HPV8 Field Cancerization in a Transgenic Mouse Model Is due to Lrig1+ Keratinocyte Stem Cell Expansion

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Abbreviations: **human papillomavirus (HPV), Epidermodysplasia Verruciformis (EV), cutaneous squamous cell carcinoma (SCC), wild type (WT), hair follicle (HF), interfollicular epidermis (IFE), keratinocyte stem cell (KSC), actinic keratosis (AK).**

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ABSTRACT

β-HPV cause near ubiquitous latent skin infection within long-lived hair follicle keratinocyte stem cells (HF KSC). In patients with epidermodysplasia verruciformis (EV), β-HPV viral replication is associated with skin keratosis and cutaneous squamous cell carcinoma (SCC). To determine the role of HF KSC in β-HPV induced skin carcinogenesis, we utilized a transgenic mouse model in which the keratin 14 promoter drives expression of the entire HPV8 early region (HPV8tg). HPV8tg mice developed thicker skin in comparison to wild type littermates consistent with a hyperproliferative epidermis. HF keratinocyte proliferation was evident within the Lrig1+ KSC population (69 vs 55%, $p<0.001$, $n=6$), and not in the CD34+, LGR5+ and LGR6+ KSC populations. This was associated with a 2.8-fold expansion in Lrig1+ keratinocytes and 3.8 fold increased colony forming efficiency. Consistent with this, we observed nuclear p63 expression throughout this population and the HF infundibulum and adjoining IFE, associated with a switch from p63 TA isoforms to ΔNp63 isoforms in HPV8tg skin. EV keratosis and in some cases actinic keratoses demonstrated similar histology associated with β-HPV virus reactivation and nuclear p63 expression within the HF infundibulum and perifollicular epidermis. These findings would suggest that β-HPV field cancerization arises from the HF junctional zone and predispose to SCC.

INTRODUCTION

More than 200 human papillomavirus (HPV) types have been DNA sequenced and thus classified into five genera (*Alpha-*, *Beta-*, *Gamma-*, *Mu-* and *Nu-papillomavirus*) (Bernard *et al*., 2010; http://pave.niaid.nih.gov/#home). High-risk α-HPV types have been established to be causative for cancer, notably in the ano-genital tract (Bosch *et al*., 2013). In contrast β-HPV types are evolutionarily distinct as they do not integrate into the host genome and cause ubiquitous latent skin infection (Quint *et al*., 2015). The likely reservoir for β-HPV latent infection is postulated to reside within long-lived hair follicle keratinocyte stem cells (HF-KSC), since plucked hair consistently demonstrates β-HPV DNA and KSC characteristics are enhanced by HPV 5 and 8 early region genes (Boxman *et al*., 1997; Bouwes Bavinck *et al*., 2008; Hufbauer *et al*., 2013). However, the precise HF-KSC populations involved in β-HPV latent infection remain to be defined (Kranjec and Doorbar, 2016). Markers of β-HPV infection are uniformly observed in Epidermodysplasia Verruciformis (EV) keratosis and cutaneous squamous cell carcinoma (SCC), which represents the prototypic model of β-HPV induced skin carcinogenesis (Borgogna *et al*., 2012, Borgogna *et al*., 2014a). EV is a rare genodermatosis that has been included in the list of primary immunodeficiency characterized by defects in innate immunity (Notarangelo *et al.*, 2004). Inactivating bi-allelic mutations of either EVER1/TMC6 or EVER2/TMC8 have been described in several families suffering from EV and account for about half of described EV cases (Ramoz *et al.*, 2002). Atypical cases of EV have been also reported and they usually display T-cell defects (Azzimonti *et al.*, 2005, Borgogna *et al.*, 2014a, Landini *et al.*, 2014). It has been postulated that β-HPV reactivation is also associated with skin carcinogenesis in organ transplant recipients (Quint *et al.*, 2015; Borgogna *et al.*, 2014b; Howley *et al.*, 2015). To determine the role of HF-KSC in β-HPV induced skin carcinogenesis, we utilized a transgenic mouse model in which the entire HPV8 early region genes are expressed under a keratin 14 promoter (HPV8tg)

(Schaper *et al*., 2005; De Andrea *et al*., 2010). HPV8tg mice develop multiple persistent papillomas within 8 weeks, of which 6% go on to develop spontaneous SCC with metastatic potential.

