgranulin B antibody (1:100 anti-Calgranulin B, clone MRP1H9; Santa Cruz, Heidelberg, Germany) and the respective secondary antibody, cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.5% Triton X-100 for 15 min. Before cell sorting, cells were filtered through a 30- μ m filter (Miltenyi Biotec). Cell analysis was performed using a FACSCanto II (BD Biosciences) or MACSQuant analyzer (Miltenyi Biotec), and cell sorting was performed using a FACSria (BD Biosciences).

Colony formation and sphere assays. For colony formation assays, 1 $\times$ 10³ cells were seeded in 5 ml medium in 25-cm² culture flasks for 7 to 10 days. The cells were fixed with paraformaldehyde and stained with crystal violet (Sigma, Steinheim, Germany). For tumor sphere assays, 1 $\times$ 10³ cells were resuspended in 0.5 ml medium containing 1% methylcellulose (Sigma). Cells were plated in 48-well plates precoated with a layer of poly(2-hydroxyethyl methacrylate) and left to grow for 2 weeks.

RNA isolation, RT, and real-time PCR. To quantify mRNA levels of cellular genes, quantitative reverse transcription (qRT)-PCR using the LightCycler system (Roche, Mannheim, Germany) was performed as described previously (46, 47). Briefly, total cellular RNA was isolated with the RNeasy kit (Qiagen, Hilden, Germany) and DNase digestion was performed on a column using RNase-free DNase. One microgram of RNA was reverse transcribed using an Omniscript kit (Qiagen) with 1 μ M oligo(dT)₂₃ primer, 10 μ M random nonamers, and 10 units RNase inhibitor. Two microliters of cDNA diluted 1:10 were used in a total volume of 20 μ l per PCR. Samples were analyzed in duplicate, together with a plasmid comprising the respective gene fragment which was used to generate a standard curve. Mean values were normalized to the hypoxanthine phosphoribosyltransferase 1 (HPRT1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. The primer sequences for the pluripotency-reprogramming genes Nanog, Oct4, c-myc, SOX2, and KLF4 and the housekeeping genes HPRT1 and GAPDH have been described previously (46, 48, 49).

Immunofluorescence staining of skin samples. Formalin-fixed, paraffin-embedded sections were deparaffinized by washing in 100% xylene and rehydrated through washing in decreasing concentrations of ethanol. For histological examination, sections were stained with hematoxylin and eosin. For immunohistochemistry, sections were treated with 0.1% pepsin (in 0.2 M HCl) for 10 min at 37°C and then blocked with 20% FCS for 60 min. Sections of organotypic skin cultures were incubated with primary antibody against EpCAM (mouse monoclonal VU1D9, 1:300 dilution in 2% FCS; Cell Signaling, Frankfurt, Germany). Sections of EV lesions were costained with an antibody against EpCAM and a rabbit anti-HPV8 E4 antibody raised against a glutathione S-transferase (GST)-E1 $\wedge$ E4 fusion protein (41) overnight at 4°C. As is generally the case with E4 polyclonals, antibodies raised against the HPV8 E1 $\wedge$ E4 protein react with the E4 proteins of related HPV types, including HPV5, -14, and -20. After extensive washes, sections were incubated with anti-mouse secondary antibody conjugated with Alexa Fluor 488 (1:500 dilution in 2% FCS; Invitrogen) and anti-rabbit secondary antibody conjugated with Alexa Fluor 594 (1:500 dilution in 2% FCS; Invitrogen) for 1 h at room temperature. After counterstaining with DAPI, staining was visualized using a Zeiss microscope and imaging software.

RESULTS

HPV8 E7 strongly increases the clonogenic activity of PM1 cells.

In order to study the clonogenic and tumorigenic activities of HPV8 oncoproteins, colony formation and sphere assays were performed using cells of the PM1 cell line, which was isolated from a premalignant skin lesion of the forehead of an immunosuppressed patient. PM1 cells are spontaneously immortalized non-invasive cells that retain the morphology and keratin expression profile of normal cultured keratinocytes and are HPV negative (38). Colony formation assays revealed a 1.3-fold increase in the number of colonies in HPV8 E2-expressing cultures, a 1.7-fold

