HUMAN PAPILLOMAVIRUSES AND POLYOMAVIRUSES IN SKIN CANCER

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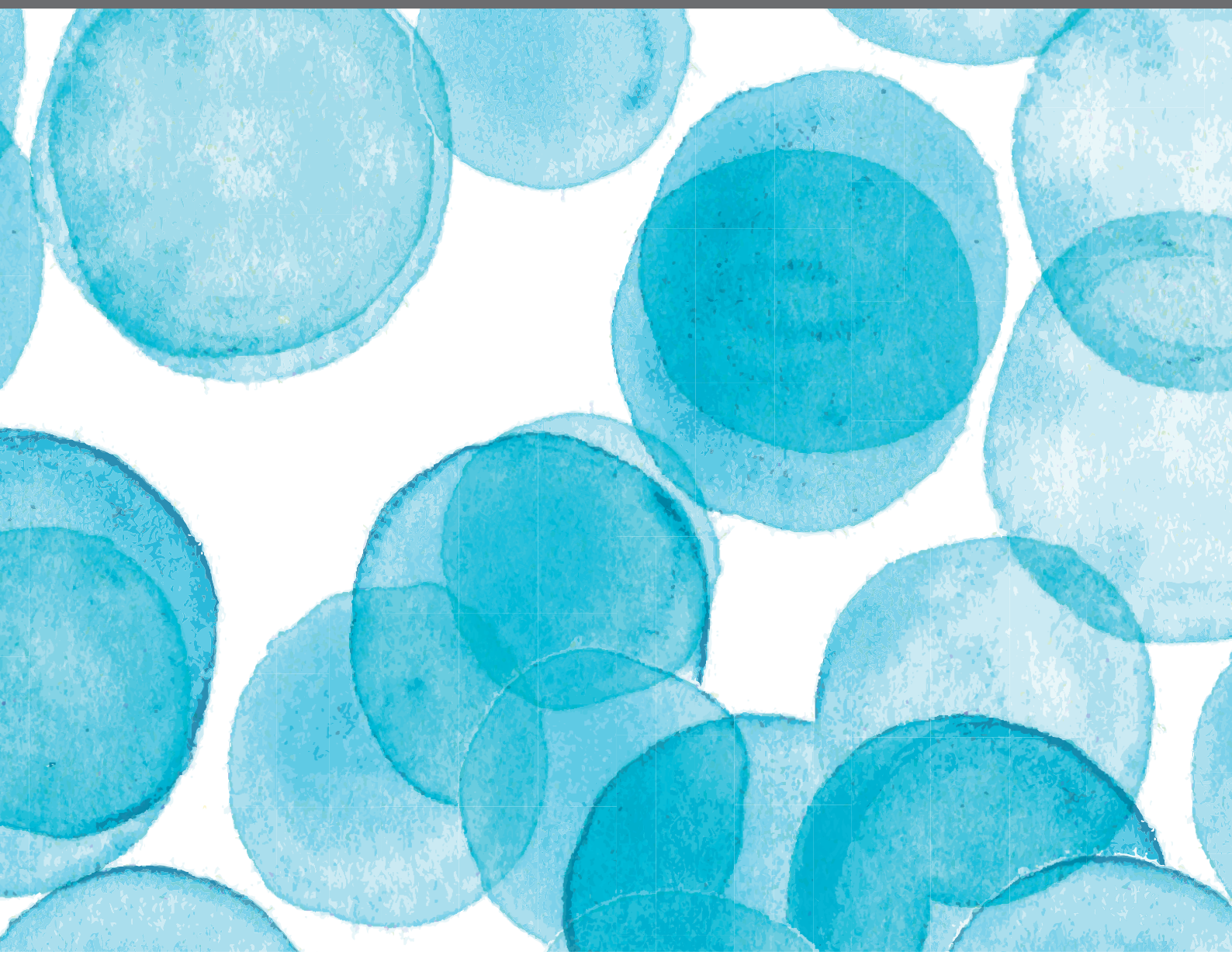

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HUMAN PAPILLOMAVIRUSES AND POLYOMAVIRUSES IN SKIN CANCER

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Nonmelanoma skin cancer (NMSC), more recently defined as keratinocyte carcinoma (KC), is the most frequent malignant tumor among Caucasians. This book deals with widely distributed cutaneous papillomaviruses and polyomaviruses, which are able to establish life-long persistent infections. They are frequently clinically inapparent in the general population, but can cause skin cancer in immunocompromised hosts or in the aging skin undergoing immunosenescence, thereby leading to major medical problems. The incidence of cutaneous squamous cell carcinoma (cSCC) in immunosuppressed organ transplant recipients is 60-250 times higher than that observed in the general population, which is highly suggestive of viral etiology. The treatment of metastatic melanoma with BRAF-inhibitors can induce cSCC formation within only 8-12 weeks. The clinicopathological characteristics of these tumors led to speculations on a possible involvement of oncogenic viruses. The evidence presented in this eBook indicates that high priority should be given to the implementation of a prophylactic and/or therapeutic vaccination program against cutaneous papillomaviruses to prevent NMSC in immunosuppressed allograft recipients.

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Editorial: Human Papillomaviruses and Polyomaviruses in Skin Cancer

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Keywords: papillomavirus, polyomavirus, nonmelanoma skin cancer, Merkel cell carcinoma, cutaneous squamous cell carcinoma

Editorial on the Research Topic

Human Papillomaviruses and Polyomaviruses in Skin Cancer

Nonmelanoma skin cancer (NMSC), more recently defined as keratinocyte carcinoma (KC), is the most frequent malignant tumor among Caucasians. NMSC comprises two major types of skin cancer: basal cell carcinoma (BCC) and cutaneous squamous cell carcinoma (cSCC), accounting for 80 and 20% of all NMSC patients, respectively. Intriguingly, the incidence of cSCC in immunosuppressed organ transplant recipients is 60–250 times higher than that observed in the general population, which is highly suggestive of viral etiology. An etiologic role of human papillomaviruses (HPV) of the genus beta (betaPV) has long been proposed due to the well-documented carcinogenicity of these cutaneous viruses in patients suffering from epidermodysplasia verruciformis (EV) (Quint et al., 2015; Hufbauer and Akgül, 2017). In addition, two epidemiologic prospective studies have recently shown the presence of anti-betaPV antibodies around the time of transplantation to be predictive of keratinocyte carcinoma development in organ transplant recipients (Genders et al., 2015), and both betaPV type diversity and viral DNA load in plucked eyebrow hair to be positively associated with an almost doubling of the risk of developing cSCC (Bouwes Bavinck et al., 2018).

Besides epidemiological evidence, mechanistic studies have reported multiple potentially oncogenic properties of betaPV proteins in keratinocyte cultures as well as transgenic mice, thus providing *in vitro* and *in vivo* functional data corroborating betaPV carcinogenicity. The proposed mechanisms indicate that betaPV play a causative role in tumor initiation and progression, but are not necessary for tumor maintenance, the so-called "hit and run" theory. This type of oncogenic activity of betaPV in cSCC differs profoundly from that of alphaPV. It is generally accepted that deregulated expression of the oncoproteins E6 and E7 of the high-risk mucosotropic alphaPVs such as HPV16 and 18 underlies neoplasia and eventual progression to anogenital or oropharyngeal cancers. The E6 and E7 oncoprotein expression is usually maintained in the full-blown cancers, indicating their necessary role in tumor maintenance. The paradigm shift between alphaPV and betaPV in terms of their role in tumor maintenance has been very important to appreciate the potential role of betaPV in skin cancer.

Two members of the human polyomavirus family (HPyV) have been associated with either malignant or benign skin tumors, respectively: the Merkel cell polyomavirus (MCPyV) has been identified and cloned from Merkel cell carcinoma (MCC) and has been reported to be monoclonally integrated in up to 80% of MCC cases, while the *Trichodysplasia spinulosa* Polyomavirus (TSPyV) is generally regarded as the causative agent of the homonymous, very rare but rather disfiguring, benign proliferative skin disease. To date, there are three more human polyomaviruses with skin-tropism but unknown pathogenicity, so far.

In summary, the Research Topic deals with widely distributed cutaneous papillomaviruses and polyomaviruses, which are able to establish life-long persistent infections. Although they are frequently clinically inapparent in the general population, they can cause skin cancer in

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immunocompromised hosts or in the aging skin undergoing immunosenescence, thereby leading to major medical problems.

Given the well-established carcinogenicity of betaPV in EV patients, the Research Topic opens with a mini review by De Jong et al. highlighting inborn errors of immunity as the genetic basis for the spectacular susceptibility of EV patients to widespread betaPV infection, resulting in cSCC formation in 30–60% of infected patients.

Among the molecular mechanisms contributing to betaPV-induced oncogenesis, attention first focused on the inhibition of cellular DNA damage repair pathways and apoptosis by the viral E6 protein, leading to genomic instability and accumulation of harmful mutations. The betaPV E6 proteins also interfere with Notch and C/EBPalpha/mir-203 signaling in keratinocytes, thereby with keratinocyte differentiation, and possibly contributing to continued proliferation of the infected cells. These activities are comprehensively reviewed and discussed by Wendel and Wallace and Meyers et al, respectively. Meyers et al. point out that the genus gamma HPV197, which was frequently detected in cSCC by next-generation sequencing, shares many cellular targets of its E6 and E7 proteins with E6/E7 of beta PV.

The Research Topic also boasts three original research contributions reporting new potentially oncogenic functions of betaPV. Marx et al. show that HPV8-E6 can interfere with cellular syntenin-2 gene regulation and its role in keratinocyte differentiation. Taute et al. report that HPV8-E6 can also stimulate epidermal growth factor receptor (EGFR) signaling in response to UV irradiation, and that this effect is required for papilloma formation in UV-irradiated mice. Lastly, Podgórska et al. demonstrate that HPV8-E2 together with C/EBPbeta strongly enhances S100A8/A9 protein expression, which in turn drives tumor promoting inflammation as observed in lesional skin from EV patients.

The following "Perspective" section by Olivero et al. identifies which betaPV target cells are critical for viral carcinogenesis. The authors show that when betaPV persistently infects adult tissue stem cells, it induces their proliferation and displacement beyond the stem cell niche as a first step toward field cancerization. Once the HPV-infected cells have acquired a number of transforming mutations, the viral DNA is not any longer required for malignant conversion and may get lost. This scenario has been revealed before by original research (Lanfredini et al., 2017). In good agreement, Borgogna et al. show here that one betaPV-positive hyperplasia from a kidney transplant recipient progressed to an HPV-negative, metastasizing SCC after xenografting in nude mice.

The thoughtful and critical review by Hasche et al. summarizes current data supporting the viral etiology of NMSC, and it also reports current work and perspectives on vaccination against cutaneous HPVs. In this regard, it is pleasantly rewarding for the scientific community to come to the realization that the viral etiology of NMSC is finally being accepted by public health institutions to a degree, which will allow the clinical development of vaccines. It goes without saying that a reduced incidence of NMSC in immunocompromised individuals after vaccination would be the litmus test of its viral etiology.

In striking contrast to what happens in cSCC in terms of betaPV infection, continued expression of the MCPyV T antigen is essential for cell proliferation in most virus-positive MCC cell lines. In this regard, Velásquez et al. here describe a newly established MCPyV-positive cell line requiring T antigen expression to proliferate. MCPyV and TSPyV encode in addition to (Large) T antigen, Middle (MT), and alternative (ALT) T antigens. Van der Meijden and Feltkamp here speculate on their roles and relevance to cancer.

The wide distribution of cutaneous papillomaviruses and polyomaviruses has raised, and still raises, a number of unsolved issues in epidemiologic case-control studies apart from MCC, where the virus is always found integrated into the cellular genome and mutated. These issues become rather obvious in the last two contributions of the Research Topic. Although Hillen et al. detect MCPyV in seborrheic keratosis, the authors conclude that this observation mainly reflects its widespread occurrence in the skin rather than representing a cause-effect link between seborrheic keratosis and the virus. Finally, Purdie et al. report a virologically broad investigation of HPV and five skin-tropic human polyomaviruses in BRAF-inhibitor (BRAFi)-induced cSCC. This drug, which has been in clinical use since 2011 for the treatment of metastatic melanoma, can induce, alongside other adverse effects, cSCC formation with a median lag time of only 8–12 weeks. Interestingly, the clinicopathological characteristics of these cSCCs lead the authors to speculate on a possible involvement of oncogenic viruses. Indeed, betaPV, MCPyV, and HPyV7 were detected in 66, 73, and 55% of cSCCs, respectively. Overall, the three viruses were co-detected in 55% of the tumors. However, they conclude that a potential role of HPV and/or HPyV in BRAFi-induced cSCC still remains uncertain and warrants further investigation.

To sum up, this volume comprises a large body of evidence attesting the viral etiology of NMSC. Nonetheless, there are still many unanswered questions awaiting further investigation. Based on the evidence hereby presented, we believe that future research efforts should take into account the following main points emerging from this volume:

1. The detection of HPV197 DNA in human skin cancer, together with the observation that its E6/E7 proteins interact with human tumor suppressor proteins, strongly suggests that this virus and possibly other gammaPVs may play a more important role in skin carcinogenesis than ever thought before;
2. In light of the proposed "hit and run" mechanism of betaPV-driven skin carcinogenesis, further research focusing on the early steps of betaPV infection is necessary to identify new diagnostic markers as well as potential targets for anticancer therapy;
3. Given the medical relevance of BRAFi-induced NMSC development, further research into the role of the HPV and HPyV identified so far and possibly other oncogenic viruses appear justified. Possible synergistic interactions between the frequently codetected HPV and HPyV would be of great interest in medical and general virology.

4. Finally, the evidence presented in this volume indicates that highest priority should be given to the implementation of a prophylactic and/or therapeutic vaccination program against cutaneous HPV to prevent NMSC in immunosuppressed allograft recipients. Consequently, basic research on viral life cycle and oncogenic functions, research on vaccine formulations, and challenge experiments in vaccinated animal models should all be given the needed resources and recognition. We strongly believe that the time is now ripe for this essential translational step in HPV-related skin cancer research.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Epidermodysplasia Verruciformis: Inborn Errors of Immunity to Human Beta-Papillomaviruses

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Epidermodysplasia verruciformis (EV) is an autosomal recessive skin disorder with a phenotype conditional on human beta-papillomavirus (beta-HPV) infection. Such infections are common and asymptomatic in the general population, but in individuals with EV, they lead to the development of plane wart-like and red or brownish papules or pityriasis versicolor-like skin lesions, from childhood onwards. Most patients develop non-melanoma skin cancer (NMSC), mostly on areas of UV-exposed skin, from the twenties or thirties onwards. At least half of the cases of typical EV are caused by biallelic loss-of-function mutations of *TMC6/EVER1* or *TMC8/EVER2*. The cellular and molecular basis of disease in TMC/EVER-deficient patients is unknown, but a defect of keratinocyte-intrinsic immunity to beta-HPV is suspected. Indeed, these patients are not susceptible to other infectious diseases and have apparently normal leukocyte development. In contrast, patients with an atypical form of EV due to inborn errors of T-cell immunity invariably develop clinical symptoms of EV in the context of other infectious diseases. The features of the typical and atypical forms of EV thus suggest that the control of beta-HPV infections requires both EVER1/EVER2-dependent keratinocyte-intrinsic immunity and T cell-dependent adaptive immunity.

Keywords: epidermodysplasia verruciformis, beta-HPV, TMC/EVER, non-melanoma skin cancer, primary immunodeficiency

INTRODUCTION

Epidermodysplasia verruciformis (EV) is a rare skin disease characterized by persistent disseminated flat warts and pityriasis versicolor-like lesions, associated with a high risk of non-melanoma skin cancer (NMSC). About 501 patients have been reported worldwide (Burger and Itin, 2014; Imahorn et al., 2017). EV was first described in Lewandowsky and Lutz (1922) as a congenital skin disease. The observation of parental consanguinity and familial cases led to the suggestion that EV might be a genetic disease with autosomal recessive inheritance

(Cockayne, 1933; Rajagopalan et al., 1972). This notion was not confirmed until 70 years later, when inactivating mutations of the *EVER1/TMC6* and *EVER2/TMC8* genes accounting for about 50% of all known cases of EV were identified (Ramoz et al., 2002; Orth, 2006, 2008; Imahorn et al., 2017). Auto- and heteroinoculation experiments performed from 1946 onward showed that the phenotype of EV was dependent on a viral infection (Lutz, 1946; Jablonska and Milewski, 1957). Viral particles were then detected in EV lesions (Ruiter and van Mullem, 1966; Jablonska et al., 1968), and, subsequently, EV-specific human papillomaviruses (HPVs) were identified, some of which, such as HPV-5 in particular, were shown to be associated with the cancers observed in EV patients (reviewed in Orth et al., 1980; Orth, 2006). EV thus results from a genetically determined susceptibility to specific skin-tropic HPVs (Orth, 2006, 2008; Imahorn et al., 2017). These viruses belong to the beta-genus, and are almost ubiquitous and apparently benign in the general population. EV patients are not prone to other viral, bacterial, or fungal infections. NMSC is the only type of cancer presenting a higher incidence in these patients than in the general population (Orth, 2006, 2008). The EVER1 and EVER2 proteins are thought to govern keratinocyte-intrinsic immunity to beta-HPVs. However, patients with cutaneous clinical symptoms of beta-HPV infection and other infectious manifestations caused by profound T-cell defects have been reported. This clinical picture is referred to as "atypical EV." The cumulative findings of almost a century of studies of typical and atypical EV suggest that both keratinocytes and T cells are required for the control of beta-HPVs. We review here the various facets of EV.

CLINICAL MANIFESTATIONS AND HISTOPATHOLOGY OF EV

EV manifests as multiple polymorphic skin lesions, which begin to appear during infancy or early childhood and persist throughout the individual's life. Flat-topped papular lesions resembling verrucae planae typically develop on the extremities. The lesions may also present as red or red-brownish papules or pityriasis versicolor-like lesions, mostly on the trunk, neck, and face (Lewandowsky and Lutz, 1922). In addition, verruca-like papillomatous or seborrheic keratosis-like lesions may develop. The frequency of verruca vulgaris is no higher in these patients than in the general population. In many patients, isomorphic lesions develop preferentially at sites of traumas (the Koebner phenomenon) (de Oliveira et al., 2003). Some patients have persistent palmar lesions similar to palmar pits and dermatoscopically consistent with plane warts (Imahorn et al., 2017). Over the course of the patient's lifetime, the phenotype of a particular skin area may change, and lesions may progress to NMSC. There is currently no cure for EV. The development of EV lesions cannot be prevented, but frequent examinations of skin lesions that might develop into skin cancers and appropriate treatment of these lesions are recommended, such as surgical removal and cryotherapy. Patients with typical EV are otherwise healthy and are not particularly susceptible to any other unusually severe infectious diseases. Provided that cancers are treated appropriately, life expectancy is similar to that in the general population; multifocal carcinomas might, however, be fatal if untreated. Histological examinations of lesions typically reveal hyperkeratosis and parakeratosis, mild acanthosis, and the presence of koilocytes, keratinocytes with pale-stained cytoplasm in the upper epidermis associated with high levels of intranuclear viral replication. On hematoxylin-eosin staining, the cytoplasm of the affected cells stains pale blue (so-called "blue cells") and numerous round basophilic keratohyalin granules are visible. This histological finding is pathognomonic for infections with HPV (Orth, 2006). Possible immune infiltration has not been comprehensively characterized in EV lesions. A number of atypical EV cases have been reported, which will be reviewed below.

CLINICAL MANIFESTATIONS OF ATYPICAL EV

Patients with atypical EV have a broader clinical phenotype than those with typical EV. The phenotype of EV, as described above, is undistinguishable between patients with typical and atypical forms. Indeed, the skin lesions develop early in life and are caused by the same beta-HPV subtypes that are found in patients with typical EV (see **Table 1**). The other clinical phenotypes, mostly infectious and auto-immune, differ between patients (Crequer et al., 2012a,b; Sanal et al., 2012; Stray-Pedersen et al., 2014; Stepensky et al., 2015; Li et al., 2016; Platt et al., 2017; Tahiat et al., 2017). The clinical features of patients with atypical EV are reviewed in **Table 1**. Some patients have growth retardation or mild developmental delay. Others present auto-immune features, which may be clinically overt and affect various tissues and organs, or covert, with detectable auto-antibodies but no clinical signs. Bacterial infections, including skin abscesses, pneumonia, and ear infections, are frequent. Infections with cutaneous herpes viruses, molluscum, mucocutaneous candidiasis, and non-beta-HPV infections have also been documented. Moreover, some patients display recurrent respiratory and gastrointestinal infections. Cancers, such as Burkitt lymphoma and EBV-lymphoma, have been reported in some patients. Finally, a few patients have been reported to display clinical manifestations of beta- and alpha-HPV co-infections in different lesions (Azzimonti et al., 2005; Borgogna et al., 2014; Landini et al., 2014). These patients display cutaneous alpha- and beta-HPV- (Borgogna et al., 2014) or genital (Landini et al., 2014) alpha-HPV-driven infections, but no broad susceptibility to infections. These cases suggest that covert immunological abnormalities might favor the development of beta- and alpha-HPV-induced cutaneous and mucosal lesions in other individuals. Based on these clinical observations, the molecular and cellular basis of typical and atypical EV phenotype appear to be different. The typical EV phenotype is, in itself, suggestive of a keratinocyte-intrinsic defect, whereas the atypical EV phenotype, with its myriad of infections and auto-immune features, is more consistent with an adaptive T-cell defect.

TABLE 1 | Immunological parameters of patients with typical and atypical EV.

Disease	Genetic etiology*	Clinical phenotypes (affected/total no. of patients)	Expression pattern	T cell counts	T cell function	Other immunological features	Reference
Typical EV	AR TMC6/EVER1	EV	Broad (including keratinocytes and T cells)	Normal	Normal	None	Orth, 2006, 2008; Imahorn et al., 2017
	AR TMC8/EVER2	EV	Broad (including keratinocytes and T cells)	Normal with slightly high proportions for skin-homing subsets	Normal	None	
Atypical EV	AR RHOH deficiency (2 siblings)	Cutaneous viral infections, bronchopulmonary disease, Burkitt lymphoma, **disseminated EV-like flat warts (histologically consistent with EV, HPV-3, -12, and -20 on Southern blotting and PCR)**	Lymphoid lineage	Naïve CD4+ T-cell lymphopenia, high memory CD4+ and CD8+ T-cell counts, low proportions of skin-homing T-cell subsets	Mildly impaired antigen-induced T-cell proliferation, no anti-CD3-induced proliferation	Normal B cell number and function, normal NK cells	Crequer et al., 2012a
	AR MST1 deficiency # (1 patient)	Recurrent respiratory infections, candidiasis, **disseminated EV-like flat warts (HPV-5 and -15 positive by Southern blotting and PCR)**, cervical adenopathy, growth retardation	Broad (including keratinocytes and T cells)	Profound CD4+ T-cell lymphopenia (naïve low, memory high), modest naïve and central memory CD8+ lymphopenia, revertant memory CD8+ T-cell counts high	Impaired mitogen (PHA, PMA/Ionomycin)- and antigen (candida, tetanus toxoid, tuberculin)-induced proliferation	Normal B and NK cells, cANCA autoantibodies, high IgA and IgE levels, poor antibody response to several vaccines	Crequer et al., 2012b
	AR CORO1A deficiency # (2 siblings)	Bronchiectasis; fatal EBV-positive lymphoma (1/2); mucocutaneous immunodeficiency syndrome with molluscum contagiosum, oral-cutaneous herpetic (HSV-1) ulcers, **disseminated EV-like HPV infection (HPV-5 and -17 positive by PCR)** and tuberculoid leprosy (1/2)	Broad (including keratinocytes and T cells)	Complete deficiency of naïve CD4+ T cells, high level of double-negative (CD3+CD4-CD8-) gd T cells	Impaired mitogen-induced proliferation, normal antigen-induced proliferation (candida and tetanus toxoid)	T-B+NK+ SCID; absent memory B cells; low NK cell counts, elevated serum IgE, normal serum IgG	Stray-Pedersen et al., 2014
	AR ARTEMIS # p.Leu123Ser (hypomorphic) (1 patient)	Recurrent respiratory and gastrointestinal infections, **persistent disseminated flat warts (histologically consistent with EV, no HPV confirmation)**	broad (including keratinocytes and T cells)	CD4+ T-cell lymphopenia, CD8+ T-cell counts normal	Impaired mitogen- and antigen-induced proliferation	T low B- leaky SCID, IgM levels normal, IgG levels high, IgA absent, normal NK cell counts	Tahiat et al., 2017
	AR DOCK8 deficiency # (4 families)	Recurrent or severe viral infections associated with cancer, atopic dermatitis, recurrent respiratory or gastrointestinal tract infections, disseminated Molluscum contagiosum; disseminated **flat-topped warts (histologically consistent with EV, HPV-5 positive by PCR),** eczema, hyperpigmentation, and folliculitis	Broad (including keratinocytes and T cells)	CD4+ T-cell lymphopenia	Normal mitogen-induced proliferation	IgM levels low, IgE levels high, variably impaired specific antibody production, normal CD8+ T-cell and B-cell numbers	Sanal et al., 2012; Liu et al., 2017

(Continued)

TABLE 1 | Continued

Disease	Genetic etiology*	Clinical phenotypes (affected/total no. of patients)	Expression pattern	T cell counts	T cell function	Other immunological features	Reference
	AR RASGRP1 # deficiency (1 patient)	Recurrent ear infections, skin abscesses, chronic non-bloody diarrhea, **disseminated warts (histologically consistent with EV, no HPV confirmation)**, severe failure to thrive, splenomegaly, diffuse lymphadenopathy, fatal EBV-positive B cell lymphoma	Broad (including keratinocytes and T cells)	CD4+ T-cell lymphopenia, CD8+ T-cell counts high	Impaired proliferation in response to mitogen (PHA) and antigen (candida and tetanus toxoid)	Normal NK cell number with reduced function, IgG levels low, IgM levels high	Platt et al., 2017
	AR LCK # c.188-2A>G (3 siblings)	Recurrent bacterial pneumonia; **pityriasis-versicolor-like lesions and flat warts on hands, abdomen, legs (histologically consistent with EV, HPV-5, -20, -38 positive by PCR)** and histologically confirmed squamous cell carcinomas, fatal in 1 sibling; genital warts (HPV-6 positive by PCR)	Lymphoid lineage	CD4+ T-cell lymphopenia	Not tested	Not tested	Li et al., 2016
	AR TPP2 deficiency (2 siblings)	Evans syndrome (immune thrombocytopenic purpura and autoimmune hemolytic anemia) (2/2); progressive leukopenia (2/2); mild viral infections (1/2); **flat, hypopigmented warts (HPV-15 positive by PCR) (1/2)**, mild developmental delay (1/2)	Broad (including keratinocytes and T cells)	Normal or slightly low CD4+ T lymphocyte counts	Senescent CD8+ T cells (impaired proliferation, enhanced staurosporine-induced apoptosis)	Premature immunosenescence (T and B cells) and antinuclear antibodies; normal IgA and IgE levels; IgG and IgM levels high	Stepensky et al., 2015

**Unless the specific mutation is listed, complete deficiency has been verified experimentally. #Other cases with the same genetic etiology but without EV-like symptoms and beta-HPV infections have been described. AR, autosomal recessive+; SCID, severe combined immunodeficiency.*

SKIN CANCER

Patients with EV have a higher than normal risk of developing actinic keratosis and NMSC, particularly cutaneous squamous cell carcinoma (cSCC), cSCC *in situ* (Bowen's disease) and, to a lesser extent basal cell carcinoma (BCC) (Lewandowsky and Lutz, 1922; Rajagopalan et al., 1972; Orth et al., 1979; Mitsuishi et al., 2008). About two third of EV patients develop NMSC, with an onset during their twenties or thirties (Lewandowsky and Lutz, 1922; Rajagopalan et al., 1972; Orth et al., 1978; Majewski and Jablonska, 1997; de Oliveira et al., 2003). NMSC typically occurs in lesions exposed to the sun, about 20 years after the appearance of the first lesions, suggesting that UV irradiation and HPV are cocarcinogens for the development of this cancer. EV patients should, therefore, pay particular attention to protecting their skin against exposure to the sun. The role of HPVs in carcinogenesis is well established. The first evidence for the oncogenic behavior of HPVs was actually documented in a study of EV (Orth et al., 1980). EV has long served as a human model for studies of viral cutaneous oncogenesis (Jablonska et al., 1972; Casanova and Abel, 2004). This oncogenic behavior of beta-HPVs led to speculations that these viruses might also contribute to the development of cSCC in the general population. However, in cases of cSCC in the general population, less than one viral genome per cell is generally detected; by contrast, viral load is very high in cSCC of EV patients, indicating that different mechanisms of cancer initiation and maintenance are probably at work (Howley and Pfister, 2015). In particular, oncogenic HPV-5 and HPV-8 have been found in EV-associated NMSC, leading to their classification, by the WHO, as possible carcinogens in EV patients (Bouvard et al., 2009). The detection of high loads of HPV-5 E6 and E7 transcripts suggested a role in the cancer induction or maintenance, although the underlying mechanism remains unclear (Orth, 1987). Radiotherapy for cSCC treatment can be effective in the general population, but is counterproductive in EV patients, as the development of more aggressive tumors after treatment has been reported (Rajabi et al., 2014; de Oliveira et al., 2015). Patients should undergo frequent physical examinations, to facilitate the identification of precancerous and cancer lesions as early as possible. However, it has been shown that infection with oncogenic HPVs known to be associated with a high risk of skin cancer is not sufficient, in itself, to cause cancer. Environmental and genetic factors thus contribute to the inter-individual variability of outcome for HPV infection.

VIRAL ETIOLOGY

More than half a century ago, EV was recognized as a virus-induced disease, based on several experimental observations. Inoculation experiments showed EV to be transmissible (Lutz, 1946; Jablonska and Milewski, 1957; Jablonska et al., 1966). The first viral particles were then detected in lesions by electron microscopy (Ruiter and van Mullem, 1966; Jablonska et al., 1968) and, several years later, the first EV-specific HPV types, all from genus beta-HPV, were isolated from EV lesions (Orth et al., 1978;

Orth, 1986). EV patients not only have high HPV loads in their lesions, they also have a high serum antibody reactivity to beta-HPVs (Michael et al., 2010). At least 25 different beta-HPV genotypes have been found in patients with EV, and patients are usually infected with multiple EV-HPV types, HPV-5 being the most prevalent genotype (Orth, 2006). HPV-5 is also the most common genotype in cases of malignant conversion (Orth, 1986; Imahorn et al., 2017). Beta-HPVs are also frequently found in the skin of healthy individuals of the general population, but mostly at low copy numbers, and they do not cause clinical disease. This suggests that beta-HPVs are commensals of the skin (Antonsson et al., 2003a,b). However, it remains a matter of debate whether these beta-HPVs are really non-pathogenic in individuals without EV, as some studies have associated EV-HPV seropositivity and viral DNA load in the eyebrow with the risk of cSCC in the general population (Neale et al., 2013; Iannacone et al., 2014). The mechanisms of persistent, asymptomatic EV-HPV infection remain largely unknown. It has been suggested that beta-HPVs lack an essential growth-promoting function, limiting their pathogenicity. Indeed, unlike the alpha- and gamma-HPVs responsible for cutaneous warts, cervical cancers, and laryngeal papillomatosis, beta-HPVs do not possess the *E5* or *E8* (also reported as E10[1]) open-reading frames typically found in pathogenic non-beta HPVs and shown to be a growth-promoting factor for keratinocytes *in vivo* (Danos et al., 1983; Giri et al., 1985; Hu et al., 2002; Nonnenmacher et al., 2006; Orth, 2006, 2008). Beta-HPVs can overcome this lack of a growth-promoting function and attain full pathogenicity, only in genetically predisposed individuals, implying that these patients have a specific cutaneous immune defect (reviewed by Orth, 2006, 2008).

HUMAN GENETICS OF TYPICAL EV

Typical EV is a Mendelian condition that is transmitted as an autosomal recessive trait and shows complete penetrance in the first decade of life, across different ethnic groups (Orth, 2006). An autosomal recessive mode of inheritance was initially proposed in Cockayne (1933), confirmed in 1974 (Rajagopalan et al., 1972) and subsequently supported by a study of 147 EV cases, in which 11% of patients were the offspring of consanguineous marriages, and 10% came from multiplex families in which 25% of the siblings were affected, with an equal sex ratio (Lutzner, 1978). Shortly after the mapping of two susceptibility loci for EV to chromosomes 17q25 (EV1) and 2p21-p24 (EV2) (Ramoz et al., 1999, 2000), bi-allelic loss-of-function mutations of two adjacent genes termed *TMC6* and *TMC8,* also named *EVER1* and *EVER2*, respectively, located within the EV1 locus, were identified (Ramoz et al., 2002). Eight different mutations of *TMC6/EVER1* and 11 mutations of *TMC8/EVER2* have been described to date, in a total of 32 patients from 19 families and 5 ethnicities reviewed in Burger and Itin (2014) and Imahorn et al. (2017). These mutations are nonsense, frameshift, or splice-site mutations with full clinical penetrance for EV in homozygotes and compound heterozygotes. The effects of these mutations on mRNA and protein levels have not been investigated in most patients. Nonsense-mediated RNA decay and undetectable TMC8/EVER2 protein have been reported in two patients with premature stop codons (Ramoz et al., 2002; Landini et al., 2012). However, splice-site mutations of *TMC8/EVER2* have been identified that have no effect on mRNA levels, despite the detection of aberrantly spliced, shorter transcripts (Miyauchi et al., 2016; Imahorn et al., 2017). Nevertheless, all mutations are assumed to be loss-of-function. By contrast, the missense polymorphisms found in the general population are not predicted to be deleterious and are too common to account for EV. Despite these genetic analyses, more than 40% of the EV families identified to date have no mutations of the genes known to be associated with this disease (Zuo et al., 2006; Arnold et al., 2011; Imahorn et al., 2017) suggesting that high-throughput sequencing studies are required to identify the causal genes in these families.

HUMAN GENETICS OF ATYPICAL EV

Inactivating mutations of *RHOH* (Crequer et al., 2012b), *STK4* (encoding the MST1 protein) (Crequer et al., 2012a), *CORO1A* (Stray-Pedersen et al., 2014), *DCLRE1C (encoding the Artemis protein)* (Tahiat et al., 2017), *DOCK8* (Sanal et al., 2012; Liu et al., 2017), *RASGRP1* (Platt et al., 2017), *LCK* (Li et al., 2016), and *TPP2* (Stepensky et al., 2015) have been reported in patients with atypical EV. Interestingly, the clinical penetrance of these genetic disorders for EV lesions is low, as only a few patients present with manifestations of EV. This is at odds with the findings for typical EV, which is a fully penetrant Mendelian trait. A complete loss of function has been reported for all mutations, except those for Artemis (hypomorphic) and Lck (no functional investigation). All these patients suffer from a classic T-cell primary immunodeficiency. Some also display impaired mitogen- and/or antigen-induced T-cell proliferation. NK cells counts are normal in all these diseases, except for CORO1A. Hyper-IgM and hyper-IgE are observed in RASGRP1 and in CORO1A and DOCK8, respectively. Autoimmunity has been reported for STK4 and TPP2 deficiencies. T-cell senescence has been reported for RHOH and TPP2 deficiencies. It remains unclear whether these observations are the cause or consequence of severe, disseminated, chronic, life-long beta-HPV and other recurrent acute and chronic infections in these patients. Few studies have addressed the specific responses of leukocytes, but a lack of autologous T-cell response to HPV-infected keratinocytes has been described (Cooper et al., 1990). Most of these genes are broadly expressed (including in T cells and other types of skin-resident cells), with the exception of *RHOH* and *LCK*, which are expressed exclusively in the lymphoid linage (**Table 1**). An additional contribution of cells other than T lymphocytes, including other types of skin-resident cells, to the pathogenesis of atypical EV cannot, therefore, be ruled out. Nevertheless, the T-cell defects underlying atypical EV are likely to be specific, as most inherited and acquired forms of T-cell deficiency do not cause clinically apparent infections with beta-HPVs.

[1] http://PAVE.niaid.nih.gov

LEUKOCYTE IMMUNITY

Atypical EV is associated with T-cell deficiency. The only immunological feature common to all known etiologies is $CD4^+$ lymphopenia. More detailed immunological studies have been performed only in RhoH-deficient patients. These patients had impaired tissue-homing T-cells marker expression, with a small decreased in the number of memory skin-homing (CLA^+) $CD4^+$ T cells and a large decrease in the number of integrin $beta7^+$ $CD4^+$ T cells. Broad immunological evaluations of patients with typical EV have proved difficult, due to the rarity of the disease, and the data available are therefore highly patchy. *TMC6/EVER1* and *TMC8/EVER2*, which are mutated in patients with typical EV, are widely expressed, including in lymphocytes and the skin (Lazarczyk et al., 2012). Patients with typical EV have normal humoral immunity and antibody responses (Jablonska et al., 1979), their Langerhans cells have normal alloantigen presentation capacity (Majewski and Jablonska, 1992), and they have largely normal NK cell activity (Majewski et al., 1986). They have been reported to display impairments of cellular immunity, including low T-cell counts, $CD4^+/CD8^+$ T-cell ratios, a lack of responsiveness to T-cell mitogens, anergy to skin antigens and sensitization to dinitrochlorobenzene (Glinski et al., 1976, 1981; Prawer et al., 1977; Majewski et al., 1986; Majewski and Jablonska, 1992; Majewski et al., 1997; de Oliveira et al., 2003), but these defects were inconsistent and further complicated the assessment of immunological dysfunction in EV patients. All these observations suggest that a role for TMC6/EVER1 and TMC8/EVER2 in skin-intrinsic immunity.

KERATINOCYTE IMMUNITY

Keratinocytes contribute to protective immunity in the skin in at least three ways. First, they form a physical barrier. Second, they secrete and respond to cytokines, chemokines, and growth factors, participating in leukocyte-mediated operations (Kupper and Fuhlbrigge, 2004). Third, keratinocyte-intrinsic mechanisms are thought to contribute to the control of keratinocyte-tropic viruses. TMC6/EVER1 and TMC8/EVER2 thus provide us a unique opportunity to study the role of keratinocytes in host defense against HPV infection. TMC6/EVER1 and TMC8/EVER2 belong to a large family of highly conserved transmembrane channel-like (TMC) proteins that are expressed in the endoplasmic reticulum, where they act as or modulate the activity of transmembrane channels (Keresztes et al., 2003; Kurima et al., 2003). It has been suggested that these two proteins interact with the zinc transporter ZnT-1, influencing intracellular Zn^{2+} concentration, and, thus, the activity of the transcription factors, such as AP-1, a key activator in the HPV life cycle and cellular proliferation (Lazarczyk et al., 2008). It has also been suggested that TMC8/EVER2 influences the cellular response to TNF-α by promoting apoptosis rather than NF-κB activation and pro-survival signaling pathways (Gaud et al., 2013; Vuillier et al., 2014). TMC6/EVER1 and TMC8/EVER2 are thus thought to serve as restriction factors for beta-HPVs in keratinocytes, through the limitation of viral replication and gene expression. Studies of TMC6/EVER1 and TMC8/EVER2 have proved technically difficult, partly due to problems raising robust antibodies against these highly hydrophobic proteins, which have 10 and 8 transmembrane domains, respectively. Known molecular functions/mechanism of TMC6/EVER1 and TMC8/EVER2 are reviewed in Orth (2008) and Lazarczyk et al. (2009). The cellular and molecular bases of beta-HPV-driven lesions in TMC6/EVER1 and TMC8/EVER2-deficient patients with EV remain unknown. The genetic defects prevent patients from controlling beta-HPV in keratinocytes (Akgul et al., 2006). The patients are susceptible to skin infections with these particular types of HPV (Boxman et al., 1999). In addition, some patients with severe combined immunodeficiency (SCID) caused by mutations of *IL2RG* or *JAK3* may develop EV. These patients develop EV only after successful bone marrow transplantation, indicating that disease development in these patients is related to a keratinocyte defect (Laffort et al., 2004).

A CLASSIFICATION OF EV INTO TWO MAJOR TYPES

In the past, EV patients rarely underwent immunological follow-up, and typical and atypical cases were treated as the same diagnostic entity. This has led to conflicting reports about the impairment of immunity in only some patients. Patients with typical EV probably have a keratinocyte-intrinsic defect allowing beta-HPVs to persist in the skin, leading to the development of clinically apparent lesions. Consistent with this notion, three TMC8/EVER2-deficient EV patients have been reported to display a largely normal partitioning of circulating T cells, possibly even with slightly larger than normal skin-homing T-cell populations (Crequer et al., 2013). These normal numbers of circulating T cells do not exclude the possibility of an impaired recognition of and response to HPV-infected keratinocytes by these cells (Cooper et al., 1990). Once it became clear that these patients had a selective susceptibility to beta-HPV infections and TMC6/EVER1 and TMC8/EVER2 deficiencies had been described as a genetic etiology, EV was identified as a primary immunodeficiency (Notarangelo et al., 2004; Orth, 2008; Casanova, 2015). It has been suggested that the TMC/EVER proteins function as antiviral restriction factors in keratinocytes, and that they control constitutive intrinsic immunity (Orth, 2008). Consistent with this hypothesis, patients with acquired or treatment-induced immunosuppression do not normally develop EV, despite the commensal nature of beta-HPVs (Antonsson et al., 2003a,b). This contrasts with common warts due to alpha-HPVs, which are more common in immunosuppressed individuals (Wieland et al., 2014). Rare cases of EV-like syndromes developing in previously unaffected adults, and known as "acquired EV," have been described in patients with suppressed cell-mediated immunity. The clinical presentations of these patients and proposed treatments have been reviewed in detail elsewhere (Zampetti et al., 2013; Ovits et al., 2017). The rarity of atypical EV in patients with broad and severe genetic T-cell deficiencies and in immunocompromised individuals suggests that immunosuppression *per se* is not

sufficient for the development of this disease. This is the case for most infectious diseases striking patients with such T-cell disorders, and it suggests that other host and environmental factors are involved in pathogenesis.

CONCLUDING REMARKS

In summary, both keratinocytes and T cells probably contribute to immunity to beta-HPV. Genetic defects affecting one or, potentially, both cell types lead to the EV-defining clinical manifestations of beta-HPV infection. All individuals with severe, recurrent, and persistent flat warts and with a diagnosis of typical or atypical EV should be tested for genetic etiologies of typical and atypical EV. The presence and extent of a systemic T-cell defect determines the spectrum of other clinical manifestations of an infectious, autoimmune, or allergic nature. Future studies should provide more conclusive demonstrations of the keratinocyte-intrinsic nature of EVER-dependent immunity to beta-HPVs. The identification of new genes associated with typical and atypical EV will pave the way for analyses of the molecular and cellular bases of EV, including studies of the respective contributions of keratinocytes and T cells to anti-beta-HPV immunity. The highly restricted susceptibility to beta-HPV observed in patients with EV has important clinical and biological implications in the fields of HPV, mucocutaneous immunology, and cell-intrinsic immunity. Elucidation of the genetic and immunological bases of EV will facilitate studies of the virulence mechanisms of other HPVs, including the role of E5 and E8 proteins produced by alpha- and gamma-HPVs, respectively. These studies will also facilitate the development of novel approaches for the diagnosis and management of patients with various conditions due to HPVs.

AUTHOR CONTRIBUTIONS

SdJ, EI, PI, GO, EJ, J-LC, and BB wrote the manuscript. SdJ, EI, PI, JU, GO, EJ, J-LC, and BB reviewed and edited the manuscript.