In this study, we provide a model of β-HPV-induced skin carcinogenesis that is based on the aberrant expansion of Lrig1+ KSC in the upper hair follicle that spill out into the surrounding epidermis. Similarly expansion of the junctional zone HF KSC population was identified by p63 labelling in human EV keratosis and actinic keratosis.

RESULTS

The HPV8tg mice skin displays HF proliferative epidermal hyperplasia

After birth, HPV8tg mice develop thicker skin in comparison to wild type (WT) littermates (Figure 1). Adult HPV8tg skin thickness of the ear was 0.6±0.1 vs 0.4±0.1 mm ($p<0.05$, n=9), there was no difference in weight and tail width. More keratinocyte layers were evident in the HF infundibulum and adjoining interfollicular epidermis (IFE) in HPV8tg, 4.2 ± 0.47 vs 2.0 ± 0.0 and 3.8 ± 0.49 vs 1.5 ± 0.43 respectively ($p<0.01$, n=5), but stratum corneum thickness measured on histological sections was not different. Consistent with a hyperproliferative epidermis, keratinocyte proliferation assessed by Ki67 positive cells per basal keratinocyte was markedly increased within the HF (41± 10.9 vs 23 ± 11.8, n=7, p=0.01) and to a lesser extent the IFE (0.46± 0.18 vs 0.31 ± 0.11, n=15, p=0.01). The expression of HPV8 early region genes in this transgenic mouse model has been previously described (Schaper *et al*., 2005; De Andrea *et al*., 2010). While this HPV8tg mouse model yield spontaneous SCC formation, other similar β-HPV transgenic models driven by K14 promoter do not develop SCC spontaneously (Viarisio *et al*. 2011). Consistent with this, levels of E6 and E7 expression in HPV8tg mouse skin were similar to that observed in HeLa cells with natural HPV18 infection (Supplementary Figure 1**).** Together these findings

suggested that HPV8 early region genes induce a proliferative epidermal hyperplasia, notably in the HF. HPV8 induced keratinocyte proliferation was greatest in the HF and immediately adjoining IFE, as determined by Ki67 expression, even though keratin 14 promoter driven HPV8 early region genes were uniformly expressed.

The Lrig1 KSC population is expanded in HPV8tg mice

Within the HF, the mean area of the infundibulum was markedly increased in HPV8tg compared to WT mice (Figure 2a), while there was no difference in HF length (Figure 2b).

To determine which hair follicle keratinocyte population become expanded in HPV8tg mice compared to wild type littermates, we labelled skin sections in whole mount analysis with a set of stem cell markers. Consistent with the observed HF infundibulum expansion, keratinocyte proliferation was evident within the Lrig1+ KSC population (69 vs 55%, $p<0.001$, n=6), and not in the CD34+ (1 vs 1%), LGR5+ (1 vs 3%) and LGR6+ (29 vs 40%) KSC populations (n=7, Figures 2c-e Figures 3a&b). Flow cytometric analysis of dissociated skin confirmed a 2.8-fold increase in Lrig1+ keratinocytes in the HPV8tg mice, 7.4% +/- 2.2% vs 2.7% +/- 0.8%, n=6, $P < 0.05$, but no difference in CD34+ KSC numbers (0.81% +/- 0.24% vs 0.73% +/- 0.37%) (Figure 2c). Flow sorted Lrig1+ and CD34+ keratinocyte sub-populations had similar levels of K14 promoter driven early region genes mRNA expression (Figure 4a), despite the observed difference in proliferation. To exclude any difference in the EVER 1 and 2 gene expression levels in HPV8tg mice versus control, qRT-PCR analysis was performed with the RNA extracted from Lrig1+ sorted cells and found comparable levels as shown in Figure 4b. Flow sorted Lrig1+ keratinocytes from HPV8tg mice also demonstrated a 3.8 fold increased colony forming efficiency (Figures 3c&d), hence Lrig1+ cells retain KSC function. There was no significant difference in colony forming efficiency from flow sorted Lrig1 negative keratinocytes from HPV8tg versus WT mice (Supplementary Figure 2). In