FIG 1 Clonogenic activities of HPV8 oncogene-expressing PM1 cells. (A to D) Colony formation (A, B) and sphere assays (C, D) were performed with PM1 cells expressing the pLXSN control or HPV8 E2, E6, or E7. (E) mRNA expression of pluripotency genes in HPV8 E7-expressing cells. The expression level of the target gene in the control culture PM1-pLXSN was set as 1. Error bars show standard deviations.

increase in HPV8 E6-expressing cultures, and a 1.9-fold increase in HPV8 E7-expressing cultures (Fig. 1A and B). Tumor sphere-forming cells are considered a surrogate for tumor stem cells and show anchorage-independent growth, self-renewal, and high tumorigenicity (50, 51). In nonadherent-sphere assays, HPV8 E2- and HPV8 E6-expressing cultures produced almost twice as many spheres as control cultures and, strikingly, the HPV8 E7-expressing cultures generated 4 times more spheres, which were also larger (Fig. 1C and D). These observations demonstrate a considerable increase in the number of cells with stem cell-like behavior in HPV8 E7-expressing cultures and a moderate increase in cul-

tures expressing HPV8 E2 and HPV8 E6. To test whether the increase in stem cell-like cells in the HPV8 E7-expressing culture was associated with increased expression of pluripotency-reprogramming genes, qRT-PCR for c-myc, Oct4, Sox2, Nanog, and KLF4 was performed. Nanog was not expressed in PM1 cells, and KLF4 was not altered in the presence of HPV8 E7 (data not shown). However, the expression of HPV8 E7 led to 1.5- to 2.5-fold increases of c-myc, Oct4, and Sox2 gene expression (Fig. 1E).

PM1 cells expressing high levels of CD44 or EpCAM show enhanced clonogenic activity. To assess whether CD44 and EpCAM are also useful markers in the nontransformed cutaneous

FIG 2 Clonogenic activities of PM1 cells. Colony formation assay of PM1 cells expressing the empty retroviral vector pLXSN and sorted for low and high levels of CD44 and EpCAM cell surface expression.

keratinocyte line PM1 to probe for subpopulations of keratinocytes with stem cell-like characteristics, cells were retrovirally transduced with the empty vector pLXSN and sorted for cells expressing high or low levels of CD44 or EpCAM. For this experiment, the 5% of cells showing the weakest or strongest expression for CD44 and EpCAM were sorted from the total population and plated at low density to characterize their ability to form colonies. As shown by the results in Fig. 2, CD44^{high} and EpCAM^{high} cell populations generated significantly more colonies than CD44^{low} and EpCAM^{low} subpopulations. These data indicated that higher CD44 and EpCAM expression correlates with colony-forming capacity in the cutaneous keratinocyte line PM1.

HPV8 oncogenes affect CD44 and EpCAM expression in PM1 cells. To test whether the expression of HPV8 oncogenes in

PM1 cells modifies the expression of CD44 or EpCAM, we characterized HPV8 E2-, E6-, or E7-expressing cells for cell surface expression of these markers. The change in the number of cells with low or high surface expression levels of CD44 or EpCAM in oncogene-positive cell lines was compared to the median expression level for control cells. The three oncogene-positive cell lines each showed a reduction in the number of cells with low CD44 expression. In HPV8 E2-, E6-, and E7-expressing cultures, the CD44^{low} fraction decreased about 22% compared to this fraction in the control (Fig. 3A). The cell population with a low EpCAM expression present in the control cells was reduced by E2 expression (Fig. 3B, graph), but the median expression level of EpCAM in the total E2-expressing culture was not altered (Fig. 3B, table). The expression of E6 led to a 25% increase and the expression of E7 to a 43% increase in the median expression level of EpCAM compared to its expression in the control culture (Fig. 3B).

CD44^{high} EpCAM^{high} cells showing stem cell-like properties are increased by HPV8 E7. To characterize the oncogenic function of HPV8 E7 cells with low and high levels of EpCAM in combination with low and high levels of CD44, we used FACS to sort for these four populations and performed colony formation and sphere assays. HPV8 E7 cells with CD44^{low} EpCAM^{low} and CD44^{low} EpCAM^{high} immunophenotypes produced the fewest colonies and spheres (Fig. 4A and B, bottom). Both the CD44^{high} EpCAM^{low} and CD44^{high} EpCAM^{high} cell fractions produced large numbers of colonies and spheres (Fig. 4A and B, top). The largest-sized colonies and spheres were found within the CD44^{high} EpCAM^{low} fraction (Fig. 4A and B, top left).