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REVIEW
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Loss of Genome Fidelity: Beta HPVs and the DNA Damage Response

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While the role of genus alpha human papillomaviruses in the tumorigenesis and tumor maintenance of anogenital and oropharyngeal cancers is well-established, the role of genus beta human papilloviruses (β-HPVs) in non-melanoma skin cancers (NMSCs) is less certain. Persistent β-HPV infections cause NMSCs in sun-exposed skin of people with a rare genetic disorder, epidermodysplasia verruciformis. However, β-HPV infections in people without epidermodysplasia verruciformis are typically transient. Further, β-HPV gene expression is not necessary for tumor maintenance in the general population as on average there is fewer than one copy of the β-HPV genome per cell in NMSC tumor biopsies. Cell culture, epidemiological, and mouse model experiments support a role for β-HPV infections in the initiation of NMSCs through a "hit and run" mechanism. The virus is hypothesized to act as a cofactor, augmenting the genome destabilizing effects of UV. Supporting this idea, two β-HPV proteins (β-HPV E6 and E7) disrupt the cellular response to UV exposure and other genome destabilizing events by abrogating DNA repair and deregulating cell cycle progression. The aberrant damage response increases the likelihood of oncogenic mutations capable of driving tumorigenesis independent of a sustained β-HPV infection or continued viral protein expression. This review summarizes what is currently known about the deleterious effects of β-HPV on genome maintenance in the context of the virus's putative role in NMSC initiation.

Keywords: beta HPV, DNA-damage response, skin cancer, genomic fidelity, tumorigenesis, epidermodysplasia verruciformis, viral oncogenesis, p300

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INTRODUCTION

Human papillomavirus (HPV) is a family of small, non-enveloped double-stranded DNA viruses that infect mucosal and cutaneous epithelia. This family is comprised of five genera (alpha, beta, gamma, nu, and mu) spanning across over 396 different HPV types potentially inhabiting human skin (Bzhalava et al., 2014). Classification of the HPV family is based on the sequence of the L1 capsid protein (Bernard et al., 2010; de Villiers, 2013). The alpha and beta genera are most widely studied because of the pathogenesis associated with some members of these genera. α-HPV causes genital warts (low risk types, HPV 6, and 11 for example) and genital cancer (high risk types, HPV 16, 18, 31, and 45 for example) (Tommasino, 2014; Doorbar et al., 2015). While β-HPV is known to cause flat warts and non-melanoma skin cancer in individuals with compromised immune systems (Cubie, 2013), there is a growing interest in determining if these viruses can cause tumors in the general population. Further, while so called "high risk" α-HPVs have been identified, the relative oncogenic potential among the beta genus of HPV is still being discussed. Moreover, despite already consisting of ∼50 types of HPV that are sub-classified in five species (Van Doorslaer, 2013), it is likely that a large portion of β-HPV types have yet to be discovered (Ekström et al., 2013;

Bzhalava et al., 2014, 2015). Because the first β-HPV types isolated were HPV 5 and HPV 8 from cutaneous squamous cell carcinomas (cSCC) of patients with the rare disorder Epidermodysplasia Verruciformis (EV) (Pfister et al., 1981; Kremsdorf et al., 1982, 1983), these two β-HPVs have been the most extensively examined.

Because β-HPVs do not generally persist in tumors, they are hypothesized to act through a "hit and run" mechanism (Pfister, 2003; Hufbauer and Akgül, 2017). Specifically, β-HPVs are believed to hinder the repair of DNA damage caused by UV radiation making mutations more likely. For a virus that infects an area frequently exposed to UV radiation and yet requires proliferating cells to complete its lifecycle, it is reasonable that β-HPVs have developed mechanisms to prevent the cell cycle arrest that accompanies repair of UV damaged DNA. However, this could have severe consequences for the host cell as the UV-induced mutations will remain after the viral infection is cleared and could drive the development of non-melanoma skin cancers (NMSCs) without continued expression of β-HPV genes. In this review, we will begin by providing a brief synapsis of the evidence that β-HPV infections cause NMSCs as well as the cellular signaling pathways that respond to UV insults. We will provide a concise discussion of α-HPV associated tumorigenesis, as these more clearly defined mechanisms of transformation provide a useful and relevant comparison for β-HPV associated oncogenesis. Then, we will move on to the *in vitro* and *in vivo* data that demonstrate the ability of β-HPV genes to disrupt the repair of DNA, destabilizing the host genome in a manner consistent with a "hit and run" mechanism of tumorigenesis.

Transformation by Alpha Genus HPV Oncogenes

Extensive evidence has established α-HPV as the causative agent in cervical cancers and in many other malignancies in the oropharynx and throughout the anogenital tract (Hobbs et al., 2006; Moody and Laimins, 2010; Carter et al., 2011; D'Souza and Dempsey, 2011; Tommasino, 2014). Infections with high risk α-HPVs can begin a multi-decade process where cells are immortalized, accumulate destabilized genomes, and are ultimately transformed into deadly malignant tumors. Two oncogenes, α-HPV E6 and E7 proteins drive this progression by activating telomerase and degrading two tumor suppressor, p53 and pRB (Dyson et al., 1989; Münger et al., 1989; Scheffner et al., 1990; Werness et al., 1990; Huibregtse et al., 1991; Boyer et al., 1996; Klingelhutz et al., 1996; Kiyono et al., 1998; Oh et al., 2001). These oncogenes also induce aberrant activation of the DNA damage response (DDR) and in some case, impair the cells ability to repair DNA damage (Patel et al., 1999; Zimmermann et al., 1999; Moody and Laimins, 2009; Sakakibara et al., 2011; Gillespie et al., 2012; Reinson et al., 2013; Hong et al., 2015; Wallace et al., 2017). In notable contrast to β-HPV associated malignancies, tumors caused by α-HPV are dependent on the continued expression of α-HPV E6 and E7 (Hwang et al., 1993; Goodwin and DiMaio, 2000; Goodwin et al., 2000). Because the β-HPV homologs of these α-HPV oncogenes are believed to be the primary contributors to NMSC, β-HPV E6, and E7 are frequently compared to α-HPV E6 and E7. When informative, we will make similar comparisons.

Beta-HPV in Epidermodysplasia Verruciformis Patients and Organ Transplant Recipients

The oncogenic potential of β-HPV is most firmly established in people with compromised immune systems. β-HPV associated cSCCs occur in 30–60% of people with the rare genetic disorder epidermodysplasia verruciformis (EV) and presented the first link between β-HPV infections and skin carcinogenesis (Orth, 1986). β-HPV+ tumors found in people with EV generally contain a high copy number (∼300 copies/cell) of viral DNA (Dell'Oste et al., 2009). In contrast, β-HPV+ tumors of non-EV patients show a very low viral DNA copy number <1 per cell (Weissenborn et al., 2005; Arron et al., 2011). The most frequently β-HPV types associated with EV are β-HPV 5 and 8 (also less often β-HPV 14 and 20) (de Oliveira et al., 2004; Dell'Oste et al., 2009). Tumors in individuals with EV occur predominantly in parts of the body frequently exposed to the sun, suggesting a role for UV in β-HPV associated tumorigenesis (Pfister, 2003). Another group at risk for β-HPV associated cSCCs are people receiving immunosuppressive therapy following organ transplant. Organ transplant recipients (OTRs) (Bouwes Bavinck et al., 2001) show an increased susceptibility to β-HPV infections, a higher prevalence of viral DNA and a greater risk of developing non-melanoma skin cancer (Boyle et al., 1984; Kiviat, 1999). This increased risk is particularly notable if they are seropositive for β-HPV, with a hazard ratio of 2.8 (Genders et al., 2015). Both, EV-patients and OTRs display a significantly elevated viral load than the immunocompetent population (Dell'Oste et al., 2009; Weissenborn et al., 2012). Further, OTRs that have similar β-HPV viral loads to patients with EV show a 100-fold increase of cSCC incidence (Weissenborn et al., 2012). Investigations in to NMSCs in EV and OTR patient groups provide strong evidence that β-HPV infections have oncogenic potential. Additionally, the OTR patient group shows that β-HPV associated cSCCs are not limited to patients with the rare EV disorder (Howley and Pfister, 2015; Tommasino, 2017). These "special" scenarios provide the proof of context specific β-HPV induced oncogenesis, but defining the breath of β-HPV's contribution to NMSC development is a critical area of research as millions of people are diagnosed with these malignancies each year.

Beta-HPV in the General Population

β-HPVs inhabit the cutaneous epithelium and are found in abundance throughout the population (Casabonne et al., 2009; de Koning et al., 2009; Weissenborn et al., 2012; Farzan et al., 2013). A particularly frequent site of infection is the hair follicles of eyebrows (Weissenborn et al., 2012; Neale et al., 2013; Iannacone et al., 2014). Unlike α-HPV infections, which are usually sexually transmitted and occur later in life, β-HPV infections often occur during early childhood through skin to skin contact (Antonsson et al., 2003; Weissenborn et al., 2009). The persistence of α- and

β-HPVs is a further differentiating factor, although the exact mechanisms driving this difference are not fully appreciated. Median β-HPV infection duration is 8.6 months in eyebrow hairs, while infections of the skin are less common but have a median persistence of 11.3 months (Hampras et al., 2014). In contrast α-HPV infections persist longer (18.3 months on average), and upon accidental genome-integration are present for decades (Richardson et al., 2003). Suggesting reinfections, β-HPV infections can persist within a family for several years without manifestation of clinical symptoms (Hsu et al., 2009). While infections first occur in infants, advanced age is a risk-factor for a β-HPV infection (Hazard et al., 2007; Weissenborn et al., 2009). Sunburn is another risk factor, potentially due to local immune suppression (Hampras et al., 2014). The evidence of β-HPV's involvement in cSCCs of EV patients led to the classification of HPV 5 and HPV 8 as possibly carcinogenic by a WHO-IARC 2009 work group (Bouvard et al., 2009).

Epidemiology

Epidemiologically, β-HPV antibody positivity is associated with an increased risk for cSCCs, especially for infections by β-HPV 38 (Bzhalava et al., 2013; Chahoud et al., 2016). There is also a difference of β-HPV prevalence by anatomical site (Hampras et al., 2017). The most common site of infection was genital skin (81.6%), followed by forearm skin (64.4%), eyebrow hairs (60.9%), oral mucosa (35.6%), and anal mucosa (33.3%). The most common type on the sunlight exposed and therefore risk associated sites eyebrows and forearm are β-HPV 38 and β-HPV 12 respectively. High loads of β-HPV DNA are statistically associated with increased cutaneous SCC incidences at an odds ratio of ∼3 in immunocompetent Australians and immunosuppressed OTRs (Bouwes et al., 2010; Neale et al., 2013). People with cSCCs more frequently test positive for viral DNA in skin as well as anti β-HPV L1 antibodies than the general population (Forslund et al., 2007; Waterboer et al., 2008; Karagas et al., 2010; Iannacone et al., 2012; Farzan et al., 2013). The involvement of β-HPV in cSCC carcinogenesis is somewhat challenged by the low relative incidence rate of β-HPV+ cSCCs considering the high prevalence of 80% for β-HPV infections. Furthermore, unlike α-HPV+ cancers, the expression of β-HPV viral proteins is not required for tumor maintenance and the viral DNA copy-number is <1 per cell in β-HPV associated cSCCs (Meyer et al., 2001; Nindl et al., 2006). Since cSCC tumors typically occur in parts of the body exposed mutagenic UV radiation from the sun, the role of β-HPV is thought to be in the initiation and acceleration of genomic destabilization.

The phrase "hit and run" was coined to describe the hypothesize contribution of β-HPV infections to NMSCs, where the virus acts as a cofactor along with UV during tumor initiation (Bavinck et al., 2008). The destabilization of the host genome caused by sun exposure is augmented by β-HPV's ability to attenuate the cellular response to UV damage and thus increase the risk of oncogenic mutations capable of driving tumor development independent of continued β-HPV gene expression. Epidemiological studies support this hypothesis (Forslund et al., 2007; Iannacone et al., 2012) as there is increased prevalence and frequency of β-HPV in precancerous, actinic keratinosis lesions (AK). AK, otherwise known as solar keratinosis, is an abnormal growth of the skin, induced by UV exposure (Moy, 2000). Consistent with a role in tumor initiation, despite being found at very low copy numbers in cSCCs, the β-HPV viral load in AK is >50 copies/cell (Weissenborn et al., 2005). Together these data form the foundation that supports the "hit and run" model of β-HPV associated skin cancer, but this hypothesis is dependent on the ability of β-HPV infections to make UV more mutagenic.

DNA Damage Response-Pathways

The most likely way that β-HPV could amplify the destabilization of the host genome induced by UV is through the aberration of the cellular DDR. The primary type of DNA damage caused by UV are DNA intrastrand crosslinks, most often cyclobutane purimidine dimers or CPDs (Yang, 2011). Failure to properly repair these lesions can result in point mutations (Brash et al., 1991; Keohavong et al., 1991). Crosslinked DNA can also cause a replication fork collapse and subsequent double strand break in DNA (DSB) (Jeggo and Löbrich, 2007). If DSBs are not repaired, the damage becomes much more deleterious, including chromosome rearrangement or loss of entire chromosome arms (Pankotai and Soutoglou, 2013). In this section, we will introduce the biochemical pathways that coordinate the cellular response to UV-damage to preserve the integrity of the human genome. A graphical representation of the cellular response to UV induced DNA damage in S-phase can be found in **Figure 1**. During S-phase, translesion synthesis (TLS) prevents UV-induced DNA crosslinks from causing replication fork collapse by facilitating the bypass of the damaged bases (Lerner et al., 2017). Depending on the position in the cell cycle, nucleotide excision repair (NER), and the Fanconi Anemia (FA) pathways repair these crosslinks (Moldovan and D'Andrea, 2009; Marteijn et al., 2014). While FA requires sister chromosomes to complete repair and is thus limited to cells that have undergone replication, NER is not bound by cell cycle position. UV-induced DSBs are the result of replication fork collapse and therefore must occur during S-phase. Although DSB repair occurs through two major pathways, non-homologous end joining (NHEJ) and homologous recombination (HR), DSBs occurring during S-phase are repaired predominantly by HR (Mao et al., 2008). Repairing DNA lesions usually requires pausing the cell cycle to avoid escalating the damage during replication (Willis and Rhind, 2009). Finally, should the cell receive more damage than can be repaired, the intrinsic apoptosis pathway will initiate program cell death (Offer et al., 2002).

Although recognized as distinct pathways, the individual proteins that make up these repair mechanisms are often shared. This is particularly true for repair kinases (Yang et al., 2003; Yan et al., 2014). For instance, ATR controls the rate limiting step of NER (through phosphorylation mediated stabilization of XPA) and facilitates translesion synthesis by phosphorylating the TLS-specific polymerase, Polη (Chen et al., 2008; Göhler et al., 2011; Lee et al., 2014). ATR's kinase activity is similarly necessary for the repair of crosslinked DNA by the FA pathway and plays a role in DSB repair via the homologous recombination pathway (Jazayeri et al., 2006; Shiotani and Zou, 2009; Shigechi et al., 2012;

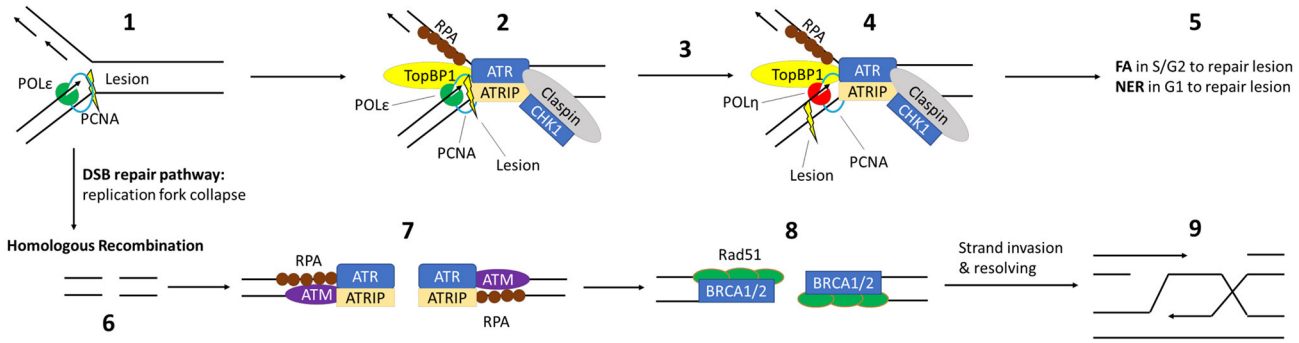

FIGURE 1 | Critical DDR Pathways for UV induced DNA Damage: (1) UV induces an intrastrand lesion, causing the replication fork to stall in S-pase. (2) Exposed ssDNA is coated with RPA, followed by recruitment of TopBP1 and ATR/ATRIP. TopBP1 accelerates autophosphorylation of ATR. Claspin and CHK1 are recruited and phosphorylated, promoting fork stabilization. (3) This leads to Rad6/Rad18 recruitment and mono-ubiquitination of PCNA (not shown), triggering the switch from high fidelity replication DNA-polymerases to y-family polymerase Polη. (4) Then, Polη replicates past the lesion and normal replication can continue after a repeated polymerase switch back to the high-fidelity replication polymerase. (5) The lesion itself is repaired by either the FA or NER pathway. (6) Prolonged stalling leads to replication fork collapse into a DSB. The MRE11, Rad50 and Nbs1 complex (not shown) is recruited to this DSB, initiating strand resection and recruitment/activation of ATM. (7) After strand resection, the exposed ssDNA is coated by RPA and ATR/ATRIP is recruited. (8) RPA is then replaced by RAD51, with the assistance of BRCA1 and BRCA2. (9) This facilitates homology-dependent single strand invasion and resolution of the lesion.

Maréchal and Zou, 2013). A similar situation exists for the related kinase, ATM. ATM phosphorylates proteins critical for the FA, NER, and HR repair pathways (Ray et al., 2013, 2016; Shiloh and Ziv, 2013). It may also play a role in translesion synthesis. Further, both ATM and ATR can stabilize p53 in response to DNA damage, causing p53-dependent DNA repair or apoptosis (Kruse and Gu, 2009; Cheng and Chen, 2010). ATM and ATR are both classically involved in DNA damage induced cell cycle arrest (Reinhardt and Yaffe, 2009). Both of these kinases also help pause the cell cycle progression by phosphorylating/activating cell cycle regulatory proteins (CHK1 and CHK2) (Branzei and Foiani, 2008). Finally, ATM and ATR also facilitate programed cell death or apoptosis should the cell's DNA be too extensively damaged. Specifically, their phosphorylation of p53 stabilizes the tumor suppressor and can result in p53-dependent apoptosis (Banin et al., 1998; Tibbetts et al., 1999; Shiloh and Ziv, 2013).

The RPA complex (RPA14, RPA32, and RPA70), BRCA1 and BRCA2 are similarly important for a myriad of DDRs (Yoshida and Miki, 2004; Maréchal and Zou, 2015). The RPA heterotrimer binds single stranded DNA (ssDNA) protecting it from degradation. Because ssDNA intermediates occur during both homologous recombination and translesion synthesis, RPA proteins are essential for these repair mechanisms. Both the FA and HR pathways require BRCA1 and BRCA2. FA pathway proteins contain the prefix "FANC" in their names indicating that many of these proteins were named after loss of the gene was shown to result in the clinical manifestation (Fanconi Anemia) from which the repair pathway derives its name, for example, FANCA, FANCB, FANC. Indicative of its requirement for FA repair an alternative name for BRCA2 is FANCD1. Moreover, BRCA1 was recently shown to be a critical component of the FA pathway (Domchek et al., 2013; Sawyer et al., 2015) and even described as the pathway's "missing link" (D'Andrea, 2013). The requirement of BRCA1 and BRCA2 in repair of DSBs by homologous recombination has been established for over a decade. Further indicative of how intertwined the cellular DNA response is, ATR and ATM both play regulatory roles in repair by phosphorylating the RPA complex, BRCA1 as well as BRCA2.

EFFECTS OF β-HPV E6 ON DNA DAMAGE RESPONSE

Inhibition of Apoptosis

The E6 protein of β-HPV can hinder both DNA repair machinery and apoptotic pathways in response to DNA damage. Unlike high risk α-HPVs, it does not commonly degrade the "guardian of the genome," p53, one of the most prominent high-risk E6 targets, directly (Scheffner et al., 1990; White et al., 2014). The tumor-suppressor, p53, is an important signaling protein that regulates two large subsets of target proteins: negative regulators of cell cycle progression (p21, 14-3-3, GADD45α) and pro-apoptotic proteins (PUMA, BAX, BAK). In normal cells, p53 has to balance on a fine line between sufficient activity to ensure genome fidelity and tumor suppression as well as avoiding hyperactivity that would induce abnormal aging by depleting stem-cell populations (Vogelstein et al., 2000; Reinhardt and Schumacher, 2012). Compared to high risk α-HPV E6s, β-HPV E6, apart from β-HPV 49, have a lower affinity for a cellular E3 ubiquitin ligase, known as E6AP or UBE3A, and are therefore not able to form the E6-E6AP complex required for proteasomal degradation of p53 (Huibregtse et al., 1991; Cornet et al., 2012). Instead, β-HPV types 17, 38, and 92 have been shown to bind to p53 directly and stabilize it (White et al., 2012), while β-HPV 23 has been shown to interfere with the phosphorylation-dependent activation of p53 by inhibiting HIPK2 (Muschik et al., 2011). β-HPV 5 and β-HPV 8 delay the stabilization and phosphorylation after UV irradiation (Wallace et al., 2014). HIPK2 is a protein kinase that can phosphorylate p53 at Ser 46, which subsequently leads to the acetylation of p53 at Lys 382 and a promotion of p53 target gene expression. More specifically, HIPK2's activity is UV-induced

and UV exposure leads to HIPK2 mediated growth arrest or apoptosis via p53. β-HPV 38 E6 induces the accumulation of ΔNp73, altering p53 functions (Accardi et al., 2006). These effects cause the alteration or loss of p53's activity as a transcriptional co-factor and impact both apoptosis- (Fas, BAX) and cell cycle checkpoint pathway (p21) gene expression (White et al., 2014). p53 is also a known transcription factor for the TLS polymerase Polη, implying the TLS DDR pathway as a suitable target for investigation (Lerner et al., 2017).

The selection between a p53-mediated checkpoint activation and p53-mediated apoptosis depends on a cell type specific threshold of p53 expression and activation (Kracikova et al., 2013). Mild DNA damage triggers p53-dependent checkpoint activation and subsequent DNA repair while moderate DNA damage causes p53-mediated senescence. Excessive DNA damage or failed cytokinesis induce apoptosis (Chen et al., 1996). β-HPV E6 can also inhibit apoptosis downstream of p53. The interaction of E6 with a pro-apoptotic protein, Bcl2 homologous antagonist killer (BAK), is a highly conserved function across HPV types and genera (Thomas and Banks, 1999; Simmonds and Storey, 2008; Underbrink et al., 2008; Jackson and Bartek, 2009; Holloway et al., 2015; Tomaić, 2016). Increases in BAK abundance are an essential step in the intrinsic apoptotic pathway (Chittenden et al., 1995). β-HPV E6 prevents the accumulation of Bak following UV-irradiation inducing DNA damage. An overview of the different pathways for β-HPV E6 mediated inhibition of apoptosis and the β-HPV types involved can be found in the upper right panel of **Figure 2**.

Interference with Checkpoint Signaling & DNA Damage Repair

Upstream of the inhibition of apoptosis, β-HPV E6 can attenuate G1 to S-phase cell cycle checkpoint induction in response to DNA damage (Wallace et al., 2012; Hufbauer et al., 2015) and continue cellular proliferation while DNA damage repair is attenuated (Giampieri and Storey, 2004). These effects arise from the interaction of β-HPV E6 with the acetyltransferase p300 (Muench et al., 2010; Howie et al., 2011). As an important coactivator of DDR gene transcription, the loss of p300 activity has far reaching consequences. β-HPV 5 and 8 have been shown to facilitate the destabilization of p300 (Howie et al., 2011; Wallace et al., 2012, 2013), while β-HPV 38 has been shown to inhibit p300 acetyltransferase activity (Muench et al., 2010). The steady state levels of the DDR kinases ATM and ATR as well as BRCA1 and BRCA2 are reduced because of the lack of p300 in β-HPV 5 and β-HPV 8 E6 expressing cells (Wallace et al., 2012, 2013, 2015). This attenuates the repair of UV induced cyclobutane pyrimidine dimer (CPD), increases the UV-induced frequency of DSBs and attenuates LINE-1 retrotransposition. Double strand breaks can be a secondary effect of UV-induced DNA damage, occurring when a replication fork collapses at an unrepaired CPD. Both the repair of CPDs (ATR dependent) as well as the repair of DSBs (ATM, BRCA1 and BRCA2 dependent) are significantly delayed by β-HPV 5 and β-HPV 8 E6 (Giampieri and Storey, 2004; Wallace et al., 2012, 2015). γH2AX is a known marker for DSBs and its foci kinetics can be utilized as indicators for DSB-repair. The (β-HPV 5 E6 and 8 E6) p300 degradation-dependent attenuation of BRCA1/BRCA2 expression and foci formation led

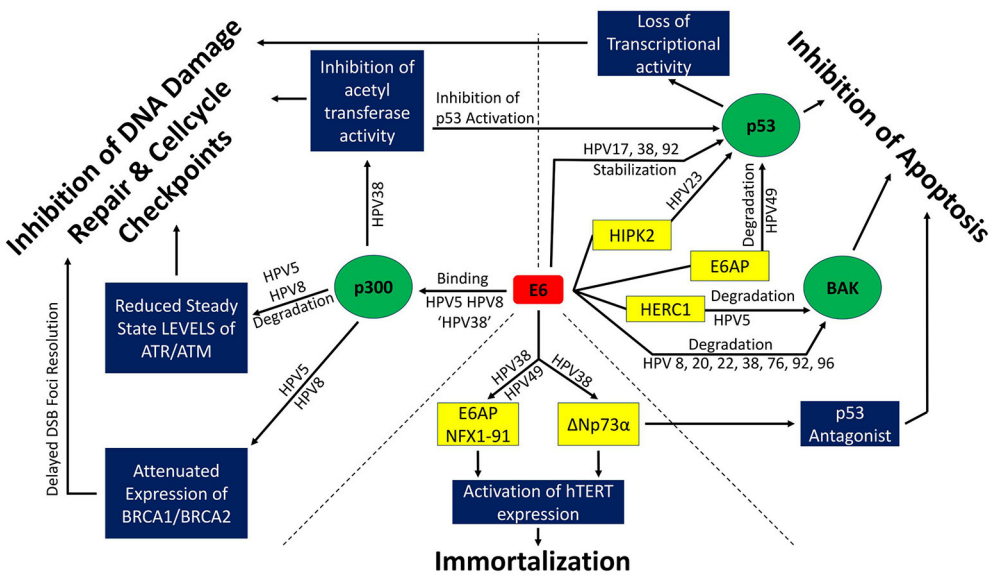

FIGURE 2 | β-HPV E6 and its effects on genome stability. Interactions among β-HPV E6s and proteins involved in DNA repair and cell cycle check points (upper left third), apoptotic signaling (upper right third), and immortalization (bottom third) are depicted. Green circles denote cellular proteins that are central to β-HPV E6's ability to alter the cellular response to DNA damage. Blue boxes depict the diversity of cellular processes altered through the manipulation of the green circular proteins. Yellow boxes represent proteins that cooperate in the manipulation of the green circular proteins or facilitate immortalization. 'HPV 38' refers to the fact that while β-HPV 38 E6 can bind p300, it does not bind it strong enough to destabilize the histone acetyltransferase.

to delayed γH2AX foci resolution after DSBs were induced by ionizing radiation (Wallace et al., 2015).

ATR and ATM activate cell cycle checkpoints by phosphorylating checkpoint kinases Chk1 and Chk2 respectively. Activation of Chk1/Chk2 induces cell cycle arrest and DDR (Reinhardt and Yaffe, 2009). β-HPV 5 and 8 E6 reduce steady state levels of ATM and ATR *in vitro* as well as reduced pATM and pATR levels *in vitro* and *in vivo* (Hufbauer et al., 2015). This leads to an attenuated Chk1 phosphorylation and impaired G1 to S-phase checkpoint activation, presenting a bypass to an important tumor-suppressing barrier. Furthermore, p300 is an important transcriptional cofactor for BRCA1, BRCA2 (Both essential proteins for HR) (Goodman and Smolik, 2000; Pao et al., 2000; Yoshida and Miki, 2004) and has been shown to be essential for proper cytokinesis and the faithful resolution of mitotic figures (Turnell et al., 2005; Xia et al., 2012). Attenuation of proper checkpoint signaling and the consequent possible unscheduled cell cycle progression leaves pre-existing DNA damage unaddressed. This allows less severe types of damage (for example, UV-induced intrastrand crosslinks) to evolve into more deleterious types of DNA damage like DSBs. β-HPV E6 expressing cells have been shown to contain a significantly larger portion of multinucleated cells *in vitro* (Wallace et al., 2014), pointing to a possible attenuation of the Hippo pathway. The Hippo pathway stabilizes p53, inactivates the oncogene YAP and induces apoptosis in the event of failed cytokinesis (Harvey et al., 2013; Ganem et al., 2014). The mechanisms through which β-HPV E6 disrupts the DDR are depicted in the upper left panel of **Figure 2**.

In addition to p300-dependent checkpoint inhibition, β-HPV 5E6 and 8E6 can bind the transcription factor SMAD3, thereby inhibiting the transforming growth factor-beta (TGF-β) pathway (Mendoza et al., 2006). TGF-β has a well-described dual role in carcinogenesis. In normal cells and early carcinomas, it acts as tumor suppressor through cytostatic effects. As the cancer progresses, the TGF-β aids proliferation, survival, invasion, and angiogenesis of tumor cells (Lebrun, 2012). Destabilization of the SMAD3 may attenuate these tumor suppressive effects by inhibiting the expression of CDK inhibitors p16, p17, p21, and p27 and possibly promoting unscheduled cell cycle progression from G1- into S-phase (Itoh et al., 2000; ten Dijke and Hill, 2004). Disruption of the TGF-β pathway has also been linked to an impaired function of DSB-repair following ionizing radiation (Kirshner et al., 2006; Bouquet et al., 2011; Kim et al., 2015).

Additional Functions of β-HPV E6

Additional notable functions of β-HPV E6 include hTERT stabilization (β-HPV 5, 20, 22, 38; Bedard et al., 2008; Gabet et al., 2008; Cornet et al., 2012), which may stabilize the genome by preventing unstably short telomers. Conversely, it could decrease genome fidelity by aborting the role of telomers as a "cellular clock" that prevent proliferation of older cells that are more likely to contain mutations (Sharma et al., 2003). Another target of β-HPV 5 and 8 E6 is the Notch signaling pathway. The Notch signaling pathway has been shown to have tumor suppressive functions in epithelial cells (Reichrath and Reichrath, 2012) and can decide cell fate (Lai, 2004). It can also act as a negative regulator of the ATM-dependent DDR (Vermezovic et al., 2015). β-HPV 5 and 8 interfere with the Notch signaling pathway through interaction with MAML1. The β-HPV E6-mediated repression of Notch signaling delays S-phase cell cycle exit and differentiation (Brimer et al., 2012; Meyers et al., 2013). Prolonged or perpetual existence in S-phase in combination with impaired DNA-damage signaling and attenuated DDR could lead to cross-amplification of these individual effects.

Summary

β-HPV E6 attenuates UV-induced apoptosis and DDR through interaction with multiple targets. BAK degradation and prevention of apoptosis is highly conserved throughout the β-HPV genus. Less common is the inhibition of apoptosis through direct interaction with and degradation of p53 (β-HPV 49, E6AP mediated) or the inhibition of p53 activation (β-HPV 23 through HIPK2 interaction). β-HPV 17, 38, and 92 E6 interact with p53 directly, leading to its stabilization but altering or restricting p53's transcriptional activity. β-HPV 5, 8, and 38 E6 interfere with DDR and checkpoint signaling through interaction with p300. A combination of inhibited apoptosis, impaired DNA damage signaling and attenuated DSB response bears a potential to destabilize the genome over time or cause carcinogenesis in combination with DNA-damaging agents that is consistent with β-HPV's proposed contribution to skin cancer. Notably, although through markedly different mechanisms, high risk α-HPV oncogenes similarly contribute to tumorigenesis by impairing the cellular response to genome destabilizing events.

E7 AND ITS INFLUENCE ON CELL CYCLE PROGRESSION AND DDR

Promotion of Cell Cycle Progression

A canonical function of the high risk α-HPV E7 oncogene is the degradation of the tumor suppressor pRb (Moody and Laimins, 2010). In normal cells, the function of pRb is the suppression of E2F transcription factor dependent genes through binding E2F and inhibiting its association with promoters (Dyson, 1998). E2F-family proteins facilitate cell cycle progression and proliferation by promoting the expression of a variety of genes encoding for proteins involved in G1/S-phase transition (Bertoli et al., 2013). High risk α-HPV E7 oncogenes bind directly to pRb via the highly conserved LXCXE binding motif (Münger et al., 1989). This leads to the disruption of the E2F—pRb complex, allows E2F to bind to its transcriptional targets (Chellappan et al., 1992). E2F target expression (for example cyclin A or cyclin E) then facilitate premature cell cycle progression and S-phase entry (Zerfass et al., 1995). Cell cycle checkpoints are meant to pause the cell cycle in case of DNA damage and allow time to repair the damage, or, if the damage surpasses a threshold, induce senescence or apoptosis. Cell cycle checkpoints are an integral part of a cells quest to maintain genome integrity. Consequently, attenuated checkpoint activation leads to a destabilization of the genome through accumulating DNA damage (Malumbres and Barbacid, 2009). α-HPV E7 allows keratinocytes with damaged DNA to progress through cell cycle check points, even when p53 is induced (Demers et al., 1994). Much like these α-HPV

E7s, several of the β-HPV E7s (β-HPV 5, 8, 38, 49) can interact with Rb (Yamashita et al., 1993; Caldeira et al., 2003; Cornet et al., 2012). However, the β-HPV E7 interactions tend to cause a hyperphosphorylation of Rb in Keratinocytes, rather than its destabilization (Caldeira et al., 2003; Cornet et al., 2012). Hyperphosphorylation of Rb inhibits its ability to bind and inactivate E2F, allowing E2F to promote transcription of its target genes (Bertoli et al., 2013). Additionally, β-HPV 24, 38, and 49 E7 severely attenuate pRb half-life in rodent fibroblasts, while β-HPV 14, 22, 23, and 36 do not affect pRb's half-life (Cornet et al., 2012). Cornet et al. also found that in the context of E6 and E7 expressing cells, β-HPV 14 and 22 can degrade pRb, but do not induce the expression of cyclin A or Cdk1. Cdk1 is essential for cell cycle progression(Santamaria et al., 2007).

Alteration of the p53-Transcriptional Network

The tumor suppressor p53 is an essential factor in the avoidance of inappropriate cell proliferation in the presence of genotoxic stress. It has transcriptional targets in several pathways, including DNA damage tolerance and apoptosis (Espinosa et al., 2003; Yu and Zhang, 2005; Beckerman and Prives, 2010). While the mechanisms of how high Risk α-HPV E7 disrupts p53 activity remain a poorly defined, significantly more is known about how some β-HPV E7 proteins disrupt this essential tumor suppressor. ΔNp73 is an important antagonist of the p53 gene family and participating in a negative feedback loop with p53 (Bailey et al., 2011). β-HPV-38 E7 promotes the accumulation and stability of ΔNp73α through both transcriptional and post-translational mechanisms (Accardi et al., 2006, p.73; Saidj et al., 2013). β-HPV 38 E7 promotes the accumulation of double monophosphorylated p53 (serine 15 and 392) in the nucleus. These dual p53 phosphorylation events increase ΔNp73α expression. β-HPV 38 E7 also mediates the nuclear translocation of the IκB kinase (IKKβ) that increases the stability of ΔNp73α by phosphorylating it at serine 422 (Accardi et al., 2011). Furthermore, IKKβ, ΔNp73α, DNMT1, and EZH2 form a transcription regulatory complex. This complex is able to bind to a subset of p53 regulated promoters and interferes with the expression of DDR pathway- and apoptosis-related genes, but not with pro-survival genes like Survivin. This implies a role of β-HPV-38 E7 in the inhibition of p53 dependent apoptosis and allows for speculation on the influence of β-HPV E7 over DDR-proteins that depend on p53 activity as a transcriptional factor (for example Polη of the TLS pathway and XPC of the NER pathway) (Fischer, 2017).

Other Functions Related to DDR

A systematic screening of E7 interacting protein has shown that E7 from β-HPVs 8, 25, and 92 can interact with the tyrosine phosphatase and tumor suppressor PTPN14. While several high- and low-risk α-HPV types have been shown to degrade PTPN14 (White et al., 2016; Szalmás et al., 2017), the role of the β-HPV E7—PTPN14 interaction is yet to be investigated. PTPN14 is a required regulator of the Hippo signaling pathway, as it is necessary for the translocation of the Hippo transcription factor, YAP1, from the nucleus to the cytoplasm (Wang et al., 2012). This indicates that β-HPV E7 may attenuate the Hippo pathway, if β-HPV E7 proves to also be capable of PTPN14 destabilization. The Hippo pathway plays a crucial role in the control of proliferation and induces apoptosis in the event of cytokinesis (Ganem et al., 2014). Phosphorylation of Yap1, a part of the hippo pathway, is a critical step in the induction of apoptosis in response to DNA damage (Levy et al., 2008). β-HPV infections occur in the skin and thus in an environment where UV-induced cell cycle arrest and apoptosis occur with some frequency. As a result, it would not be surprising if there yet undiscovered abilities of β-HPV E7 to disconnect the cellular response to UV in manners that promote the β-HPV lifecycle.

MOUSE MODELS: EVIDENCE FOR A ROLE OF β-HPV IN CARCINOGENESIS VIA A HIT AND RUN MECHANISM

Mouse models can be an effective tool for elucidating the oncogenic potential of β-HPVs. The most common of these is the transgenic (tg) mouse model that expresses can individual and groups of β-HPV genes (E2, E6, E7, E6/E7) or the entire early region of the virus under a keratin promoter. Placing the expression under a keratin promoter restricts expression to keratinocytes, the cell type infected by β-HPV. The result is that genes are only expressed at anatomical sites relevant in the context of β-HPV infections. Our review of the literature shows that β-HPV 8 is the most frequently investigated β-HPV type, followed by β-HPV 38, β-HPV 49, and β-HPV 20. Additionally, one study has investigated β-HPV 5 E7 in a nude mouse/artificial skin graft model (Buitrago-Pérez et al., 2012).

β-HPV 8 Transgenic-Mouse Models

Schaper et al. established the first β-HPV tg-model using the Keratin 14 (K14) promoter to express the entire early gene region of β-HPV 8. Transgene expression was the highest for E2, followed by E6 and E7 (Schaper et al., 2005). While the tg-negative littermates did not develop lesions on the skin or other organs, 91% of the β-HPV 8 tg-positive mice developed single- or multifocal benign tumors. Of this population, 25% showed varying degrees of dysplasia and finally 6% developed SCCs spontaneously. The SCCs developed without additional DNA-damaging events. This established an *in vivo* link between β-HPV 8 and carcinogenesis. A caveat, however, is the stable oncogene expression in tg-mice. β-HPV infections, and therefore expression of β-HPV genes in the immunocompetent population, are transient.

In a follow up study, a possible role of β-HPV 8 E2 in skin tumor induction was revealed when 60% of the E2 positive population, but none of the E2 negative population developed spontaneous ulcerous lesions of the skin (Pfefferle et al., 2008). Moreover, 3 weeks after UV irradiation, 87% of a tg-mouse line with high E2 expression levels and 36% of a tg-mouse line with lower E2 expression levels developed skin tumors. On the other hand, irradiation of tg-negative mice did not lead to tumor formation. This could point toward a potential synergy between β-HPV 8 E2, E6, and E7 for their role in carcinogenesis.

Later, by expressing E6 individually under the K14 promoter, it was shown that β-HPV 8 E6 is the major driving force behind both spontaneous tumor development in tg-mice and that tumor formation could be prevented via DNA vaccination (Marcuzzi et al., 2009, 2014). Rapid tumor development by induction, either through UV irradiation or wounding, was demonstrated. This observation that persistent β-HPV 8 infections combined with UV exposure and wound healing processes pose a significant risk factor for skin cancer is consistent with observations in individuals with persistent β-HPV 8 infections due to EV. A potential role of the signal transducer and activator of transcription 3 (Stat3) was demonstrated in tg mice expressing the early region of β-HPV 8 that additionally had an epidermis restricted ablation of Stat3 (Andrea et al., 2010, p.3). The Stat3 heterozygous line was significantly less prone to spontaneous tumor development and these tumors did not progress to malignancy. Hufbauer et al. showed that β-HPV 8 E6 expressing tg-mice dysregulate mi-RNA expression (Hufbauer et al., 2011). Following UV induced inflammation and wound healing, the levels of mi-RNA for regulatory targets including cell-cycle (Rb) and apoptosis (PTEN, PDCD4) remained dysregulated, while dysregulation in wt-mice was transient. Hufbauer et al. also demonstrated that β-HPV 8 E6 expression leads to an impaired DNA-damage response in tg-mice following UV irradiation (Hufbauer et al., 2015). β-HPV 8 E6 expressing mice were shown to be thymine-dimer positive for an extended period while wt-mice efficiently repaired the UV induced DNA damage within 48 h. More importantly, probing for the DSB marker, γH2AX, revealed that the persistence of UV induced lesions in β-HPV 8 E6 expressing cells ultimately led to the formation of highly mutagenic and more dangerous DSBs. Immunohistochemical analysis of tg-mouse skin biopsies showed an increased signal for γH2AX at both, early (3d, 5d) and later (13d, 24d) timepoints. This not only demonstrates β-HPV 8 E6's attenuation of DDR, but also its ability to enhance the cells tolerance of persistent and increasingly severe DNA-damage. UV radiation typically causes intrastrand lesions that are tolerated by TLS- and repaired by NER-pathway. If this type of damage persists it will lead to increased rates of DSBs. The emergence and persistence of DSBs could point toward a broad influence of β-HPV 8 E6 on the DNA-damage response machinery, stretching from pathways meant to handle minor DNA damage (TLS, NER) to pathways that control and repair highly mutagenic DSBs (NHEJ, HR).

The role of β-HPV 8 E7 has also been investigated in tg-mice (Sperling et al., 2012; Heuser et al., 2016). It was shown that β-HPV 8 E7, but not β-HPV 8 E6 enables an escape from the immune response of β-HPV 8^+ lesions. β-HPV 8 E7 achieved this by inhibiting chemotactic signaling. β-HPV 8 E7 binds to C/EBPβ and inhibits its interaction with the CCL20 promoter, leading to decreased levels of CCL20, a chemoattractant for Langerhans cells. β-HPV 8 E7 is also critical for the invasiveness of hyperproliferating keratinocytes by dysregulating cell-cell interactions.

β-HPV 38 Transgenic-Mouse Models

While β-HPV 8 tg-mice demonstrated spontaneous tumor formation, tg-models of other β-HPV types do not exhibit the same behavior. Dong et al. showed that a mouse model for β-HPV 38 E6/E7 expressing mice under the Keratin 10 promoter exhibit hyperproliferation of the skin, but require external stimuli for carcinogenesis (Dong et al., 2005). Furthermore, ΔNp73α has been identified as integral to the attenuation of cell cycle arrest after UV exposure (Dong et al., 2007). Loss of p53 attenuated ΔNp73α expression and partially restored cell cycle arrest while loss of p73 lead to loss of ΔNp73α and consequently to high levels of p21 expression and cell cycle arrest after UV exposure. Differential susceptibility to chemically induced (DMBA/TPA) carcinogenesis correlates with differential oncogene expression levels and indicates a dose-dependency for risk of carcinogenesis. Irradiation of wt-mice with a single UV-dose led to accumulation of p21 and cell cycle arrest in the epidermis, while β-HPV 38 E6/E7 expressing mice inhibited cell cycle arrest through attenuation of p21 accumulation. Chronic irradiation of tg-mice with UV led to the formation of AK-like lesions that are considered precursors to SCCs in humans, while wt-mice did not develop lesions. Some of the AK-like lesions progressed to SCCs after 22 weeks (Viarisio et al., 2011). This provides evidence for the carcinogenesis-risk amplifying potential of the β-HPV 38 oncogenes E6 and E7.