keeping with Lrig1+ expansion and proliferation, we observed nuclear p63 expression throughout this population and the emanating keratinocytes of HF infundibulum and adjoining IFE (Figure 3e). RT-PCR and western blotting analysis confirmed the switch from p63 TA isoforms to ΔNp63 isoforms in HPV8tg skin (Figures 5a-c), consistent with earlier reports indicating HPV8 early proteins induce p63 expression (Meyers *et al*., 2013). Thus Lrig1+ KSC proliferation through induction of ΔNp63 in HPV8tg skin resulted in KSC expansion into the overlying infundibulum and adjoining IFE.

β-HPV associated expansion of the HF junctional zone KSC population in human skin field cancerization

The dilated HF infundibulum with increased keratinocyte layers and the crowded perifollicular epidermis with hyperkeratosis were consistently observed in the HPV8tg mice, and resembled Freudenthal's funnel, the pathognomonic histological finding in actinic keratosis (n=28 mice, Supplementary Figure 3). EV keratosis uniformly demonstrated similar histology associated with β-HPV virus reactivation and nuclear p63 expression within the HF infundibulum and perifollicular epidermis (Figure 5d, 6 patients with 44 lesions, Supplementary Table 1). Likewise, in some cases of actinic keratosis from non-EV patients, we were able to detect nuclear p63 expression within the infundibulum and perifollicular epidermis in areas where β-HPV virus reactivation was well evident, as detected by expression of the viral marker E4 (Figure 5d, n=2 of 25). Hence, our findings suggest that similar to HPV8 mice, EV and some patients with AK demonstrate expansion of a junctional zone HF KSC population identified by p63 labelling. These findings would suggest that β-HPV field cancerization arises from the HF infundibulum and predispose to SCC. Indeed, we observed p63 positive cells in HPV8tg and human SCC (Figure 5e, n=5); the latter were from immunocompetent patient from sun-exposed sites.

DISCUSSION

The concept of “field cancerization” as introduced by Slaughter et al. was initially used to describe an area of upper aerodigestive tract in situ SCC, within which develop multiple invasive SCC foci (Slaughter *et al*., 1953). In the skin, similar pre-neoplastic field cancerization is observed and is characterized by the presence of multiple AK’s (Stockfleth *et al*., 2011). Although ultraviolet (UV) light induced DNA damage is the prime cause of AK, other factors, including β-HPV infection can also contribute to development of this skin disorder, especially in immunocompromised patients (Taguchi *et al*., 1994; Weissenborn *et al*., 2005; Banerjee *et al*., 2008). Patients with EV and related primary T cell immunodeficiency syndrome, are predisposed to develop skin field cancerization with similar keratoses that are associated with re-activation of latent β-HPV infection (Azzimonti *et al*., 2005; Landini *et al*., 2014). In all cases of skin field cancerization, UV light is the key driver for transformation to SCC, as tumors typically arise on sun-exposed sites and contain p53 UV signature mutations (Jonason *et al*., 1996). Intriguingly, we have previously observed β-HPV reactivation at the clinically unaffected skin and in situ carcinoma at the periphery of SCC lesions from EV and organ transplant recipient, as well as within EV SCC (Borgogna *et al*., 2014b). In some cases, 2 of 25 cases studied of non-EV AK, β-HPV reactivation was observed. The HPV8tg mouse model with constitutive epithelial HPV8 expression develops keratosis like skin changes, with 6% of lesions progressing to spontaneous SCC (Schaper *et al*., 2005). The rate of conversion to SCC is greater in HPV8tg than in EV patients and may reflect the higher expression of early region genes. As in human EV, UV radiation rapidly leads to SCC formation in HPV8tg and other HPV mouse models (Marcuzzi *et al*., 2009; Viarisio *et al*., 2011).