The CD44^{high} EpCAM^{high} cell fraction was found to be significantly increased in HPV8 E7-expressing cultures (Fig. 5A) and

FIG 3 Effects of HPV8 oncogenes E2, E6, and E7 on CD44 or EpCAM expression. FACS profiles of PM1-pLXSN vector and HPV8 E2-, E6-, and E7-expressing cells tested for CD44 (A) and EpCAM (B) cell surface expression. Changes in expression are summarized in the tables. The figures presented are representatives from three independent experiments.

FIG 4 Cell-transforming activities of HPV8 E7-expressing cells double sorted for CD44 and EpCAM. Colony formation assay (A) and sphere assay (B) of HPV8 E7-expressing cells sorted for different levels of cell surface CD44 and EpCAM expression.

also in PM1 cultures expressing E7 of HPV5 and HPV20 (both members of species *Betapapillomavirus 1*). The expression of HPV4 E7 (genus *Gammapapillomavirus*), however, did not change the number of CD44^{high} EpCAM^{high} cells but did lead to an increase of CD44^{low} EpCAM^{low} cells (Fig. 5B). To confirm the findings for HPV8 E7 obtained using the immortalized keratinocyte line PM1, we also tested the effects of the expression of HPV8 E6 and E7 oncoproteins on CD44 and EpCAM in a pool of primary human skin keratinocytes of several healthy donors. In these cells, an increase in the CD44^{high} EpCAM^{high} population due to HPV8 E7 and E6E7 expression was found (Fig. 5C). HPV8 E6 alone, however, induced a shift of the cell population from CD44^{low} EpCAM^{low} (41% with pLXSN and 24% with E6) to CD44^{high} EpCAM^{low} (12% with pLXSN and 32% with E6). The shifting toward higher CD44 cell surface expression and the resulting reduction in the number of terminally differentiating CD44^{low} cells in the corresponding cell lines suggested an interference of HPV8 oncoproteins with keratinocyte differentiation. The expression of HPV8 E6, E7, or E6E7 caused a decrease in the percentage of cells expressing high levels of the epithelial differentiation marker Calgranulin B, indicating that HPV8 oncoproteins, besides upregulating epithelial stem cell markers, simultaneously suppress the entry of primary keratinocytes into differentiation (Fig. 5D).

Expression of EpCAM in organotypic skin cultures of HPV E7-expressing cells and skin lesions of EV patients. To confirm EpCAM induction by E7, we analyzed its levels in organotypic skin cultures of E7-positive primary skin keratinocytes by immunofluorescence staining. After 14 days in the air-liquid interface, conditions that induce epidermal differentiation, the cultures were paraffin embedded and 4- μ m sections were stained. As shown by the results in Fig. 6, EpCAM induction was not detected in HPV4 E7-expressing epithelia but was present in the suprabasal cell layers of epithelia expressing HPV5 E7 and HPV8 E7, as well as E7 of HPV16 (genus *Alphapapillomavirus*). These data indicate that EpCAM is induced in suprabasal keratinocytes expressing E7 of oncogenic alpha and beta HPV types but not by the wart-associated type HPV4. Finally, using clinical skin samples, we found EpCAM induction localized with a band-like appearance in the most-superficial cell layers of dysplastic EV skin (2/2) (Fig. 6a, b, and d), where betaPV positivity was identified by staining for the viral E4 protein (Fig. 6c and d). Increased expression of EpCAM in a punctuated pattern was found in E4-positive areas of 4/4 EV

SCCs and in one proliferative keratosis (images representative for EV SCC are shown in Fig. 6e to g). However, E4-positive EpCAM-negative areas were also present (data not shown).