Other β-HPV Transgenic-Mouse Models

The K14 tg-model for β-HPV 49 E6/E7 does neither show spontaneous tumor development nor tumor development after UV-irradiation (Viarisio et al., 2016). However, the β-HPV 49 tg-mice lines where susceptible to chemically induced carcinogenesis of the upper digestive tract. When exposed to 4 nitroquinoline 1-oxide (4NQO), 87% of the β-HPV 49 tg-mice developed tumors in the upper digestive tract while 4NQO treatment had little effect on β-HPV 38 tg-mice, which are susceptible to UV-triggered skin carcinogenesis.

A model for β-HPV 20 E6/E7 showed enhanced proliferation and papilloma formation in the evaluated transgenic lines, when compared to non-transgenic controls (Michel et al., 2006). Chronic exposure to UV radiation led to the development of SCCs and proliferation was enhanced for several weeks after UV treatment. The tg-lines also showed a reduced expression of differentiation markers (involucrin & loricrin and irregular patterns of p53 expression post UV, while controls showed a continued expression of proliferation markers and even expression of p53.

Summary

Mouse models provide a valuable *in vivo* tool to dissect the relative ability of β-HPV genes to induce tumorigenesis. They also allow a distinction between different carcinogenic potentials exhibited by E2, E6, or E7 to be accessed. The mouse models make it evident that β-HPV E6 carries a particularly high potential due to its effects on both apoptotic pathways and the DDR. The tg-mouse models also support the "hit and run" hypothesis regarding β-HPV induced malignancies, since most models require external stimuli to induce malignant tumors. β-HPV 8 has the potential for spontaneous transformation in tg-models, but it remains unclear whether the potential persists when β-HPV 8 early gene expression is transient, as it is the

case in most humans. It also should be pointed out that the average daily UV dose of an American is ∼94 J/m^2 (Godar et al., 2001), while the dosages used in the β-HPV tg-model studies discussed here reach 45-fold great levels. Although constrains in experimental design may require doses of this magnitude, it is a necessary caveat to considered when evaluating tg models of β-HPV induced skin cancer.

CONCLUSIONS

The impact of genus β-HPV on the DNA damage as well as the ability of these viruses to induce carcinogenesis is diverse and manifest in both *in vitro* and *in vivo* investigations. Generally, β-HPV viruses effect on DDR may be explained by their interest of uninterrupted proliferation, despite the presence of DNA damage. While most β-HPV E6 inhibit apoptosis by degrading the pro-apoptotic protein BAK, only a subset (β-HPV 5 and 8 E6) bind p300 strongly enough to destabilize the histone acetyltransferase, causing delayed repair of thymine dimers and double strand breaks (Giampieri and Storey, 2004; Muench et al., 2010; Howie et al., 2011; Hufbauer et al., 2011; Wallace et al., 2012, 2013, 2015). A larger subset of β-HPV E6 proteins (β-HPV 5, 8, 17, 23, 38, 49, and 92) interfere with p53 activity, but they do so through different mechanisms. Continuing the theme of diversity among β-HPVs, β-HPV 38 E6 immortalizes cells through a ΔNp73α-dependent mechanism, while β-HPV 49 E6 acts more similarly to high risk α-HPV E6, immortalizing cells through interactions with E6AP and NFX1-91 (Caldeira et al., 2003; Gabet et al., 2008; Muench et al., 2010). **Figure 2** depicts these diverse effects of β-HPV E6.

There are notable differences between β-HPV E7 proteins as well. A large cohort of these viral proteins (β-HPV 5, 8, 24, 38, and 49 E7) impair pRB function. The sole mechanism of pRB interference by β-HPV 5 and 8 E7 is through binding and induction of hyperphosphorylation of pRB. In addition to promoting the phosphorylation of pRB, β-HPV 38 and 49 E7 also decrease the half-life of pRB. β-HPV 24 E7 is only known to reduce pRB's half-life. Further, β-HPV 38 E7 has the ability to interfere with p53 activity by stabilizing the p53 antagonist, ΔNp73α, and increase inhibitory post-translational modifications of p53.

Not surprisingly, *in vivo* transgenic mouse models of β-HPV associated tumorigenesis reflect the varied ability of β-HPV to degrade the fidelity of host cells (Dong et al., 2005; Michel et al., 2006; Viarisio et al., 2011). The tg-mouse models of β-HPV 8 show spontaneous tumor formation (Schaper et al., 2005; Pfefferle et al., 2008; Marcuzzi et al., 2009). While carcinogenesis is augmented by UV exposure in β-HPV 8 mouse models, similar models of other β-HPV induced cancers (β-HPV 20, 38, and 49) require external stimulants to develop tumors. The required stimulants vary as well. Expression of β-HPV 20 and 38 proteins cause cancer in mice exposed to UV, but only β-HPV 49 proteins cause upper digestive tract tumors after exposure to 4NQO. Notably, the transient nature of β-HPV infections in immunocompetent individuals is not reflected in the mouse models discussed above and the ability of persistent β-HPV infection to cause cancer is generally accepted. Further, the amount of UV exposure necessary to cause tumors in β-HPV tg mice is often well above what humans typically receive. Thus, new *in vivo* models will be quintessential to drive the field forward and may include mice infected with the murine papillomavirus (MmuPV1) that recapitulates some but not all of the characteristics of β-HPV (Meyers et al., 2017).

The bulk of evidence for β-HPV infections playing a role in NMSCs suggests that β-HPV 5, 8, and 38 are the most tumorigenic, with a particular and unsurprising emphasis on

TABLE 1 | Characteristics and differences of several β-HPV strands and high risk α-HPVs.

	HPV5	HPV8	HPV38	HPV49	high risk αHPVs
Impact on DDR	•Delay UV repair •Destabilize p300 •ATM, ATR, BRCA1/2 ↓	•Delay UV repair •Destabilize p300 •ATM, ATR, BRCA1/2 ↓	•Delayed UV repair •ΔNp73α ↑ •Inhibit p300	•Unknown	•Attenuate and repurpose several DDR proteins
Prevention of Apoptosis	•Degrade BAK	•Degrade BAK	•Stabilize p53 •Alter p53 activity •Degrade BAK	•Degrade p53	•Degrade p53
Proliferation	•Hyperphosphorylate pRb	•Hyperphosphorylate pRb	•Attenuate p21 accumulation •Phosphorylate pRB	•Hyperphosphorylate pRb	•Degrade pRb
Immortalization	•No	•No	•Yes	•Yes	•Yes
Tumor maintenance	•Not required	•Not required	•Not required	•Not required	•Required
E6 motif	•No PDZ domain	•No PDZ domain	•No PDZ domain	•No PDZ domain	•PDZ domain present
Animal Models	•Artificial skin graft model •UV-induced lesion formation	•Spontaneous tumor formation	•UV DNA damage induced tumor formation	•No cSCC formation •Chemically-induced carcinogenesis in digestive tract	•Spontaneous tumor formation

This table compares the impact of several β-HPVs and high risk α-HPVs on DDR and other pathways relevant to carcinogenesis. ↑ and ↓ indicate increased or decreased abundance, respectively.

the homologous of high risk α-HPV oncogenes (E6 and E7). These viruses are expected to promote carcinogenesis via the "hit and run" model that is dependent on their amplification of UV's genome destabilizing effects and otherwise decreasing genome fidelity. Since the mechanisms of abrogating genome stability vary among β-HPVs, it is reasonable to expect that the carcinogenic potential of individual β-HPVs depends on their cellular environment during infection. As a result, not only is the clear delineation of pathways and proteins impacted by β-HPV necessary to understand their oncogenic potential, but also the relative ability among β-HPVs to disrupt these pathways. A major knowledge gap regarding β-HPV induced oncogenesis is the extent that the reduced availability of ATM, ATR, BRCA1, and BRCA2 affects the activity of these proteins in each of the repair pathways that they participate in. This could expand the known ability of β-HPV proteins to disrupt DNA repair to include nearly every cellular response to damaged DNA. Alternatively, if the impairment of these repair proteins was context specific, it could highlight particularly deleterious situations for a β-HPV infection to occur. Further, there is a diversity of genome destabilizing events beyond DNA damage (failed cytokinesis, centrosome duplication errors, etc.) that if disrupted would fit the ascribed "hit and run" model of tumorigenesis. Defining the mutagenic potential of β-HPV infections is essential to take next steps in preventing disease associated with these viruses. **Table 1** compares β-HPV 5, 8, 38, and 49 as well as high-risk α-HPV oncogenes regarding their oncogenic potential. A possible intervention is the development of β-HPV specific vaccines. While β-HPV infection occurs in early childhood, a vaccine against specific "high-risk" types may prevent re-infection. Additionally, immunity acquired through vaccination may be more effective than immunity through infection. The FDA-approved technology to make the current vaccine could be readily adapted to prevent β-HPV infections or specific inhibitors of β-HPVs could be developed and added to sunscreens to precisely target the intersection of UV and viral infection. A commonly stated challenge to the development of a vaccine against β-HPV is the fact that these infections are initially acquired soon after birth and that β-HPV infections do not illicit a protective immune response. Although true, vaccination could mitigate disease by preventing reinfection and potential initial infection with particularly oncogenic β-HPVs not necessarily acquired at birth. Further, vaccination is effective against members of the alpha HPV genus despite poor natural adaptive immune responses to those infections. Ultimately, these ambitious goals are dependent on advancements in the dissection of β-HPV biology and its ability to hinder the cellular DDR.

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REVIEW
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Inhibition of TGF-β and NOTCH Signaling by Cutaneous Papillomaviruses

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Infections with cutaneous papillomaviruses have been linked to cutaneous squamous cell carcinomas that arise in patients who suffer from a rare genetic disorder, epidermodysplasia verruciformis, or those who have experienced long-term, systemic immunosuppression following organ transplantation. The E6 proteins of the prototypical cutaneous human papillomavirus (HPV) 5 and HPV8 inhibit TGF-β and NOTCH signaling. The *Mus musculus* papillomavirus 1, MmuPV1, infects laboratory mouse strains and causes cutaneous skin warts that can progress to squamous cell carcinomas. MmuPV1 E6 shares biological and biochemical activities with HPV8 E6 including the ability to inhibit TGF-β and NOTCH signaling by binding the SMAD2/SMAD3 and MAML1 transcription factors, respectively. Inhibition of TGF-β and NOTCH signaling is linked to delayed differentiation and sustained proliferation of differentiating keratinocytes. Furthermore, the ability of MmuPV1 E6 to bind MAML1 is necessary for wart and cancer formation in experimentally infected mice. Hence, experimental MmuPV1 infection in mice will be a robust and valuable experimental system to dissect key aspects of cutaneous HPV infection, pathogenesis, and carcinogenesis.

Keywords: viral oncogenesis, squamous cell carcinoma, epidermodysplasia verruciformis, keratinocyte differentiation, hit-and-run carcinogenesis

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INTRODUCTION

As of January 2018, more than 300 human papillomavirus (HPV) genotypes are listed in the PAVE database[1] (Van Doorslaer et al., 2017). HPVs have ∼8 kb circular double stranded DNA genomes, do not generally infect heterologous hosts and exhibit a preference for infecting either mucosal or cutaneous epithelia. Mucosal HPVs are generally transmitted by sexual contact and mucosal infections with α-genus HPVs are the most common sexually transmitted disease with a prevalence of 70 million cases and an annual incidence of 14 million new transmissions in the United States (CDC, 2015). Mucosal HPVs mostly include members of the α-genus. Clinically, α-HPVs can be designated either as "low-risk" or "high-risk." Low-risk α-HPV infections trigger

[1] pave.niaid.nih.gov

benign genital warts, whereas high-risk α-HPVs cause intraepithelial lesions that can undergo malignant progression. Almost 100% of cervical carcinoma are caused by high-risk HPVs and infections with these viruses also contribute to a large percentage of anogenital tract as well as oral cancers (Schiffman et al., 2007). Globally, high-risk α-HPVs infections cause approximately 5% of all human cancers. Despite the availability of efficacious prophylactic vaccines for the most abundant high-risk α-HPVs, there will be approximately 4,170 cervical cancer deaths in 2018 in the United States alone, which means that approximately every 2 h one woman will succumb to cervical cancer (Siegel et al., 2018). Because cancers arise years to decades after the initial infection, a significant impact of these prophylactic vaccines on cervical cancer rates is not expected for another 20 years (Frazer, 2004; Schiller and Lowy, 2012). Unfortunately, there are no antiviral compounds to combat HPV infections (Hellner and Munger, 2011).

Cutaneous HPVs include the β-, γ-, μ-, and ν-genera and some α-HPVs also have a preference for infecting cutaneous epithelia. Cutaneous HPV infections are very frequent and have been linked to development of warts, actinic keratosis, keratoacanthoma, psoriasis, and non-melanoma skin cancers. In Australia, up to 24% of 16- to 18-year-olds have skin warts (Kilkenny et al., 1998). The prevalence of actinic keratosis in the United States is ∼20% and resulted in 47 million dermatology visits in 2006 (Del Rosso et al., 2014). Keratoacanthoma are low grade, rapidly growing skin tumors that, unlike squamous cell carcinomas, are derived from cells located in the hair follicle. Cutaneous HPV genomes are readily detected in hair follicle cells harvested by plucking hair, and a high viral load is a predictor of cancer development (Neale et al., 2013). Keratoacanthoma mostly occur in sun-exposed skin in middle aged patients. The incidence has been estimated at approximately 1:1,000 (Chuang et al., 1993). Cutaneous HPVs have also been frequently detected in psoriasis patients (Mahe et al., 2003). In patients suffering from a rare inherited disease, *epidermodysplasia verruciformis* (EV), and in the rapidly increasing population of chronically immune-suppressed individuals, such as organ transplant patients, lesions caused by cutaneous HPV infections can progress to cutaneous squamous cell carcinomas (cSCCs) (Pass et al., 1977; Orth et al., 1978; Neale et al., 2013). Indeed, cSCCs arising in EV patients were the first human tumors that were conclusively linked to HPV infections. More recently, β- and γ-HPV genomes have also been detected at mucosal sites, suggesting the possibility that these viruses may also infect mucosal epithelia (Bottalico et al., 2011; Hampras et al., 2017; Smelov et al., 2017).

The incidence of cSCCs in the general population has been linked to UV exposure and is rising rapidly with more than 700,000 cases and 8,000 deaths yearly in the United States (Alam and Ratner, 2001; Deady et al., 2014). The estimated annual costs for cSCC treatments in the United States are at $3.8 billion (Guy et al., 2015). Whether and how HPVs may be involved in cSCCs development in immunocompetent individuals, however, remains a matter of debate (Feltkamp et al., 2008), even though a recent meta-analysis provided evidence for a modestly increased overall risk of 1.42 (1.18–1.72) for β-HPV infection and cSCC development (Chahoud et al., 2016).

Hence, β-HPV infections are highly associated with cSCC development in EV patients, correlated in the case of cSCCs arising in immune suppressed patients, and only slightly or sporadically associated with cSCCs in immune competent patients. HPV sequences are not retained and expressed in every cSCC cell. This indicates that, unlike what has been shown for high-risk α-HPV-associated cancers, cutaneous HPVs may not be necessary for tumor maintenance. Cutaneous HPV genomes are almost universally detected in asymptomatic patients (Antonsson et al., 2003a; Kohler et al., 2007), but it is not clear whether this is reflective of active and/or persistent infection. Detection of HPV RNA or protein expression may provide a better estimation of the frequency of active HPV infection in non-neoplastic skin (Borgogna et al., 2012, 2014) and it has been debated whether cutaneous HPVs function as carcinogenic drivers, or if they appear as innocuous passengers in cSCCs that arise in immunocompetent individuals (Quint et al., 2015). A recent study with a transgenic mouse model, has provided evidence for the alternative model that some cutaneous HPVs are drivers of cancer initiation but are not necessary for tumor maintenance (Viarisio et al., 2017).

CARCINOGENIC ACTIVITIES OF β-HPVs

Because of their isolation from cSCCs in EV patients, much of the early research efforts with the β-HPVs focused on HPV5 and HPV8. Somewhat surprisingly, HPV5 and HPV8 scored as only weakly transforming or non-transforming in standard cell-based assays (Yamashita et al., 1993; Tommasino, 2017). However, experiments with genetically engineered mice where HPV8 sequences were expressed in basal epithelial cells, impressively demonstrated the oncogenic activity of this HPV. While these mice spontaneously develop skin cancer without the requirement of any additional treatment, they also recapitulated the co-carcinogenic effect of UV exposure (reviewed in Howley and Pfister, 2015). These experiments showed that HPV8 E6, E7 and, uniquely, also E2 could each independently contribute to skin cancer formation when ectopically expressed in basal epithelial cells of a transgenic mouse (Schaper et al., 2005; Pfefferle et al., 2008; Marcuzzi et al., 2009).

The β-HPVs are phylogenetically diverse and as of January 2018, 64 β-HPVs are listed in the PAVE database (see text footnote 1; Van Doorslaer et al., 2017). These are further classified into phylogenetic species. HPV5 and HPV8 together with 19 additional HPVs are classified as species 1, there are 22 members classified as species 2, four are species 3, one is species 4, three are species 5, and 13 have not yet been classified by the HPV reference center.

The E6 and E7 proteins of other β-HPVs, including HPV types 20, 38, and 48 also exhibit carcinogenic activities in transgenic mouse models. Mice expressing β1-HPV20 E6 or E7 from keratin K10 promoter and mice with β2-HPV38 E6/E7 expression from a keratin K14 promoter developed skin cancers when exposed to UV. In contrast, mice expressing the β3-HPV49 E6/E7 genes from a keratin 14 promoter were not susceptible to skin carcinogenesis after UV exposure but developed upper digestive

tract carcinomas when treated with the chemical carcinogen 4-nitroquinoline 1-oxide (Michel et al., 2006; Viarisio et al., 2011, 2016). Some of these more recently detected β-HPVs also exhibit transforming activities in cell-based assays. Ectopic expression of the β1 HPV24 or HPV36 E6/E7 proteins causes life span extension of primary human foreskin keratinocytes (HFKs), whereas expression of the β2 HPV38 and β3 HPV49 E6/E7 proteins can trigger HFK immortalization (Cornet et al., 2012).

Despite similar genomic organization, there are some differences between the β- and high-risk α-HPVs (McLaughlin-Drubin et al., 2012). Most strikingly, β-HPVs do not encode E5 proteins, which are oncogenic in high-risk α-HPVs (Genther et al., 2003; Maufort et al., 2007, 2010). Moreover, while high-risk α-HPV-associated cervical carcinomas are generally non-productive, cSCCs arising in EV patients are productive infections. Despite these differences, early mechanistic analyses of the oncogenic activities of β-HPVs were modeled after studies with high-risk α-HPV E6 and E7 proteins. Like high-risk α-HPV E6 and E7 proteins, β-HPV E6 and E7s are low molecular weight, cysteine-rich, metal binding proteins of approximately 150 and 100 amino acids, respectively. They lack intrinsic enzymatic activities, are not known to directly bind specific DNA sequences and presumably exert their biological activities by interacting with and functionally modifying cellular regulatory proteins (Munger et al., 2004; Roman and Munger, 2013; Vande Pol and Klingelhutz, 2013). The best studied cellular targets of the high-risk α-HPV E6 and E7 proteins are the TP53 and RB1 tumor suppressors, respectively. The transforming activities of papillomaviruses are a reflection of their life cycles, specifically their need to secure the availability of cellular replication factors in terminally differentiated epithelial cells that would have normally withdrawn from the cell division cycle (Harden and Munger, 2017). Papillomaviruses need to uncouple cell-cycle withdrawal from epithelial differentiation, effectively allowing for unlicensed proliferation in suprabasal keratinocytes. Aberrant DNA synthesis can be sensed by cells and triggers cell abortive responses, which will need to be inhibited by the virus. The strategies that specific papillomaviruses have evolved to enable their productive replication cycles largely determine their oncogenic potential (McLaughlin-Drubin and Munger, 2009; Moody and Laimins, 2010).

The high-risk HPV E7 proteins cause S-phase competence in differentiating cells by targeting the retinoblastoma tumor suppressor, RB1, for degradation. Uncontrolled S-phase entry as a consequence of RB1 inactivation causes activation of the TP53 tumor suppressor, which triggers a transcriptional program to eliminate such rogue cells by apoptosis. This simple, albeit likely not entirely correct, model (Munger et al., 2004; Munger and Jones, 2015) also served as a blueprint for many studies with the EV-associated β-HPVs, even though it became clear very quickly that it was unlikely to apply to these viruses (McLaughlin-Drubin et al., 2012; Meyers and Munger, 2014; Tommasino, 2017). HPV8 E6 does not detectably interact with TP53 or the UBE3A (E6AP) E3 ubiquitin ligase that is necessary for high-risk α-HPV E6 mediated TP53 degradation (Elbel et al., 1997; White et al., 2012a; Brimer et al., 2017). HPV5 and HPV8 E7s weakly bind and do not degrade RB1, similar to the low-risk α-HPV E7s (Yamashita et al., 1993).

All known β-HPV E7 proteins contain canonical LXCXE (L, leucine; C, cysteine; E, glutamate; X, any amino acid)-based binding sites for the retinoblastoma tumor suppressor family of proteins and many have been documented to bind RB1, RBL1 (p107), and RBL2 (p130). Some β-HPV E6 and E7 in fact interfere with RB1 and TP53 stability, reminiscent of cervical cancer associated, high-risk α-HPVs (Caldeira et al., 2003; Accardi et al., 2006; Cornet et al., 2012; White et al., 2014). The interactions described for β-HPV E6 proteins with TP53 are particularly complex as some have been reported to stabilize TP53 (HPV17, HPV38, and HPV92), while others destabilize it (HPV49) and some have been reported to interfere with TP53 activation and/or TP53 mediated activation of certain target genes (HPV5, HPV8, and HPV38) (Cornet et al., 2012; Wallace et al., 2014; White et al., 2014). Whether humans infected with cutaneous HPVs that target RB1 and TP53 similar to the high-risk α-HPVs are at a particularly high risk for developing cSCC, remains to be determined.

Given the fact that most β-HPV-associated tumors arise in sun exposed areas, it was investigated whether these viruses might interfere with UV-induced apoptosis and/or DNA damage repair. Consistent with expectations, several groups reported that β-HPV E6 proteins cause degradation of BAK, a pro-apoptotic BCL2 family member that is released from the mitochondria following UV exposure (Jackson et al., 2000). BAK degradation by β-HPV E6 proteins is predicted to inhibit apoptosis and allow for survival of UV-damaged cells that may have possibly acquired oncogenic mutations (Leverrier et al., 2007; Hufbauer et al., 2015).

Other studies showed that the repair of UV-damaged DNA damage is inhibited in HPV8 E6 expressing cells. HPV5 and HPV8 E6 proteins were reported to bind the EP300/CREBBP acetyltransferases and target them for degradation thereby inhibiting the Ataxia Telangiectasia Mutated (ATM) and Ataxia Telangiectasia and Rad3-Related (ATR) kinases, key sensors, and mediators of double strand DNA break repair. By abrogating ATM/ATR mediated sensing and/or repair of double strand DNA breaks, cellular mutations may accumulate in β-HPV infected cells, thereby driving carcinogenesis (Howie et al., 2011; Wallace et al., 2012; Wendel and Wallace, 2017). These findings support a "hit-and-run" mechanism for β-HPV carcinogenesis where viral gene expression contributes to cancer initiation by increasing the mutational burden caused by UV exposure but is not necessary for tumor maintenance. This would explain why viral gene expression is not maintained in all tumor cells (Arron et al., 2011). Such a model was recently recapitulated with HPV38 transgenic mice (Viarisio et al., 2018). Interestingly, unbiased proteomic analyses of the HPV38 E6 associated proteins provided no evidence for EP300/CREBBP binding to HPV38 E6 (White et al., 2012a), suggesting that inhibition of DNA break repair by β-HPV E6 proteins may not be necessarily linked to EP300/CREBBP degradation.

As mentioned previously, β-HPV genomes have been detected in hair follicles, which contain epithelial stem cells and HPV8

has been reported to increase the number of keratinocytes with stem cell characteristics both in cell- and animal-based model systems (Hufbauer et al., 2013; Lanfredini et al., 2017; Marthaler et al., 2017). Several studies have shown that the TP53 family member and epithelial stem cell marker TP63 is dysregulated in HPV8 E6 expressing keratinocytes. Specifically, higher levels of the amino terminally truncated ΔNp63α and lower levels of full-length TAp63 isoforms were detected in HPV8 E6 expressing human and murine keratinocytes (Lanfredini et al., 2017; Marthaler et al., 2017). NOTCH inhibition decreased TP63 levels in HPV8 E6 expressing undifferentiated keratinocytes, but TP63 expression remained high in differentiating HPV8 E6 expressing keratinocytes, suggesting that there are NOTCH independent pathways that cause deregulated TP63 expression (Meyers et al., 2013). One of these pathways involves down-regulation of microRNA-203 (miR-203) by HPV8 E6 by inhibiting EP300 which in turn affects CCAAT/enhancer-binding protein a (CEBPA) transcription factor activity that controls miR-203 expression. Significantly, up-regulation of miR-203 during calcium-induced differentiation is abrogated in HPV8 E6 expressing keratinocytes (Marthaler et al., 2017). High-level TP63 and low levels of miR-203 were also detected throughout the lesional tissue of an HPV8 positive EV patient (Marthaler et al., 2017). Since EP300 binding is not shared by all β-HPV E6 (White et al., 2012a) it remains to be determined whether this pathway is universally targeted by β-HPV E6 proteins.

THE γ-HPVs; BENIGN FLORA OR DANGEROUS VILLAINS?

As of January 2018, a total of 171 γ-HPVs have been detected, which are classified into 27 different species. The number of species may increase, since 93 γ-HPVs have not yet been classified into species by the HPV reference center (see text footnote 1; Van Doorslaer et al., 2017). The enormous phylogenetic diversity of γ-HPVs is also reflected in their genome organization; members of the γ6 species, for example, do not encode recognizable E6 proteins and some γ-HPVs encode E7 proteins that lack the canonical LXCXE binding site for the RB1 tumor suppressor and the related p107 (RBL1) and p130 (RBL2) proteins. The γ-HPVs were initially isolated from cutaneous epithelia and are often regarded as components of the normal skin viral flora (Antonsson et al., 2003a,b; Forslund, 2007; Chen et al., 2008; Foulongne et al., 2012). More recent studies also detected γ-HPV DNA at mucosal sites (Bottalico et al., 2011; Smelov et al., 2017).

A "deep sequencing" study of human skin cancers and matched normal tissues showed that HPV197, a γ24-HPV, was the most frequently detected HPV genome in cSCCs and was not present in normal skin (Arroyo Muhr et al., 2015). HPV197 could not have been previously detected because the genome is not recognized by the "consensus" PCR primers that are frequently used for HPV analysis of clinical specimens (Arroyo Muhr et al., 2015). To this date, however, there is no evidence that HPV197 sequences are actively expressed in skin cancers and/or that HPV197 or potentially other γ-HPVs contribute to cSCC development in immunosuppressed or immunocompetent individuals.

Analysis of potential cellular targets by affinity purification followed by mass spectrometry (AP/MS) revealed that HPV197 E6 shares many cellular targets with β-HPV E6 proteins, particularly the β1 HPVs that include HPV5 and HPV8 (Grace and Munger, 2017). The HPV197 E7 protein lacks an LXCXE-based RB1 binding site, yet, consistent with earlier work with the γ1 HPV4 E7 protein (Wang et al., 2010), HPV197 E7 interacts with RB1 (Grace and Munger, 2017). Interestingly, however, HPV197 E7 does not bind to RBL1 or RBL2 (Grace and Munger, 2017). The detection of HPV197 DNA in human skin cancers but not normal skin, and the interactions of the E6 and E7 proteins with human tumor suppressor proteins are consistent with the possibility that HPV197 and potentially other γ-HPVs may be associated with skin cancer development. Currently, however, only very few studies have explored the biological activities of γ-HPVs in cell-based or animal models.

MmuPV1, AN ANIMAL MODEL FOR CUTANEOUS HPV INFECTIONS

Papillomaviruses are species specific and generally do not infect heterologous hosts (Knipe and Howley, 2013). This has severely hindered the study of PV pathogenesis *in vivo*. Research has also been stymied by the lack of a papillomavirus that naturally infects and replicates in a genetically tractable laboratory animal. The isolation of *Mus musculus* papillomavirus 1, MmuPV1, from skin warts on immunodeficient nude mice promises to revolutionize PV pathogenesis studies (Ingle et al., 2011; Joh et al., 2011). The ability to cause wart formation upon experimental infection with quasivirus or by naked DNA inoculation was originally observed in mouse strains that have T-cell deficiencies or are temporarily immune suppressed (Handisurya et al., 2014; Joh et al., 2016; Uberoi et al., 2016). Lesions arising due to MmuPV1 infection have malignant potential and can progress to cSCCs. Upon UVB-exposure, experimental MmuPV1 infection also causes skin warts that can progress to cSCCs in immunocompetent, standard laboratory mouse strains (Uberoi and Lambert, 2017). Importantly, there is a correlation between immunosuppression due to UV irradiation and onset of MmuPV1 dependent cutaneous papillomas (Uberoi et al., 2016).

MmuPV1 is a member of the Pi genus, which encompasses rodent PVs (Van Doorslaer et al., 2017) and is related to γ- and β-HPVs (Joh et al., 2011). Consistent with these phylogenetic relationships, the MmuPV1 E6 protein has similar cellular targets as the β-HPV8 E6 protein (Meyers et al., 2017). The MmuPV1 E7 protein is related to the γ-HPV197 E7 protein in that it lacks the canonical LXCXE-based RB1 binding site (Joh et al., 2011; Grace and Munger, 2017). Moreover, transcriptomic analyses revealed that similar to cutaneous HPVs, MmuPV1 expresses E6 and E7 from two separate early promoters (Xue et al., 2017).

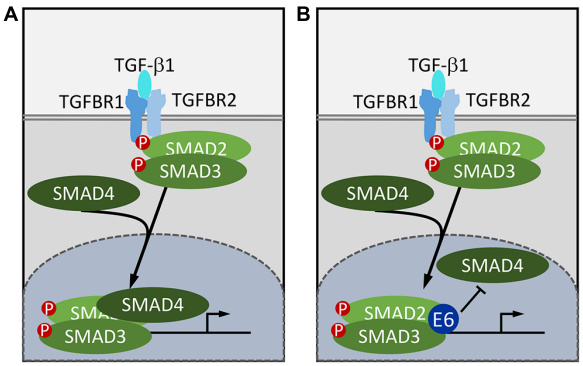

FIGURE 1 | Subversion of TGF-β signaling by HPV8 and MmuPV1 E6. In normal cells, TGF-β binding to the heterotetrameric serine/threonine kinase receptor complex triggers a phosphorylation cascade resulting in phosphorylation of receptor the SMADs, SMAD2, and SMAD3. Phosphorylated receptor SMADs disassociate from the receptor, form a complex with the co-SMAD, SMAD4, and enter the nucleus. The SMAD2/SMAD3/SMAD4 complex then binds to SMAD binding sites to stimulate target gene transcription **(A)**. HPV8 and MmuPV1 E6 proteins bind SMAD2 and SMAD3 and inhibit TGF-β signaling by preventing the formation of the active DNA bound SMAD2/SMAD3/SMAD4 complex **(B)**.

Because of its ability to infect cutaneous epithelia and cause lesions capable of malignant progression upon UV exposure, MmuPV1 may be an important model for determining the potential roles of β- and some γ-HPVs in skin cancer development.

Interestingly, MmuPV1 can productively infect both cutaneous and mucosal tissues (Cladel et al., 2013, 2016; Hu et al., 2015) and thus may also model aspects of mucosal pathogenesis. In any case, results from infection experiments with mutant MmuPV1 genomes can be combined with infection studies of relevant, genetically engineered mouse models. This will enable studies to define mechanisms of action of viral proteins and to determine how viral targeting of specific cellular signaling pathways contribute to the life cycle, pathogenesis and carcinogenicity of a papillomavirus in a natural host.

SUBVERSION OF TGF-β AND NOTCH SIGNALING BY CUTANEOUS PAPILLOMAVIRUSES

While inhibition of UV-mediated apoptosis and/or DNA damage sensing/repair provides a satisfying model to mechanistically support a hit-and-run mechanism of cutaneous HPV mediated carcinogenesis, it does not explain how these viruses may cause warts, a necessary precursor to cSCCs. Moreover, studies with MmuPV1 showed that UV-mediated immune suppression rather than mutagenesis was important for pathogenesis and tumor development (Uberoi et al., 2016).

Inhibition of TGF-β Signaling

Multiple developmental and cellular processes are regulated by TGF-β signaling. The core components of this signal transduction pathway consist of the ligand, TGF-β, heterotetrameric receptors with serine/threonine kinase activity, receptor associated SMAD (R-SMADs) proteins, and co-activator SMADs (co-SMADs). The tetrameric receptor consists of one dimer of the ligand interacting TGF-β type II receptor (TGFBR2), and one dimer of TGF-β type I receptor (TGFBR1). TGF-β binding triggers a phosphorylation cascade from TGFBR2 to TGFBR1 to R-SMADs (Chaikuad and Bullock, 2016). Activin/Nodal and TGFBR1 receptors phosphorylate the R-SMAD pair SMAD2 and SMAD3, whereas the Bone Morphogenetic Protein (BMP) type I receptors activate the R-SMADs SMAD1, SMAD5, and SMAD8 (Miyazawa et al., 2002). After phosphorylation, R-SMADs complex with the co-SMAD, SMAD4, enter the nucleus, bind specific DNA sequences, and activate target gene transcription (**Figure 1A**). Transcriptional activation of the CDK2 inhibitors $p21^{CIP1}$ (CDKN1A) and $p27^{KIP1}$ (CDKN1B), and the CDK4/CDK6 inhibitor $p15^{INK4B}$ (CDKN2B) have been linked to TGF-β mediated G1 cell cycle arrest (Datto et al., 1995; Reynisdottir et al., 1995). Hence, TGF-β functions as a tumor suppressor in epithelial cells, and TGF-β pathway loss of function mutations are frequent in epithelial tumors (Wang et al., 2000; Qiu et al., 2007; Fleming et al., 2013). Importantly, however, TGF-β signaling also induces expression of factors involved in epithelial to mesenchymal transition (EMT) and causes increased production of matrix metalloproteases, which results in enhanced migration and invasion (Valcourt et al., 2016). This is consistent with an oncogenic activity of TGF-β as was originally discovered in fibroblasts (Roberts et al., 1985). Hence, the NOTCH and TGF-β signaling pathways each have tumor suppressor activities at early stages of carcinogenesis and can function as oncogenes during later stages of carcinogenesis.

The first evidence that cutaneous HPVs inhibit TGF-β signaling was provided by Michel Favre's group who reported that HPV5 E6 could target SMAD3 for proteasomal degradation (Mendoza et al., 2006). Consistent with this report, AP/MS experiments provided evidence for SMAD2 and SMAD3 association with HPV8 E6 (Meyers et al., 2017). Similarly, the MmuPV1 E6 protein was also shown to bind SMAD2 and SMAD3 (Meyers et al., 2017). Unlike many other papillomavirus E6 binding cellular proteins, SMAD2 and SMAD3 do not contain helical LXXLL (L, leucine; X, any amino acid) motifs (Chen et al., 1998). There is no evidence for SMAD2, SMAD3, or SMAD4 destabilization by HPV8 E6 or MmuPV1 E6 either under basal or TGF-β stimulated conditions (Meyers et al., 2017). Moreover, there is no defect in SMAD2/SMAD3 phosphorylation and nuclear translocation following TGF-β stimulation in HPV8 or MmuPV1 E6 expressing human keratinocytes. The HPV8 and MmuPV1 E6 proteins, however, were shown to reduce SMAD4 association with SMAD2 and SMAD3 after TGF-β stimulation and to inhibit SMAD2/3 and SMAD4 binding to target DNA. Hence cutaneous papillomavirus E6 proteins inhibit TGF-β signaling by blocking productive binding of the SMAD2/3/4 complex to TGF-β responsive promoters (Meyers et al., 2017; **Figure 1B**). It will be interesting to determine whether these E6 proteins can also inhibit signaling by TGF-β related cytokines. Interestingly, TGF-β signaling is also inhibited by α-HPVs and

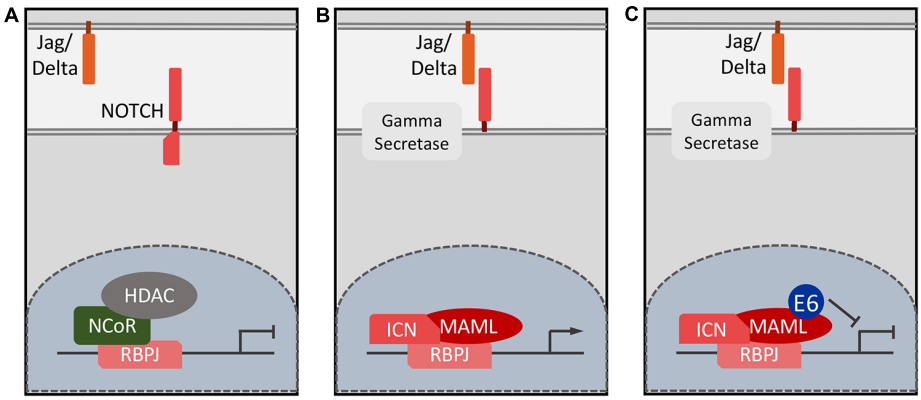

FIGURE 2 | Schematic of NOTCH target gene expression. In the absence of ligand binding, NOTCH responsive genes remain repressed and RBPJ is in a DNA bound, nuclear complex with repressors such as the N-CoR complex **(A)**. Ligand binding leads to NOTCH cleavage and ICN enters the nucleus, displaces repressors and recruits MAML and other co-activators such EP300/CREBBP **(B)**. HPV8 and MmuPV1 E6 proteins inhibit NOTCH signaling by forming a complex with the DNA bound ICN/MAML1 complex thereby preventing formation of a functional transcriptional activator complex **(C)**.

high-risk HPV16 has been reported to inhibit TGF-β through E7 (Pietenpol et al., 1990; Lee et al., 2002) as well as E5 (French et al., 2013), but not through E6. Hence the cutaneous and mucosal HPVs share the ability to inhibit TGF-β signaling although they have evolved different mechanisms.

Inhibition of NOTCH Signaling

Similar to TGF-β, NOTCH regulates multiple processes during embryogenesis and in the adult organism. Of particular interest to papillomavirus biology, NOTCH signaling drives epithelial differentiation and is required for proper formation of the skin barrier (Rangarajan et al., 2001). NOTCH signaling is triggered through cell-to-cell contact where membrane anchored ligands of the Delta or Jagged family bind to NOTCH family receptors. In humans there are four NOTCH receptors (NOTCH1–4), which exhibit some overlapping functions, though this may be context specific (Kojika and Griffin, 2001). Receptor–ligand binding induces conformational changes in NOTCH that result in a series of proteolytic cleavages ultimately releasing an intracellular fragment of the NOTCH receptor, termed Intracellular NOTCH (ICN; De Strooper et al., 1999). ICN translocates to the nucleus where it associates with Recombination Signal Binding Protein for Immunoglobulin Kappa J Region (RBPJ), which binds to specific DNA sequences. In the absence of ICN, RBPJ is bound to the DNA in association with co-repressors, thus repressing target gene expression (Oswald et al., 2002; **Figure 2A**). ICN displaces these co-repressors and the ICN–RBPJ complex recruits a member of the transcriptional co-activators mastermind-like (MAML) family (Nam et al., 2006). The ICN/RBPJ/MAML complex represents the core NOTCH transcriptional activator complex and MAML then recruits additional co-activators (**Figure 2B**; Saint Just Ribeiro and Wallberg, 2009). Classical NOTCH targets include the Hairy and Enhancer of Split (HES) gene, which belongs to a family of basic helix-loop-helix transcriptional repressors, which are particularly important for lateral inhibition and patterning during development (Iso et al., 2003). NOTCH also regulates other targets such as c-myc (MYC; Weng et al., 2006), cyclin D1 (CCND1; Cohen et al., 2010), and the $p21^{CIP1}$ (CDKN1A) cyclin dependent kinase inhibitor (Rangarajan et al., 2001). Due to the variety of cell types where NOTCH signaling is important, NOTCH target genes are lineage specific and critical NOTCH targets that drive keratinocyte differentiation have not been well-characterized.

Human cSCCs frequently harbor loss of function mutations in the NOTCH pathway, implicating NOTCH activity as tumor suppressive in epithelial cells (Nicolas et al., 2003; Agrawal et al., 2011; Stransky et al., 2011; Wang et al., 2011; South et al., 2014). The tumor suppressive effects of NOTCH in keratinocytes are largely thought to be due to a differentiation-induced block of cell proliferation and the activation of keratinocyte cell death (Zhang et al., 2016; Aster et al., 2017). NOTCH, however, also functions as an oncogenic driver in a number of human tumors, particularly hematopoietic cancers such as T-cell acute lymphoblastic leukemia (T-ALL) where gain of function mutations are commonly observed (Demarest et al., 2008).

MAML1 was originally identified in a yeast-two hybrid screen with HPV16 E6 as a bait (Wu et al., 2000). Like many cellular E6 binding proteins, MAML1 contains LXXLL sequence motifs and several groups detected MAML1 as a cellular interactor of β-HPV E6 proteins in AP/MS experiments (Brimer et al., 2012; Rozenblatt-Rosen et al., 2012; Tan et al., 2012; White et al., 2012a). Similarly, the bovine papillomavirus type 1 (BPV1) E6 protein was also shown to bind MAML1 (Brimer et al., 2012; Tan et al., 2012). Despite the original identification as an HPV16 E6 associated cellular protein (Wu et al., 2000), there is no evidence that mucosal α-HPV E6 proteins detectably associate with MAML1 in mammalian cells (Brimer et al., 2017).

Biological Consequences of TGF-β and NOTCH Inhibition by Cutaneous Papillomaviruses

Given that the TGF-β and NOTCH pathways are critical in regulating epithelial differentiation and function as tumor

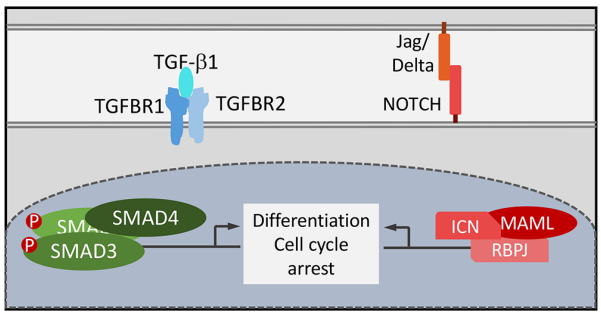

FIGURE 3 | Functional consequences of NOTCH and TGF-β signaling by HPV8 E6 and MmuPV1 E6. TGF-β and NOTCH signaling are critical for cell cycle arrest and keratinocyte differentiation that are tightly coupled in normal squamous epithelial cells. Functional uncoupling of these two processes is a necessary prerequisite for papillomavirus progeny formation in terminally differentiated epithelial cells.

suppressors in a variety of epithelial cancers, inhibition of these two pathways by cutaneous papillomavirus E6 proteins may be key to the transforming activities of these viruses.