KSC reside within each compartment of the skin: the IFE, HF, sebaceous and sweat glands. Different mouse KSC pools are distributed along the HF, defined by the expression of

cell surface proteins that facilitate isolation and thus characterization. To date KSC have been identified within four HF regions: upper (Lrig1) and lower (LGR6) junctional zone, the bulge (CD34 and K15) and bulb (LGR5) (Solanas & Benitah, 2013; Kretzschmar & Watt, 2014) (Figure 6). In this study, we have identified the HF Lrig1+ KSC population as the putative target in HPV8tg mice skin carcinogenesis. Lrig1 expression defines a distinct multipotent stem cell population in mammalian epidermis, which resides in the HF junctional zone in mice (Jensen *et al*., 2009). Although Lrig1 expression defines an IFE KSC population in humans, it is not expressed in the reciprocal HF junctional zone (Supplementary Figure 4) and so prevented further characterization of the β-HPV target in humans (Jensen and Watt, 2006). Reporter mice studies have shown that junctional zone Lrig1 cells give rise to keratinocytes in the infundibulum and adjoining IFE, akin to the expansion of this population in our HPV8tg mice, human EV keratosis and AK.

p63 is a p53 protein family member expressed primarily in epithelia as six distinct isoforms, due to alternative transcription and C terminus splicing (Yang *et al*., 1998). Three p63 isoforms contain an N-terminal transcriptional activation (TA isoforms) sequence, while the other three do not (ΔN isoforms). All of the p63 isoforms, TA and ΔN, are transcriptionally active and the ΔN isoforms repress TA isoforms as dominant-negative molecules (Koster. 2010). Multiple lines of evidence support the role of p63 in KSC maintenance (Melino *et al*., 2015): i) p63 null mice demonstrate a terminally differentiated epidermis with no proliferative basal layer containing stem cells (Yang *et al*., 1999); ii) epidermal p63 knockdown induces differentiation, which is induced by TAp63 (Truong *et al*., 2006); iii) ΔNp63 expression maintains the proliferative basal layer (Truong *et al*., 2006); and iv) p63 nuclear accumulation is prominent in KSC and holoclones (Pellegrini *et al*., 2001). UV directly and via p53 mutation results in p63 downregulation and loss of transcriptional activity (Liefer et al., 2000). Recently it has been proposed that UV induced concomitant

activation of oncogenic Ras and TGF-β pathways can restore p63 activity in p53 mutant cells to promote tumor progression (Vasilaki et al., 2016). In keeping with this, UV induced AK and cutaneous SCC frequently demonstrate nuclear p63 (Abbas et al., 2011).

The frequency of nuclear p63 positivity is greater in HPV associated SCC of the oral and anogentital regions, promoted by HPV E6 degradation of p53 (Melino, 2011).

β-HPV early region genes E2, E6 and E7 can induce KSC proliferation through inhibition of Notch signaling and subsequent induction of p63 (Hufbauer *et al*., 2013; Meyers *et al*., 2013, Pfefferle *et al.,* 2008) providing a putative mechanism for this β-HPV induced field cancerization. Hence, we propose that β-HPV early region genes initiate proliferation of Lrig1+ KSC causing their expansion into the overlying HF infundibulum and overlying epidermis. β-HPV driven KSC proliferation results in EV keratosis and occasionally non-EV AK, which are predisposed to transformation into SCC whereupon the β-HPV episome and so gene expression is lost (Figure 6).

MATERIAL AND METHODS

Transgenic Mouse Model

HPV8tg and wild type litter mates were housed and managed under conditions approved by the Italian Animal Care Committee. Age and sex matched mice ear thickness and tail width were measured using Vernier caliper.

Whole mount skin preparation

Tail and back skin was cut into 0.5 cm^2 pieces and dissociated using 2.5 U/ml Dispase (Roche, UK) over night at 4°C. The epidermis was gently removed and fixed in 10% neutral buffered formalin for 2 hours at room temperature, tissue was labelled and mounted as previously described (Braun K *et al.*, 2003).