DISCUSSION

We have been able to demonstrate, using *in vitro* colony formation and tumor sphere assays, that HPV8 E7-expressing keratinocytes and, to a lesser extent, HPV8 E2- or E6-positive cells, show greater clonogenicity and ability for sphere formation. The expression of HPV8 E7 was also accompanied by an increase in transcription of the pluripotency-reprogramming genes *c-myc*, *Oct-4*, and *Sox2*, indicating a shift toward maintenance of stemness. CD44 and EpCAM, molecules previously shown to be expressed on stem cells in both normal and malignant epithelial tissues (31, 52, 53), are both expressed by cells of the cutaneous keratinocyte line PM1. In this line, the CD44^{high} or EpCAM^{high} cell population generated more colonies and spheres than the CD44^{low} or EpCAM^{low} fraction. The expression of HPV8 E2, E6, and E7 led to a shift of the total population from low toward higher CD44 or EpCAM cell surface expression and, in turn, pointed to a reduction in the number of terminally differentiated cells. The reduction of the epithelial differentiation marker Calgranulin B in HPV8 E6-, E7-, and E6E7-expressing primary keratinocytes confirmed this assumption and revealed a blockage of the commitment of cells to differentiation. HPV8 oncogene-expressing PM1 lines, especially E7-positive ones, contained a greater number of cells with behavioral characteristics typical of stem cells and, by cell sorting, this behavior was shown to correlate with a CD44^{high} EpCAM^{high} or CD44^{high} EpCAM^{low} immunophenotype. This phenotype was not limited only to HPV8 E7 but could also be found on cells expressing E7 of the betaPV types HPV5 or HPV20, which are both, like HPV8, members of species *Betapapillomavirus 1*. A recent study of the oral cancer cell line CA1 has shown that CD44^{high} EpCAM^{high} cells represent a population of proliferative cancer stem cells (31). The significant increase found in the number of CD44^{high} EpCAM^{high} cells suggests that HPV8 E7 enlarges this population of proliferative stem cell-like cells. In this context, it is worth mentioning that HPV8 E7, but not HPV8 E2 or E6, induced hyperproliferation and invasion of primary human adult keratinocytes in organotypic skin cultures (45, 54).

Upregulation of EpCAM in organotypic skin cultures was found in the suprabasal layers of differentiating keratinocytes expressing E7 of HPV5, HPV8, and HPV16 but not in cultures ex-

FIG 5 FACS profiles of human keratinocytes double labeled for CD44 and EpCAM cell surface expression or Calgranulin B. (A and B) FACS profiles of PM1 cells expressing E7 of HPV8 (A) or of HPV4, HPV5, or HPV20 (B). (C) CD44 EpCAM profiles of primary human keratinocytes expressing HPV8 E6 and E7 or E6E7. (D) Calgranulin B expression levels in primary human keratinocytes expressing HPV8 E6 and E7 individually or in combination. Changes in expression are summarized in the table.

pressing HPV4 E7. Based on these findings, it may be hypothesized that the overexpression of EpCAM is associated only with oncogenic HPV types. During the betaPV life cycle in a productive EV lesion, small amounts of E7 transcript were detected in basal and suprabasal signals, with the highest signals in the most-super-

ficial layers (55). This E7 expression pattern correlates with regions of enhanced EpCAM protein levels found in dysplastic EV lesions and may indicate the generation of virus-induced transformed cells in suprabasal layers. Cells with strong EpCAM expression were also found in virus-positive areas of EV SCCs and

FIG 6 EpCAM expression in organotypic skin cultures expressing E7 of HPV4, -5, -8, and -16 and in lesions from epidermodysplasia verruciformis patients. Immunofluorescence staining: EpCAM (green), HPV E4 (red), DAPI (blue). (a) Histology of dysplastic EV lesion. (b to d) Costaining for EpCAM and HPV E4 on dysplastic EV lesion. (e, f) Histology of EV SCC. (g) Costaining for EpCAM and HPV E4 on EV SCC. Magnification: 400× for all images except image e, which is 100×.

may have contributed to betaPV-mediated carcinogenic processes in these patients.

Taken together, our findings lead to the conclusion that the expression of betaPV inhibits the entry of keratinocytes into differentiation and that the maintenance of basal cells in an undifferentiated state may increase the pool of cells available for the accumulation of damage that can persist and lead to the generation of stem cells with malignant properties.

ACKNOWLEDGMENTS

This study was supported by grants from Köln Fortune (grant no. 87/2010) and the Deutsche Krebshilfe (grant no. 109524).