Consistent with this model, it was shown that keratinocyte differentiation is inhibited in HPV8 E6 expressing epithelial cells. Chromatin immunoprecipitation experiments showed that E6 binds to and inhibits the DNA bound RBPJ/ICN/MAML1 activator complex (Meyers et al., 2013). HPV8 E6 binds to an LXXLL motif within the C-terminal transactivation domain (TAD) 2 of MAML1 (Tan et al., 2012; Meyers et al., 2017). The mechanism of action of TAD2 is unknown; EP300 and related co-activators associate with MAML through its TAD1 domain (Saint Just Ribeiro and Wallberg, 2009; Gerhardt et al., 2014). The MmuPV1 E6 protein also interacts with MAML1 and inhibits NOTCH signaling thereby inhibiting keratinocyte differentiation (Meyers et al., 2017; **Figure 2C**). MAML1 binding defective HPV8 and MmuPV1 E6 mutants were identified using a structure guided approach (Meyers et al., 2017). Of note, many of the HPV8 E6 mutants that were previously used to characterize the biological relevance of the interaction between cutaneous HPV E6 protein and EP300/CREBBP, were found to be MAML1 binding defective, thus further complicating the interpretation of published studies investigating the biological consequences of the EP300/CREBBP interactions with HPV5 and HPV8 E6 (Meyers et al., 2017). Association of E6 with MAML and inhibition of NOTCH signaling is independent of EP300/CREBBP binding, however, since MAML1 binding defective HPV8 E6 mutants retained EP300/CREBBP association and MmuPV1 E6 does not detectably bind EP300/CREBBP (Meyers et al., 2017). Indeed, the notable absence of EP300/CREBBP peptides in AP/MS experiments with MmuPV1 E6 is consistent with a model whereby E6 binding to TAD2 of MAML1 may inhibit expression of NOTCH transcriptional targets by interfering with recruitment or by displacing EP300/CREBBP from MAML1 TAD1. This model, however, awaits experimental validation.

The presence of an intact binding site for MAML1 in MmuPV1 E6 was shown to be necessary for formation of papillomas and carcinomas in experimentally infected mice (Meyers et al., 2017). While these studies cannot definitively exclude the possibility that association of E6 with other LXXLL domain proteins may also contribute to this phenotype, this is unlikely given a recent study that showed that UBE3A and MAML1 are the major LXXLL targets of papillomavirus E6 proteins and, consistent with the published E6/LXXLL structures (Zanier et al., 2013), the various papillomavirus E6 proteins displayed mutually exclusive binding to either UBE3A or MAML1 (Brimer et al., 2017).

Despite the fact that the E6 proteins of mucosal, high-risk α-HPVs have a strong preference for UBE3A binding and do not detectably interact with MAML1 (White et al., 2012a; Brimer et al., 2017), they have also been reported to inhibit NOTCH. HPV16 E6 can inhibit NOTCH signaling indirectly through degradation of the TP53 tumor suppressor (Dotto, 2009) and/or the TAp63β protein (Ben Khalifa et al., 2011). More recent studies showed that NOTCH inhibition is relevant to the biology of these viruses as HPV16 E6 mediated NOTCH inhibition was shown to decrease the commitment of keratinocytes to differentiation (Kranjec et al., 2017).

Hence, similar to TGF-β, inhibition of keratinocyte differentiation by targeting NOTCH signaling by cutaneous HPVs is shared with mucosal HPVs but is mediated through different mechanisms. Conceptually this is rather satisfying as papillomaviruses all had to evolve to render normally postmitotic, terminally differentiated epithelial cells conducive for supporting the viral life cycle (**Figure 3**). Interestingly however, different papillomaviruses have evolved mechanistically distinct strategies to solve this problem.

"Hit and Run" Carcinogenesis: A Consequence of E6 Mediated NOTCH and TGF-β Inhibition?

Hit and run carcinogenesis is a concept that historically has generated some skepticism, also with the authors of this article. The failure to detect viral gene expression in the majority of cancer cells may also imply that β- and γ-HPVs are biologically innocuous passengers that do not drive the carcinogenic process. Given, however, that at least some of these papillomaviruses are carcinogenic in transgenic mouse models and inhibit TGF-β and NOTCH signaling as well as other tumor suppressor pathways, it is more likely that these viruses provide oncogenic hits that drive at least some early aspects of epithelial carcinogenesis. In addition to many other biological activities of cutaneous HPVs, which include stem cell expansion, abrogating apoptosis, sensing and repair of UV-induced double strand DNA breaks, inhibition of the TGF-β and NOTCH tumor suppressors provide plausible oncogenic "hits" during early phases of carcinogenesis. Since the NOTCH and TGF-β pathways are oncogenic at later stages and drive malignant progression (Massague, 2008; Aster et al., 2017) loss of papillomavirus gene expression will re-activate NOTCH and TGF-β. This may drive malignant progression, thereby providing the mechanistic underpinnings for the "run" component of hit and run carcinogenesis.

It is important to note that the β- and γ-HPVs are phylogenetically and biologically diverse (Cornet et al., 2012;

Van Doorslaer et al., 2017) and that this diversity is also reflected in the cellular interactomes of their E6 and E7 proteins (White et al., 2012a,b). Hence, findings with one specific β- or γ-HPV type may not be applicable to other HPVs, even within the same genus. The accumulation of UV-induced mutations in a recently published HPV38 transgenic skin cancer model that recapitulates hit-and-run carcinogenesis, for example, is unlikely to be caused through an EP300/CREBBP dependent mechanism as no EP300/CREBBP binding has been detected in unbiased AP/MS studies with HPV38 E6 (White et al., 2012a; Viarisio et al., 2018).

The wide application of AP/MS to define cellular interactomes of papillomavirus proteins has already generated interesting hypotheses, many of which await rigorous validation (White and Howley, 2013; McBride, 2017). Next generation sequencing experiments may detect HPV DNA and/or RNA sequences in unexpected anatomic locations and tentatively link them to unanticipated pathologies (Chen et al., 2017). Such studies may also lead to the identification of HPVs that evaded detection by standard PCR-based techniques. The frequent detection of the novel γ-HPV197 genome in a deep sequencing study of human skin cancers, but not in normal skin, while not implying an etiologic link, should provide at least an impetus to study the biology of γ-HPVs in greater detail (Arroyo Muhr et al., 2015). Given that the HPV197 E7 protein, similar to MmuPV1, lacks an LXCXE-based canonical RB1 binding domain, and binds RB1 through a different sequence, suggest that some aspects of HPV197 E7 can be studied with the MmuPV1 animal model. These and other studies might also reveal whether HPV197, and potentially other γ-HPVs, have oncogenic activities.

AUTHOR CONTRIBUTIONS

JM and KM drafted the manuscript. AU, PL, and MG provided editorial and substantive input. KM wrote the final version of the manuscript.

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HPV8-E6 Interferes with Syntenin-2 Expression through Deregulation of Differentiation, Methylation and Phosphatidylinositide-Kinase Dependent Mechanisms

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The E6 oncoproteins of high-risk human papillomaviruses (HPV) of genus alpha contain a short peptide sequence at the carboxy-terminus, the PDZ binding domain, with which they interact with the corresponding PDZ domain of cellular proteins. Interestingly, E6 proteins from papillomaviruses of genus beta (betaPV) do not encode a comparable PDZ binding domain. Irrespective of this fact, we previously showed that the E6 protein of HPV8 (betaPV type) could circumvent this deficit by targeting the PDZ protein Syntenin-2 through transcriptional repression (Lazic et al., 2012). Despite its high binding affinity to phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$), very little is known about Syntenin-2. This study aimed to extend the knowledge on Syntenin-2 and how its expression is controlled. We now identified that Syntenin-2 is expressed at high levels in differentiating and in lower amounts in keratinocytes cultured in serum-free media containing low calcium concentration. HPV8-E6 led to a further reduction of Syntenin-2 expression only in cells cultured in low calcium. In the skin of patients suffering from Epidermodysplasia verruciformis, who are predisposed to betaPV infection, Syntenin-2 was expressed in differentiating keratinocytes of non-lesional skin, but was absent in virus positive squamous tumors. Using 5-Aza-2′-deoxycytidine, which causes DNA demethylation, Syntenin-2 transcription was profoundly activated and fully restored in the absence and presence of HPV8-E6, implicating that E6 mediated repression of Syntenin-2 transcription is due to promoter hypermethylation. Since Syntenin-2 binds to $PI(4,5)P_2$, we further tested whether the $PI(4,5)P_2$ metabolic pathway might govern Syntenin-2 expression. $PI(4,5)P_2$ is generated by the activity of phosphatidylinositol-4-phosphate-5-kinase type I (PIP5KI) or phosphatidylinositol-5-phosphate-4-kinase type II (PIP4KII) isoforms α, β and γ. Phosphatidylinositide kinases have recently been identified as regulators of gene transcription. Surprisingly, transfection of siRNAs directed against PIP5KI and PIP4KII resulted in higher Syntenin-2 expression with the highest effect mediated by siPIP5KIα. HPV8-E6 was able to counteract siPIP4KIIα, siPIP4KIIβ and siPIP5KIγ mediated Syntenin-2 re-expression but not siPIP5KIα. Finally,

we identified Syntenin-2 as a key factor regulating PIP5KIα expression. Collectively, our data demonstrates that Syntenin-2 is regulated through multiple mechanisms and that downregulation of Syntenin-2 expression may contribute to E6 mediated dedifferentiation of infected skin cells.

Keywords: human papillomavirus (HPV), E6 oncoprotein, Syntenin-2, methylation, differentiation, phosphatidylinositol-4,5-bisphosphate

INTRODUCTION

Papillomaviruses are a family of non-enveloped DNA tumor viruses that infect the skin or mucosa of their vertebrate hosts (Bravo et al., 2010). Human papillomaviruses (HPV) are associated with the development of epithelial cancers and high-risk HPV of genus alpha (alphaPV) have been proven to be a necessary causative factor for cervical and oropharyngeal cancer (Hübbers and Akgül, 2015). HPV types of genus beta (betaPV) have been implicated in the development of cutaneous squamous cell carcinomas (SCC) and actinic keratosis. The association between betaPV and skin cancer was first reported in patients with Epidermodysplasia verruciformis (EV) (Majewski and Jablonska, 2006). Patients suffering from this condition have a specific susceptibility to infections with betaPV, which is characterized by virus-positive cutaneous lesions. These lesions mainly occur in sun-exposed areas and harbor a considerable risk of malignant transformation into SCC in affected individuals (Akgül et al., 2006; Borgogna et al., 2012; Howley and Pfister, 2015). Viral gene expression and replication proceed in a tightly controlled fashion regulated by keratinocyte differentiation, and lead to the expression of several non-structural viral early (E) proteins. Expression of the viral E4 protein during a productive infection contributes to virus release by perturbing the cytokeratin network. However, it may also represent a marker for viral infection during betaPV-associated skin cancer progression in EV patients (Borgogna et al., 2012; Quint et al., 2015). The HPV E6 and E7 oncoproteins are necessary for malignant conversion (Wallace and Galloway, 2015). A main feature of E6 oncoproteins from high-risk alphaHPV, e.g., HPV16, is their PDZ binding motif [identified as a domain interacting with PSD-95, Dlg, and ZO-1 proteins (hence the name PDZ (Ganti et al., 2015))], through which they promote the proteasomal degradation of a variety of cellular substrates harboring PDZ domains. Such PDZ proteins play a major role in maintenance of cell polarity, growth and differentiation as well as scaffolding proteins in multi-protein signaling complexes (Pim et al., 2012). Their degradation by the HPV E6 protein may cause loss of cell polarity and morphological conversion accompanied by an epithelial-mesenchymal transition, leading to transformation and carcinogenesis (Yugawa and Kiyono, 2009). The ability of E6 to bind to PDZ proteins correlates with its oncogenic potential (James and Roberts, 2016). In addition, the interactions between E6 and PDZ proteins also results in a significant stabilization of the E6 protein, which may ensure that sufficient E6 levels are maintained (Nicolaides et al., 2011). As previously mentioned, E6 of betaPV, e.g., HPV8, do not encode for a PDZ binding motif. We recently found, that the PDZ protein Syntenin-2, but not Syntenin-1, is transcriptionally downregulated in primary human keratinocytes by HPV8-E6. These results identified Syntenin-2 as the first PDZ protein being controlled by a betaHPV at mRNA level (Lazic et al., 2012). The PDZ protein family of Syntenins comprises Syntenin-1 and Syntenin-2. Syntenin-1 was originally identified as a protein that binds directly to the cytoplasmic domain of the syndecan family of heparan sulfate proteoglycans (Grootjans et al., 1997). These are implicated in cell adhesion and several growth factor signaling pathways (Zimmermann and David, 1999; Couchman, 2003; Egea-Jimenez et al., 2016; Fares et al., 2016). Although Syntenin-1 and -2 show different preferences, as far as protein binding is concerned, both proteins share the ability to interact with phosphatidylinositol-4, 5-bisphosphate ($PI(4,5)P_2$) (Mortier et al., 2005). However, the precise mechanism by which Syntenin-2 is physiologically regulated and how transcription is modulated by HPV8-E6 remained elusive.

This study aimed to extend the understanding of transcriptional regulation controlling Syntenin-2 expression and how E6 interferes with these mechanisms. Our results suggest an interaction of E6 with Syntenin-2 expression through interference of cell differentiation, promoter-methylation and $PI(4,5)P_2$ generating kinases.

MATERIALS AND METHODS

Syntenin Sequence Analysis

For generation of the Syntenin evolutionary trees, representative Syntenin sequences were obtained from The UniProt Consortium (2017), aligned by the L-Ins-I algorithm of the MAFFT package (Katoh et al., 2002), and subjected to tree calculation by the T-Rex server (Boc et al., 2012) using the RAxML method (Stamatakis, 2006). Uniprot accession numbers for sequences analysis: human Syntenin-1 (SDCB1_HUMAN), human Syntenin-2 (SDCB2_HUMAN), murine Syntenin-1 (SDCB1_MOUSE), murine Syntenin-2 (SDCB2_MOUSE), zebrafish Syntenin-1 (Q6PUR1_DANRE), zebrafish Syntenin-2 (Q803J6_DANRE), xenopus Syntenin-1 (Q66JA3_XENLA), xenopus Syntenin-2 (Q801P2_XENLA), anolis Syntenin-1 (G1KE37_ANOCA), anolis Syntenin-2 (H9GDS9_ANOCA), chicken Syntenin-1 (Q5ZHM8_CHICK), chicken Syntenin-2 (R4GGF6_CHICK), mosquito Syntenin (Q7PK99_ANOGA), tick Syntenin (B7PNL5_IXOSC), nematostella Syntenin (A7S2D9_NEMVE), sponge Syntenin (I1FJB9_AMPQE), capsaspora Syntenin (A0A0D2VN74_CAPO3), monosiga Syntenin (A9UPR7_MONBE).

Retroviral Expression Vectors and Infection of Keratinocytes

We have previously described the generation of the pLXSN-based retroviral vectors coding for HPV8-E6 (Akgül et al., 2010). PLXSN-HPV8-E6 based mutants were generated by site-directed mutagenesis using specific primers. For retroviral infection, 3×10^5 cells were seeded into 6 cm dishes and treated as described in Hufbauer et al. (2013). Selection of infected cells was started 2 days later using 250 μg/ml G418. Positive clones were pooled and expanded.

Cell Lines and Treatments

RTS3b (Purdie et al., 1993) and PM1 keratinocytes (Proby et al., 2000) were maintained in RM+ media [consisting of a 3:1 ratio of DMEM:F12 with 10% fetal bovine serum (FBS), 1% glutamine, 0.4 μg hydrocortisone, 10^{-10}M cholera toxin, 5 μg/ml transferrin, 2×10^{-11}M liothyronine, 5 μg/ml insulin, 10 ng/ml epidermal growth factor, 1× Penicillin/Streptomycin mixture] (Akgül et al., 2003). HaCaT cells (Boukamp et al., 1988) were grown in DMEM with 10% FBS. N/TERT cells (Dickson et al., 2000) were either cultivated in KGM-Gold (which contains low concentrations of calcium to prevent differentiation) or in RM+ media. All cell lines were cultivated at 37°C and 6% CO_2. For global demethylation of DNA, N/TERT cells were treated with 5-aza-2′-deoxycytidine (5-Aza) (Sigma–Aldrich, Steinheim, Germany) diluted in dimethyl sulfoxide (DMSO) with different final concentrations of 0 or 10 μM for 72 h with media renewal every 24 h. Differentiation of N/TERT cells grown in KGM-Gold media was accomplished by treatment with 2 mM $CaCl_2$ for up to 8 days with 24 h media renewal.

siRNA Transfection

Silencing of PI(4,5)P_2 generating kinase gene expression was achieved through transfection of siRNA-SMARTpools (Dharmacon, GE Healthcare, Freiburg, Germany) with the RNAiMAX kit (Thermo Scientific, Waltham, MA, United States) according to the manufacturer's protocol (siPIP4KIIα: M-006778-01; siPIP4KIIβ: M-006779-03; siPIP5KIα: M-004780-02; siPIP5KIγ: M-004782-00). These siRNAs have previously been successfully used to decrease kinase expression (Bultsma et al., 2010; Chao et al., 2010). The siRNA against Syntenin-2 had the following sequence: siSyn-2: GCAACGGGCTCCTCACCAATT. Cells transfected with a scramble siRNA (Cat. Nr.: 98129_A, Biospring, Frankfurt, Germany) were used as controls.

Western Blotting

For Western blot analysis, cells were trypsinized, pelleted by centrifugation and lysed on ice in LSDB buffer [50 mM Tris/HCl (pH 8.0), 20% Glycerol, 100 mM KCl, 0,1% NP40, 1 mM DTT, 50 mM NaF, 1 mM orthovanadate, 1 mM PMSF] supplemented with 1× Cocktail Protease Inhibitors (Roche Diagnostics, Mannheim, Germany). The resulting extracts were sonicated, and protein concentration was determined using the Biorad Protein Assay (Biorad, Munich, Germany). Cell extracts were resolved by SDS–PAGE and transferred to a nitrocellulose membrane. After the membrane had been blocked with 5% skimmed milk in TBST [10 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20] for 1 h, the blots were probed with antibodies against Syntenin-2 (rabbit polyclonal, Cat. no. 10407-1-AP, diluted 1:1500; Proteintech, Chicago, IL, United States), Loricrin (rabbit polyclonal, Cat. no. ab85679, diluted: 1:1000, Abcam, Cambridge, United Kingdom), PIP5K1α (rabbit polyclonal, Cat. no. PA5-29405, diluted 1:500, Thermo Scientific) and Tubulin (rat monoclonal, Cat. no. YL1/2, diluted 1:10,000; Abcam), which was used as loading control. Immunoreactive proteins were visualized using horseradish peroxidase-coupled IgG secondary antibodies (Dako, Hamburg, Germany) and the BM Chemiluminescence Blotting Substrate Detection System (Roche Diagnostics). The blots were exposed to autoradiographic films and signals were quantified using the ImageJ software.

Immunofluorescence Staining of Skin Samples

Anonymized EV skin lesions were obtained from routine surgical excisions. The use of the EV tissue for "studies on the mechanisms of HPV oncoprotein action in cutaneous oncogenesis" was approved by the local ethics-committee at the Medical University of Warsaw. HPV typing of these lesions was performed as described in Heuser et al. (2016). Four-micrometer formalin-fixed, paraffin-embedded sections were deparaffinized with xylene. Samples were hydrated through a descending alcohol series (100, 90, and 70%; 5 min each) and endogenous peroxidases were inactivated by incubation in 3% H_2O_2 in Methanol for 20 min. Antigen unmasking was performed by boiling the tissue sections in 10 mM citric acid buffer for 3 min in a beaker in a microwave followed by 15 min resting at RT. Further incubation steps were performed in a humid chamber to prevent drying-out. Blocking of unspecific antigen sites was achieved with 50% goat serum (Thermo Scientific) in PBS for 1 h at RT. Incubation with primary antibody against rabbit-anti-Syntenin-2 (ProteinTech) was done in a dilution of 1:250 in 2% goat serum over night at 4°C, followed by three washes with PBS. Sections of EV lesions were stained with rabbit anti-HPV8 E4 antibodies raised against a glutathione S-transferase (GST)-E1^E4 fusion protein (Borgogna et al., 2012). Detection of primary Syntenin-2 antibody was achieved by incubating the sections with a HRP-conjugated goat-anti-rabbit-IgG antibody (Dako, Santa Clara, CA, United States) diluted 1:2000 in 2% goat serum for 1 h at RT, followed by three washes with PBS. The fluorescence staining was performed with the "TSA™-Plus Fluorescein System" (PerkinElmer, Waltham, MA, United States). For E4 detection, sections were incubated with anti-rabbit secondary antibody conjugated with Alexa Fluor 488 (1:500 dilution in 2% FCS; Invitrogen) for 1 h at room temperature. The sections were counterstained with DAPI and embedded with Immumount (Thermo Fisher Scientific, St. Leon-Rot, Germany). Specific signals were visualized on the Leica DMI 6000B fluorescence microscope.

Quantitative Reverse Transcription-PCR (qRT-PCR)

RNA was isolated from cells using the RNeasy kit, and DNase digestion was performed on-column using RNase-free DNase according to the manufacturer's instructions (Qiagen, Hilden, Germany) (Lazic et al., 2011; Hufbauer et al., 2015). One microgram of total RNA was reverse transcribed using the Omniscript RT kit (Qiagen, Hilden, Germany) with 10 μM random nonamers (TIB MOLBIOL, Berlin, Germany) and 1 μM oligo(dT_{23}) primer (Sigma), as well as 10 units of RNase inhibitor (Thermo Fisher Scientific). Quantitative PCR (qPCR) was performed using the Light-Cycler system (Roche Diagnostics). The total copy number of target genes was normalized to the total copy number of the housekeeping gene encoding hypoxanthine phosphoribosyltransferase 1 (HPRT1). One PCR mixture contained 2 μl of 1:10 diluted cDNA in a total volume of 20 μl, 1.25 units Platinum *Taq* polymerase and the buffer provided in the kit (Invitrogen, Karlsruhe, Germany), 4 mM $MgCl_2$, 1.6 μl of a 1:1000 dilution of SYBR® Green (Sigma), 5% DMSO, 0.5 μM each forward and backward primer, 500 ng/μl non-acetylated bovine serum albumin (Thermo Fisher Scientific), and 0.2 mM deoxynucleotide triphosphate. To generate absolute standards, PCR fragments, amplified with primers also used for subsequent qPCR analysis, were cloned into pJET1.2 (Thermo Fisher Scientific). Samples were analyzed in duplicates together with a 10-fold dilution series of standard plasmid. The cycling protocol conditions were 60 s at 95°C, followed by 40 cycles of 1 s at 95°C (20°C/s), 5 s at annealing temperature (melting temperature of primer minus 5°C) (20°C/s), and 15 s at 72°C (20°C/s). The primers used in this study had the following 5′-to-3′ sequences:

Syntenin-2-fw: GTGGACGGGCAGAATGTTAT,
Syntenin-2-bw: ATGGAGATTCTGGCCACG;
HPRT1-fw: CCTAAGATGAGCGCAAGTTGAA,
HPRT1-bw: CCACAGGACTAGAACACCTGCTAA;
PIP4KIIα-fw: ATGGAATTAAGTGCCATGAAAAC,
PIP4KIIα-bw: GCATCATAATGAGTAAGGATGTCAAT;
PIP4KIIβ-fw: TGCATGTGGGAGAGGAGAGT,
PIP4KIIβ-bw: TCAGCTGTGCCAAGAACTCA;
PIP5KIα-fw: CCAACATAAAGAGGCGGAAT,
PIP5KIα-bw: AGGGTTCTGGTTGAGGTTCAT;
PIP5KIγ-fw: AAGGAGGCCGAGTTCCTG,
PIP5KIγ-bw: CGGGTTCTGGTTGAGGTTC;
HPV8-E6-fw: CCGCAACGTTTGAATTTAATG,
HPV8-E6-bw: ATTGAACGTCCTGTAGCTAATTCA.

Immunocytochemistry

Cells were seeded on coverslips and fixed in 4% paraformaldehyde for 10 min at RT and further permeabilized with 0.1% Triton X-100 for 5 min at RT. After blocking in 10% goat serum (diluted in PBS) for 30 min at RT, cells were incubated with primary antibodies against Syntenin-2 (ProteinTech) diluted 1:250 in 2% goat serum over night at 4°C. On the following day cells were incubated with corresponding fluorescently labeled secondary antibodies, counterstained with DAPI and embedded using Immumount (Thermo Fisher Scientific). Fluorescent signals were visualized on a Leica DMI 6000B fluorescence microscope.

Statistical Analysis

All qRT-PCR experiments were repeated a minimum of three times in duplicates. The results are expressed as mean ± SEM. Western blots were repeated in $n = 3$ independent experiments. The data presented as immunoblots or images of immunofluorescence analysis are from a representative experiment, which was qualitatively similar in the replicate experiments. Statistical significance was determined with unpaired 2-tailed Student's *t*-test. The asterisks shown in the figures indicate significant differences of experimental groups ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). Non-significant changes are labeled as "ns."

RESULTS

Papillomavirus Evolution Corresponds with Syntenin Gene Duplication

The PDZ protein family of Syntenins is comprised of two family members, Syntenin-1 and Syntenin-2. Retrieving Syntenin sequences from UniProt and constructing phylogenetic trees, we identified that both genes are the result of a gene duplication event that occurred during vertebrate evolution from a single ancestral Syntenin gene. Since the host range of papillomaviruses seems to be restricted to vertebrates (Rector and Van Ranst, 2013; Lopez-Bueno et al., 2016), the proposed timing of Syntenin gene duplication correlates with the evolution of the first papillomaviruses (**Figure 1A**). In addition, Syntenin-1 and Syntenin-2 show extensive structural similarity within the different vertebrate lineages (**Figure 1B**). Thus, Syntenin gene duplication may have contributed to both divergent and convergent evolution of Syntenin-1 and Syntenin-2 during vertebrate evolution.

Differentiation Dependent Syntenin-2 Expression Is Repressed by betaPV

To address the mechanisms through which HPV8-E6 regulates gene expression in a cell line model, Syntenin-2 protein levels were determined in different keratinocyte cell lines. Total cell extracts of the HPV-negative PM1, HaCaT and RTS3b cells were analyzed by Western blotting in addition to N/TERT cells grown either in RM+ ($N/TERT^{RM+}$) or in KGM-Gold ($N/TERT^{KGM}$) media. As shown in **Figure 2A**, PM1, HaCaT and RTS3b cells showed expression of Syntenin-2. However, compared to isogenic $N/TERT^{RM+}$ cells, Syntenin-2 was found to be downregulated in $N/TERT^{KGM}$. These data indicated that Syntenin-2 expression is culture media and differentiation dependent. To further confirm differentiation dependence, $N/TERT^{KGM}$ were exposed to high extracellular calcium concentrations (2 mM) for up to 8 days. Parallel to the induction of the differentiation marker loricrin, confirming the differentiation-inducing cell culture conditions, Syntenin-2

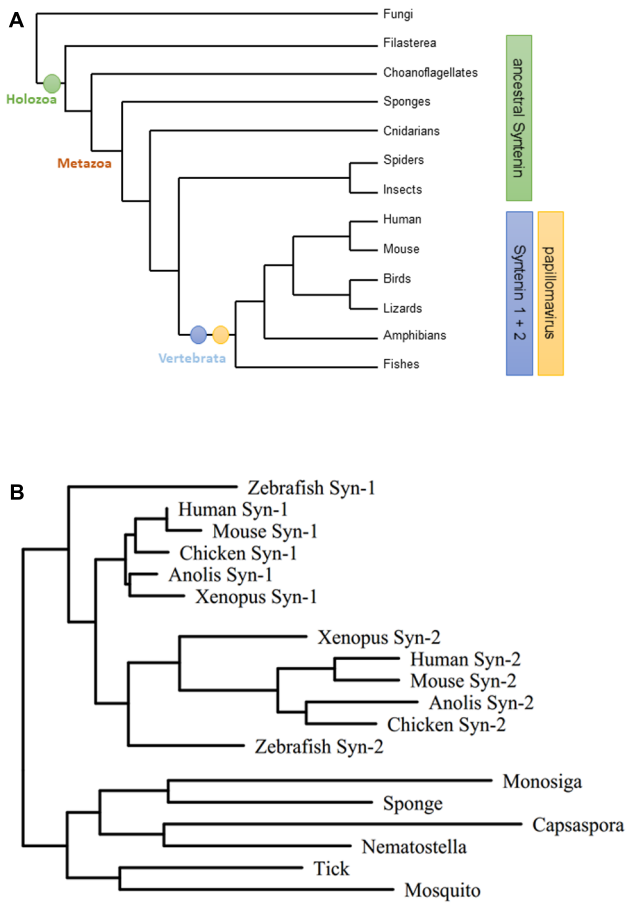

FIGURE 1 | Evolution of the Syntenin gene family. **(A)** Phylogram of major opisthokont lineages [tree simplified after (Torruella et al., 2015)]. Host range of papillomaviruses corresponds to Syntenin-1/Syntenin-2 evolutionary split. **(B)** Maximum likelihood tree for the Syntenin gene family. Representative Syntenin sequences were obtained from UniProt (The UniProt Consortium, 2017), aligned by the L-Ins-I algorithm of the MAFFT package (Katoh et al., 2002), and subjected to tree calculation by the T-Rex server (Boc et al., 2012) using the RAxML method (Stamatakis, 2006) (Syn-1: Syntenin-1; Syn-2: Syntenin-2).

was found to be upregulated in a calcium- and time-dependent manner (**Figure 2B**).

To further evaluate the expression pattern of Syntenin-2 in skin tumors induced by betaPV infection, we stained HPV8 positive skin tumors of EV patients. Staining against the viral E4 protein, which can have both a nuclear and cytoplasmic staining pattern in betaPV-induced tumors was used as surrogate marker for betaPV infection. To visualize E4, a broadly cross-reactive polyclonal antibody raised against the E4 protein of HPV5 and HPV8 was used (Borgogna et al., 2012). As demonstrated in **Figure 2C**, Syntenin-2 is expressed in the cytoplasm and nuclei of suprabasal keratinocytes in E4 negative EV skin and is absent in E4 positive lesional skin. Taken together, these findings implicated that Syntenin-2 expression is dependent on cell differentiation and is downregulated in skin with productive betaPV infection.

HPV8-E6 Represses Syntenin-2 Expression in Keratinocytes with a Basal Cell Phenotype

Given that HPV8-E6 mediated Syntenin-2 repression was previously identified in primary human keratinocytes (Lazic et al., 2012), we next tested whether E6 may also interfere with Syntenin-2 regulation in keratinocyte cell lines. As shown in **Figure 3A**, E6 mediated repression of Syntenin-2 transcription was only found to be significant in N/TERT^{KGM} and was not observed in HPV8-E6 positive N/TERT^{RM+}, RTS3b, HaCaT and PM1 cells. Immunocytochemical analysis revealed nuclear and cytoplasmic localization of Syntenin-2 in empty vector transduced N/TERT^{KGM} keratinocytes and a marked reduction of mainly cytoplasmic Syntenin-2 in HPV8-E6 positive cells (**Figure 3B**). To further elucidate the role of conserved amino acids in HPV8-E6 involved in regulation of Syntenin-2 expression, seven mutants of E6 were generated. To test whether repression of Syntenin-2 is a direct consequence of the previously described function of E6 to inhibit Mastermind-like protein 1 (MAML1) dependent differentiation processes (Brimer et al., 2012; Tan et al., 2012; Meyers et al., 2013), the mutants L61A, W63A and L61A/W63A were used. We could show in another study, that the E6 mutants L61A and W63A are still able to bind to MAML1 whereas the double mutant L61A/W63A is deficient in MAML1 binding (own unpublished data). Based on previously published data on HPV5-E6 mutants, the HPV8-E6 mutants V68A, D96A and D126A represent functional mutants, which are still able to inhibit UV-induced apoptosis but are incapable to induce proteasomal degradation of the pro-apoptotic protein Bak (Simmonds and Storey, 2008). The HPV8-E6-K136N mutant is, in contrast to wild type E6, deficient in inhibiting UV-induced DNA damage repair (Hufbauer et al., 2015). In line with wild type E6, the mutants L61A, W63A and the double mutant L61A/W63A (all positions are located within the first zinc-finger) were able to repress Syntenin-2 expression, whereas mutations located near the C-terminus (V68A, D96A, D126A, K136N) failed to inhibit Syntenin-2 expression (**Figures 3C,D**). This data provided evidence that E6 mediated downregulation of Syntenin-2, is a mechanism that is independent of E6 mediated blockade of MAML1 dependent differentiation processes.

Syntenin-2 Gene Repression in HPV8-E6 Cells Is Released by 5-Aza-2′-deoxycytidine

Hypermethylation of promoter regions can be involved in transcriptional regulation of cellular genes and E6 mediated promoter hypermethylation has previously been described for HPV16-E6 (D'Costa et al., 2012; Schütze et al., 2015; Yin et al., 2017). To test the hypothesis that Syntenin-2 promoter activity may also be methylation-dependent, the effect of 5-Aza-2′-deoxycytidine (5-Aza) mediated inhibition of DNA methylation on Syntenin-2 promoter regulation was measured in the presence and absence of HPV8-E6. E6 expression in cells used for these assays was confirmed by qRT-PCR (**Supplementary Figure S1**).

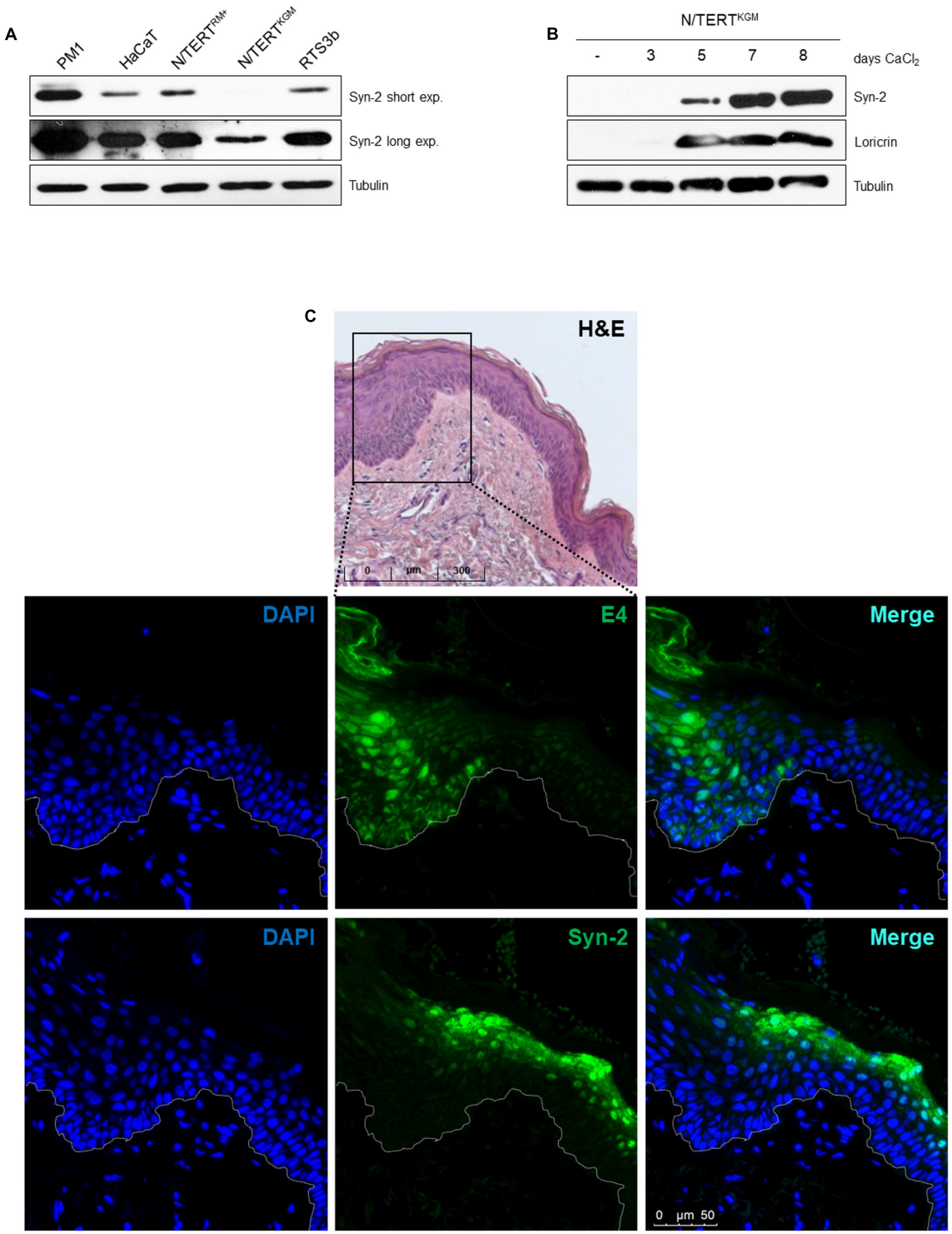

FIGURE 2 | Differentiation dependent expression of Syntenin-2 in skin keratinocytes. **(A)** Representative Western blot showing Syntenin-2 levels in total cell extracts of the keratinocyte cell lines PM1, HaCaT, RTS3b and isogenic N/TERT cells either cultured in RM+ (N/TERT^{RM+}) or in KGM-Gold (N/TERT^{KGM}). Equal loading was confirmed by immunoblotting for Tubulin. **(B)** Representative Western blot of Syntenin-2 levels in total cell extracts of N/TERT^{KGM} treated with 2 mM $CaCl_2$ for up to 8 days. Calcium induced keratinocyte differentiation was confirmed by Western blotting for Loricrin. Equal loading was confirmed by immunoblotting for Tubulin. **(C)** Representative immunofluorescence staining images detecting Syntenin-2 in EV-derived skin lesions. The skin area with productive betaPV infection was identified by anti-E4 staining. Nuclear counterstain was performed using DAPI. Syntenin-2 expression is present in suprabasal keratinocytes of non-lesional/E4-negative EV skin and absent in an E4-positive skin tumor. Top lane: H&E staining of the EV tissue analyzed.

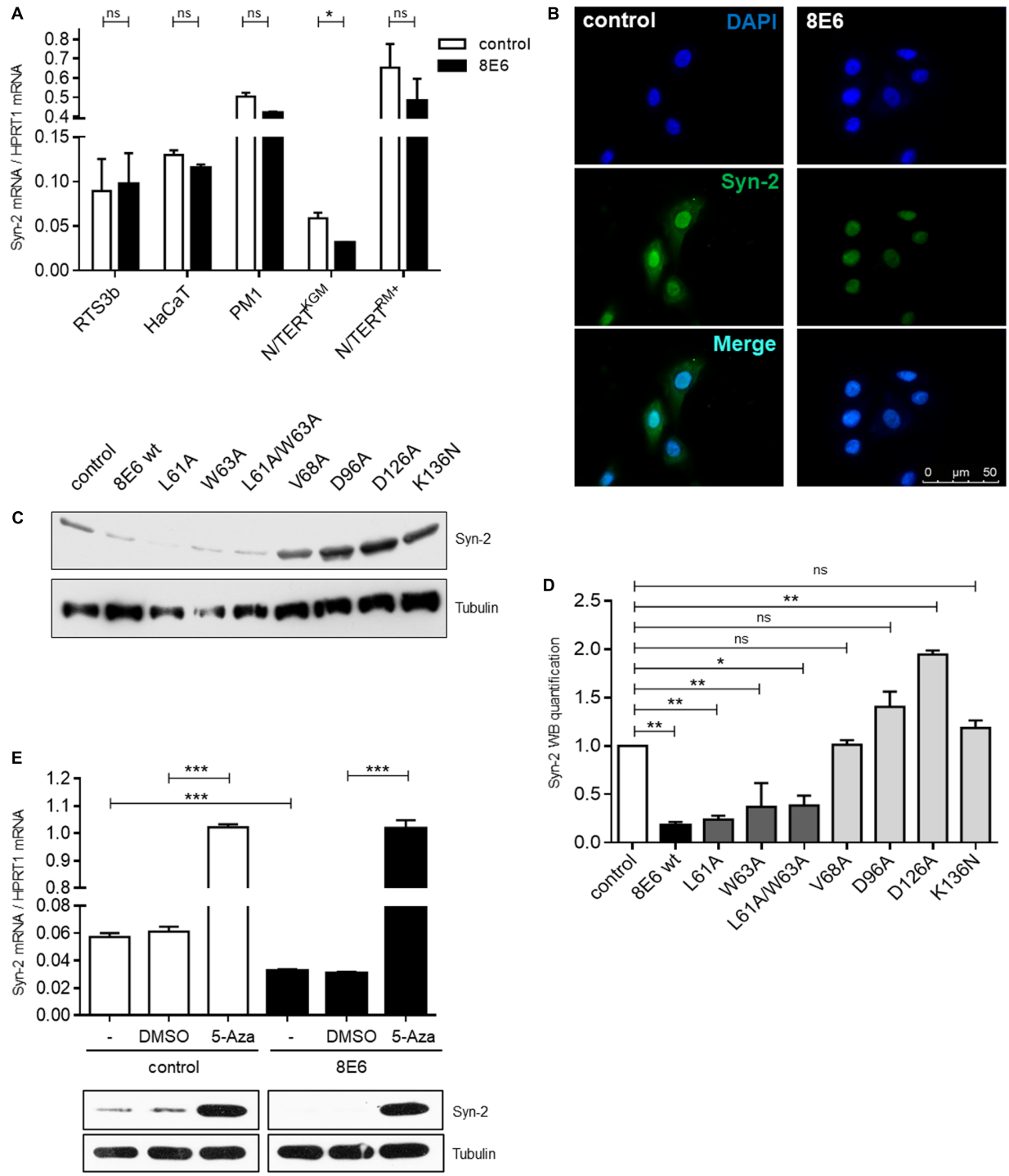

FIGURE 3 | Syntenin-2 expression is repressed by HPV8-E6 in keratinocytes with basal cell characteristics through promoter hypermethylation. **(A)** Quantification of Syntenin-2 mRNA expression by qRT-PCR in empty vector and HPV8-E6 expressing RTS3b, HaCaT, PM1 and isogenic N/TERT keratinocytes cultured either in RM+ or KGM. **(B)** Representative immunocytochemical staining of Syntenin-2 in N/TERT^{KGM}-control (Left) and N/TERT^{KGM}-8E6 (Right) demonstrating reduction of mainly cytoplasmic Syntenin-2 in HPV8-E6 positive cells (blue: DAPI; green: Syntenin-2). **(C)** Representative Western blot showing Syntenin-2 in total cell extracts of N/TERT^{KGM} expressing either HPV8-E6 wild type or the E6 mutants L61A, W63A, L61A/W63A, V68A, D96A, D126A or K136N. Loading was confirmed by immunoblotting for Tubulin. **(D)** Quantification of Syntenin-2 protein levels from **(C)** normalized to Tubulin protein levels. **(E)** Quantification of Syntenin-2 mRNA expression in N/TERT^{KGM}-control and N/TERT^{KGM}-8E6 cells after treatment with 10 µM 5-Aza or DMSO ($n = 3$ independent experiments, measured in duplicate, Upper). Data are presented as mean ± SEM (***$p < 0.001$). Western blot analysis of Syntenin-2 in extracts from cells treated with 5-Aza. Equal loading was confirmed by immunoblotting for Tubulin (Lower).