Immunofluorescence labelling

Immunofluorescence on whole mount, frozen and paraffin embedded section was performed using standard techniques as previously described (Borgogna *et al*. 2012). For the list of antibodies used see Supplementary Table 2.

Single cell suspension for flow cytometry and colony forming efficiency

Tail and back skin was cut into 0.5 cm^2 pieces and dissociated using 2.5 U/ml Dispase (Roche, UK) over night at 4°C. The epidermis was gently removed and further dissociated with TrypLE™ Express Enzyme (ThermoFisher, UK), and the supernatant passed through a 70 μm cell strainer (BD bioscience, UK). Enzymes were inactivated with DMEM with 10% FBS and keratinocytes cell suspension were re-suspended as required.

Flow cytometry, cell sorting and colony forming efficiency assay

Samples were analysed and flow sorted using BD LSRFORTESSA and BD FACSAria™ Fusion (BD Biosciences, USA). The data were analyzed using Flowjo software (Tree Star Inc). Keratinocytes were flow sorted for CD34-/Lrig1+ population and 3000 cells per well were seeded in 6-well plate and cultured for 15 days. Rheinwald and Green Media was

changed every 3 days. The colonies were stained with crystal violet 0.05%, scanned with GelCount™ (Oxford Optronix), and analyzed using ImageJ software (NIH, USA).

RT-PCR

Mouse skin was homogenized in Trizol (ThermoFisher Scientific, UK). RNA isolation from sorted cells and homogenized tissues was performed with RNeasy kit and cDNA was synthetized using QuantiTect Reverse Transcription Kit (Qiagen, UK). Semi-quantitative reverse transcription-PCR (RT-PCR) reactions were carried out using GoTaq® G2 Green Master Mix (Promega, UK) and specific primers sequence are listed in Supplementary Table 3. Real-time quantitative reverse transcription analysis (qRT-PCR) was performed on QuantStudio™ 7 Flex Real-Time PCR System (ThermoFisher Scientific UK). For the determination of E6 and E7 gene expression levels, SyGreen (PCRBIOSYSTEM, UK) was used. Cycling condition were previously described in Schaper et al., 2005, De Andrea et al., 2010. Total mouse-specific β-Actin was used as the housekeeping gene.

EVER1, EVER2, TAp63 and ΔNp63 transcription levels were analyzed with Taqman probes at standard conditions. Total mouse-specific GAPDH was used as the housekeeping gene. Primers details are described in Supplementary Table 3 and 4.

Western blotting

100 mg of shaved back skin was homogenized in 1 ml of RIPA buffer containing 1% Triton X-100, 1% Sodium Deoxycholate, 0.1% SDS, 1 mM EDTA, 160 mM NaCl, 20 mM Tris-HCl (pH 7.4) and 25 µl/ml Protease Inhibitor Cocktail (Sigma, UK). Protein concentrations were analyzed using Pierce BCA Protein Assay (ThermoFisher, UK). Thirty µg of protein lysate was loaded on 8% SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Millipore, UK). For the list of antibodies used see Supplementary Table 2.

Human tissue samples

EV patients' keratosis formalin-fixed and paraffin embedded (FFPE) tissue sections were analyzed according to the protocol approved by the ''Maggiore Hospital'' Research Ethics Committee, Italy. Non-EV actinic keratosis (14-NW-1272) and squamous cell carcinoma (09-WSE-02-1) tissues were obtained after UK NHS R&D, Local Research Ethics Committee approval and informed written consent.

Statistical analysis

Paired t-tests were used to compare HPV8 and wild type litter mates, using GraphPad software (Prism).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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FIGURE LEGENDS

Figure 1: Phenotypic and histological characterization of HPV8tg mice.

a, Schematic representation of the HPV8 transgenes, showing the human cytokeratin-14 gene promoter upstream of the open reading frames of the HPV8 early region genes. **b,** Hematoxylin and eosin staining of paraffin embedded skin sections from WT (left panel) and HPV8tg (right panel) mouse skin. **c,** Toluidine blue staining of mouse whole mount skin including the hair follicle and overlying epidermis from WT (left panel) and HPV8tg (right panel) from the tail. **d,** Ear thickness (upper graph) and tail width (bottom graph) measured using calipers on age matched WT and HPV8tg littermates (n=8), with mean+/-SD (*,$p<0.05$; unpaired t test). All the images were processed using ImageJ software (NIH, USA). All scale bars =100μm.