REFERENCES

- zur Hausen H. 2002. Papillomaviruses and cancer: from basic studies to clinical application. *Nat. Rev. Cancer* 2:342–350.
- Arias-Pulido H, Peyton CL, Joste NE, Vargas H, Wheeler CM. 2006. Human papillomavirus type 16 integration in cervical carcinoma in situ and in invasive cervical cancer. *J. Clin. Microbiol.* 44:1755–1762.
- Cullen AP, Reid R, Campion M, Lorincz AT. 1991. Analysis of the physical state of different human papillomavirus DNAs in intraepithelial and invasive cervical neoplasm. *J. Virol.* 65:606–612.
- Ziegert C, Wentzensen N, Vinokurova S, Kisseljov F, Eichenkel J, Hoeckel M, von Knebel Doeberitz M. 2003. A comprehensive analysis of HPV integration loci in anogenital lesions combining transcript and genome-based amplification techniques. *Oncogene* 22:3977–3984.
- Weissenborn SJ, De Koning MN, Wieland U, Quint WG, Pfister HJ. 2009. Intrafamilial transmission and family-specific spectra of cutaneous betapapillomaviruses. *J. Virol.* 83:811–816.
- Dell'Oste V, Azzimonti B, De Andrea M, Mondini M, Zavattaro E, Leigh G, Weissenborn SJ, Pfister H, Michael KM, Waterboer T, Pawlita M, Amantea A, Landolfo S, Gariglio M. 2009. High beta-HPV DNA loads and strong seroreactivity are present in epidermodysplasia verruciformis. *J. Invest. Dermatol.* 129:1026–1034.
- Nindl I, Gottschling M, Stockfleth E. 2007. Human papillomaviruses and non-melanoma skin cancer: basic virology and clinical manifestations. *Dis. Markers* 23:247–259.
- Weissenborn SJ, Nindl I, Purdie K, Harwood C, Proby C, Breuer J, Majewski S, Pfister H, Wieland U. 2005. Human papillomavirus-DNA loads in actinic keratoses exceed those in non-melanoma skin cancers. *J. Invest. Dermatol.* 125:93–97.
- Boxman IL, Berkhout RJ, Mulder LH, Wolkers MC, Bouwes Bavinck JN, Vermeer BJ, ter Schegget J. 1997. Detection of human papillomavirus DNA in plucked hairs from renal transplant recipients and healthy volunteers. *J. Invest. Dermatol.* 108:712–715.
- Schmitt A, Rochat A, Zeltner R, Borenstein L, Barrandon Y, Wettstein FO, Iftner T. 1996. The primary target cells of the high-risk cottontail rabbit papillomavirus colocalize with hair follicle stem cells. *J. Virol.* 70:1912–1922.
- Münger K, Basile JR, Duensing S, Eichten A, Gonzalez SL, Grace M, Zaczyn VL. 2001. Biological activities and molecular targets of the human papillomavirus E7 oncoprotein. *Oncogene* 20:7888–7898.
- Bonnet D, Dick JE. 1997. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat. Med.* 3:730–737.
- Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, Jones DL, Visvader J, Weissman IL, Wahl GM. 2006. Cancer stem cells—perspectives on current status and future directions: AACR Workshop on cancer stem cells. *Cancer Res.* 66:9339–9344.
- Reya T, Morrison SJ, Clarke MF, Weissman IL. 2001. Stem cells, cancer, and cancer stem cells. *Nature* 414:105–111.
- Abbott DE, Postovit LM, Seftor EA, Margaryan NV, Seftor RE, Hendrix MJ. 2007. Exploiting the convergence of embryonic and tumorigenic signaling pathways to develop new therapeutic targets. *Stem Cell Rev.* 3:68–78.
- Foijer F, te Riele H. 2006. Check, double check: the G2 barrier to cancer. *Cell Cycle* 5:831–836.
- Bissell MJ, Labarge MA. 2005. Context, tissue plasticity, and cancer: are tumor stem cells also regulated by the microenvironment? *Cancer Cell* 7:17–23.
- Diehn M, Cho RW, Clarke MF. 2009. Therapeutic implications of the cancer stem cell hypothesis. *Semin. Radiat. Oncol.* 19:78–86.
- Huang EH, Heidt DG, Li CW, Simeone DM. 2007. Cancer stem cells: a new paradigm for understanding tumor progression and therapeutic resistance. *Surgery* 141:415–419.
- Fillmore CM, Kuperwasser C. 2008. Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. *Breast Cancer Res.* 10:R25. doi:10.1186/bcr1982.
- Harper LJ, Piper K, Common J, Fortune F, Mackenzie IC. 2007. Stem cell patterns in cell lines derived from head and neck squamous cell carcinoma. *J. Oral Pathol. Med.* 36:594–603.
- Li H, Chen X, Calhoun-Davis T, Claypool K, Tang DG. 2008. PC3 human prostate carcinoma cell holoclones contain self-renewing tumor-initiating cells. *Cancer Res.* 68:1820–1825.
- Locke M, Heywood M, Fawell S, Mackenzie IC. 2005. Retention of intrinsic stem cell hierarchies in carcinoma-derived cell lines. *Cancer Res.* 65:8944–8950.
- Patrawala L, Calhoun T, Schneider-Broussard R, Li H, Bhatia B, Tang S, Reilly JG, Chandra D, Zhou J, Claypool K, Coghlan L, Tang DG. 2006. Highly purified CD44+ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells. *Oncogene* 25:1696–1708.
- Daley GQ. 2004. Chronic myeloid leukemia: proving ground for cancer stem cells. *Cell* 119:314–316.
- Liang Y, Zhong Z, Huang Y, Deng W, Cao J, Tsao G, Liu Q, Pei D, Kang T, Zeng YX. 2010. Stem-like cancer cells are inducible by increasing genomic instability in cancer cells. *J. Biol. Chem.* 285:4931–4940.
- Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. 2003. Prospective identification of tumorigenic breast cancer cells. *Proc. Natl. Acad. Sci. U. S. A.* 100:3983–3988.
- Dalerba P, Dylla SJ, Park IK, Liu R, Wang X, Cho RW, Hoey T, Gurney A, Huang EH, Simeone DM, Shelton AA, Parmiani G, Castelli C, Clarke MF. 2007. Phenotypic characterization of human colorectal cancer stem cells. *Proc. Natl. Acad. Sci. U. S. A.* 104:10158–10163.

29. Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF, Simeone DM. 2007. Identification of pancreatic cancer stem cells. *Cancer Res.* 67:1030–1037.
30. Prince ME, Sivanandan R, Kaczorowski A, Wolf GT, Kaplan MJ, Dalerba P, Weissman IL, Clarke MF, Ailles LE. 2007. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proc. Natl. Acad. Sci. U. S. A.* 104:973–978.
31. Biddle A, Liang X, Gammon L, Fazil B, Harper LJ, Emich H, Costea DE, Mackenzie IC. 2011. Cancer stem cells in squamous cell carcinoma switch between two distinct phenotypes that are preferentially migratory or proliferative. *Cancer Res.* 71:5317–5326.
32. Shigeishi H, Biddle A, Gammon L, Emich H, Rodini CO, Gemenetzidis E, Fazil B, Sugiyama M, Kamata N, Mackenzie IC. 7 May 2013. Maintenance of stem cell self-renewal in head and neck cancers requires actions of GSK3beta influenced by CD44 and RHAMM. *Stem Cells*. doi:10.1002/stem.1418.
33. Munz M, Kieu C, Mack B, Schmitt B, Zeidler R, Gires O. 2004. The carcinoma-associated antigen EpCAM upregulates c-myc and induces cell proliferation. *Oncogene* 23:5748–5758.
34. Osta WA, Chen Y, Mikhitarian K, Mitas M, Salem M, Hannun YA, Cole DJ, Gillanders WE. 2004. EpCAM is overexpressed in breast cancer and is a potential target for breast cancer gene therapy. *Cancer Res.* 64:5818–5824.
35. Gonzalez B, Denzel S, Mack B, Conrad M, Gires O. 2009. EpCAM is involved in maintenance of the murine embryonic stem cell phenotype. *Stem Cells* 27:1782–1791.
36. Lu TY, Lu RM, Liao MY, Yu J, Chung CH, Kao CF, Wu HC. 2010. Epithelial cell adhesion molecule regulation is associated with the maintenance of the undifferentiated phenotype of human embryonic stem cells. *J. Biol. Chem.* 285:8719–8732.
37. Ng VY, Ang SN, Chan JX, Choo AB. 2010. Characterization of epithelial cell adhesion molecule as a surface marker on undifferentiated human embryonic stem cells. *Stem Cells* 28:29–35.
38. Proby CM, Purdie KJ, Sexton CJ, Purkis P, Navsaria HA, Stables JN, Leigh IM. 2000. Spontaneous keratinocyte cell lines representing early and advanced stages of malignant transformation of the epidermis. *Exp. Dermatol.* 9:104–117.
39. Akgül B, Bauer B, Zigrino P, Storey A, Mauch C, Pfister H. 2011. Upregulation of lipocalin-2 in human papillomavirus-positive keratinocytes and cutaneous squamous cell carcinomas. *J. Gen. Virol.* 92:395–401.
40. Westphal K, Akgül B, Storey A, Nindl I. 2009. Cutaneous human papillomavirus E7 type-specific effects on differentiation and proliferation of organotypic skin cultures. *Cell Oncol.* 31:213–226.
41. Borgogna C, Zavattaro E, De Andrea M, Griffin HM, Dell'Oste V, Azzimonti B, Landini MM, Peh WL, Pfister H, Doorbar J, Landolfo S, Gariglio M. 2012. Characterization of beta papillomavirus E4 expression in tumours from epidermodysplasia verruciformis patients and in experimental models. *Virology* 423:195–204.