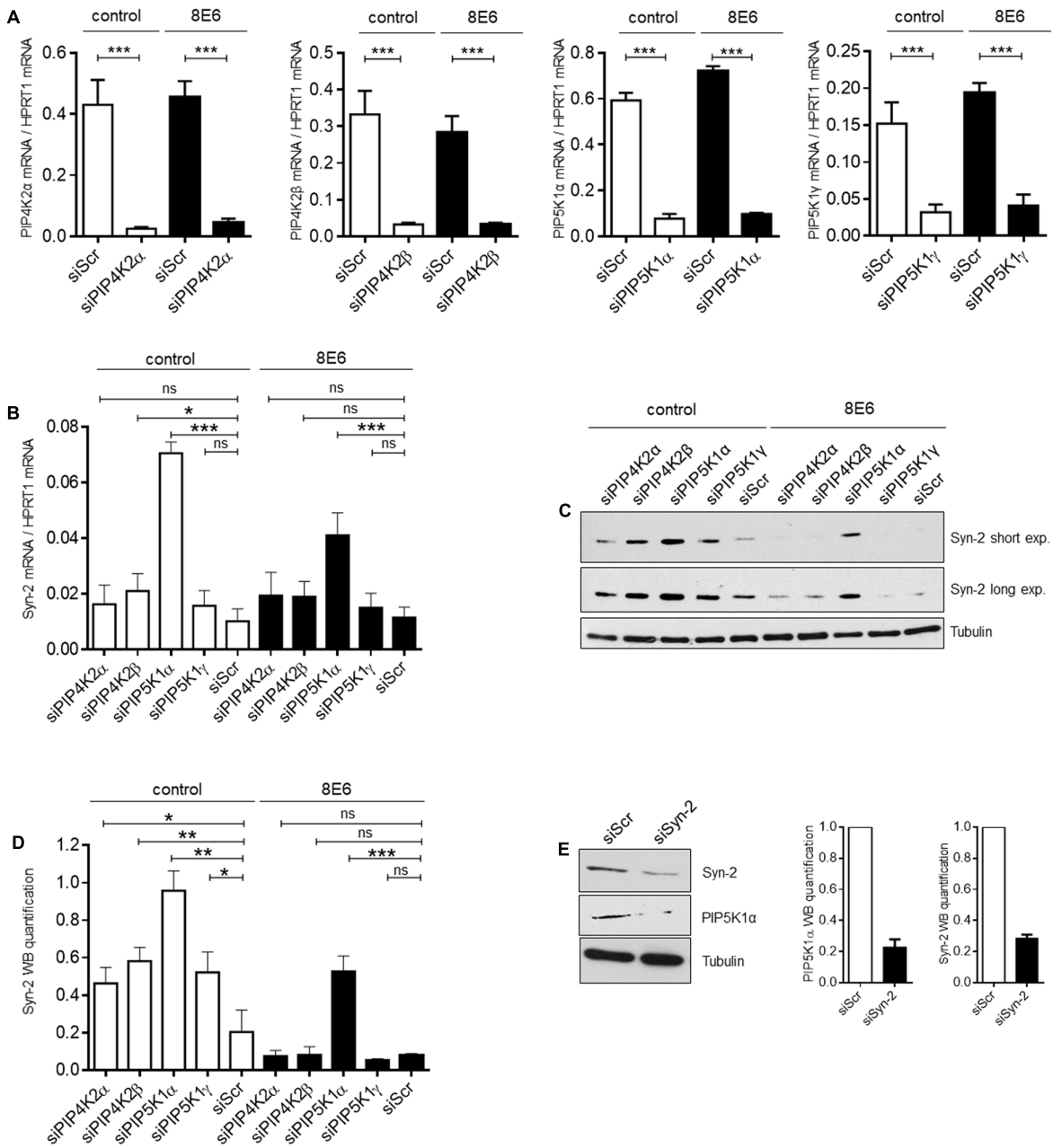

FIGURE 4 | PI(4,5)P_2 generating kinases are involved in control of Syntenin-2 expression. **(A)** Control (siScr), siPIP4KIIα, siPIP4KIIβ, siPIP5KIα or siPIP5KIγ transfected N/TERT^{KGM}-control and N/TERT^{KGM}-8E6 cells were harvested 48 h post transfection and mRNA expression of kinase isoforms was determined by qRT-PCR ($n = 3$ independent experiments, measured in duplicate). Data are presented as mean ± SEM (***$p < 0.001$). **(B)** Quantification of Syntenin-2 mRNA expression by qRT-PCR in N/TERT^{KGM}-control and N/TERT^{KGM}-8E6, in which the kinase isoforms PIP4KIIα, PIP4KIIβ, PIP5KIα and PIP5KIγ were silenced by siRNA transfection. **(C)** Representative Western blot showing Syntenin-2 expression in total cell extracts from N/TERT^{KGM}-control and N/TERT^{KGM}-8E6, in which kinase isoforms were silenced by siRNA transfection. Equal loading was confirmed by immunoblotting for Tubulin. **(D)** Quantification of Syntenin-2 protein levels in cells treated with siRNAs for specific kinase isoforms ($n = 3$ independent experiments). Data are presented as mean ± SEM. **(E)** Representative Western blot demonstrating downregulation of PIP5KIα protein levels in cells transfected with specific siRNA against Syntenin-2 (Left). Bars presenting the quantification of PIP5KIα and Syntenin-2 protein levels are shown in the Middle and Right ($n = 3$ independent experiments). Data are presented as mean ± SEM.

Syntenin-2 mRNA levels were low in DMSO treated cells but significantly increased following treatment with 5-Aza in control cells. This increase in mRNA was also mirrored on the protein level (**Figure 3E**). Syntenin-2 mRNA levels were lower in HPV8-E6 expressing cells compared to empty vector positive and DMSO treated keratinocytes. Syntenin-2 transcription levels were increased about 30-fold following 5-Aza treatment in E6 positive cells, restoring them to the same level that was detected in

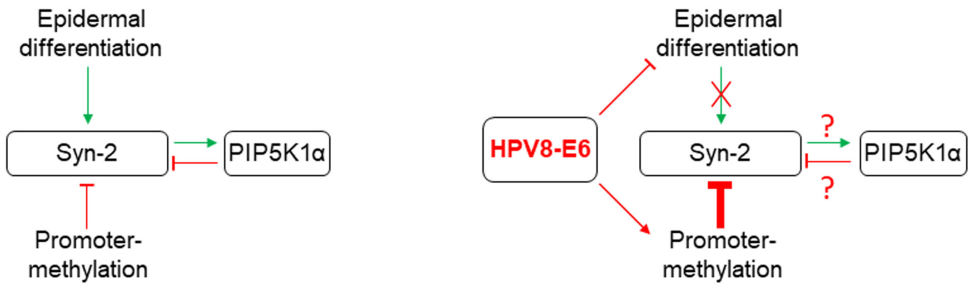

FIGURE 5 | Graphical summary of the regulatory pathways identified in this study controlling Syntenin-2 expression in normal keratinocytes (Left). Known HPV8-E6 mediated interference with these mechanisms is shown on the Right.

5-Aza treated control cells (**Figure 3E**). This complete restoration supports hyper-methylation dependent regulation of Syntenin-2 expression by HPV8-E6.

Phosphatidylinositol Metabolic Pathway Controls Syntenin-2 Gene Expression

It has been shown by Mortier et al. (2005), that nuclear $PI(4,5)P_2$ is required for the sub-nuclear enrichment of Syntenin-2. $PI(4,5)P_2$ generating kinases are present in the nucleus and have an impact on gene expression by contributing to chromatin unfolding and transcriptional regulation (Delage et al., 2013). In principle, $PI(4,5)P_2$ is synthesized by either the phosphorylation of phosphatidylinositol-4-phosphate (PI4P) by the lipid kinase phosphatidylinositol-4-phosphate-5-kinase type I (PIP5KI) or by the phosphorylation of phosphatidylinositol-5-phosphate (PI5P) through phosphatidylinositol-5-phosphate-4-kinase type II (PIP4KII) (van den Bout and Divecha, 2009). Each of these kinases comprises three isoforms, given the designations α, β and γ. To evaluate the functional correlation between the $PI(4,5)P_2$ pathway and Syntenin-2 gene expression, we next analyzed the impact of $PI(4,5)P_2$ generating kinases on Syntenin-2 expression through silencing of the single kinase isoforms. The kinase isoform PIP4KIIγ is not a nuclear protein and was therefore not studied (Itoh et al., 1998; Clarke et al., 2008) in addition to PIP5KIβ, which is not expressed in $N/TERT^{KGM}$ (data not shown). A strong knockdown of the kinase isoforms could be achieved for all four tested enzymes using kinase specific siRNA pools in E6 negative and positive cells (**Figure 4A**). The expression of HPV8-E6 in these cells was confirmed by qRT-PCR and was not significantly changed in all siRNA treated cells (**Supplementary Figure S2**). Knockdown of PIP4KIIβ and particularly PIP5KIα led to a significant upregulation of Syntenin-2 mRNA expression in wild type keratinocytes. Interestingly, HPV8-E6 was able to counteract siPIP4KIIβ and siPIP5KIα mediated re-activation of Syntenin-2 transcription (**Figure 4B**). On protein level, kinase silencing led to the induction of Syntenin-2 expression in siPIP4KIIα, siPIP4KIIβ, siPIP5KIα and siPIP5KIγ transfected cells with siPIP5KIα showing the strongest effect in empty vector positive $N/TERT^{KGM}$ cells. HPV8-E6 blocked Syntenin-2 protein re-expression in siPIP4KIIα, siPIP4KIIβ and siPIP5KIγ treated cells and counteracted siPIP5KIα-mediated re-expression of Syntenin-2 by 50% (**Figures 4C,D**). To further determine whether, on the other hand, Syntenin-2 is a regulator of PIP5K1α expression, we also measured protein levels in keratinocytes treated with Syntenin-2 specific siRNA. Unexpectedly, knockdown of Syntenin-2 led to a strong downregulation of PIP5K1α protein expression (**Figure 4E**).

DISCUSSION

We recently identified Syntenin-2 as the first PDZ protein to be targeted by HPV8-E6 on transcriptional level in primary keratinocytes cultured in low calcium media (Lazic et al., 2012). In this study we elucidated the mechanisms controlling Syntenin-2 gene expression. We now could show that Syntenin-2 is expressed in different keratinocyte cell lines cultured in RM+ media. It was, however, significantly downregulated in N/TERT cells grown in KGM media, under cell culture conditions in which keratinocytes do not differentiate and phenotypically resemble basal epidermal cells. Using $N/TERT^{KGM}$ keratinocytes as a cell line model, we demonstrate that HPV8-E6 is capable of suppressing Syntenin-2 expression only in these keratinocytes. We previously demonstrated, that Syntenin-2 is absent in three-dimensional organotypic skin cultures of HPV8-E6 positive keratinocytes, which show loss of normal stratification and the absence of stratum corneum formation. In addition, downregulation of Syntenin-2 through shRNA expression inhibited differentiation of normal keratinocytes in skin cultures (Lazic et al., 2012). The interference of E6 with normal keratinocyte differentiation represents an oncogenic mechanism that has recently been linked to the ability of HPV8-E6 to bind to MAML1 and thus blocks NOTCH-dependent differentiation regulation (Brimer et al., 2012; Tan et al., 2012; Meyers et al., 2013). HPV8-E6 is also known to prevent pro-Caspase-14 cleavage, which is involved in the regulation of late terminal differentiation of keratinocytes (Kazem et al., 2011). We now show, that Syntenin-2 expression is absent in suprabasal cell layers of HPV8 positive EV skin tumors. The fact that the L61A/W63A mutant, which is not able to bind to MAML1 still represses Syntenin-2 indicated that Syntenin-2 downregulation is not linked to E6-mediated inhibition of MAML1/NOTCH-dependent differentiation. This

led to the conclusion that reduction of Syntenin-2 by E6 may contribute to betaPV-mediated alteration of keratinocyte differentiation independent of MAML1 inhibition.

Another key observation is, that the Syntenin-2 promoter is regulated by DNA methylation. The observation, that Syntenin-2 mRNA and protein levels could completely be restored by 5-Aza treatment in HPV8-E6 expressing cells, led us to the conclusion, that E6 mediated suppression of Syntenin-2 is mediated through promoter hypermethylation. The exact mechanism how HPV8-E6 induces hypermethylation of cellular gene promoters and how the C-terminal part of E6 contributes to this needs still to be investigated.

Syntenin-2 is known to bind nuclear $PI(4,5)P_2$ with high affinity (Mortier et al., 2005). Phosphatidylinositides within the nucleus appear to influence many steps of transcriptional regulation, including reading and writing of the histone code, polymerase-dependent transcription, splicing and polyadenylation, and finally mRNA export (Lewis et al., 2011; Shah et al., 2013). In addition to our observation that differentiation and methylation pathways are involved in Syntenin-2 regulation, we also provide evidence, that Syntenin-2 is a target of the $PI(4,5)P_2$ metabolic pathway (**Figure 5**). Particularly, the downregulation of PIP5KIα resulted in re-expression of Syntenin-2 and this specific Syntenin-2 upregulation could only partially be counteracted by E6. Whether shortage of $PI(4,5)P_2$, accumulation of PI4P or PIP5KIα functions during keratinocyte differentiation (Xie et al., 2009; Shrestha et al., 2016) contribute to transcriptional repression of Syntenin-2 remains unclear. In addition, the observation that Syntenin-2 silencing using siRNAs resulted in inhibition of PIP5KIα protein expression points to a negative feedback loop between these two factors (**Figure 5**).

Known interaction partners of Syntenin-1 do not interact with Syntenin-2, which may imply that they are functionally not redundant (Koroll et al., 2001). Both Syntenins have evolved by gene duplication from an ancestral Syntenin gene during vertebrate evolution. Gene duplication has been linked to many aspects of genome evolution and is a potent way to create new biological functions (Wagner et al., 2007), which may also account for Syntenin proteins. Syntenin-1 is predominantly located in cell – cell adherens junctions and cytoplasm and is involved in diverse functions including human tumorigenesis (Beekman and Coffer, 2008; Friand et al., 2015; Philley et al., 2016). Interestingly, Syntenin-1 has been linked with the endocytic pathway controlling HPV entry into the host cell, controlling the delivery of internalized viral particles to endosomes and viral capsid disassembly in a CD63-Syntenin-1-ALIX dependent manner (Grassel et al., 2016). Although human Syntenin-2 is highly related to Syntenin-1 in its overall domain organization and sequence, both proteins show higher similarities with the homolog protein of other vertebrate species than to the variants expressed in an individual organism. It is tempting to speculate that during evolution Syntenin-1 may have provided the platform for virus-host specification of an ancestral papillomavirus as a key event in the evolution determinant of papillomaviruses (Gottschling et al., 2011; Van Doorslaer, 2013). However, at the same time, papillomaviruses may need to downregulate Syntenin-2 through E6 to achieve tissue dedifferentiation to enable efficient viral propagation.

CONCLUSION

We have advanced the understanding of Syntenin-2 gene regulation in normal cells and how HPV8-E6 can interfere with these mechanisms. Elucidation of the Syntenin-2 regulated pathways will provide further knowledge on its role in regulating keratinocyte differentiation.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: BM and BA. Performed the experiments: BM, DM-L, KH, and MH. Analyzed the data: BM, DM-L, MH, and BA. Approved the final version of the manuscript: BM, DM-L, KH, SM, JD, MH, and BA. Contributed reagents/materials: SM and JD. Wrote the paper: BA.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01724/full#supplementary-material

FIGURE S1 | Measurement of HPV8-E6 mRNA levels by qRT-PCR in N/TERT^{KGM}-control and N/TERT^{KGM}-8E6 cells treated with DMSO or 5-Aza. Total E6 mRNA was normalized to HPRT1 mRNA levels.

FIGURE S2 | Measurement of HPV8-E6 mRNA levels by qRT-PCR in N/TERT^{KGM}-control and N/TERT^{KGM}-8E6 cells transfected with a Scramble siRNA (siScr) or with siRNAs directed against PIP4KIIα, PIP4KIIβ, PIP5KIα or PIP5KIγ. Total E6 mRNA was normalized to HPRT1 mRNA levels.

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frontiers in Microbiology

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Induction of Tyrosine Phosphorylation of UV-Activated EGFR by the Beta-Human Papillomavirus Type 8 E6 Leads to Papillomatosis

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Epidemiological evidence is accumulating that beta-human papillomaviruses (HPV) synergize with UV-light in the development of precancerous actinic keratosis, and cutaneous squamous cell carcinomas (cSCC), one of the most common cancers in the Caucasian population. We previously demonstrated the tumorigenic activity of beta-HPV type 8 (HPV8) in the skin of transgenic mice and its cooperation with UV-light. Analysis of underlying mechanisms now showed that in keratinocytes expressing the HPV8E6 protein a transient increase of tyrosine phosphorylated epidermal growth factor receptor (EGFR) in response to UV-irradiation occurred, while EGFR tyrosine phosphorylation, i.e., receptor tyrosine kinase (RTK)-activity was hardly affected in empty vector control cells. FACS and immunofluorescences revealed that the EGFR was internalized into early endosomes in response to UV-exposure in both, HPV8E6 positive and in control cells, yet with a higher rate in the presence of HPV8E6. Moreover, only in HPV8E6 expressing keratinocytes the EGFR was further sorted into CD63+ intraluminal vesicles, indicative for trafficking to late endosomes. The latter requires the ubiquitination of the EGFR, and in correlation, we could show that only in HPV8E6 positive keratinocytes the EGFR was ubiquitinated upon UV-exposure. HPV8E6 and tyrosine phosphorylated EGFR directly interacted which was enhanced by UV-irradiation. The treatment of K14-HPV8E6 transgenic mice with Canertinib, an inhibitor of the RTK-activity of the EGFR, suppressed skin papilloma growth in response to UV-irradiation. This confirms the crucial role of the RTK-activity of the EGFR in HPV8E6 and UV-mediated papillomatosis in transgenic mice. Taken together, our results demonstrate that HPV8E6 alters the signaling of the UV-activated EGFR and this is a critical step in papilloma formation in response to UV-light in transgenic mice. Our results provide a molecular basis how a beta-HPV type may support early steps of skin tumor formation in cooperation with UV-light.

Keywords: beta-HPV8, E6 oncoprotein, cutaneous squamous cell carcinomas, UV-light, papillomatosis, EGFR signaling and trafficking

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INTRODUCTION

The cutaneous squamous cell carcinoma (cSCC) is one of the most prevalent skin tumors in the Caucasian population and particularly frequent after organ transplantation. Since cSCC is a cancer of the elderly population with a mean age of 75 years it is estimated that its incidence will augment in the future due to the increased life expectation (Hillen et al., 2014). The precancerous actinic (solar) keratosis (AK) is an intraepidermal squamous neoplasm of sun-damaged skin. The transition to invasive cSCC is reported in 5–10% and with immunosuppression in 30% of patients. On the other side, 80% of the cSCC have AK with a variable grade of dysplasia in their periphery. Immunosuppressed organ transplant recipients (OTR) have a 100-fold increased risk to develop an invasive cSCC and 250-fold increased risk for AK (Lomas et al., 2012). Most AK and cSCC appear on sun exposed skin areas, which clearly points to the etiologic role of UV-light as initiator and tumor promoter of AK and cSCC. This relies on DNA damage including inactivating mutations of p53, and on effects of UV-light on signal transduction pathways that regulate cell proliferation and survival such as the signaling by the epidermal growth factor receptor (EGFR).

Evidence is accumulating that human papillomaviruses (HPV) belonging to the genus beta (β-HPV) synergize with UV-light in the development of cSCC. This was initially recognized in patients suffering from the rare disease Epidermodysplasia verruciformis (EV). CSCC in EV-patients harbor multiple, extrachromosomal genome copies of specific β-HPV types, especially HPV5 or HPV8 (reviewed in Howley and Pfister, 2015). β-HPVs are wide spread in humans but cause no apparent lesions in the skin of the general population. These viruses replicate in the hair follicles and are regarded as part of the commensal skin flora. Iatrogenic immunosuppression in OTR allows a more active replication of the commensal β-HPV spectrum in the entire skin with the result of higher viral load (Weissenborn et al., 2012). A high β-HPV DNA load in plucked eyebrow hairs was shown to imply a significant risk for the development of cSCC (Neale et al., 2013). The viral load of β-HPV in cSCC of the general population is lower compared to EV with usually less than 1 viral genome per cell (Weissenborn et al., 2005) and no viral transcripts have been detected (Arron et al., 2011). Though, the DNA loads of β-HPVs are higher in the precancerous AK (Weissenborn et al., 2005) and active viral replication and gene expression could be demonstrated (Borgogna et al., 2014). These findings are consistent with a role of β-HPV at early steps in non-melanoma skin cancer development by acting as a co-factor that enhances the carcinogenic potential of UV light.

The tumorigenic activity of β-HPV was demonstrated in transgenic mouse studies. We generated K14-HPV8CER transgenic mice that express the complete early region (CER) of HPV8 in the basal layer of their epidermis. Nearly all K14-HPV8CER transgenic mice spontaneously developed papillomas with varying degrees of epidermal dysplasia within 1 year. CSCC appeared in 6% of the animals (Schaper et al., 2005). After a single UV-irradiation or mechanical wounding, benign skin tumors grew within 3 weeks in all transgenic animals, which was consistently preceded by an enhanced expression of the viral oncogenes (Marcuzzi et al., 2009; Hufbauer et al., 2010). K14-HPV8E6 transgenic animals expressing only the E6 oncoprotein had the same phenotype as the K14-HPV8CER mice, indicating that HPV8E6 is the major oncogene in the murine skin (Marcuzzi et al., 2009). Transgene expression turned out to be crucial for tumor development since siRNA-mediated suppression of HPV8E6 reduced the papilloma incidence in these mice (Hufbauer et al., 2010). Thus, in this mouse model the HPV8 early genes cooperate with UV-light in the induction of skin tumors and the HPV8E6 oncogene is sufficient for this.

UV-irradiation transiently activates the EGFR which leads to increased proliferation, suppression of cell death and hyperplasia (El-Abaseri et al., 2006). Studies on mouse models deciphered the importance of EGFR signaling and downstream targets in the UV-induced skin tumorigenesis (Wan et al., 2001; El-Abaseri et al., 2005) and normal skin homeostasis (reviewed in Schneider et al., 2008). Ligand-, such as EGF, dependent activation initiates EGFR dimerization, which activates the receptor tyrosine kinase (RTK) resulting in the auto-phosphorylation of intracellular localized tyrosine residues of the EGFR. These phospho-tyrosines serve as binding motifs for a number of effectors which initiate the activation of downstream signaling pathways including the phosphatidylinositol 3 kinase (PI3K)/AKT, the Ras/mitogen-activated protein kinase (MAPK) pathways and Rac1 (Zhu et al., 2015) to promote cell survival and proliferation (reviewed in Lemmon and Schlessinger, 2010). Full activation of the EGFR as well as termination of the signaling depends on receptor endocytosis and intracellular trafficking. EGF-activated EGFR is rapidly internalized into early endosomes. Further downstream processing to late endosomes/lysosomes depends on the ubiquitination of the EGFR, which is initiated by binding of the E3 ubiquitin ligase Cbl to phospho-tyrosine residues of the EGFR. Ubiquitination is a prerequisite for the recognition by the endosomal sorting complex required for transport (ESCRT) that drives EGFR sorting from early endosomes via intraluminal vesicles (ILVs) to late endosomes and lysosomes leading to the proteasomal degradation. Dysregulation of this sorting process contributes to oncogenesis (reviewed in Tomas et al., 2014). Many intrinsic and iatrogenic cellular stresses such as UV-light, the cytokine TNFα or the cancer therapeutic cisplatin activate the EGFR in a tyrosine-kinase-independent way. UV-irradiation triggers a rapid clathrin-mediated internalization of the EGFR, which is ligand and RTK-activity independent and results in the accumulation of the UV-activated EGFR within the endosomal compartment without degradation (Zwang and Yarden, 2006).

Here, we addressed the effects of HPV8E6 on the UV-activated EGFR signaling and the implications for HPV8E6 mediated skin tumor formation in transgenic mice.

MATERIALS AND METHODS

Mice

The K14-HPV8E6 mice were in the FVB/N background and express the HPV8E6 oncogene under control of the keratin 14 promoter (Marcuzzi et al., 2009). All mice were irradiated with UV as described previously at a dose of 1 J/cm^{-2} for UVB

and 10 J/cm^{-2} for UVA (Marcuzzi et al., 2009). UV-radiation was generated by a UV device (UV 801; Waldmann). Prior to UV-exposure, mice were anesthetized and their back area was shaved with an electric shaver. A 2 cm^2 area was irradiated while the rest of the skin was covered with a UV-impermeable sheet. The Canertinib group was administered once 20 mg/kg Canertinib (CI-1033, Selleckchem, S1019) solved in 150 µl PBS by gavage and the control group obtained 150 µl PBS 4 h prior UV-irradiation. After UV, the Canertinib group was treated with 10 mg/kg Canertinib solved in 150 µl PBS and the control group with 150 µl PBS daily for 24 days, respectively.

Cell Culture

Normal human epithelial keratinocytes (NHEK) were obtained from PromoCell and were cultivated in KGM2 supplemented with growth factors and penicillin and streptomycin (S/P). HaCaT (provided by Professor N. Fusenig, German Cancer Research Center, Heidelberg, Germany) and C33A cells (from ATCC) were grown in DMEM. The immortalized keratinocyte cell line RTS3b [obtained from I. Leigh (Purdie et al., 1993)] and N/TERT (obtained from the ATCC) were cultivated in E-Medium. DMEM and E-medium were supplemented with 10% FCS and S/P. Transduction with pLXSN or pLXSN-HPV8E6 and selection by G418 was performed as previously protocol (Leverrier et al., 2007). RTS3b cells were transiently transfected with the X-treme gene reagent from Roche and C33A cells by the $CaCl_2$ precipitate method. UV irradiation was performed at a dosage of 30 or 40 mJ/cm^2 with UVB light using a UVP CL-1000 ultra-violet cross-linker with F8T5 bulbs giving a spectral peak at 320 nm. Cells were grown in media containing Canertinib, (4 or 7 µM), AG478 (10 µM) (Selleckchem) or vehicle for 2 h before UV-irradiation.

Plasmids

The expression vectors pLXSN, pLXSN-HPV8E6, and pXJ41-FLAG-HPV8E6 have been described previously (Müller-Schiffmann et al., 2006; Leverrier et al., 2007). The expression vector for EGFR-GFP (Addgene Plasmid 32751) was a gift from Alexander Sorkin (Carter and Sorkin, 1998).

Antibodies

The antibodies against the EGFR (D38B1) uncoupled or coupled to sepharose beads or to Alexa Flour (AF) 488, the phospho-EGFR-Y1068 (D7A5) and -Y1045 (#2237), -T669 (#8808) and the K48-polyubiquitin (12805), as well as the rabbit IgG isotype control (DA1E) were from Cell Signaling. The anti-EEA1 (BD Clone 14) and CD63 (BD clone H5C6) were purchased from BD Biosciences and M2-FLAG-affinity Gel and antibodies from Sigma and the GFP-tag antibody from Thermo-Scientific (A-11122). The guinea pig polyclonal antibody against HPV8E6 was kindly provided by Janet L. Brandsma and is described in Hufbauer et al. (2010).

Western Blots and Co-IP

Cells were washed once in ice cold PBS and lyzed in low salt lysis buffer (20% glycerol, 50 mM Tris pH 7.9, 150 mM NaCl, 0.1% Nonidet-P40, 1 mM DTT) supplemented with protease inhibitors PMSF, aprotinin, leupeptin, and pepstatin and the phosphatase inhibitors NaF and Na-orthovanadate on ice for 15 min followed by sonification. IPs, blotting, and WB were done according to standard procedures. Blots were either developed with the use of X-ray films and scanned for digitalization or with the ChemiDoc XRS System (Bio-Rad, Dreieich, Germany), further processed with Adobe Photoshop CS6 (Adobe Systems Inc., Dublin, Ireland). Quantification was done with ImageJ software.

Immunostainings and FACS-Analysis

Skin lesions were excised from transgenic mice, fixed in 4% paraformaldehyde and processed for paraffin embedding. Four micrometers thick sections were mounted and processed. IHC staining was performed with the Vectastain Universal Elite ABC Kit (Vector Laboratories, Peterborough, United Kingdom). Paraffin sections were stained for hematoxylin/eosin (H/E) and Giemsa following standard protocols. Sections were analyzed with the Leica DM4000B light microscope equipped with a KY-F75U digital camera (JVC) and Diskus 4.50 Software. For IFTs, cells were grown on coverslips, fixed in 4% paraformaldehyde for 15 min, washed with PBS three times and blocked for 60 min in PBS/10% horse serum/0.3% Triton-X-100, followed by incubation with the antibodies against EEA1 or CD63 (both diluted 1:200 in PBS, 5% horse serum, 0.15% Triton-X-100) for 2 h at rt. After three PBS washes for 5 min, the anti-mouse-IgG-Rhodamine (Santa Cruz), (1:100) and the anti-EGFR-AF488 (Cell Signaling, 1:150) antibodies were mixed and incubated for 2 h, followed by three PBS wash steps with DAPI being added to the last PBS wash. Coverslips were mounted on object plates and analyzed by a DMI6000B microscope equipped with a fluorescence Leica DFC365FX camera. The overlays were processed by the Leica LASX software. Pictures were further adapted with Adobe Photoshop CS6 (Adobe Systems Inc., Dublin, Ireland). Cells for flow cytometry were trypsinized, washed in PBS and fixed in 4% paraformaldehyde for 10 min at 37°C followed by 1 min on ice. After two washing steps with PBS/0.5% BSA, the cells were incubated with the anti-EGFR antibody (1:100) or the rabbit isotype control, both coupled to AF488 for 1 h at rt. After two times washing, the cells were resuspended in PBS and analyzed by FACS in a MACS Quant analyzer (Miltenyi, Germany) and the FlowJo software.

RNA Processing

Skin samples were stored at −20°C in RNALater until RNA was isolated with the RNeasy kit (Qiagen, Hilden, Germany). One microgram of RNA was reverse transcribed using the GoTaq-RT-PCR System (Promega). Quantitative PCR were performed with the Go-Taq qPCR-Sybr-Green System (Promega) and a LightCycler480 (Roche).

Ethics Statement

The generation of the transgenic mice and the UV irradiation protocols were approved by the governmental animal care office North-Rhine-Westphalia (protocol no. 84-02.04.2014.A087) and were in accordance with the German Animal Welfare Act as well

as the German Regulation for the protection of animals used for experimental purposes.

RESULTS

The Expression of HPV8E6 Leads to Increased Tyrosine Phosphorylation of the EGFR in Response to UV-Light in Keratinocytes

To decipher the effects of UV and HPV8E6 on the activation of the EGFR we used keratinocytes expressing the HPV8E6 protein under control of the rather weak long terminal repeat (LTR) promoter of pLXSN-retroviruses to match the low level of the E6 protein as observed during natural HPV infections (Wallace et al., 2014). The expression of HPV8E6 in the stably transduced immortalized keratinocyte lines HaCat, RTS3b, and N/TERT, and in primary NHEK was confirmed by qRT-PCR (data not shown). All types of keratinocytes were kept 24 h under low serum conditions (i.e., 0.2% FBS), followed by UV-irradiation (30 mJ/cm^2) and a recovery period of 30 min prior to be harvested. The stimulation of the RTK-activity in the UV-treated cells was analyzed by detecting the phosphorylation of the EGFR at tyrosine at pos. 1068 (pEGFR-Y1068) by WB. All unirradiated keratinocytes, except HaCat cells, had low, but detectable level pEGFR-Y1068 (**Figure 1A**, lanes 1, 5, and 13). These were hardly affected by UV-irradiation. In HaCat cells, pEGFR-Y1068 was only detectable after UV-irradiation. Importantly, in all four tested keratinocytes the signals corresponding to pEGFR-Y1068 were stronger in the presence of HPV8E6 compared those of empty vector cells 30 min after UV-irradiation (**Figure 1A**, lanes 4, 8, 12, and 16). The quantifications of the ratio of total EGFR versus pEGFR-Y1068 revealed that in the presence of HPV8E6 UV-exposure enhanced the RTK-activity of the EGFR, which ranged from 2.5-fold (in HaCat cells) to more than 4-fold (in RTS3b, N/TERT and primary keratinocytes). This implies that HPV8E6 increases the RTK-activity of the EGFR in response to UV-irradiation. We consistently observed a slight shift in the migration of the EGFR after UV-exposure (**Figure 1A**, compare lanes 1 and 2, 5 and 6, 13 and 14). This may be attributed to the UV-dependent phosphorylations of S or T residues which included the threonine at pos. 669 (T669). These phosphorylations have been found to be required for the internalization of the UV-exposed EGFR (Zwang and Yarden, 2006). The UV-light induced phosphorylation of the EGFR at T669 was confirmed in all four types of keratinocytes we investigated here (**Figure 1A**).

To address the effect of HPV8E6 on the tyrosine phosphorylation of the EGFR in more detail, we performed a kinetic. In empty vector control NHEK the level of pEGFR-Y1068 was hardly affected by UV-light within 60 min following the treatment. We regularly observed an enhanced tyrosine phosphorylation 90 or 120 min after UV-exposure in empty vector cells, which may be triggered by UV-induced p38MAPK activity, as suggested previously (Tomas et al., 2015). In HPV8E6 expressing primary keratinocytes, UV-irradiation led to a marked increase of the level of pEGFR-Y1068 30 min and 45 min after UV-treatment. Then, the amounts declined and finally returned to background level (**Figure 1B**, upper part). A similar

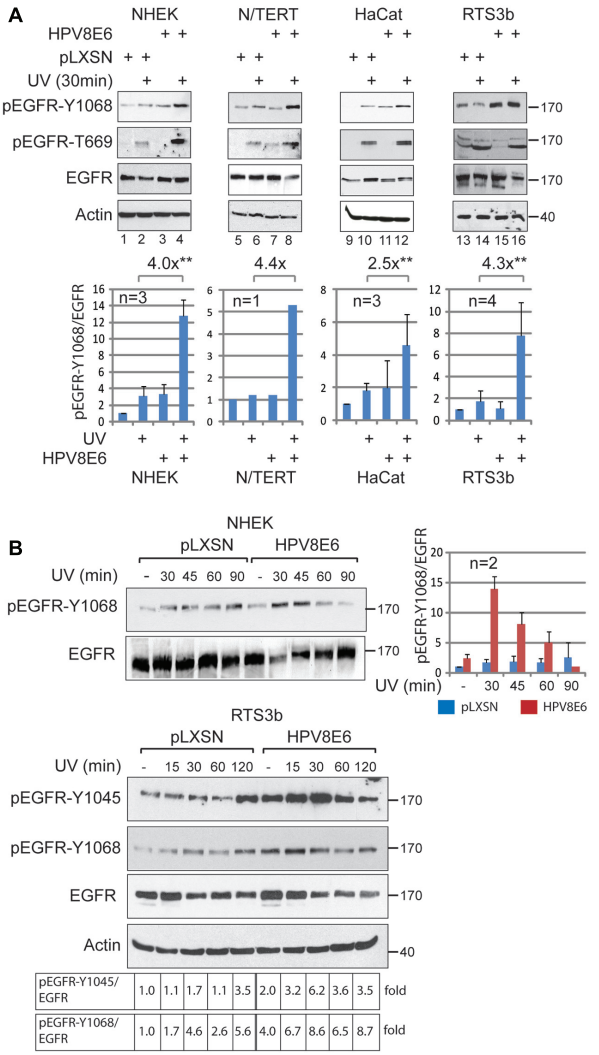

FIGURE 1 | HPV8E6 increases the rate of tyrosine-phosphorylation of the EGFR after UV-irradiation. **(A)** Normal human epithelial keratinocytes (NHEK), N/TERT, HaCat and RTS3b cells, harboring either pLXSN or pLXSN-HPV8E6, respectively, were left untreated or irradiated with UV light and further cultivated for 30 min. Protein extracts were used for a WB developed with antibodies as indicated in the figure. Shown is one representative blot for each cell line. The signals were quantitated and the average ratio of pEGFR-Y1068 versus total EGFR obtained with three to four independent experiments, as indicated, were given in the graph for NHEK, RTS3b, and HaCat cells. The fold changes of the pEGFR-Y1068 versus total EGFR in UV-irradiated cell due to the expression of HPV8E6 are given. The ratios with N/TERTS are from one experiment (**$p < 0.05$). **(B)** PLXSN-HPV8E6 or empty vector harboring NHEK and RTS3b cells were UV-irradiated and harvested at the indicated time points later. The pEGFR-Y1068, (pEGFR-Y1045 in the case of RTS3b) and the total EGFR were detected by WB. Shown is one representative example out of two experiments using NHEK, which both were used for quantifications shown in the figure. The values of the quantification of WB with the RTS3 cells are given as well. The positions of the molecular weight markers are given (in kDa).

kinetic of UV- and HPV8E6-mediated increase of the pEGFR-Y1068 was observed in RTS3b cell (**Figure 1B**, lower part). In addition, UV-irradiation transiently triggered the pEGFR-Y1045 detectable 30 min and 45 min later in HPV8E6 expressing cells. These results support the notion that the expression of HPV8E6 transiently increases the RTK-activity of the EGFR in response to UV-irradiation.

HPV8E6 Alters the Intracellular Trafficking of the UV-Activated EGFR

UV-light induces an RTK independent EGFR transactivation and internalization, which results in an arrest within the early endosome without the ubiquitination and proteasomal degradation of the UV-activated EGFR within the first hour post-UV (reviewed in Tan et al., 2016). In order to investigate whether the internalization and the downstream sorting of the UV-activated EGFR was modulated by the expression of HPV8E6 we initially monitored the cell surface expression of the UV-exposed EGFR by FACS analysis with non-permeabilized N/TERT and HaCat keratinocytes. In both cell types, the amounts of EGFR at the cell surface were reduced 30 min after UV-irradiation, independently whether HPV8E6 was expressed or not (see **Figure 2A**). After a 75 min recovery period, however, in control cells the EGFR surface level returned to those as in untreated cells, while in HPV8E6 positive N/TERT and HaCat the amount of the surface EGFR remained reduced (**Figure 2A**). This implies that the intracellular processing of the UV-activated, internalized EGFR is altered in the presence of HPV8E6.

We further addressed this by immunofluorescence staining of UV-irradiated cells. In unirradiated control and HPV8E6 expressing N/TERT the EGFR was diffusely distributed along the plasma membrane (**Figure 2B**). It is noticeable that in HPV8E6 expressing keratinocytes a specific EGFR staining was detectable particularly at the adhesion sites, which was not further addressed here. Following UV-irradiation a substantial portion of the EGFR was redistributed from the plasma membrane into early endosomes, indicated by a co-localization of the early endosomal associated protein 1 (EEA1) and the EGFR (**Figure 2B**). This was detectable as early as 15 min up to 75 min post-UV exposure in both, control and HPV8E6 expressing cells, respectively (**Figure 2B**). Quantifications by counting the number of yellow spots within the cytoplasm per cell documented, however, that in the presence of HPV8E6 more EGFR was associated with early endosomes which was most obvious early after UV-irradiation (**Figure 2C**), implying that HPV8E6 enhances the internalization of the UV-activated EGFR.

To address the processing of the EGFR downstream of the early endosome we performed co-staining with CD63, also known as Lamp3, a well-established component of intraluminal vesicles (ILVs) and of late endosomal and lysosomal membranes. As early endosomes mature they can accumulate ILVs through inward budding of the membrane. The number of ILVs increases during endosome maturation. These finally fuse with the late endosomes and lysosomes and are then exposed to lysosomal hydrolysis (Oksvold et al., 2001). Interestingly, in the HPV8E6 positive cells a significant co-localization of CD63 positive structures and the EGFR was observed, indicated by the yellow spots which were not detectable in empty vector cells (**Figure 2B**). This was most pronounced 15 min after UV-irradiation, mostly at perinuclear sites. This co-localization progressively decreased 30 min and 75 min post UV, as confirmed by the quantification of the number of yellow spots (**Figures 2B,C**). No significant co-localization was obvious in the empty vector cells. Thus, UV-activated EGFR is further processed to ILVs in UV-irradiated HPV8E6 expressing cells while in control cells the UV-activated EGFR stays within the early endosome, in agreement with the findings described in the literature (Oksvold et al., 2001; Tomas et al., 2015).

The Association of HPV8E6 with the EGFR Is Enhanced by UV-Light and Requires RTK-Activity

Ubiquitination of the EGFR is a prerequisite for ESCRT-mediated sorting into ILVs followed by proteasomal degradation of the tyrosine phosphorylated EGFR (Tomas et al., 2014). Since we observed that in the presence of HPV8E6 the UV-exposed EGFR is processed downstream of the early endosome into CD63 positive structures we analyzed whether the EGFR is ubiquitinated. We used UV-irradiated RTS3b cells, since these immortalized keratinocytes can be transiently transfected to high rate. After the transient transfection of a vector expressing FLAG-HPV8E6 under control of the strong CMV promoter the endogenous EGFR was precipitated. A subsequent WB with an antibody against K48-linked polyubiquitin-chains demonstrates that the EGFR underwent poly-ubiquitination in HPV8E6 positive cells after UV-exposure (**Figure 3A**, lane 4), in contrast to cells transfected with the empty vector. Beyond that, probing the blot with the antibody against HPV8E6 revealed that HPV8E6 specifically co-precipitated with the EGFR after UV-irradiation. This was confirmed by a co-immunoprecipitation of the EGFR with the FLAG-antibody, recognizing the FLAG-tagged HPV8E6 (**Figure 3A**, lanes 5–8). Again, the HPV8E6-associated EGFR was highly ubiquitinated. A specific interaction between the EGFR and HPV8E6 in response to UV-irradiation could also be demonstrated with extracts from the RTS3b-pLXSN-HPV8E6 cell line, which expresses much lower levels of HPV8E6 (**Figure 3B**). These results demonstrate that UV-irradiation enhances the binding of HPV8E6 to the endogenous EGFR and that the HPV8E6-associated EGFR is ubiquitinated. Since the ubiquitination of the EGFR requires its prior tyrosine phosphorylation, we analyzed whether the RTK-activity modulates the interaction with HPV8E6 and cultivated the transiently transfected cells in medium containing the irreversible RTK-pan-ErbB-inhibitor Canertinib, also known as CI-1033 (Smaill et al., 2000). As shown in **Figure 3C**, the binding of HPV8E6 to the EGFR was abolished in a dose dependent manner in the presence of Canertinib even when the cells have been exposed to UV-light. No tyrosine phosphorylated EGFR could be detected in the input confirming the efficiency of the RTK-inhibitor. The amount of total EGFR and HPV8E6 were hardly affected by Canertinib and by UV-irradiation, respectively (**Figure 3C**). We

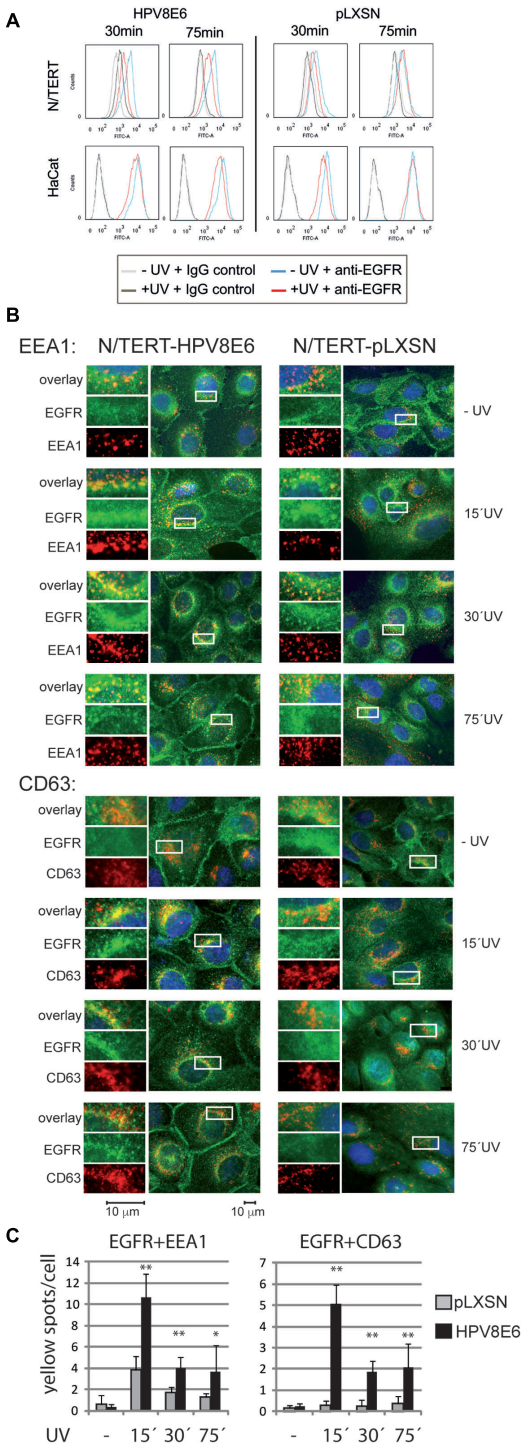

FIGURE 2 | HPV8E6 alters the intracellular trafficking of the EGFR after UV-irradiation. **(A)** FACS analysis of fixed and non-permeabilized pLXSN-empty vector or HPV8E6-NTERT and HaCat cells. Cells were left untreated or irradiated with a dose of UV-light of 40 mJ/cm^2 and harvested 30 or 75 min after UV-exposure. The cells were stained with the anti-EGFR-AF488 or the isotype control IgG-AF488 coupled antibody. The graphs represent the overlays of the EGFR-positive non-irradiated cells (blue line) with those harvested 30 min or 75 min post UV (red line), respectively. *(Continued)*

FIGURE 2 | Continued
The light and dark gray graphs represent the cells stained with the isotype control. **(B)** Immunofluorescence analysis. N/TERT harboring the pLXSN or pLXSN-HPV8E6 were fixed and permeabilized after UV-exposure, as indicated. Immunofluorescence was performed with anti-EGFR-AF488 (green) and with the anti-EEA1 (red, upper part) or the anti-CD63 (red, lower part) antibody. Nuclei were stained with DAPI. The photos represent representative images, respectively. A more detailed section in a higher magnification is provided including the single stains. The scale bars are given at the bottom (magnification 1:1000). **(C)** Quantification of the number of EGFR positive early endosomes (EGFR and EEA1 positive), and of the CD63 and EGFR positive ILVs (in each case indicated by yellow spots). Bar graphs represent the fold changes of the number of yellow spots per cell, indicative for a co-localization of EGFR and EEA1 or CD63 15, 30, and 75 min after UV-irradiation. The values were obtained by counting the yellow spots in a total of 70 to 130 cells, obtained from two independent experiments. Error bars indicate the standard deviations, the asterisks indicate the significance (**$p < 0.001$, *$p = 0.052$).

further confirmed these results with recombinant proteins. C33A cells were transiently transfected with expression vectors for GFP-tagged EGFR and FLAG-HPV8E6 and treated in addition to Canertinib also with the EGFR specific inhibitor tyrphostin AG-1478 followed by UV-irradiation. AG-1478 competitively binds to the ATP pocket of the EGFR to inhibit its RTK-activity (Gan et al., 2007). Here, FLAG-HPV8E6 co-precipitated with the GFP-tagged EGFR. This interaction was clearly reduced in the presence of AG-1478 and abolished by Canertinib. It has to be mentioned that the endogenous EGFR present in C33A cells could not be precipitated and detected under these conditions (data not shown). The residual binding may result from marginal Y-1068 phosphorylation of the overexpressed EGFR in the presence of AG-1478, which was still visible after long exposures (data not shown). Thus, HPV8E6 directly binds to the EGFR, which requires its Y-phosphorylation. From this we conclude that HPV8E6 enhances the RTK-activity of the UV-exposed EGFR, which promotes its interaction with HPV8E6 and leads to ubiquitination and to altered intracellular trafficking of the EGFR.