Figure 2: HPV8 transgenes induce hair follicle changes in HPV8tg mice.

a, b, Adult mice whole mount skin were photographed and analyzed for the area of HF regions and length, WT and HPV8tg were compared, with mean+/-SD, using an unpaired t-test (n=20, ** $p<0.01$). **c,** FACs analysis WT and HPV8tg mice skin keratinocyte isolates (n=6), labelled with Lrig1-FITC and CD34-647 antibodies, with DAPI to select live cells. **d,** The number of Lrig1 positive cells determined by FACs (n=6). (**, $p<0.01$; unpaired t test), with mean+/-SD **e,** Whole mount immunofluorescence of adult WT and HPV8tg tail skin for Ki67 (red) and HF-KSC markers (green). All the images were processed using ImageJ software (NIH, USA). All scale bars = 100 μm.

Figure 3: Lrig1 keratinocyte stem cell proliferation in HPV8tg

a, Whole mount adult WT and HPV8tg (n=6) skin sections labelled with Ki67 (red), Lrig1 (green) antibodies and DAPI (blue). **b,** Number of proliferating cells within KSC populations (Lrig1, LGR6, CD34, and LGR5) was enumerated, with mean+/-SD, in WT and HPV8tg tissue sections (n=7); ** $p<0.01$. **c,** Keratinocyte colony forming assays from flow sorted Lrig1 cells from WT and HPV8tg (n=6) skin dissociates, with mean+/-SD and **d,** A representative image. **e,** p63 labelled frozen sections from adult WT and HPV8tg mice (n=3), the broken line indicates the basal layer. Immuno-labelled tissue sections were visualized and photographed by fluorescent microscope with x20 magnification then processed using ImageJ software (NIH, USA). All scale bars are 100μm.

Figure 4: HPV8 transgenes are uniformly expressed in the epidermis of HPV8tg mice.

a, qRT-PCR of early region genes of WT and HPV8tg mouse skin isolated KSC populations, with mean+/-SD. **b,** qRT-PCR of murine homologues of EVER1 (TMC6) and EVER2 (TMC8) genes of WT and HPV8tg mouse skin isolated Lrig1+ KSC populations, with mean+/-SD.

Figure 5: β-HPV induced keratinocyte stem cell expansion results in keratosis that are predisposed to SCC

a&b, RT-PCR with mean+/-SD (**, $p<0.01$), and **c,** Western blot gels of p63 isoforms from RNA and protein isolates respectively from WT, HPV8tg skin, papilloma (pap) and SCC (n=6). **d,** Images of tissue sections of HPV8tg mouse skin, human EV keratosis, and AK labelled with p63 (green), β-HPV E4 (red) specific antibodies, and DAPI (blue). **e,** Images of tissue sections of HPV8tg mouse and human SCCs labelled with K14 (red), p63 (green) specific antibodies and DAPI (blue). Immuno-labelled tissue sections were visualized and

photographed by fluorescent microscope with x20 magnification then processed using ImageJ software (NIH, USA). All scale bars are 100μm.

Figure 6: β-HPV Field Cancerization Model

The mouse HF (left image) is composed by at least 4 different KSC populations. In the WT HF, the LGR5 is expressed in the hair bulb, CD34+ and K15+ KSC are located in the bulge, and the Lrig1 and LGR6 KSC are located in the upper and lower junctional zone respectively. In HPV8tg, the Lrig1 KSC population expand beyond the junctional zone niche and no longer express Lrig1 but they maintain KSC function. Similarly, in human β-HPV reactivation (right image), the expanded population occupies the hair follicle infundibulum and adjoining interfollicular epidermis, as shown by the yellow arrow. These changes culminate in the histological phenotype known as Freudenthal's funnel, the pathognomonic finding in actinic keratosis and skin field cancerization.