42. Landini MM, Zavattaro E, Borgogna C, Azzimonti B, De Andrea M, Colombo E, Marengo F, Amantea A, Landolfo S, Gariglio M. 2012. Lack of EVER2 protein in two epidermodysplasia verruciformis patients with skin cancer presenting previously unreported homozygous genetic deletions in the EVER2 gene. *J. Invest. Dermatol.* 132:1305–1308.
43. O'Shaughnessy RF, Akgül B, Storey A, Pfister H, Harwood CA, Byrne C. 2007. Cutaneous human papillomaviruses down-regulate AKT1, whereas AKT2 up-regulation and activation associates with tumors. *Cancer Res.* 67:8207–8215.
44. Leverrier S, Bergamaschi D, Ghali L, Ola A, Warnes G, Akgül B, Blight K, Garcia-Escudero R, Penna A, Eddaoudi A, Storey A. 2007. Role of HPV E6 proteins in preventing UVB-induced release of pro-apoptotic factors from the mitochondria. *Apoptosis* 12:549–560.
45. Akgül B, Garcia-Escudero R, Ghali L, Pfister HJ, Fuchs PG, Navsaria H, Storey A. 2005. The E7 protein of cutaneous human papillomavirus type 8 causes invasion of human keratinocytes into the dermis in organotypic cultures of skin. *Cancer Res.* 65:2216–2223.
46. Hufbauer M, Lazic D, Akgül B, Brandsma JL, Pfister H, Weissenborn SJ. 2010. Enhanced human papillomavirus type 8 oncogene expression levels are crucial for skin tumorigenesis in transgenic mice. *Virology* 403:128–136.
47. Lazic D, Alborzi F, Marcuzzi GP, Angel P, Hess J, Pfister H, Akgül B. 2011. Enhanced StefinA and Spr2 expression during papilloma formation in HPV8 transgenic mice. *J. Dermatol. Sci.* 62:84–90.
48. Akgül B, Bostanci N, Westphal K, Nindl I, Navsaria H, Storey A, Pfister H. 2010. Human papillomavirus 5 and 8 E6 downregulate interleukin-8 secretion in primary human keratinocytes. *J. Gen. Virol.* 91:888–892.
49. Park IH, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, Lerou PH, Lensch MW, Daley GQ. 2008. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* 451:141–146.
50. Cao L, Zhou Y, Zhai B, Liao J, Xu W, Zhang R, Li J, Zhang Y, Chen L, Qian H, Wu M, Yin Z. 2011. Sphere-forming cell subpopulations with cancer stem cell properties in human hepatoma cell lines. *BMC Gastroenterol.* 11:71. doi:10.1186/1471-230X-11-71.
51. Visvader JE, Lindeman GJ. 2008. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat. Rev. Cancer.* 8:755–768.
52. Sengupta A, Cancelas JA. 2010. Cancer stem cells: a stride towards cancer cure? *J. Cell Physiol.* 225:7–14.
53. Wray H, Mackenzie IC, Storey A, Navsaria H. 2012. alpha6 integrin and CD44 enrich for a primary keratinocyte population that displays resistance to UV-induced apoptosis. *PLoS One* 7:e46968. doi:10.1371/journal.pone.0046968.
54. Akgül B, Ghali L, Davies D, Pfister H, Leigh IM, Storey A. 2007. HPV8 early genes modulate differentiation and cell cycle of primary human adult keratinocytes. *Exp. Dermatol.* 16:590–599.
55. Haller K, Stubenrauch F, Pfister H. 1995. Differentiation-dependent transcription of the epidermodysplasia verruciformis-associated human papillomavirus type 5 in benign lesions. *Virology* 214:245–255.
56. Akgül B, Cooke JC, Storey A. 2006. HPV-associated skin disease. *J. Pathol.* 208:165–175.