Inhibition of the RTK-Activity of the EGFR Suppresses UV-Induced Skin Papillomatosis in K14-HPV8E6 Mice

We asked whether the activation of the RTK-activity of EGFR by HPV8E6 is relevant for the induction of papilloma growth in transgenic mice. Initially, we determined the level of pEGFR-Y1068 in hyperplastic skin lesions that appeared 13 days after UV-irradiation in K14-HPV8E6 transgenic mice by immunohistochemistry with the specific antibody. A faint specific staining of the epidermal cells associated with the plasma membranes and intracellular sites was readily visible indicating that the EGFR is in a tyrosine phosphorylated state (**Figure 4A**). To address its functional contribution to HPV8E6 and UV-mediated tumor formation, we treated K14-HPV8E6 transgenic mice with the RTK-inhibitor Canertinib. Seventeen K14-HPV8E6 transgenic mice obtained orally once 20 mg/kg body-weight Canertinib 4 h prior UV-irradiation and then once daily 10 mg/kg Canertinib for a period of 24 days while 12

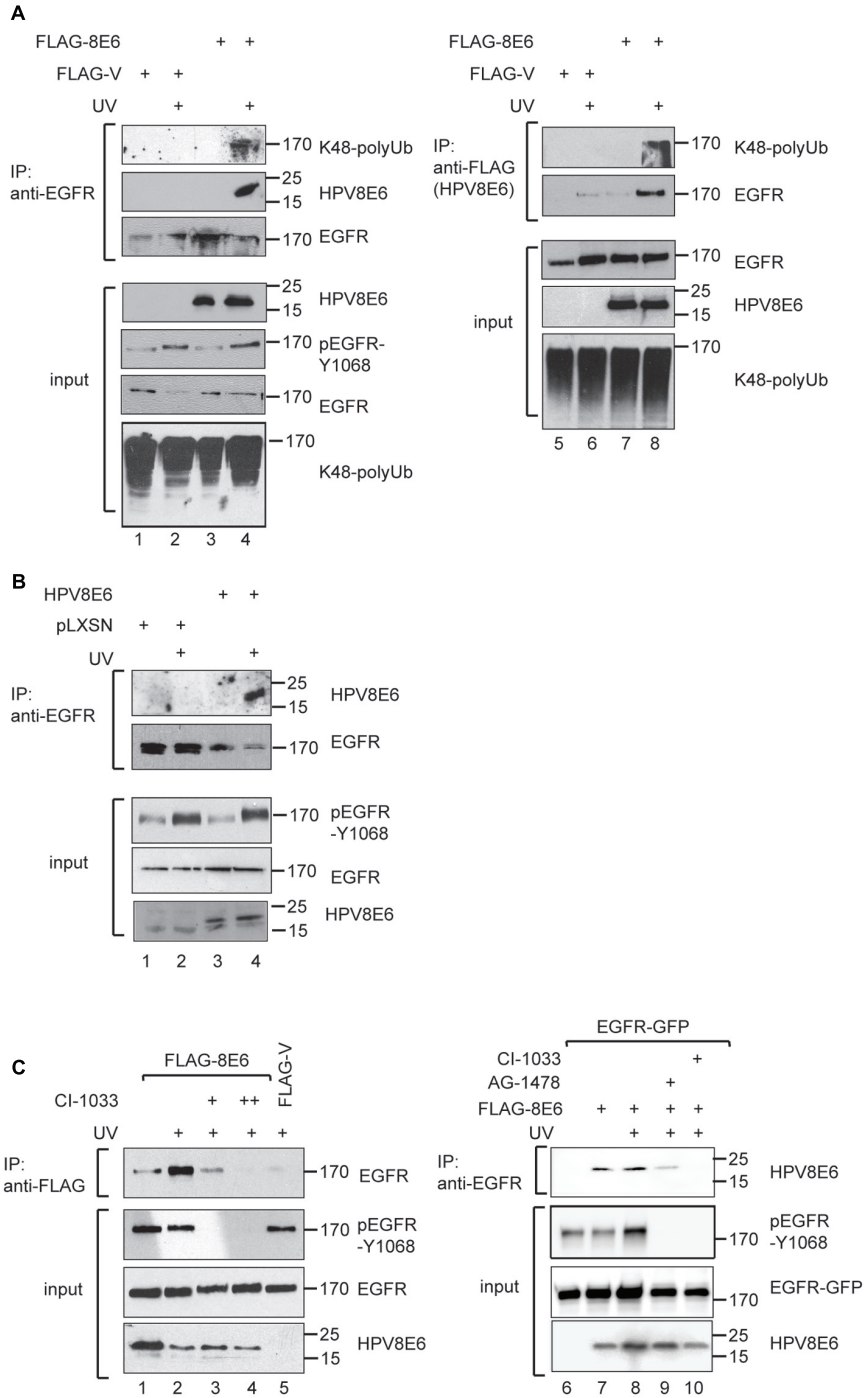

FIGURE 3 | HPV8E6 directly binds to the ubiquitinated EGFR in an UV-dependent manner. **(A)** RTS3b cells were transiently transfected with a vector directing the expression of FLAG-tagged HPV8E6 under control of the CMV-promoter or the empty vector. Where indicated the cells have been irradiated with UV-light 30 min prior harvesting. 400 μg of extracts were incubated with the anti-EGFR-antibody (lanes 1–4) or with the FLAG-antibody (lanes 5–8), both coupled to sepharose, washed four times in 0.3 M KCl-buffer prior analysis by WB and 40 μg of the extracts were used in the input blots. **(B)** IP with anti-EGFR sepharose and 800 μg of extracts derived from the stable RTS3b-pLXSN and RTS3b-HPV8E6 lines, either left untreated or UV-irradiated 30 min prior harvesting. FLAG-HPV8E6 and EGFR present in the precipitate as well as in the input were detected by WB. **(C)** Extracts from UV-irradiated RTS3b cells that have been transiently transfected with the FLAG-HPV8E6 or the FLAG-vector were incubated either with DMSO (lanes 1, 2, 5), 3 μM (lane 3) or 7 μM (lane 4) Canertinib for 30 min. The IP was performed with the FLAG-antibody. All WB were developed with the indicated antibodies. In lanes 6–10, C33A cells were transiently transfected with expression vectors for EGFR-GFP and FLAG-HPV8E6, and UV-irradiated, where indicated, prior harvesting 30 min later. EGFR-GFP was precipitated from 400 μg of extract by the EGFR antibody and bound HPV8E6 was detected by the FLAG-antibody. The input blots were developed with the antibodies against pEGFR-Y1068, GFP, and FLAG. The positions of the molecular weight markers are provided (in kDa).

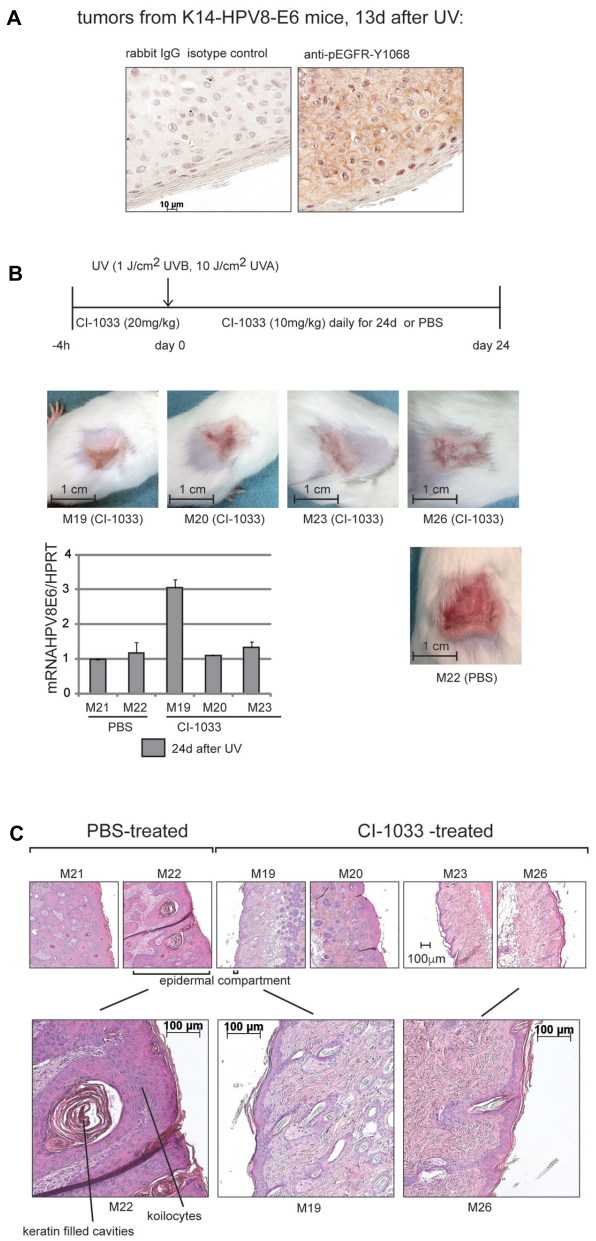

FIGURE 4 | Inhibition of the RTK-activity of the EGFR by the pan-ErbB-inhibitor Canertinib (CI-1033) suppresses UV-induced papillomatosis in K14-HPV8E6 transgenic mice. **(A)** Immunohistochemical staining of paraffin embedded sections obtained from skin of K14-HPV8E6 transgenic mice 13 days after UV-exposure with the anti-pEGFR-Y1068 or rabbit IgG isotype control (magnification 1:400). **(B)** Transgenic mice obtained either Canertinib (CI-1033) (20 mg/kg weight) or the same volume of PBS 4 h prior UV-irradiation followed by applications of Canertinib once daily at a dose of 10 mg/kg weight or 150 µl PBS for 24 days, as given in the time line. The pictures represent examples of the lesions from four mice treated with CI-1033 (M19, 20, 23, and M26) and from one control mouse which obtained PBS (M22). The graph beneath shows the quantification of RT-PCR results with RNA isolated from two PBS and three Canertinib-treated animals to demonstrate the expression of HPV8E6. **(C)** Histology with H/E staining of skin section obtained from two PBS-control (M21 and M22) and four Canertinib (CI-1033)-treated animals (M19, M20, M23, and M26) 24 days after UV-irradiation.

UV-irradiated K14-HPV8E6 transgenic animals, representing the control group, were administered the solvent PBS at the same time points (see the time scale in **Figure 4B**). All K14-HPV8E6 transgenic mice as well as two wt mice (which were included as control) developed a sun burn 2–3 days after UV-irradiation according to our previously established protocol (Marcuzzi et al., 2009). While the sun burn healed after about 7 days in the wt mice, as described previously (Marcuzzi et al., 2009), papillomas started to grow in the transgenic mice demonstrating the effect of HPV8E6 which is expressed in the skin of these animals. After 24 days, all 12 animals in the control group had developed large, exalted papillomas covering the initially irradiated skin sections. In contrast, in all 17 animals treated with Canertinib the initially UV-irradiated skin-areal, which had developed a sun burn, had shrunk and none of the lesions was exalted (**Figure 4B**). The affected skin within the irradiated area appeared to be renewed and tender. RT-PCR with RNA isolated 24 days after UV-treatment from the lesions excluded that the expression of the HPV8E6 was diminished due to the application of Canertinib (**Figure 4B**). H/E-staining of lesions from two PBS treated animals revealed histology of papillomatosis, with multiple cavities filled with lamellated concentric keratin masses in the epidermal compartment. In the upper layers, intracytoplasmic keratohyalin granules were present and in some areas koilocytosis occurred, as observed previously (Schaper et al., 2005; Marcuzzi et al., 2009). The histology of the lesions from four animals of the Canertinib group illustrates that the epidermis from three Canertinib treated animals (M19, M23, and M26) was composed of two to three cell layers (**Figure 4C**). In the section of one treated mouse, the epidermal compartment was thickened but was still much thinner compared to the lesions of the control animals (M20, **Figure 4C**). These results demonstrate that the RTK-activity of the EGFR is required for UV-induced papilloma formation in K14-HPV8E6 transgenic mice.

DISCUSSION

We demonstrate here that HPV8E6 transiently increases the RTK-activity of the EGFR shortly after UV-exposure. We observe an increased tyrosine phosphorylation of the EGFR as a consequence of the expression of HPV8E6 in three different immortalized keratinocyte lines as well as in primary keratinocytes. This excludes cell-lineage dependent effects and points to a specific impact of HPV8E6 on the RTK-activity. We provide evidence that the effect of HPV8E6 has consequences on the downstream processing of the EGFR. The UV-activated EGFR is ubiquitinated only in HPV8E6 positive cells indicating that the HPV8E6-dependent increased tyrosine phosphorylation of the UV-exposed EGFR is sufficient to recruit the E3 ubiquitin-ligase Cbl and its associated Grb2. Indeed we observed a co-precipitation of Grb with the UV-stimulated EGFR in the presence of HPV8E6 (data not shown). Moreover, there was an enhanced internalization of the UV-activated EGFR into early endosomes in HPV8E6 cells compared to control cells and the presence of the EGFR in structures downstream of the early endosome even entirely depended on HPV8E6.

Tyrosine phosphorylated, ubiquitinated and endocytosed EGFR was shown to interact with Eps15 and AP-2, two endocytotic adaptor proteins to form clathrin coated endocytic vesicles (Zwang and Yarden, 2009). The complex is then targeted by the ESCRT and further sorted to CD63 positive ILVs beyond the early endosome, which fuse with lysosomes to complete the degradation of the EGFR and attenuate the signaling (Tomas et al., 2014). This supports the notion that UV-activated EGFR is prone to lysosomal degradation in HPV8E6 expressing cells, and indeed we occasionally observed reduced amounts of EGFR in the HPV8E6 cells up to 30 min after UV-irradiation where the highest level of tyrosine phosphorylation was present (see **Figure 1A**, lanes 7, 8, 15, 16 and **Figure 1B**). The distinct mode of endosomal EGFR trafficking induced by HPV8E6 was underlined by the lack of detectable ubiquitination and of co-localization with CD63 of the UV-activated EGFR in HPV8E6 negative cells. Thus, in agreement with previous reports the UV-light resulted in EGFR internalization with an arrest in the early endosome in HPV8E6 negative cells (Tomas et al., 2014; Peng et al., 2016).

The altered intracellular trafficking of the UV-activated EGFR induced by HPV8E6 may prolong the signaling and support cell survival. Activation of EGFR-signaling pathways not only occurs from the plasma-membrane. For instance, EGFR endocytosis to early endosomes was found to be required for the full stimulation of EGF-induced ERK1/2 and PI3K/AKT signaling (Vieira et al., 1996). Particularly, perinuclear endosomes have been shown to provide a spatial compartment for prolonged EGFR signaling (Huang et al., 2016). Our data showing a co-localization of the UV-activated EGFR and CD63 predominantly at perinuclear regions in HPV8E6 N/TERTs may thus reflect a prolonged signaling. A stimulation of the EGFR-mediated MAPK-Ras signaling pathway was suggested to occur from late endosomes and lysosomes as well (Oksvold et al., 2001; Jiang and Sorkin, 2002; Fehrenbacher et al., 2009; German et al., 2011). Moreover, continuous EGFR signaling from late endosomes was shown to contribute to sustained downstream AKT and STAT3 activation and the endosomal accumulation of the activated EGFR increased tumor cell survival (Huang et al., 2016).

In accordance with these observations, we report here that the functional interaction between HPV8E6 and the UV-activated EGFR which we have characterized here has a crucial role in the UV- and HPV8E6-induced skin tumor induction *in vivo* since the pharmacological inhibition of the RTK-activity of the EGFR suppressed papillomatosis in transgenic mice. The contribution of HPV8E6 is reflected by the facts that the UV-induced skin lesions, i.e., sun burn, healed in wt mice, while in HPV8E6 positive mice a massive tumor growth was induced, as observed previously (Marcuzzi et al., 2009). Since these mice have the same phenotype as the K14-HPV8CER mice, expressing all early HPV8 proteins, it is obvious that HPV8 E6 is the major oncogene in the mouse skin and can function on its own to stimulate papilloma growth in cooperation with UV-light. In addition, it is also necessary since its suppression reduced tumor formation in the HPV8CER mice (Hufbauer et al., 2010). Nevertheless, it has to be admitted that with this setting we used here potential interaction between the other early viral proteins E7, E2 and E1 with E6 or with the EGFR or the corresponding signaling pathways, which may occur during natural infections, have not been considered and cannot be excluded.

A previous study suggested that the short lived UV-induced activation of the EGFR is a powerful promoter of skin tumorigenesis of initiated keratinocytes (El-Abaseri et al., 2005). Similarly, the HPV8E6-mediated transient boost of the RTK-activity of the EGFR may support papilloma growth. It has to be mentioned that although our *in vivo* results obtained with transgenic mice imply that the functional stimulation of the EGFR-activity by HPV8E6 is essential for tumor induction, they do not prove it. The pan-ErbB inhibitor Canertinib has highest activity against the EGFR but also blocks the activity of ErbB2/Her2, which was shown to be expressed and to be tumorigenic in mouse skin as well (Kiguchi et al., 2000; Krahn et al., 2001). Thus, it cannot be excluded that a possible functional interaction of HPV8E6 with ErbB2 may contribute to the UV-induced papilloma formation.

What might be the molecular basis for the HPV8E6-mediated enhancement of the UV-activated EGFR? In agreement with published data (Zwang and Yarden, 2006), we found that UV-treatment induced S/T phosphorylations including that of the T in pos. 669, which were shown to be dependent on the UV-activated p38MAPK. These triggered a gradual and weak transient RTK-activation of the internalized EGFR which was detectable within the following 2–4 h (Tomas et al., 2015). However, others have reported weak tyrosine phosphorylations within minutes after UV-exposure (El-Abaseri et al., 2006; Singh et al., 2009). Since the binding of HPV8E6 to the EGFR depended on its tyrosine phosphorylation and was enhanced by UV-light, one might speculate that low level of tyrosine phosphorylations early after UV-exposure will initiate the recruitment of HPV8E6. This may somehow stabilize the phosphorylated EGFR form by a not yet characterized feedback mechanism. It has been reported that EGFR ubiquitination requires a certain threshold of receptor tyrosine phosphorylations, namely the simultaneous presence of two phosphotyrosines, of the pY1045 and either one of pY1068 or pY1086, on the same EGFR moiety. These mechanistically led to the cooperative recruitment of the E3 ligase Cbl in complex with Grb2, to the EGFR (Sigismund et al., 2013). Thus, HPV8E6 might promote to pass the required threshold of tyrosine phosphorylation allowing then EGFR-ubiquitination, with the consequence of altered endosomal trafficking and signaling of the UV-activated EGFR as discussed. Further studies are necessary to elucidate the mechanism how HPV8E6 stimulates the RTK-activity of the EGFR and whether the interaction will affect the activity of E6. Moreover, a functional interaction of HPV8E6 with other ErbB family members may participate, as discussed above, which has to be analyzed as well. The observation that the pharmacological inhibition of the RTK-activity suppressed papillomatosis mediated by HPV8E6 and UV-light was surprising in view of multiple, pleiotropic effects described for β-HPV E6 proteins. In common they have the capacity to inhibit MAML, which acts as co-factor for Notch.

In this way, E6 may suppress keratinocyte differentiation via inhibiting Notch (Tan et al., 2012; Meyers et al., 2013) and thus further amplify the negative effect of EGFR signaling on Notch (Kolev et al., 2008). The E6 proteins of HPV8 and other β-HPV-types are able to inhibit apoptosis (Jackson and Storey, 2000; Jackson et al., 2000; Struijk et al., 2008) and interfere with the repair of UVB-induced DNA damage and repair (Wallace et al., 2012; Hufbauer et al., 2015) and thus favor the accumulation of mutations including UV-induced inactivating mutations of p53, which are thought to be a crucial step in the development of AKs and cSCC also in humans (Brash et al., 1991; Kemp et al., 1993; Jiang et al., 1999). These broader activities of HPV8E6 may support the progression of the benign papillomas, which depend on the RTK-activity of the EGFR as we have demonstrated here, to cSCC (Schaper et al., 2005; Marcuzzi et al., 2009).

Targeting the EGFR signaling seems to be a common theme for HPV. Previously, the E5 and the E6 oncoproteins of the high risk genital HPV16 belonging to the genus alpha were found to interfere with EGF stimulated EGFR signaling pathways and thus protect cells from apoptosis and induce migration, respectively (Zhang et al., 2005; Spangle and Munger, 2013). Here, we demonstrate for the first time, to our best knowledge, a functional interaction of an HPVE6 protein with the UV-activated EGFR. Remarkably, β-HPV types do not encode an E5 protein and E6 may have overtaken the role to modify signaling by UV-activated EGFR. This may reflect the association of β-HPV with the sun exposed cutaneous skin. It will be interesting to analyze whether other β-HPVE6 proteins are able to bind the Y-phosphorylated EGFR and to stimulate the UV-dependent activation of the EGFR as well and whether this is linked to E6 proteins of HPV types that are cancer associated.

Our data obtained with the use of transgenic mice and human keratinocytes provide a molecular basis how HPV8 may cooperate with UV-light in the induction of papillomas and also support a role of β-HPV in the development of proliferative skin lesions in humans, as implied by epidemiological studies. These findings also provide a molecular mechanism explaining the contribution of β-HPVs to the early steps in the cSCC development in human, particularly in immunosuppressed patients. In these patients, higher loads of β-HPV in the skin and the hair follicles due to their immune-suppression will result in elevated levels of E6, which then alter UV-induced EGFR signaling according to our data. Although this effect will be transient, reiterated UV irradiation may lead to sustained activation of the EGFR and downstream signaling pathways and thus trigger hyperproliferation of the epidermis and the development of AK, the precursor lesions of cSCC.

AUTHOR CONTRIBUTIONS

ST, HP, and GS planned and designed the experiments. ST conducted most of the experiments and analyzed the results. GS conducted and analyzed some of the experiments. GS wrote the manuscript. All the authors reviewed the manuscript.

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Chronic Inflammatory Microenvironment in Epidermodysplasia Verruciformis Skin Lesions: Role of the Synergism Between HPV8 E2 and C/EBPβ to Induce Pro-Inflammatory S100A8/A9 Proteins

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Persistent genus β-HPV (human papillomavirus) infection is a major co-factor for non-melanoma skin cancer in patients suffering from the inherited skin disease epidermodysplasia verruciformis (EV). Malignant EV lesions are particularly associated with HPV type 5 or 8. There is clinical and molecular evidence that HPV8 actively suppresses epithelial immunosurveillance by interfering with the recruitment of Langerhans cells, which may favor viral persistence. Mechanisms how persistent HPV8 infection promotes the carcinogenic process are, however, less well understood. In various tumor types chronic inflammation has a central role in tumor progression. The calprotectin complex consisting of S100A8 and S100A9 proteins has recently been identified as key driver of chronic and tumor promoting inflammation in skin carcinogenesis. It induces chemotaxis of neutrophil granulocytes and modulates inflammatory as well as immune responses. In this study, we demonstrate that skin lesions of EV-patients are massively infiltrated by inflammatory cells, including $CD15^+$ granulocytes. At the same time we observed a very strong expression of S100A8 and S100A9 proteins in lesional keratinocytes, which was mostly confined to the suprabasal layers of the epidermis. Both proteins were hardly detected in non-lesional skin. Further experiments revealed that the HPV8 oncoproteins E6 and E7 were not involved in S100A8/A9 up-regulation. They rather suppressed differentiation-induced S100A8/A9 expression. In contrast, the viral transcription factor E2 strongly enhanced PMA-mediated S100A8/A9 up-regulation in primary human keratinocytes. Similarly, a tremendous up-regulation of both S100 proteins was observed, when

minute amounts of the PMA-inducible CCAAT/enhancer binding protein β (C/EBPβ), which is expressed at low levels in the suprabasal layers of the epidermis, were co-expressed together with HPV8 E2. This confirmed our previous observation that C/EBPβ interacts and functionally synergizes with the HPV8 E2 protein in differentiation-dependent gene expression. Potent synergistic up-regulation of S100A8/A9 was seen at transcriptional and protein levels. S100A8/A9 containing supernatants from keratinocytes co-expressing HPV8 E2 and C/EBPβ significantly induced chemotaxis of granulocytes in migration assays supporting the relevance of our finding. In conclusion, our data suggest that the HPV8 E2 protein actively contributes to the recruitment of myeloid cells into EV skin lesions, which may support chronic inflammation and progression to skin cancer.

Keywords: HPV, E2, epidermodysplasia verruciformis, inflammation, C/EBP, S100A8/A9

INTRODUCTION

Human papillomaviruses (HPVs) infect keratinocytes of mucosa and skin and cause a wide range of clinical outcomes from benign to malignant lesions. Mucosal HPV types have a well-established role in anogenital tumors, particularly in cervical carcinoma. Cutaneous HPVs of the genus beta (β-HPV) were first discovered in patients with epidermodysplasia verruciformis (EV) (Majewski and Jablonska, 2006). This rare genetic skin condition mostly results from recessive mutations in *EVER1* or *EVER2* genes, which confer predisposition to persistent β-HPV infection (Shamanin et al., 1994; Ramoz et al., 2002). EV begins early in childhood with widespread flat warts and macules, which can, later in life, evolve to cutaneous squamous cell carcinoma (SCC), mainly at sun-exposed sites. Multiple HPVs, e.g., HPV5, 8, 17, 20, 27, 38, or 47, can be found within the EV skin lesions but HPV5 or 8 are the most frequently detected in EV-associated skin carcinomas (Orth et al., 1978; Orth, 1986; Pfister, 2003).

Viral persistence is a major prerequisite for HPV-induced tumor development in cervix uteri and potentially in the skin and it requires an escape of the virus from host immune surveillance (zur Hausen, 2009; Smola, 2017; Smola et al., 2017). Recently, we have proposed a novel molecular mechanism how HPV8 might disrupt innate immunity in skin. We found reduced numbers of antigen-presenting Langerhans cells in EV lesions and showed that it is a consequence of HPV8-mediated suppression of CCL20, which is a chemokine attracting Langerhans cells (Sperling et al., 2012; Smola, 2014).

A set of *in vivo* data employing transgenic mouse models established a role of EV-associated HPVs in the development of skin tumors. HPV38, 20, and 27 transgenic mice expressing E6 and E7, the main viral oncoproteins, displayed increased susceptibility to carcinogenesis after UV or chemical treatment (Michel et al., 2006; Viarisio et al., 2011). In mice expressing the complete early region of HPV8 under the keratin 14 (K14) promoter even spontaneous development of skin cancers was observed. Transcript analysis in the affected tissue revealed high mRNA levels of the viral transcription factor E2, which prevailed over E7 and E6 and did not change during tumor progression (Schaper et al., 2005). Interestingly tumor development was also observed in transgenic mice expressing only the HPV8 E2 gene under the K14 promoter and this process was accompanied by a massive stromal infiltration of immune cells (Pfefferle et al., 2008). These findings emphasized the importance of the E2 protein in tumor formation and raised the hypothesis that E2 might augment tumor-promoting inflammatory responses in skin.

Recently, S100A8 (calgranulin A, MRP-8) and S100A9 (calgranulin B, MRP-14) have been identified as key drivers of skin carcinogenesis in a mouse model (Gebhardt et al., 2008). Both proteins belong to the S100 multigenic family of non-ubiquitous Ca^{2+}-binding low molecular weight proteins. They associate in a higher-ordered heteromeric complex called calprotectin, which is essential for their biological activity (Leukert et al., 2006). In skin their expression has been observed under inflammatory conditions, such as wound healing or psoriasis (Kerkhoff et al., 2012). Beside the intracellular activity of S100A8 and S100A9 proteins, they are also found in the extracellular milieu, where they function as damage-associated molecular pattern molecules (DAMPs) or alarmins and induce immune cell migration, particularly of granulocytes (Ryckman et al., 2003).

In this report, we demonstrate that S100A8/A9 proteins are potently up-regulated in HPV8-positive EV lesions and S100 expression was paralleled by a strong inflammatory response. Detailed molecular analysis revealed that not the HPV8 E6 or E7 oncoproteins, but the viral transcription factor E2 plays a major role in S100A8/A9 induction.

MATERIALS AND METHODS

Ethics Statement

This study was carried out in accordance with the recommendations of the Declaration of Helsinki with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Bioethics Committee at the Medical University of Warsaw, Poland, and the Saarland University at the Saarland Ärztekammer.

Immunohistochemistry, Immunofluorescence, and HPV Genotyping

Formalin fixed paraffin-embedded (FFPE) skin specimens from EV-lesions were obtained from the Department of Dermatology, Medical University of Warsaw, Warsaw, Poland. The presence of HPV8 was confirmed by quantitative real-time PCR (qRT-PCR) as described in Weissenborn et al. (2010). Sections were stained with mouse monoclonal anti-CD15 antibody (clone Carb-3, Dako, Glostrup, Denmark), rabbit monoclonal anti-S100A8 antibody (clone EPR3554; Novus Biologicals, Cambridge, United Kingdom), rabbit polyclonal anti-S100A9 antibody (H-90, sc-20173, Santa Cruz Biotechnology, Heidelberg, Germany) or mouse anti-CD45 (Abcam, Cambridge, United Kingdom). Staining was performed using the Dako instrument Autostainer Plus (Dako) or Immpress AP Reagent kit (Vector, Burlingame, CA, United States). For immunofluorescence, cells were grown on cover slips, fixed with 2% buffered paraformaldehyde, permeabilized with 0.1% Triton X-100 and stained with rabbit anti-HPV8 E2 (from Mart Ustav, University of Tartu, Tartu, Estonia) and mouse monoclonal anti-pan-cytokeratin (clone C11; Sigma-Aldrich, Steinheim, Germany) and secondary goat anti-rabbit Alexa Fluor 546 goat and goat anti-mouse Alexa Fluor 488 (Life Technologies, Eugene, OR, United States), respectively.

Plasmid Constructs

Expression vectors of HPV8 E2 and E6/E7 cloned in pLXSN, HPV8 E2, E6, E7, HPV16 E2, C/EBPβ, cloned in pcDNA3.1+ and HPV8 E2ΔC in pXJ42 were reported previously (Hadaschik et al., 2003). S100A8 in pcDNA3.1/myc-His(-) and S100A9 in pcDNA3.1+ expression vectors were kind gifts from Peter Angel (German Cancer Research Centre, Heidelberg, Germany). The S100A8 luciferase reporter construct containing a fragment (−917 to +456 bp) of the murine S100A8 promoter region cloned upstream of the firefly luciferase gene in the pGL2-basic vector was a kind gift from Kenneth Hsu (Sydney, NSW, Australia). It comprises a functional C/EBPβ binding site, which is highly conserved in mammalian S100A8 promoters, particularly in human and murine promoters, and has been shown to bind to C/EBPβ by chromatin immunoprecipitation (Miao et al., 2012).

Cell Culture and Retroviral Infection

Normal human foreskin keratinocytes (NFKs) were cultured in KBM-Gold medium with supplements (Lonza, Basel, Switzerland). NFK stably expressing HPV8 E2 or E6/E7 were generated by retroviral gene transfer as previously described (Oldak et al., 2010). Viral gene expression was confirmed by HPV8 E2-, E6-, and E7-specific qRT-PCR. The HPV-negative skin SCC-derived RTS3b cell line (Purdie et al., 1993) was grown as previously reported (Hadaschik et al., 2003). Organotypic 3-dimensional cultures (OC) were generated as described in Smola et al. (1993) with minor modifications. Dermal equivalents were prepared by seeding 0.5×10^6 primary foreskin fibroblasts (usually between passage 3 and 6) in 4 mg/ml rat-tail collagen. Next day 0.5×10^6 RTS3b cells were seeded onto the fibroblast-collagen matrix. Twenty-four hours later OC were lifted on metal grids at the air-medium interphase, grown for 14 days, fixed with 4% buffered paraformaldehyde, embedded in paraffin and sectioned.

Granulocyte Isolation and Migration Assay

Granulocytes were obtained from whole blood of healthy volunteers by dextran-sedimentation of red blood cell pellets. The purity of granulocyte isolation was 93–99% as determined by CD15 and CD66 surface expression (mouse anti-human CD15-PE BD Biosciences, Heidelberg, Germany and mouse anti-human CD66abce-APC, Miltenyi Biotec, Bergisch Gladbach, Germany). Contaminating monocytes, T- and B-cells in granulocyte isolates were discriminated with CD14-FITC, CD3-FITC, and CD19-PE antibodies. Prior to the assessment of migration, granulocytes were cultured for 2 h under endotoxin-free conditions in RPMI 1640 supplemented with 5% FCS, 1% penicillin-streptomycin and 1% sodium pyruvate. 0.25×10^6 granulocytes were then seeded onto transwell chambers placed in 24-well plates (3 μm pore size, Corning Costar Corp., Corning, NY, United States). The migration capacity of granulocytes was assessed after 2 h. Background migration in culture medium only was subtracted.

Transient Transfection and Luciferase Assay

Normal human foreskin keratinocytes were transfected with TransFast reaching transfection efficiencies between 10 and 15% (Promega, Mannheim, Germany) and RTS3b cells with Lipofectamine 2000 reagent reaching transfection efficiencies between 50 and 70% (Invitrogen, Karlsruhe, Germany) according to the manufacturers' protocols. Twenty-four hours later NFKs were stimulated with PMA for another 24 h (Sigma-Aldrich, Taufkirchen, Germany). Afterward, the cells were lysed and assayed for luciferase activity as described previously (Marthaler et al., 2017). The values of luciferase activities were normalized to protein content of the respective lysates.

RNA Isolation and qRT-PCR

RNA was isolated using NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) and cDNA was generated from 0.5 to 1 μg of RNA with the Maxima Reverse Transcriptase (Thermo Fisher Scientific, Rockford, IL, United States). qRT-PCR was performed using gene-specific primers and the Universal Probe Library System (Roche, Mannheim, Germany) as follows: S100A8: 5′-CAAGTCCGTGGG CATCAT-3′, 5′-GACGTCGATGATAGAGTTCAAGG-3′, probe #78, S100A9: 5′-GTGCGAAAAGATCTGCAAAA-3′, 5′-CCA GCTGCTTGTCTGCATTT-3′, probe #85, RPL13A: 5′-AGCG GATGAACACCAACC-3′, 5′-TTTGTGGGGCAGCATACTC-3′, probe #28, HPV8 E2: 5′-GACGGCGATCAACCTCAA-3′, 5′-CTCCCCTTTGTGACCGTTT-3′, probe #22, HPV8 E6: 5′-CCGCAACGTTTGAATTTAATG-3′, 5′-ATTGAACGTCCT GTAGCTAATTCA-3′, probe #13 and HPV8 E7: 5′-AGG AATTACCAAACGAACAGGA-3′, 5′-CACGGTGCAACAATTT TGAATA-3′, probe #63. Expression levels were measured using

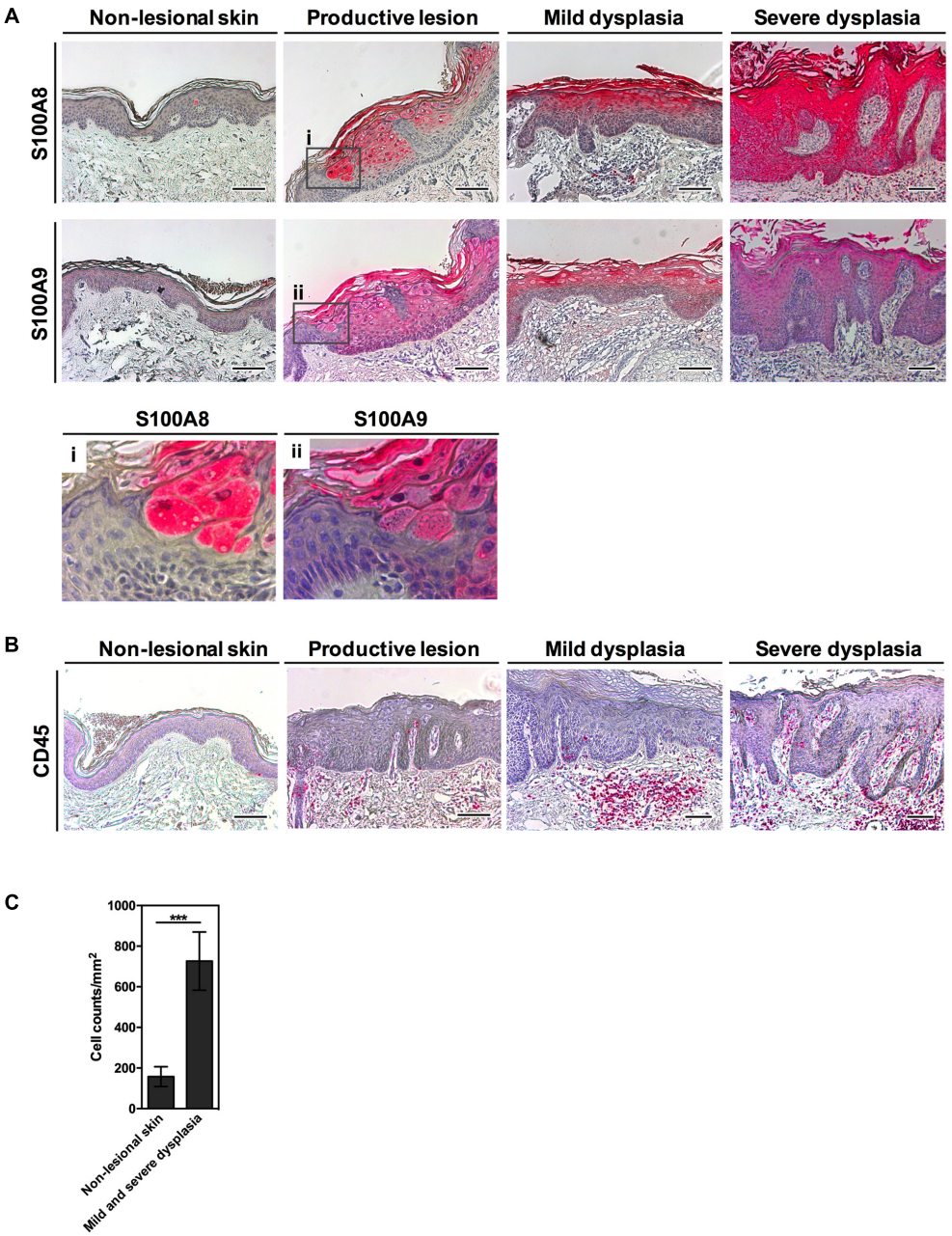

FIGURE 1 | Strong expression of S100A8/A9 in HPV8-positive EV-lesions corresponds to an inflammatory infiltrate. **(A)** Serial sections of HPV8-positive EV-lesions were analyzed by IHC for S100A8 and S100A9 (magnifications in **i** and **ii**) or **(B)** CD45 (all red) and counterstained with hematoxylin. Scale bars 100 μm. **(C)** Infiltrating immune cells were counted in the stroma of mild and severe dysplasia of EV-lesions versus non-lesional skin of EV-patients based on hematoxylin counterstaining and are given as cell counts per mm^2. Values represent counts ± SD from six different areas. ***$p < 0.001$, unpaired *t*-test.

LightCycler 480 II instrument (Roche) and normalized to RPL13A housekeeping gene expression (Marthaler et al., 2017).

Western Blot Analysis and ELISAs

The cells were lysed in buffer containing 62.5 mM Tris-Cl pH 6.8, 10% glycerol and 2% SDS. Thirty micrograms of the samples were separated by 9.5–20% gradient SDS-PAGE and subjected to Western blotting. Anti-S100A8, anti-S100A9, mouse monoclonal anti-actin (clone AC-15, Sigma-Aldrich, St. Louis, MO, United States) as well as horseradish peroxidase-labeled goat anti-rabbit and rabbit anti-mouse (both Sigma-Aldrich) antibodies were used followed by chemiluminescence detection (Thermo Fisher Scientific). ChemiDoc XRS$^+$ Molecular Imager and Quantity One analysis software (both Bio-Rad, Philadelphia, PA, United States) were used for quantification. Interleukin-8 (IL-8, CXCL8), Epithelial-derived Neutrophil-Activating peptide

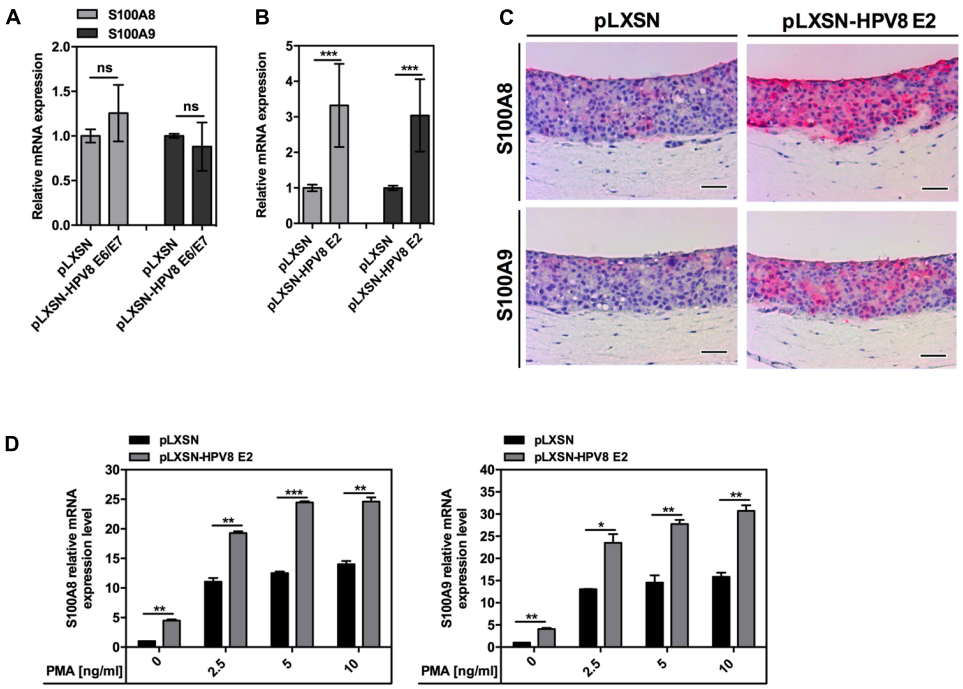

FIGURE 2 | Not HPV8 E6/E7 oncoproteins but the E2 transcription factor induces both S100A8 and S100A9. S100A8/A9 mRNA levels in NFK stably expressing **(A)** HPV8 E6/E7 or **(B)** HPV8 E2 and corresponding control pLXSN cells were measured by qRT-PCR in relation to RPL13A. The amount of S100A8 and S100A9 in pLXSN control cells was set at 1. Shown are the mean values ± SD from $n \geq 2$ independent experiments performed in duplicates. **(C)** Serial sections of OC of HPV8 E2-expressing or control pLXSN RTS3b cells were analyzed by IHC for S100A8 and S100A9 (both red) and counterstained with hematoxylin. Scale bars 100 μm. **(D)** NFK stably expressing HPV8 E2 or control pLXSN cells were stimulated with PMA or DMSO as a vehicle control and 24 h later S100A8 and S100A9 mRNA levels were measured by qRT-PCR in relation to RPL13A. Shown are the mean values ± SD from one representative experiment out of three performed in duplicates. ns, not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, unpaired *t*-test.

78 (ENA-78, CXCL5), Neutrophil Activating Protein-2 (NAP-2, CXCL7) and Growth-Regulated Oncogene-α (GRO-α, CXCL1) concentrations were measured with DuoSet (R&D Systems, South Beloit, IL, United States) according to the manufacturer's protocols.

Statistical Analysis

Statistical differences were determined with unpaired *t*-test using Prism 5 software (GraphPad Software, La Jolla, CA, United States). Significances are indicated by asterisks ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

RESULTS

In this study, we investigated S100A8 and S100A9 protein expression in the skin of EV-patients. In non-lesional skin both proteins were occasionally detectable in the keratinocytes of the granular layer or were completely lacking (**Figure 1A**). HPV8-positive lesional skin of EV-patients, however, revealed a dramatic induction of S100A8 and S100A9 proteins in keratinocytes of suprabasal spinous and granular layers. In productive EV-lesions staining of both proteins was particularly prominent in keratinocytes displaying viral cytopathic effects as demonstrated in higher magnifications (**Figures 1A i** and **ii**). Also in mild and severe dysplasia S100A8 and S100A9 staining was strongly enhanced and mostly confined to the suprabasal layers of infected epidermis. Notably, S100A8 and S100A9 expression in lesional epidermis was paralleled by significant stromal infiltration with immune cells as shown by CD45 staining, a pan-marker for leukocytes, and quantification of stromal infiltrating cells (**Figures 1B,C**).

To investigate the potential impact of HPV8 early proteins on S100A8 and S100A9 induction, normal human keratinocytes (NFKs) and the RTS3b skin keratinocyte cell line were engineered to stably express HPV8 E2 or combined E6/E7 genes by retroviral gene transfer. Expression of HPV8 E2, E6, and E7 genes was confirmed by qRT-PCR and E2 additionally on protein level by double immunofluorescence (Supplementary Figure S1). Presence of HPV8 E6/E7 in NFK did not influence S100A8 and S100A9 expression (**Figure 2A**). However, a significant increase in S100A8/A9 mRNA levels was observed in HPV8 E2-expressing NFK (**Figure 2B**). This was further validated at the protein level by strong S100A8 and S100A9 IHC staining in OC of HPV8 E2-expressing RTS3b keratinocytes compared to control cells with empty pLXSN vector only (**Figure 2C**).

Since in EV-lesions S100A8 and S100A9 staining was confined to the suprabasal more differentiated layers, we were interested whether HPV8 E2 might cooperate with differentiation-regulating factors to up-regulate the calgranulins. To test

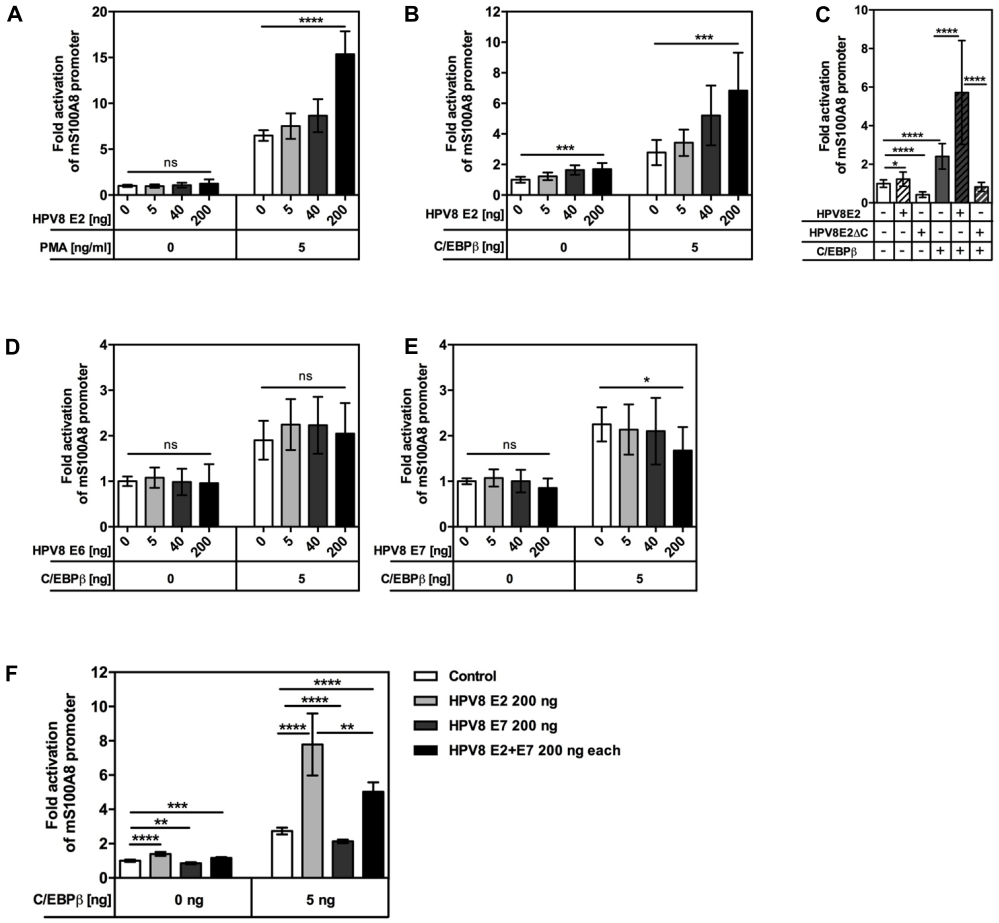

FIGURE 3 | HPV8 E2 enhances PMA- or C/EBPβ-induced activation of the S100A8 promoter in human keratinocytes. **(A)** 0.1 × 10^6 NFKs were seeded onto 12-well plates, next day co-transfected with 0.2 μg S100A8 reporter vector and 0.005, 0.04, or 0.2 μg HPV8 E2 expression vector and 6 h later stimulated with PMA or DMSO as a vehicle control. 0.61 × 10^5 RTS3b cells were seeded onto 12-well plates and next day co-transfected with 0.2 μg S100A8 reporter vector together with 0.005 μg C/EBPβ and in **(B)** 0.005, 0.04, or 0.2 μg HPV8 E2, **(C)** 0.2 μg HPV8 E2 or HPV8 E2ΔC, **(D)** 0.005, 0.04, or 0.2 μg HPV8 E6, **(E)** 0.005, 0.04, or 0.2 μg HPV8 E7 or **(F)** HPV8 E2 and E7 expression vectors (0.2 μg each). Total amount of DNA in all transfections was adjusted up to 0.8 μg with pcDNA3.1+ empty vector. 24 h post-transfection the luciferase activity was measured and normalized to protein concentration. Control transfection was set at 1. Shown are the mean values ± SD from $n \geq 3$ independent experiments performed in triplicates. ns, not significant, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, unpaired *t*-test.

this hypothesis, HPV8 E2-expressing NFKs were stimulated with phorbol 12-myristate 13-acetate (PMA), a well-known activator of protein kinase C (PKC) and inducer of keratinocyte differentiation (Agarwal et al., 1999; Balasubramanian et al., 2000; Goethe et al., 2007). PMA significantly enhanced the effect of HPV8 E2 on S100A8 and S100A9 mRNA in NFK from two different donors (**Figure 2D** and Supplementary Figure S2).

To study whether regulation took place at the promoter level, the interplay of PMA activation and HPV8 E2 expression was examined in reporter gene assays. In NFK, transient expression of HPV8 E2 alone had only a minor effect, while PMA stimulation strongly synergized with HPV8 E2 in S100A8 promoter activation (**Figure 3A**). Others and we have previously demonstrated that PMA potently induces the transcription factor C/EBPβ in primary human keratinocytes (Park et al., 2008; Sperling et al., 2012). Furthermore, our previous data had shown that HPV8 E2 can bind to and synergize with C/EBPβ to transactivate the involucrin promoter (Hadaschik et al., 2003). Notably, a functional C/EBPβ binding site is also highly conserved in mammalian S100A8 promoters, particularly in human and murine promoters (Miao et al., 2012). Since C/EBPβ is expressed at low levels in suprabasal layers of the skin (Sperling et al., 2012), minute amounts of C/EBPβ (5 ng) were co-transfected together with the HPV8 E2 expression vector in RTS3b cells. In these cells transient transfection of HPV8 E2 alone significantly activated the murine S100A8 promoter up to 1.64-fold while co-expression of HPV8 E2 and C/EBPβ significantly increased promoter activity in a dose-dependent manner up to 7-fold, similar to the results obtained with the involucrin promoter (**Figure 3B**) (Hadaschik et al., 2003). HPV8 E2 interacts with C/EBPβ via its C-terminus. We therefore investigated the impact of the C-terminal E2 deletion mutant (HPV8 E2ΔC) on S100A8 promoter activity. While full length HPV8 E2 strongly synergized with C/EBPβ, this was not observed

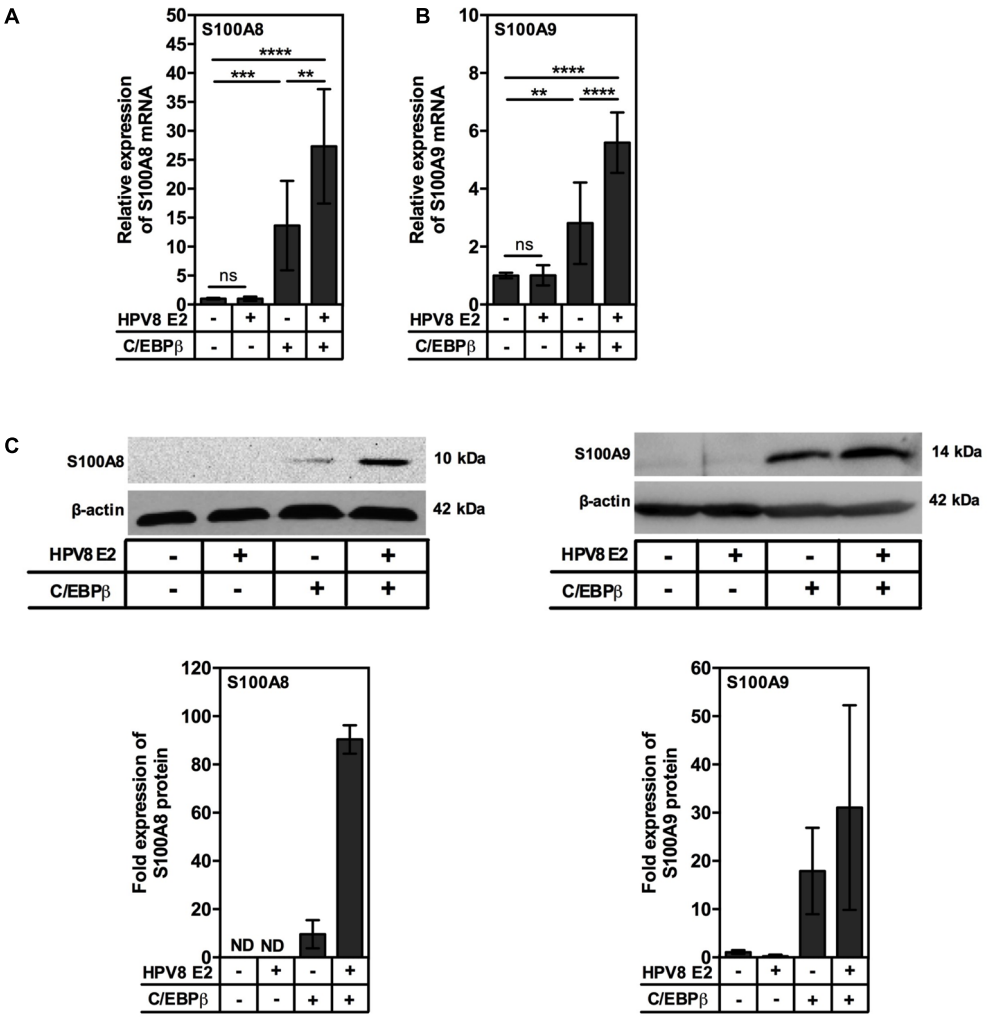

FIGURE 4 | HPV8 E2 synergizes with minute amounts of C/EBPβ to up-regulate S100A8 and S100A9 in human keratinocytes. RTS3b cells were seeded onto 6-cm dishes at a density of 0.4×10^6 cells per dish and transiently co-transfected with 2.64 μg HPV8 E2 and 0.1 μg C/EBPβ expression vectors and 24 h later were analyzed for **(A)** S100A8 and **(B)** S100A9 mRNA expression by qRT-PCR in relation to RPL13A. Shown are the mean values ± SD from $n = 3$ independent experiments performed in duplicates. **(C)** For Western blot analysis 1×10^6 RTS3b cells were seeded onto 10-cm dishes and next day co-transfected with 0.27 μg C/EBPβ and 7.2 μg HPV8 E2 expression vectors. Forty-eight hours later whole-cell extracts were analyzed for S100A8 (left) and S100A9 (right) protein expression. β-actin served as a loading control. Shown is one representative experiment out of $n = 3$. Diagrams summarize $n = 3$ experiments. ND, not detected; ns, not significant, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, unpaired *t*-test.

for the E2 mutant lacking the C-terminal C/EBPβ-interacting domain (**Figure 3C**). To investigate the influence of HPV8 E6 and E7 on C/EBPβ-induced S100A8 promoter activity, we performed co-transfection experiments in RTS3b cells. Neither E6 nor E7 alone increased C/EBPβ-induced promoter activity. HPV8 E7 even led to a suppression of C/EBPβ-induced promoter activity (**Figures 3D,E**). This corresponded well to our previous observation that HPV8 E7 can interfere with C/EBPβ-mediated induction of the Langerhans cell attracting chemokine CCL20 (Sperling et al., 2012). Since E7 is predominantly expressed in the granular layer of EV-lesions, we investigated its potential impact on the synergism between C/EBPβ and E2 on the S100A8 promoter. HPV8 E7 alone was able to reduce C/EBPβ-dependent promoter activity. When equal amounts of HPV8 E2 and E7 were co-transfected together with C/EBPβ, however, the positive E2 activity prevailed over the negative effect of HPV8 E7 (**Figure 3F**).

Next, we analyzed the impact of transient HPV8 E2 and C/EBPβ expression on the regulation of endogenous S100A8 and S100A9. Expression of HPV8 E2 led to a strong enhancement of C/EBPβ-mediated S100A8 induction (more than 25-fold, **Figure 4A**) and S100A9 (up to 6-fold, **Figure 4B**) at mRNA level. In parallel experiments the cells were subjected to Western blot analysis. While control cells expressed minimal levels of calgranulins, HPV8 E2 enhanced the impact of C/EBPβ on the levels of endogenous S100A8 and S100A9 proteins up to 90- and 31-fold, respectively, as shown by quantification (**Figure 4C**).

This effect was not seen for its mucosal counterpart HPV16 E2, which did not enhance but rather suppressed

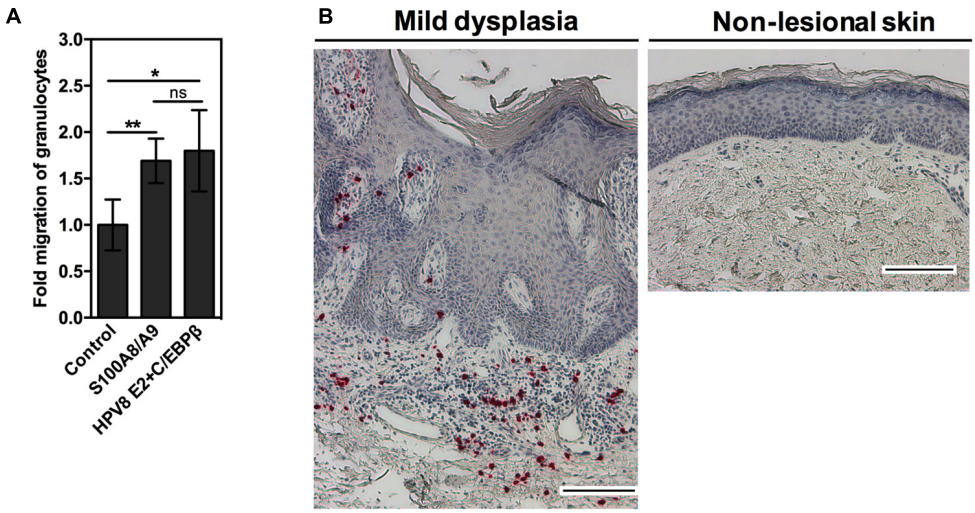

FIGURE 5 | Co-expression of HPV8 E2 and C/EBPβ induces migration of granulocytes *in vitro*. **(A)** Granulocyte migration toward conditioned media collected from RTS3b cells co-transfected with S100A8 and S100A9 or HPV8 E2 and C/EBPβ expression vectors or control vectors was measured by transwell chemotaxis assay. Number of migrated granulocytes toward control conditioned media was set at 1. Shown are the mean values ± SD from $n = 3$ independent experiments performed in duplicates. **(B)** Sections of EV-lesions were stained with anti-CD15 antibody by IHC (red). Counterstaining with hematoxylin reveals the segmented appearance of nuclei in CD15-positive cells indicating infiltration with granulocytes. Scale bars 100 μm. ns, not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, unpaired *t*-test.

C/EBPβ-activated S100A8 promoter activity and S100A8/A9 mRNA levels (Supplementary Figure S3).

Since the S100A8/A9 complex is chemotactic for neutrophils, we set up an *in vitro* migration assay. Freshly prepared conditioned media from transiently transfected keratinocytes were applied onto transwells containing granulocytes. Keratinocytes ectopically expressing S100A8/A9 significantly induced the migration of granulocytes. Notably, conditioned media obtained from the keratinocytes co-transfected with HPV8 E2 and minute amounts of C/EBPβ induced migration of granulocytes at least as strong as overexpressed S100A8/A9 (**Figure 5A**).

To validate this finding *in vivo*, the EV-lesions were further investigated. We observed that the stroma of HPV8-positive EV-lesions was strongly infiltrated with CD15-positive granulocytes in comparison to non-lesional skin, where granulocytes were not detected (**Figure 5B**).

DISCUSSION

In this study, we demonstrate that HPV8-positive skin lesions of EV patients are characterized by a dramatic increase of suprabasal alarmin S100A8/A9 expression and stromal inflammation starting in productive lesions and still observed in mild and severe dysplasia. Analysis of the underlying molecular mechanism revealed that the viral transcription factor HPV8 E2 synergizes with the cellular differentiation-dependent transcription factor C/EBPβ to up-regulate S100A8/A9 expression and to enhance recruitment of granulocytes. Our data indicate that the HPV8 E2 protein hijacks a differentiation-associated pathway to create a chronic inflammatory microenvironment in EV-patients that may pave the way for carcinogenic progression (**Figure 6**).

Epidermodysplasia verruciformis patients are highly susceptible to β-HPV-associated skin carcinogenesis at sun-exposed sites (Smola, 2014). However, the HPV-mediated molecular mechanisms that support the carcinogenic process are poorly understood so far. Our study strongly indicates that the cutaneous β-HPV type 8 can actively trigger inflammation, a hallmark of cancer (Hanahan and Weinberg, 2011). In human organ transplant recipients as well as in mouse models of skin carcinogenesis, this involves the induction of the alarmins S100A8/A9 promoting a pro-tumorigenic inflammatory microenvironment (Gebhardt et al., 2008; Iotzova-Weiss et al., 2015). S100A8/A9 proteins gained a unique interest over the last decades with respect to their role in cancer progression since they can propel cancer cell proliferation, migration, and invasion (Bresnick et al., 2015). Despite the well-defined role of S100A8/A9 in carcinogenesis, however, little is known about factors altering their expression, particularly in HPV-associated carcinogenesis.

Our data clearly demonstrate that S100A8/A9 expression is accompanied by strong immune cell infiltration starting during the productive phase of viral infection in EV-lesions. Tumor-promoting inflammation was previously also observed in mucosal HPV-driven carcinogenesis (Schroer et al., 2011; Walch-Ruckheim et al., 2015, 2016; Smola, 2017; Smola et al., 2017). From published data it was unclear, whether β-HPV oncoproteins contribute to increased inflammatory cytokine production (De Andrea et al., 2007; Akgul et al., 2010). For HPV8 E7, it was rather shown that it interferes with Langerhans recruitment via repression of the chemokine CCL20 (Sperling et al., 2012). This is consistent with observations in anogenital

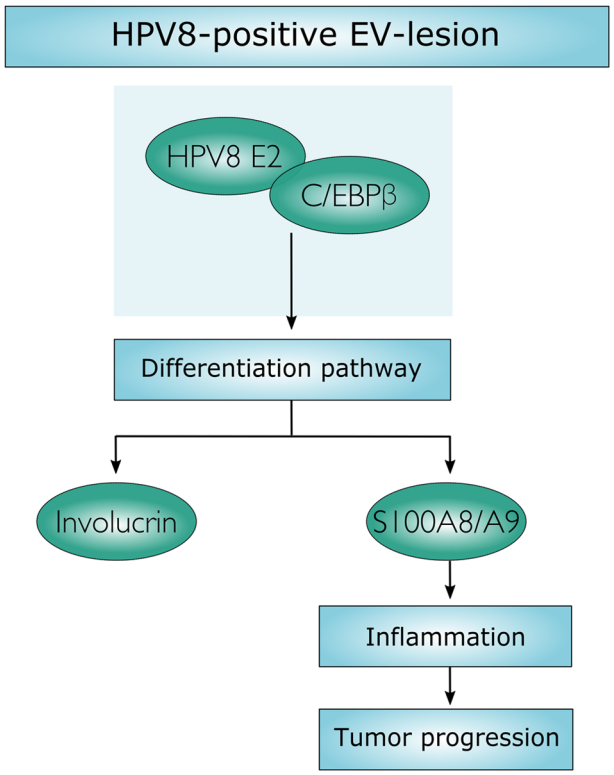

FIGURE 6 | Schematic presentation. Molecular mechanism, by which HPV8 E2 synergizes with C/EBPβ to induce keratinocyte differentiation and to up-regulate S100A8/A9 expression.

HPV infection, where mucosal high-risk HPV oncoproteins were shown to suppress inflammatory responses in keratinocytes (Guess and McCance, 2005; Smola-Hess and Pfister, 2006; Karim et al., 2011, 2013). Here, we demonstrate that neither expression of the HPV8 E6 nor the E7 protein in primary human keratinocytes resulted in higher levels of S100A8 and S100A9. Rather, our study provides evidence that the β-HPV type 8-encoded transcription factor E2 up-regulates S100A8 and S100A9 on mRNA and protein levels. The relevance of this finding was further supported by the observation that calgranulin expression converges spatially with HPV5 E2 mRNA (Haller et al., 1995) in EV-lesions that display characteristic cytopathic effects of β-HPV infection. Notably, this activity seemed to be a unique feature of cutaneous HPV E2, since mucosal high-risk HPV16 E2 was unable to induce S100A8/A9 mRNA expression.

The HPV E2 protein regulates viral transcription through direct binding via its C-terminal domain to the palindromic $ACCN_6GGT$ motif in the HPV non-coding regulatory region, which can result either in gene induction or repression (McBride, 2013), and HPV8 E2 was additionally shown to bind to a non-classical site $ATCGN_4CGAT$ (Akgul et al., 2003). We have previously demonstrated that HPV8 E2 can also modulate the expression of a cellular gene, β4-integrin, via E2-binding sites (Oldak et al., 2004, 2010). E2-expressing cells lose β4-integrin expression, which normally anchors keratinocytes to the basement membrane. This may push E2-expressing cells into suprabasal layers, where they are destined to differentiate. Applying bioinformatic tools, however, we neither detected any classical E2 binding site in the S100A8 and S100A9 promoter regions nor a non-canonical site completely matching with $ATCGN_4CGAT$ (Akgul et al., 2003). Only in the human S100A8 promoter, a site with some similarities (ATCTGGCTGGAT) was detectable at positions −168 to −156, while not being present in the murine counterpart that was used in this study for reporter assays.

As an alternative mechanism to regulate cellular gene expression, HPV E2 engages cellular transcription factors via direct protein-protein interactions. This was first shown for cellular transcription factors of the C/EBP family (Hadaschik et al., 2003) and also for other factors (McBride et al., 2004; McPhillips et al., 2006; Wu et al., 2016). It was shown that E2 can form a ternary complex with C/EBP factors in a DNA-bound state at a C/EBP-specific binding site within the human involucrin gene, a classical marker of keratinocyte differentiation. As a consequence of its interaction with C/EBP factors, E2 leads to potent activation of the involucrin promoter (Hadaschik et al., 2003). Notably, C/EBPβ also directly binds to the S100A8 promoter region as demonstrated by chromatin immunoprecipitation (Miao et al., 2012). In fact, in our transient transfection experiments in RTS3b cells, HPV8 E2 alone was able to activate the murine S100A8 promoter containing the C/EBP but no putative E2-binding site. Moreover, in both, primary human keratinocytes as well as RTS3b cells, HPV8 E2 significantly enhanced PMA- or C/EBPβ-induced promoter activation, respectively. This strongly supported the notion that the molecular mechanism underlying the synergism between HPV8 E2 and C/EBPβ does not necessarily depend on a classical or non-classical E2-binding site but rather resembles the situation previously described for the human involucrin promoter (Hadaschik et al., 2003). However, with respect to the human S100A8 promoter, we cannot exclude that the ATCTGGCTGGAT sequence might further contribute to the E2 effect. In addition, human and murine S100A8 promoter regions are very similar with respect to their C/EBPβ binding site (Miao et al., 2012) but they are not identical. Therefore, we cannot exclude that the promoter region of the murine gene, we used for the reporter assays, can also bind to other transcription factors than those involved in the regulation of the human gene.

S100A8/A9 are potent pro-inflammatory mediators. Our preliminary analysis indicates that calgranulins are not the only pro-inflammatory factors that are up-regulated in cells co-expressing E2 and C/EBPβ, since we could also detect several other neutrophil attracting chemokines in their supernatants. These included IL-8, ENA-78 and NAP-2. The chemokine GRO-α was expressed constitutively and only slightly but non-significantly up-regulated in the E2 and C/EBPβ expressing cells (Supplementary Figure S4). The exact mechanisms underlying the regulation of these chemokines remain to be determined in the future. In our preliminary analyses, recombinant S100A8/A9

did not lead to induction of these chemokines indicating that their up-regulation was parallel to and not via S100A8/A9. In contrast, it has been demonstrated that calgranulins are involved in the induction matrix-metalloproteinases, such as MMP-9, which are involved in tumor growth and angiogenesis (Moon et al., 2008; Saha et al., 2010; Kerkhoff et al., 2012; Lim et al., 2016). From this it can be speculated that the E2-C/EBPβ-S100A8/A9 axis might exert further pleiotropic effects during carcinogenesis.

Apart from this, S100A8/A9, loss of β4-integrin and the induction of involucrin are all involved in keratinocyte differentiation. A role for HPV8 E2 in keratinocyte differentiation was also supported by observations in transgenic mice expressing the HPV8 E2 protein under the K14-promoter (Pfefferle et al., 2008). Morphologically, their skin presented obvious ulcerations and thinning of the epidermis. This was discussed as a putative consequence of premature keratinocyte differentiation and a reduced pool of proliferative keratinocytes consistent with our previous observation of β4-integrin suppression and involucrin induction (Hadaschik et al., 2003; Oldak et al., 2004, 2010; Pfefferle et al., 2008). Importantly, the skin of HPV8 E2-transgenic mice was also characterized by chronic immune cell infiltration and it was associated with spontaneous tumorigenic progression in a significant proportion of the mice (Pfefferle et al., 2008).

Collectively, these data provided evidence for a role of the E2 protein not only in differentiation but also in inflammation and skin tumor formation. This raised the question, how HPV8 E2 might augment tumor-promoting inflammation in skin.

The data presented in this study may provide this missing link. We demonstrate that HPV8 E2 engages the differentiation-inducing C/EBPβ pathway to up-regulate S100A8/A9 factors, which are not only part of the epidermal differentiation complex (Kuruto-Niwa et al., 1998; Miao et al., 2012) but also part of an inflammatory response promoting tumorigenesis (**Figure 6**).

AUTHOR CONTRIBUTIONS

SS, MP, and MO: conceptualization. SS and SL: funding acquisition. SS, MP, MO, AM, SL, and AW: investigation. SS, MP, AM, MO, CM, and BW-R: methodology. SS, CM, TV, MU, MM, and SM: resources. SS: supervision. SS, MP, MO, SL, and BW-R: validation. MP: visualization. SS and MP: writing the original draft. SS, MP, and SL: reviewing and editing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00392/full#supplementary-material

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HPV-Induced Field Cancerisation: Transformation of Adult Tissue Stem Cell Into Cancer Stem Cell

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Field cancerisation was originally described as a basis for multiple head and neck squamous cell carcinoma (HNSCC) and is a pre-malignant phenomenon that is frequently attributable to oncogenic human papillomavirus (HPV) infection. Our work on β-HPV-induced cutaneous squamous cell carcinomas identified a novel Lrig1+ hair follicle junctional zone keratinocyte stem cell population as the basis for field cancerisation. Herein, we describe the ability for HPV to infect adult tissue stem cells in order to establish persistent infection and induce their proliferation and displacement resulting in field cancerisation. By review of the HPV literature, we reveal how this mechanism is conserved as the basis of field cancerisation across many tissues. New insights have identified the capacity for HPV early region genes to dysregulate adult tissue stem cell self-renewal pathways ensuring that the expanded population preserve its stem cell characteristics beyond the stem cell niche. HPV-infected cells acquire additional transforming mutations that can give rise to intraepithelial neoplasia (IEN), from environmental factors such as sunlight or tobacco induced mutations in skin and oral cavity, respectively. With establishment of IEN, HPV viral replication is sacrificed with loss of the episome, and the tissue is predisposed to multiple cancer stem cell-driven carcinomas.

Keywords: HPV, field cancerization, adult tissue stem cells, skin cancer stem cells, squamous cell carcinoma

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INTRODUCTION

Human papillomavirus (HPV) infection is associated with oropharyngeal and anogenital cancers in both men and women. Approximately 90% of all cervical cancers are attributed to high-risk alpha-genus HPV (α-HPV) infections, also ∼60% of squamous cell carcinomas (SCC) of the vulva, vagina, anus and penis are due to infection of α-HPV (Crow, 2012). HPV infection is considered to be responsible for the rise in head and neck squamous cell carcinoma (HNSCC), particularly in cancers of the oropharynx and base of tongue (Marur et al., 2010; Leemans et al., 2011). Cutaneous HPVs, which are clustered in the evolutionarily distinct β-genus, have been also associated with the development of cutaneous SCC, especially in the immunosuppressed setting (Howley and Pfister, 2015; Quint et al., 2015).

Sequential genetic and epigenetic changes occur over several years and provide the transformational basis for both intraepithelial neoplasia (IEN) and ensuing epithelial cancers (carcinoma). The proportion of transformed cells within IEN can be graded and used to define the risk of invasive disease (FIGO Committee on Gynecologic Oncology, 2014). Progression to invasive carcinoma from IEN can take many years and there is often evidence of IEN at the excised tumor margins (Mao et al., 1996; Scholes et al., 1998).

As an entity, carcinoma account for over 70% of all malignancies and over 70% of all cancer mortality (Cancer Research UK, 2017[1]), hence the American Association for Cancer Research Task Force on the treatment and prevention of IEN recognizes the importance of early treatment to prevent invasive disease (O'Shaughnessy et al., 2002). Intriguingly, IEN can spontaneously regress. Although more often, IEN will progress to invasive malignancies (Dakubo et al., 2007). In epithelia susceptible to HPV infection, HPV early genes can cause IEN, notably E6 and E7 expression, and is therefore a mechanistic link to cancer, as such it represents a target for cancer prevention and is therefore the basis of this review.

FIELD CANCERISATION

Field cancerization, as a concept, was coined by Slaughter et al. (1953) to explain the occurrence of multiple foci of HNSCC. In all 783 HNSCC cases studied, the authors noted IEN at the peripheral margins of the resected primary malignancy. Where the tumor depth was less than 1 cm, they identified a second primary SCC focus in 43 of 88 cases. It is now clear that establishment of a premalignant epithelium, field cancerisation, is the basis for HNSCC, skin and cervical SCC.

Within field cancerisation, cells harbor a substantial number of mutations including those within known tumor suppressor genes, most frequently within the p53 gene (Bartkova et al., 1995; Ortiz et al., 2001; van Houten et al., 2002; Elgazzar et al., 2005; Merrick et al., 2006; Hu et al., 2012). In many tissues, the presence of mutant p53 clones is widely accepted as the hallmark of field cancerisation (**Figure 1**) (El-Naggar et al., 1995; Franklin et al., 1997). However, in cervical IEN, p53 is less frequently mutated (Akasofu and Oda, 1995). Within the early stage of field cancerisation there are multiple clones, but with increasing dysplasia severe field cancerisation becomes monoclonal (**Figure 2**) (Chung et al., 1995; Enomoto et al., 1997; Tate et al., 1997). Therefore, field cancerisation at its outset is polyclonal, implying that multiple cell lineages contribute to its occurrence as observed in active HPV infection.

Increasing mutational burden and greater dysplasia result in clonal selection, with a tendency toward mono-clonality (**Figure 2**). Clonal selection and expansion may result in a single clone in continuous epithelia (skin, oral and cervical tissues) or multiple clones in discontinuous epithelia (breast and lung) (Prevo et al., 1999; Simon et al., 2001; Larson et al., 2002; Tabor et al., 2002; Smeds et al., 2005). Within continuous epithelia, wherein HPV infection occurs, field cancerisation and ensuing cancers exhibit common epigenetic gene silencing, chromosomal anomalies, loss of heterozygosity, single nucleotide polymorphism, mutations, changes in mitochondrial genome, and altered gene expression (transcripts and proteins) (Ha et al., 2002; Tabor et al., 2004; Shen et al., 2005; Sui et al., 2006). Hence, severe IEN (part of the field cancerisation spectrum) that gives rise to multiple cancer, has limited numbers of clones.

The ensuing SCC that arise within continuous stratified squamous epithelia are clonal with respect to the underlying field cancerisation and severe IEN (**Figure 2**) (Sheu et al., 1995; Kim et al., 1996; Enomoto et al., 1997; Tate et al., 1997). However, the proliferative explosion of SCC cells results in multiple evolving clones, from acquisition of new mutations, which similarly undergo Darwinian evolutionary selection (McGranahan and Swanton, 2017). As a consequence, tumors arising from within IEN are genetically distinct (Nakashima et al., 1995; Shinmura et al., 1998). Hence, Darwinian evolutionary clonal selection determines the loss of clones in field cancerisation and determines the size of multiple clones within the emerging SCC.

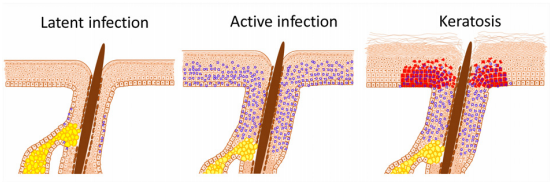

FIGURE 1 | HPV infection induced stem cell expansion. Latent HPV infection persists within epithelial stem cells and, in the case of β-HPV, within hair follicle keratinocyte stem cells; characterized herein by the presence of viral episomes (blue circles). Active replication, as occurs after immunosuppression or trauma, results in proliferation of stem cells beyond the niche; in the case of the skin into the interfollicular epidermis (IFE). Expansion and displacement of keratinocyte stem cells into the IFE renders them susceptible to UV induced transformation, resulting in field cancerisation. The archetypal initial lesion is clonal p53 mutation expression, within actinic keratoses (red cells).

HPV INFECTION AND STEM CELL EXPANSION

Human papillomavirus (HPV) binds epithelial cell heparan sulfate proteoglycans and cell specific receptors to gain entry by both clathrin-dependent and -independent endocytosis (McMillan et al., 1999; Day et al., 2003; Shafti-Keramat et al., 2003; Spoden et al., 2008; Schelhaas et al., 2012; Day and Schelhaas, 2014). Infection leads to the establishment of the HPV circular double-stranded genome as a stable episome within some cells of the basal layer (Dell et al., 2003). In the case of alpha-HPV, the viral genome can integrate into the host genome, whereas for beta-HPV, the viral genome remains episomal (Quint et al., 2015). Viral replication proteins E1 and E2 are required for the maintenance of the viral genome in the basal layer (Frattini et al., 1996; Stubenrauch et al., 1998; McBride, 2013). HPV infection of epithelial basal cells may be non-selective and by chance may involve adult tissue stem cells that reside in this layer.

[1]http://www.cancerresearchuk.org/health-professional/cancer-statistics/incidence/common-cancers-compared

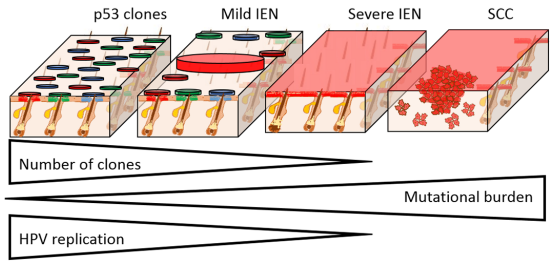

FIGURE 2 | Field cancerisation in the setting of HPV infection. The progression from IEN to SCC formation is characterized by the clonal selection, acquisition of mutations, and loss of HPV replication. Skin is used here as a HPV susceptible tissue, as we have previously shown that clonal expansion occurs from the hair follicle junctional zone. In mild IEN there are many clones, which in the case of p53 mutation susceptible tissues, may harbor individual distinct mutations as shown (circles with different colors). Progression of IEN is characterized by the selection and expansion of individual clones that have gained a proliferative advantage from additional mutations, culminating in severe IEN with full thickness epidermal dysplasia that is genetically uniform. With increasing mutational load, epidermal dysplasia increases and HPV episomes are lost. In the case of β-HPV types, the entire viral infection is lost, which accounts for the "hit and run" mechanism of transformation. From within the severe IEN, foci of SCC develop as shown, resulting in invasion into the underlying tissue.

Most HPV infections are spontaneously cleared. For example, the risk of α-HPV female genital infection over a lifetime is up to 80% (Syrjanen et al., 1990), but within 1–2 years most individuals clear the virus (Rodríguez et al., 2008). Although HPV may not specifically bind epithelial adult tissue stem cells for infection, as discussed earlier, persistent and or latent infection is presumed to involve epithelial adult tissue stem cells, but has not been determined for all tissues as stem cell markers are lacking (Schmitt et al., 1996; Boxman et al., 1997; Maglennon et al., 2011; Kranjec and Doorbar, 2016). Notably, the proposed reservoir for latent β-HPV infection has been the hair follicles (Boxman et al., 1997; De Koning et al., 2007; Galloway and Laimins, 2015; Quint et al., 2015; Hufbauer and Akgül, 2017; Tommasino, 2017). Animal models substantiated these clinical findings and moreover showed that the sub-populations of infected hair follicle cells have increased clonogenic potential, a hallmark of adult tissue stem cells (Schmitt et al., 1996; Lanfredini et al., 2017). In the oncogenic β-HPV8 transgenic mouse model we observed skin thickening (acanthosis), which was evident from birth and attributable to an expansion of the Lrig1 hair follicle adult tissue stem population (Lanfredini et al., 2017). In the absence of overt cutaneous lesions, such as papilloma or carcinoma, both unsorted and Lrig1+ keratinocytes demonstrated increased colony forming efficiency (increased clonogenicity) consistent with an expansion in keratinocyte stem cell numbers. Similarly, earlier studies on the cottontail rabbit model of HPV infection had also demonstrated the hair follicle to be the site of persistent HPV infection and, through similar colony forming assays, an expansion of the hair follicle junctional zone keratinocyte stem cells was reported (Schmitt et al., 1996). In these two studies, papilloma arose as a result of continued keratinocyte stem cell expansion into the adjacent overlying epidermis. For example, human benign cutaneous viral warts similarly result from keratinocyte stem cells expansion (Egawa, 2003). It is possible that the immune privilege provided by the stem cells niche prevents immune attack, thereby facilitating long term infection.

HPV-INDUCED EPIDERMAL PROLIFERATION

In cervical lesions caused by the α-HPVs, the viral oncogenes E6 and E7 increase proliferation of suprabasal epithelial cells. Along with E1 and E2, viral replication requires E6 and E7 for entry into S-phase. Upon leaving the basal layer, keratinocytes enter into a program of terminal differentiation in order to produce a protective barrier. However, in HPV infection, suprabasal cells continue to proliferate and are prevented from entering terminal differentiation (Sherman et al., 1997; Doorbar, 2006). Oncogenic viruses, including HPV, deregulate cell growth by disruption of pRb (retinoblastoma protein) binding to the E2F family of transcription factors though E7 binding pRb. Host p21 and p27 cyclin-dependent kinase inhibitors moderate the ability of E7 to drive cell proliferation in some cells (Doorbar, 2006; Tomaić, 2016). Inactive complexes with E7 and cyclin E occur within differentiating keratinocytes that express high levels of p21 and p27 (Noya et al., 2001; Akgül et al., 2006). In synchrony, high-risk α-HPV E6 prevent growth arrest or apoptosis by binding to p53, thus leading to p53 ubiquitination and degradation. In benign infections, proliferating cells remain in the epithelial basal layer, including within the hair follicle. As the infected cell enters the suprabasal cell layers of the epidermis, virus production is switched on resulting in viron assembly (Peh et al., 2002).

HPV INFECTION INDUCES STEM CELL EXPANSION AND SELF-RENEWAL PATHWAYS

Fluorescent labeling studies in mice using lineage tracing have concluded that stem cell division is prominently (∼90%) asymmetric; in which there is renewal of the stem cell and a daughter cell that is committed to terminal differentiation (Clayton et al., 2007; Doupé et al., 2012). Stochastic cell division in basal cells, including stem cells, can lead to HPV infection clearance. Mathematical modeling together with epidemiological data suggests that natural stem cell dynamics contributes >80% toward viral clearance rather than rejection by the immune system (Ryser et al., 2015). Thus, factors that promote adult tissue stem cell symmetrical cell division resulting in an increase in stem cell numbers may perpetuate infection accounting for the correlation between the increased risk of persistent infections associated cervical cancer and long-term use of combined oral contraceptives (Muñoz et al., 2006). This may also explain the basis for why trauma, ultraviolet light and repetitive exposure to the virus are essential in maintaining site-specific HPV infection (Kranjec and Doorbar, 2016).

Adult tissue stem cell expansion, as proposed for the mechanism of HPV-induced field cancerisation, is dependent on symmetrical division of existing stem cells. As discussed, HPV viral oncogenes will drive proliferation of infected adult tissue stem cells by targeting p53 or pRb. Importantly, the binding of E7 to pRb releases repression of both sex determining region Y-box 2 (Sox2) and octamer-binding transcription factor 4 (Oct4) (Kareta et al., 2015). Similarly, α-HPV E6 mediated degradation of p53 results increased Nanog expression, which is normally transcriptionally repressed by p53 (Lin et al., 2005). Thus, HPV early region genes promote self-renewal pathways.

In addition, high-risk α-HPV E7 induces expression of the key transcription factor Oct4 and also directly to enhance activation of its target genes (Brehm et al., 1997, 1999; Organista-Nava et al., 2016). Another key transcription factor, Kruppel-like factor 4 (Klf4), is also upregulated and hypoSUMOylated by high risk α-HPV E6 (Gunasekharan et al., 2016). Simultaneously, β-HPV E6 blocks differentiation by inhibition of C/EBPα, Notch signaling and Hes1 upregulation (Tyagi et al., 2016; Kranjec et al., 2017; Marthaler et al., 2017; Meyers et al., 2017). β-HPV E6 specifically binds to a cellular target MAML1, resulting in the inhibition of Notch-mediated transcription, which is important to keep infected keratinocytes in a proliferative state (Meyers et al., 2017). α-HPV E7 also prevents histone3 Lysine27 (H3K27) trimethylation and therefore maintains adult tissue stem cells in a permissive epigenetic state (McLaughlin-Drubin et al., 2011). Thus, HPV causes proliferation of adult tissue stem cells and maintains stemness of these cells as they egress from the stem cell niche, consistent with expression of stem cell proteins and observations *in vitro* of increased colony forming efficiency (Hufbauer et al., 2013; Lindquist et al., 2014).

TRANSITION FROM HPV-INDUCED STEM CELL EXPANSION TO IEN

The earliest evolution of HPV-induced stem cell expansion into visible lesions is the presence of dysregulated stratification within the epidermis, resulting in benign keratoses (the archetypal lesion in epidermodysplasia verruciformis) or cutaneous warts. Similarly, mucosal HPV lesions include condyloma or leukoplakia within the genitalia and oral mucosa (Cubie, 2013). In addition, persistent infections with high-risk HPV types simultaneously trigger neoplastic change (Rodríguez et al., 2010).

The transition from benign to premalignant lesion has been characterized by TP53 immunostaining, resulting from mutation acquisition, and manifesting as a small micro-clonal expansion comprising of 60–3000 cells presenting clinically as an actinic (solar) keratosis or leukoplakia (Jonasson et al., 1996; Ren et al., 1966; Ponten et al., 1997; Waridel et al., 1997; Garcia et al., 1999; van Houten et al., 2002). In the skin, these p53 micro-clonal patches were larger and more frequent in sun-exposed than sun-shielded areas, suggesting that mutations arise from UV. In addition, HPV is able to inhibit DNA repair through E6 protein expression, facilitating acquisition of p53 mutations (Wallace et al., 2012; Hufbauer et al., 2015; McKinney et al., 2015). Gain-of-function p53 mutation acquisition results in persistence of the protein within cells to promote transformation (Caulin et al., 2007).

Progression of field cancerisation toward severe IEN is associated with loss of the viral episome. In HPV infection, such as benign warts, epithelial proliferating cells remain in the basal layers, with genome amplification and virion assembly occurring within the suprabasal cell layers (Peh et al., 2002; Middleton et al., 2003). In the case of the high-risk HPV types the relative thickness of the basal layers is increased, presumably due to expansion in the number of adult tissue stem cells. Progression to IEN is characterized by a loss of terminal differentiation and therefore the expression of viral coat proteins is retarded (**Figure 2**) (Middleton et al., 2003). For example in cervical IEN, increasing dysplasia is associated with reduced virion production and loss of viral episomes. This phenomenon is even more evident in the case of skin infection by β-HPV types, which do not integrate into the host genome, and do not maintain viral DNA in the late stages of skin cancer progression. For example, SCC that develop within HPV associated Organ Transplant Recipient (OTR) field cancerisation no longer express β-HPV proteins (Borgogna et al., 2014) Similarly, HPV expression was lost during actinic keratosis transformation to SCC in a nude mouse xenograft model (Borgogna et al., 2018). Hence, the progression to cancer from IEN occurs independent of virus production, and for the beta genotypes in the skin, this is referred to as the "hit and run" mechanism of carcinogenesis (Howley and Pfister, 2015; Quint et al., 2015).

Field cancerisation emerging from HPV induced amplification of adult tissue stem cells results from additional environmental induced mutations. The area of IEN can be large, in the oral cavity it can be over 7 cm in diameter and is predisposed to multiple primary HNSCC and therefore poor prognosis (Tabor et al., 2002, 2004; Baxi et al., 2014). Intriguingly, HPV associated HNSCC demonstrate a favorable response to chemotherapy (Hayes et al., 2015; Vokes et al., 2015). Likewise, HPV and non-HPV vulvar SCC have distinct mutational profiles and moreover multiple primaries developing from within HPV IEN demonstrate separate clonal basis (Rosenthal et al., 2002; Hampl et al., 2007). Hence, HPV-induced adult tissue stem cell expansion risks the generation of IEN that in turn is predisposed to further transformation resulting in multiple primary tumors.

HPV INFECTION DRIVEN CANCER STEM CELLS

Many cancers exhibit hierarchical growth with evidence of differentiation consistent with the cancer stem cell model (Colmont et al., 2012). Wherein a subset of cancer cells, called "cancer stem cells", which continue to exhibit stem cell characteristic, serve to promote tumor growth through self-renewal with symmetric and asymmetric cell division (Patel et al., 2012; Colmont et al., 2013). There is evidence of active self-renewal in HPV-induced female genital tract cancers, cervical and vulval cancers, which characteristically express the stem cell transcription factors Sox 2, Oct4, and Hes1

(Brustmann and Brunner, 2013; Kim et al., 2015; Napoletano et al., 2016; Gut et al., 2018). In cervical cancer, HPV gene E6 can enhance self-renewal associated hedgehog transcription factor Gli1 expression and therefore increase cancer stem cell numbers (Vishnoi et al., 2016).

Head and neck squamous cell carcinoma (HNSCC) identification and characterisation of cancer stem cells has been supported by *in vitro* and *in vivo* assays (Prince et al., 2007). Similar to HPV-induced female genital tract cancers, the self-renewal associated transcription factor Sox2 was found expressed in HPV associated HNSCC, resulting from HPV E6/7 associated PI3K-AKT pathway activation (Keysar et al., 2016; Xi et al., 2016). The ensuing HPV-associated HNSCC retain cancer stem cell markers, CD44, CD24, ALHD1, and functional side population characteristics (Tang et al., 2013; Lindquist et al., 2014; Pullos et al., 2015). Overall, HPV associated HNSCC has favorable outcome compared to non-HPV associated HNSCC, and intriguingly this has been attributed to reduced cancer stem cell frequency in HPV HNSCC (Rietbergen et al., 2014; Vlashi et al., 2016). High numbers of cancer stem cells in HNSCC, irrespective of HPV status, is associated with poor outcome and lack of response to both radiotherapy and chemotherapy (Linge et al., 2016; Modur et al., 2016). Hence, the role of HPV to cause both normal stem cell and cancer stem cell expansion, may establish the basis for cancer stem cell driven tumor growth and influence cancer outcome.

CONCLUSION

This review has focused on HPV infection, notably oncogenic genotypes from both the alpha and beta genus. Within the tropic tissue that was breached to allow viral entry, persistent infection requires that resident adult tissue stem cells are infected. HPV-infected adult tissue stem cells, similar to other HPV-infected cells are forced to proliferate, leading to their expansion as adult tissue stem cells beyond their native niche. This expansion renders them susceptible to environmental carcinogens. In the case of skin, β-HPV genotypes induce hair follicle junctional zone keratinocyte stem cells to proliferate and expand into the overlying epidermis, whereupon they are susceptible to UV-induced mutations. Transformational mutations result in field cancerisation, with additional driver mutations, causing clonal selection as IEN progresses from mild to severe. Additional mutations then can give rise to multiple cancers. Hence, HPV-induced stem cell expansion predisposes to and, through viral oncogene expression, induces the generation of cancer stem cells, which in turn can define the fate of tumor and patient prognosis. Hence, we propose that the ability of oncogenic HPV infection to manipulate adult tissue stem cells underpin its ability to drive cancer growth through promotion of cancer stem cells.

AUTHOR CONTRIBUTIONS

CO, SL, and GP conceived the idea. CO, SL, CB, MG, and GP drafted the manuscript with inputs from all authors. All authors have made final approval for the final version to be submitted.

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β-HPV Infection Correlates with Early Stages of Carcinogenesis in Skin Tumors and Patient-Derived Xenografts from a Kidney Transplant Recipient Cohort

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Many malignancies that occur in high excess in kidney transplant recipients (KTRs) are due to viruses that thrive in the setting of immunosuppression. Keratinocyte carcinoma (KC), the most frequently occurring cancer type in KTR, has been associated with skin infection by human papillomavirus (HPV) from the beta genus. In this report, we extend our previous investigation aimed at identifying the presence of active β-HPV infection in skin tumors from KTRs through detection of viral protein expression. Using a combination of antibodies raised against the E4 and L1 proteins of the β-genotypes, we were able to visualize infection in five tumors [one keratoacanthoma (KA), three actinic keratoses (AKs), and one seborrheic keratoses (SKs)] that were all removed from two patients who had been both transplanted twice, had developed multiple KCs, and presented with a long history of immunosuppression (>30 years). These infected tissues displayed intraepidermal hyperplasia and increased expression of the ΔNp63 protein, which extended into the upper epithelial layers. In addition, using a xenograft model system in nude mice displaying a humanized stromal bed in the site of grafting, we successfully engrafted three AKs, two of which were derived from the aforementioned KTRs and displayed β-HPV infection in the original tumor. Of note, one AK-derived xenograft, along with its ensuing lymph node metastasis, was diagnosed as squamous cell carcinoma (SCC). In the latter, both β-HPV infection and ΔNp63 expression were no longer detectable. Although the overall success rate of engrafting was very low, the results of this study show for the first time that β-HPV$^+$ and ΔNp63$^+$ intraepidermal hyperplasia can indeed progress to an aggressive SCC able to metastasize. Consistent with a series of reports attributing a causative role of β-HPV at early stages of skin carcinogenesis through ΔNp63 induction and increased keratinocytes stemness, here

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we provide *in vivo* evidence that these events are also occurring in the affected skin of KTRs. Due to these β-HPV-driven molecular pathways, the nascent tumor cell is able to acquire a high enough number of carcinogenic insults that its proliferation and survival will eventually become independent of viral gene expression.

Keywords: β-HPV, skin cancer, xenograft, carcinogenesis, organ transplant recipients

INTRODUCTION

The prolonged use of immunosuppressive drugs in kidney transplant recipients (KTRs) is a recognized risk factor for infectious diseases and ensuing complications, including infection-related malignancies (Vajdic and van Leeuwen, 2009; Krynitz et al., 2013; Geissler, 2015). Keratinocyte carcinoma (KC), which includes basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), is the most frequent cancer type in KTRs, with an incidence rate ranging from 10 to 30% at 10 years post-transplantation (Kovach and Stasko, 2009; Madeleine et al., 2012).

Cutaneous human papillomaviruses (HPVs) from the β-genus, such as HPV5 and HPV8, have long been associated with KC in patients with a specific genetic background and in long-term immunosuppressed individuals (Genders et al., 2015; Howley and Pfister, 2015; Quint et al., 2015). Currently, more than 200 different HPV types have been described, which can be clustered into five genera (i.e., alpha-, beta-, gamma-, mu- and nu-papillomavirus) based on their taxonomy (http://pave.niaid.nih.gov/#home) (Bernard, 2013; Hufbauer and Akgül, 2017; Tommasino, 2017). Among them, mucosal α-genus HPV types are able to infect both oral and genital mucosa epithelia and are generally ranked as low-risk and high-risk types according to the tendency of the resulting neoplasm to undergo malignant progression (Kranjec and Doorbar, 2016). On the other hand, cutaneous HPVs, which are clustered in the evolutionarily distinct β-genus, can cause widespread unapparent or asymptomatic infections in the general population (Howley and Pfister, 2015; Quint et al., 2015). However, in immunosuppressed patients and in individuals suffering from the rare inherited disease epidermodysplasia verruciformis (EV), these viruses can spread unchecked and promote the development of KC. For example, β-HPV-associated wart-like lesions in EV patients are at high risk for progression to KC (Borgogna et al., 2012; Landini et al., 2014), and the ensuing tumors are positive for β-HPV DNA and protein expression (Dell'Oste et al., 2009).

Unlike cancers associated with mucosal HPV infections, cutaneous HPV-associated KC arises mainly in sun-exposed body areas, and hence these viruses are thought to cooperate with UV to induce cancer (Bouwes Bavinck et al., 2010, 2017; Quint et al., 2015). A series of reports have indeed demonstrated that expression of either β-HPV5 or 8 E6 substantially increases the mutagenic potential of UVB exposure (Wallace et al., 2012). For example, HPV8 E2, E6, and E7 each have oncogenic activities when expressed in basal epithelial cells of transgenic mice (Schaper et al., 2005; Pfefferle et al., 2008; Marcuzzi et al., 2009; Heuser et al., 2016). Furthermore, β-HPV E6s can alter Notch-mediated keratinocyte differentiation, thereby fostering a cellular environment conducive to viral replication (Meyers et al., 2013; Wallace and Galloway, 2014).

Although recent publications have, for the most part, characterized *in vitro* the effects of β-HPV oncogenes on host signaling pathways, it has become increasingly apparent that the dissection of the mechanisms underlying viral pathogenesis and oncogenesis in the affected skin areas during long-term HPV persistence would further our understanding of how such tissues are primed for cancer progression. Unfortunately, the exquisite host specificity of HPVs, which prevents experimental infections of heterologous hosts, has so far hindered the development of tractable animal models of infection. Moreover, it appears that β-HPV sequences are not essential for the maintenance of the transformed state as they are apparently lost from cancer cells, especially in non-EV tumors, at later stages of disease (Weissenborn et al., 2005).

In an effort to correlate β-HPV infection with disease pathology during progression of premalignant lesions to KC, we have set up an immunofluorescence protocol that allows both visualization of productive HPV infection in affected skin areas and evaluation of skin homeostasis through detection of the minichromosome maintenance complex component 7 (MCM7), a cellular proliferation marker (Borgogna et al., 2012, 2014). Using this protocol, in a previous study of a single center KTR cohort (Borgogna et al., 2014), we could observe active β-HPV infection in hyperplastic edges of skin SCC and BCC as well as in premalignant lesions, such as actinic keratoses (AK). Of note, while the $E4^+$ areas were mainly found within the disorganized epithelium of AK lesions, in more advanced tumors, such as skin SCC and BCC, they were always localized in the adjacent pathological epithelium. Furthermore, we detected MCM7 expression extending into the upper epithelial layers of E4 expressing areas ($E4^+$), indicating that epithelial cells were actively proliferating in areas of productive viral infection. Thus, based on these findings, we hypothesized that β-HPV-induced basal cell proliferation in the immunosuppressed setting alongside other transforming agents, such as UVB irradiation, might play a causative role at early stages of neoplastic transformation, while it may not be necessarily involved at later stages of the disease (Quint et al., 2015).

Emerging evidence also indicates that β-HPV can perturb p63 expression, a master regulator of development and maintenance of multilayered epithelia, including the epidermis (Meyers et al., 2013; Marthaler et al., 2017). p63, predominantly the ΔNp63 isoform, is selectively distributed in basal cells of stratified epithelia where it is required for the replenishment of stem cells (Senoo et al., 2007; Romano et al., 2009; Koster, 2010; Melino et al., 2015). In this regard, we have recently shown

that HPV8-induced field cancerization in a transgenic mouse model is due to Lrig1$^+$ keratinocyte stem cell expansion, which is accompanied by aberrant nuclear p63 expression in the expanded infundibulum and adjoining interfollicular epidermis. EV and non-EV keratoses displayed similar histology associated with β-HPV reactivation and nuclear p63 expression (Lanfredini et al., 2017).

In this study, we have further extended our previous analysis of a single center cohort of KTRs by (i) analyzing 128 additional skin lesions excised from 29 KTRs; (ii) correlating ΔNp63 expression levels with those of E4 and MCM7 in skin areas of productive HPV infection; and (iii) establishing and characterizing patient-derived xenografts as models to study skin cancer progression of β-HPV-infected skin lesions. In particular, we provide evidence of two KTRs with multiple β-HPV$^+$ skin tumors and demonstrate progression of a β-HPV$^+$ AK lesion into SCC in a patient-derived xenograft.

MATERIALS AND METHODS

Sample Collection

Tissue sections were obtained from 171 formalin-fixed and paraffin-embedded blocks (FFPE) corresponding to either the core or the edges of the tumor, collected from 128 skin lesions excised from 29 KTRs attending the dermatology unit in the University Hospital-Novara. Thirty-two tumors were derived from KTRs already described in our previous work (patients 1M, 3M, 9M, 14M, 15M, and 17M respectively; Borgogna et al., 2014).

The study was approved by the local ethic committee: Comitato Etico Interaziendale Novara (Prot. CE 168/15) and written informed consent was obtained by all subjects according to the Declaration of Helsinki.

Transplantation of Human Skin Tumors

The immunodeficient mice used for xenograft development were male athymic Nude-Foxn1nu (Envigo, Huntingdon, UK). Mice were housed under pathogen-free conditions in our animal facilities in accordance with The Guide for the Care and Use of Laboratory Animals and the experimentation was approved by the CESAPO (Comitato Etico Sperimentazione Animale Piemonte Orientale, Prot. 20093). Mice were anesthetized with an Ohmeda TEC4 Vaporizer using Isoflurane Vet (Merial, Ingelheim am Rhein, Germany) and Gelfoam dressing (Johnson & Johnson, New Brunswick, NJ) was implanted into the dorsal sub-cutaneous space together with primary human fibroblasts suspended in 100 μl Matrigel (BD Biosciences, Milano, Italia), and wounds were closed with surgical stitches (Ethicon, Johnson & Johnson, New Brunswick, NJ). After 14 days, mice were anesthetized and human skin tumors (∼0.5 cm^3) were xenografted into the stromal bed together with 10^6 primary human fibroblasts suspended in 100 μl Matrigel (Patel et al., 2012). Xenograft growth was weekly monitored by measuring the longer diameter of the tumor mass by caliper. After 3 or 6 months, mice were sacrified *via* CO_2 inhalation and tumors were removed: half of them was fixed in 10% neutral-buffered formalin and embedded in paraffin blocks, while the other half was snap frozen and stored at −80°C. Five-μm sections were cut from the FFPE blocks and stained with hematoxylin and eosin (H&E) as performed for human skin biopsies.

HPV Genotyping

Total DNA was extracted from the frozen xenograft or plucked hair bulbs from eyebrows using QIAamp DNA Mini Kit following the manufacturer's instructions (Qiagen, Milan, Italy). β-HPV DNA analysis was carried out using broad spectrum PCR (PM-PCR) in combination with a reverse hybridization system [RHA Kit Skin (beta) HPV (Diassay, Rijswijk, the Netherlands)] as previously described (de Koning et al., 2006).

Immunofluorescence Labeling

DNA-FISH and immunofluorescence detection of β-HPV E4, MCM7, β-HPV L1 were performed on formalin-fixed and paraffin-embedded skin biopsies or xenografts using standard techniques as previously described (Borgogna et al., 2012, 2014). Briefly, DNA-FISH was performed using as probe the complete biotin-labeled (Biotin-nick translation kit, Roche Diagnostics GmbH, Mannheim, Germany) genomic HPV plasmid DNA (100 ng/ml). For the other markers analyzed (β-HPV E4, MCM7, β-HPV L1, and ΔNp63), antigen unmasking was performed by heating the slides in a conventional decloaking chamber for 10 min at 121°C in 10 mM citrate buffer at pH 6.0 (Vector Laboratories, Burlingame, CA, USA). Immunofluorescence was carried out using the following antibodies: rabbit anti-β-HPV E4 (kindly provided by John Doorbar, 1:1,000), mouse anti-MCM7 (1:200, Neomarkers Fremont, Fremont, CA), rabbit anti-ΔNp63 antibody (clone Poly6190) (BioLegend, San Diego, CA, USA) (1:500) and rabbit anti-β-HPV L1 (1:1,000). Images were acquired using a digital scanner (Pannoramic MIDI; 3D Histech Kft., Budapest, Hungary). For the assessment of histological features, the slides analyzed by immunofluorescence were disassembled and stained with H&E. The production of polyclonal antibodies to β-HPV E4 (HPV5E4) and β-HPV L1 has been previously described (Borgogna et al., 2012, 2014). Briefly, the broad spectrum anti-L1 antibodies were raised against the highly conserved region between amino acid 200 and 300 of the L1 protein, while those against the HPV5E4 crossreact with many genotypes from species 1 only, including HPV8, 14, 20, 24, 25, and 36.

RESULTS

Visualization of β-HPV Viral Protein Expression in Skin Tumors from the KTR Cohort

In our previous work, we had already analyzed 111 FFPE blocks obtained from 79 skin lesions of 17 patients from a cohort of KTRs for the presence of active β-HPV infection by immunostaining with anti-E4 and anti-L1 antibodies. Among those specimens, we could find E4$^+$ areas in six FFPE blocks from four patients corresponding to four AK lesions and the adjacent pathological area of one SCC and one BCC (**Table 1**). In this study, we have analyzed 171 additional FFPE blocks from 128 skin lesions excised from 29 KTRs following the same procedure. Within this second group, two patients, who were not included

TABLE 1 | Overview of the skin biopsy blocks from the KTR cohort study examined by immunohistochemistry for the presence of β-HPV infection and grouped according to pathology diagnosis.

	Seborrheic keratoses, n = 18	*Keratoacanthoma, n = 16*	*Bowen's disease, n = 3*	*Actinic keratoses, n = 54*	*Basal cell carcinoma, n = 77*	*Squamous cell carcinoma, n = 39*	*Total Lesions, n = 207*
	FFPE blocks n = 20	*FFPE blocks n = 21*	*FFPE blocks n = 3*	*FFPE blocks n = 71*	*FFPE blocks n = 107*	*FFPE blocks n = 60*	*Total FFPE blocks n = 282*
β-HPV-positive lesions	1	1		7	1	1	11
Previous study (Borgogna et al., 2014)				4	1	1	6
This study	1	1		3			5

The skin biopsies found positive for β-HPV E4 expression in our previous study (Borgogna et al., 2014) and this current one are indicated separately.

in the previous survey, presented with multiple skin lesions some of which displayed areas of β-HPV infection.

The first case was a female KTR who underwent a second kidney transplant (KTx) in 2016 due to the failure of her first one after 25 years. She displayed many flat, reddish papular lesions widespread in the total body skin as shown by the representative picture of the forehead (**Figure 1A**) that were very much resembled the EV keratoses, and her clinical history reported the development of many proliferative skin lesions (>15) in different body sites starting approximately 10 years after her first kidney transplant, which was carried out in 1980. A total of 22 FFPE blocks corresponding to the 13 tumors excised from this patient from 2014 in our hospital, each diagnosed as SCC ($n = 5$), BCC ($n = 3$), AK ($n = 2$), or keratoacanthoma (KA) ($n = 3$), were made available for immunofluorescence analysis. β-HPV$^+$ areas were found in one KA located on the leg, and in two AKs located on the hand and the leg (**Table 1**). As shown in representative (**Figures 1C,D**), E4$^+$ cells were clearly evident in the areas of an AK lesion presenting with hyperkeratosis and parakeratosis; some of them were trapped in the keratin layers where L1$^+$ nuclei were also observed (data not shown). The cellular proliferation marker MCM7, a subunit of DNA helicase, was more evident in the E4$^+$ area compared with the adjacent epithelium, and extended to suprabasal layers, indicating that cells were primed to enter the cell cycle. In the AK found on the hand, E4 expression was not detected, while nuclei stained with the β-HPV late capsidic protein (L1) were well evident in the upper layers of the epithelium where some differentiation was still occurring, indicating the presence of productive viral infection (data not shown).

The second case was a male KTR born in 1960 who underwent kidney transplant twice, with the first one taking place in 1996. From 2012 onwards, the patient developed five skin cancers in different body sites diagnosed as BCC ($n = 2$), AK ($n = 2$) and seborrheic keratoses (SK; $n = 1$). Positivity for β-HPV markers was found in one AK and in the SK removed from the back and the left forearm, respectively. The virus-positive AK displayed highly keratotic epithelium with clear signs of β-HPV-related cytopathic effects (**Figures 2A,B**). Immunofluorescence analysis showed large areas of β-HPV E4 expression in the most superficial layers of the epithelium and increased MCM7 expression in the basal and suprabasal layers (**Figure 2C**). Likewise, in the SK we found a large number of E4$^+$ cells spread throughout the entire lesion as shown in **Figures 2E–G**. Consistent with the benign nature of this skin tumor, MCM7 expression in the basal layer was less evident, while it was highly expressed in the suprabasal cells overexpressing cytoplasmic E4. These cells located in the mid to upper epithelial layers had likely re-entered the cell cycle to support viral genome amplification as E4 expression usually occurs in cells where viral DNA is replicating.

ΔNp63 Expression in Skin Tumors

To ascertain whether p63 induction could also be visualized in virus-positive skin cancers from the KTR cohort, tissue sections from all the skin lesions excised from the two aforementioned patients ($n = 18$) were co-labeled with antibodies against ΔNp63 and MCM7. In addition, six skin lesions (SCC $n = 1$, BCC $n = 1$ and AK $n = 4$) with areas positive for E4 expression from our previous survey were similarly analyzed. As depicted in the representative **Figure 3** showing a KTR-derived BCC identified in the first round of screening (patient 5M, Borgogna et al., 2014), the ΔNp63 protein was highly expressed in the neoplastic area in comparison with the non-pathological adjoining epithelium (**Figure 3D** vs. **Figure 3J**), and its expression generally overlapped with that of MCM7 (**Figures 3C,I** respectively). This staining pattern was reproducibly observed in the high-grade lesions, including SCC ($n = 6$) and BCC ($n = 6$). Of note, in the adjacent β-HPV-infected hyperplastic epithelium as determined by E4 staining (**Figure 3F**), ΔNp63 expression extended in the middle-superficial layers, higher than those expressing MCM7, indicating that ΔNp63 was expressed in cells either proliferating or with a great potential of proliferation although not yet transformed. A similar pattern of ΔNp63 expression was also observed in the benign SK shown in **Figure 2**, where it spread across the entire epithelium despite the fact that MCM7 was restricted to some cells in the basal layer and some cells in the intermediate layers (**Figures 2G,H**). Also in the case of AK ($n = 8$) and KA ($n = 3$), ΔNp63 expression exceeded that of MCM7 as it was still detectable in the more superficial nucleated cells. Quantification of ΔNp63 and MCM7 positive cells confirmed the increase

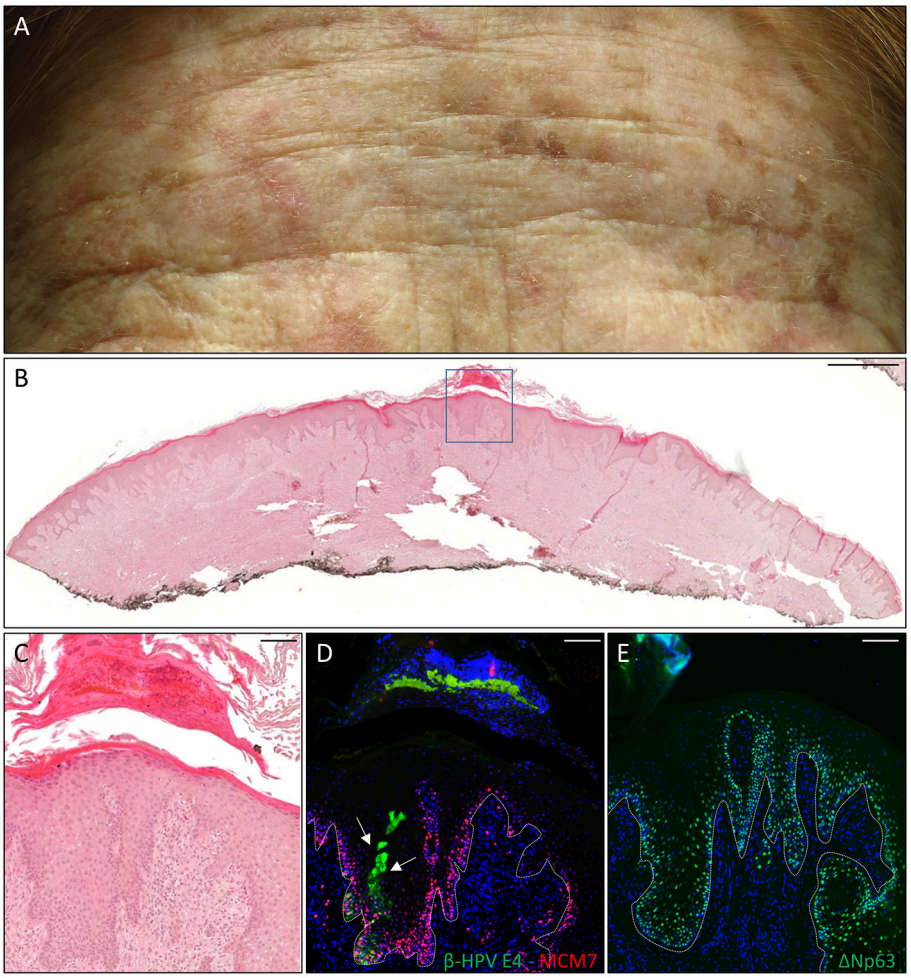

FIGURE 1 | Histopathological analysis of an actinic keratoses (AK) from a female kidney transplant recipient (KTR). **(A)** Flat whitish and reddish papular lesions on her forehead. **(B)** Hematoxylin and eosin (H&E) staining of a representative tissue section (scale bar: 1,000 μm) from one AK lesion on the leg of the patient. **(C)** Magnification of the region highlighted by the blue box shown in panel B (scale bar: 100 μm). **(D)** Immunofluorescence staining for beta human papillomavirus (β-HPV) E4 and minichromosome maintenance complex component 7 (MCM7) (β-HPV E4 in green; MCM7 in red) of the same tissue section shown in **(B,C)**. The white arrows indicate E4 staining. **(E)** Immunofluorescence staining for ΔNp63 (green) of a serial section. The white dotted line indicates the basal layer. Sections in **(D,E)** were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue) to visualize cell nuclei.

in $\Delta Np63^+$ cells in both β-HPV-infected cells and tumors vs. non-pathological epithelium (Supplementary Figure 1). In the case of β-HPV-infected cells, the number of $\Delta Np63^+$ cells was significantly higher than that of $MCM7^+$ cells.

Development of Xenografts in Athymic Nude Mice

A well-established model to study tumor progression and clinical outcome is represented by patient-derived xenograft (PDX) models. PDXs are developed by implanting tumor samples obtained directly from patients in immunodeficient mice. In this regard, we have previously shown that the creation of a humanized stromal bed, by first implanting Gelfoam dressing along with HDF, facilitates primary human skin SCC xenograft growth in nude mice (Patel et al., 2012).

Thus, we asked whether premalignant skin lesions could be similarly engrafted so that we could monitor progression or regression of a lesion that otherwise would be surgically removed from the patient. For this purpose, we engrafted all skin lesions available from the cohort study in the period of observation (3 years), including those that clinically appeared as benign and/or premalignant. From a total of 42 lesions engrafted following this procedure, we were able to collect 38 xenografts—in four cases no tumor growth was detected. The main characteristics of the KTRs whose skin tumors were grafted into nude mice are shown in Supplementary Table 1, and the histological features of the tumors are listed in **Table 2**. At the beginning, tumors were removed 6 months after the graft (15 out of 38). Following this timeline, growth was obtained for two BCCs whose xenograft histology closely mirrored that found in the original tumor. The human origin of the xenograft tumors was confirmed by staining

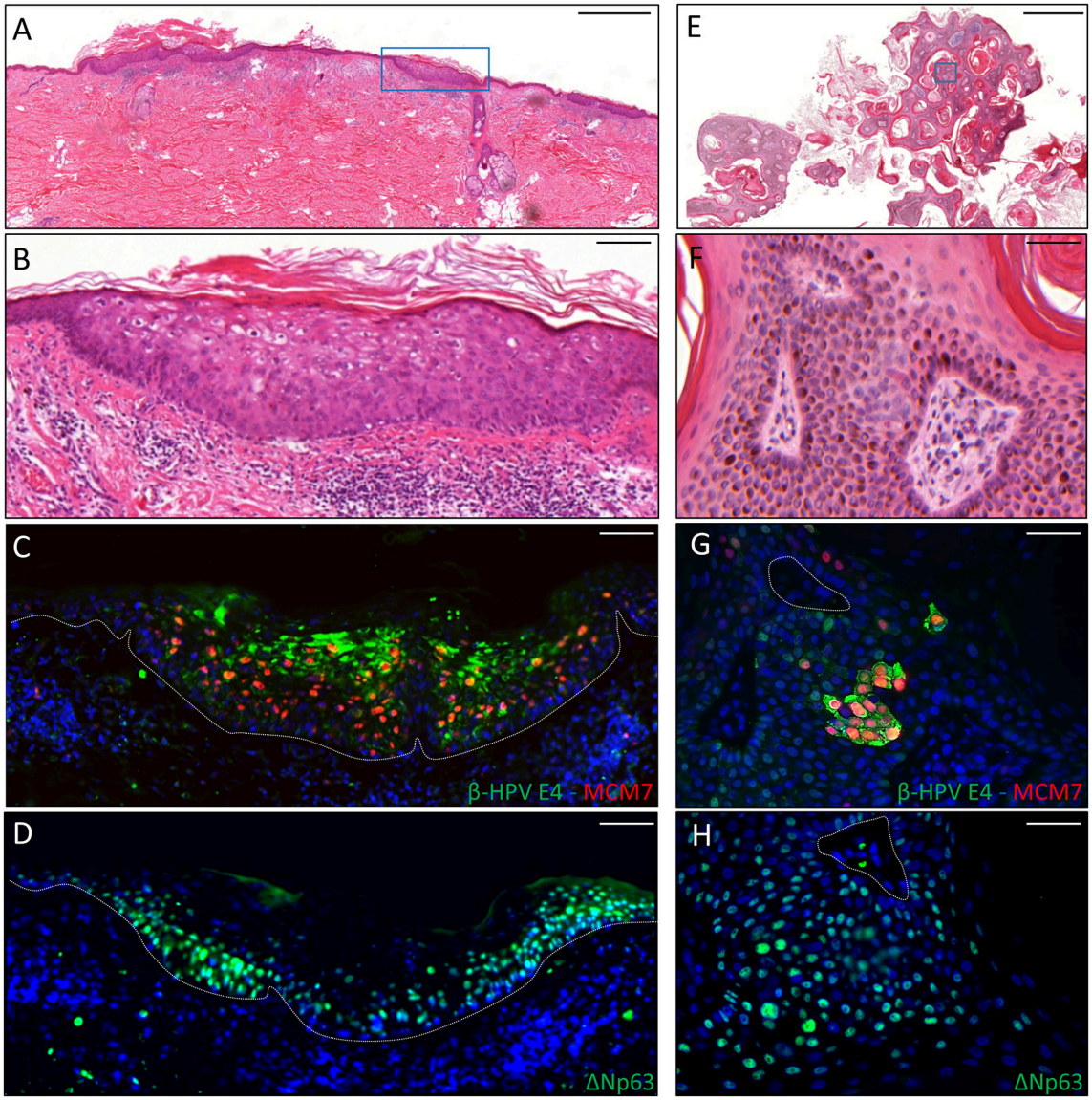

FIGURE 2 | Immunohistochemical staining for β-HPV E4, MCM7, and ΔNp63 in one AK lesion **(A–D)** and one seborrheic keratoses (SK) lesions **(E–H)** derived from a male KTR. **(A)** H&E staining of a representative tissue section (scale bar: 1,000 μm) from one AK lesion on the back of the patient. **(B)** Magnification of the region highlighted by the blue box shown in **A** (scale bar: 100 μm). **(C)** Immunofluorescence staining for β-HPV E4 (green) and MCM7 (red) of the same tissue section shown in **(A,B)**. **(D)** Immunofluorescence staining for ΔNp63 (green) of a serial section. The white dotted line indicates the basal layer. **(E)** H&E staining of a representative tissue section (scale bar: 1,000 μm) from one SK lesion on the forearm of the patient. **(F)** Magnification of the region highlighted by the blue box shown in **E** (scale bar: 50 μm). **(G)** Immunofluorescence staining for β-HPV E4 (green) and MCM7 (red) of the same tissue section shown in **(E,F)**. **(H)** Immunofluorescence staining for ΔNp63 (green) of a serial section. Sections **(C,D,G,H)** were counterstained with DAPI (blue) to visualize cell nuclei.

with human cytokeratin (data not shown). Unfortunately, all other cases ($n = 13$) turned out to be epidermal cysts filled with cornified material and surrounded by dying epithelial cells. This pitfall prompted us to shorten the length of the growing period in nude mice to 3 months. Using this modified timeline, three out of the 23 skin tumors engrafted gave rise to a tumor mass with well-defined histological features. Three AKs successfully grew in nude mice; two of them displayed a growth curve similar to that of the two BCCs, while one grew faster, especially after week 8 (Supplementary Figure 2). While the formers xenografts retained the original histological features, the latter, which was derived from the aforementioned male KTR, was diagnosed as SCC along with its ensuing lymph node metastasis (**Figure 4**). Although β-HPV markers were well evident in multiple areas of the original AK (**Figures 2A–D**), they were no longer detectable in the SCC, not even by PCR amplification of xenograft total DNA using broad spectrum primers for beta genotypes (data not shown, de Koning et al., 2006). This SCC turned out to be

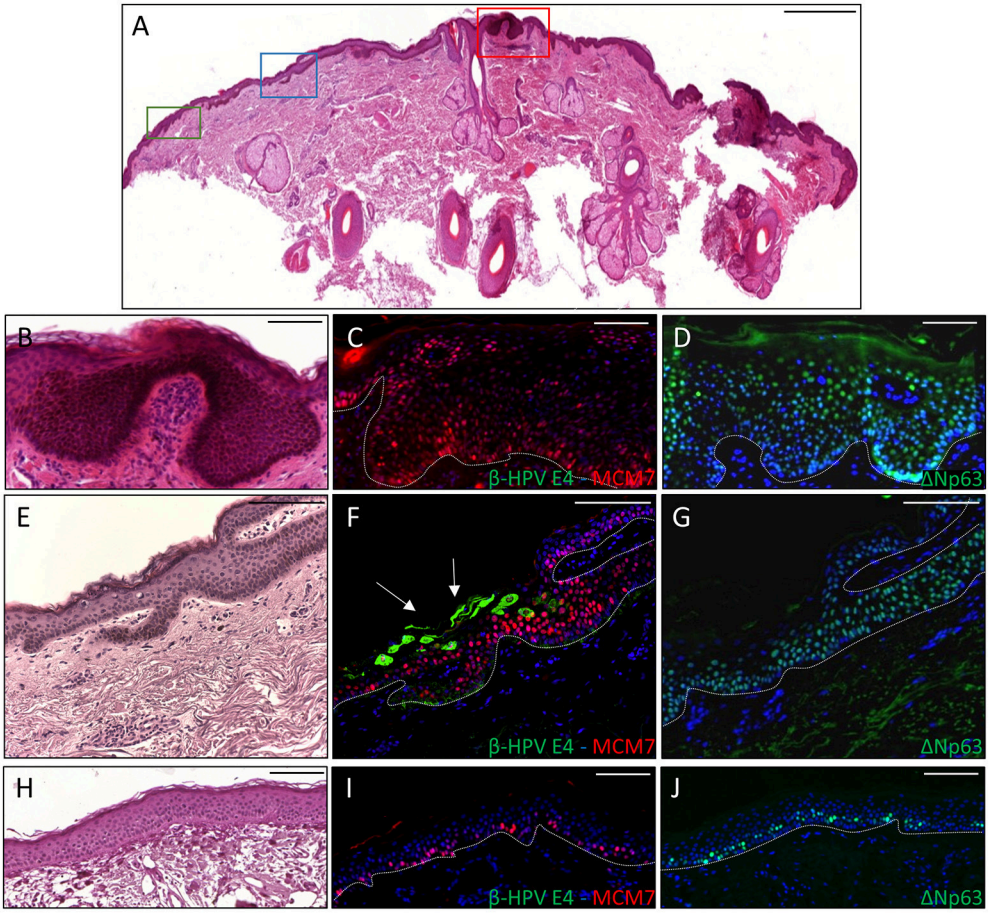

FIGURE 3 | Immunohistochemical staining for β-HPV E4, MCM7, and ΔNp63 in one basal cell carcinoma (BCC) from a KTR (patient 5M, Borgogna et al., 2014). **(A)** H&E staining of a representative tissue section (scale bar: 1,000 μm). The red square highlights the BCC, the blue square indicates the HPV-infected area in the adjacent epithelium, and the green square shows non-pathological epithelium. **(B,E,H)** Magnification of the regions highlighted by the boxes shown in **A** (**B**: red square; **E**: blue square; **H**: green square). Scale bar: 100 μm. **(C,F,I)** Serial sections obtained from the same specimens depicted in panels B, E, and H, respectively, were subject to double staining using antibodies against β-HPV E4 (green) and MCM7 (red). The white arrows indicate E4 staining. **(D,G,J)** Serial sections obtained from the same specimens depicted in **(B,E,H)**, respectively, were stained with antibodies against ΔNp63 (green). The white dotted line indicates the basal layer. All sections were counterstained with DAPI (blue) to visualize cell nuclei.

negative for ΔNp63 expression by immunohistochemistry (data not shown), while it was strongly positive for MCM7. From the same patient, we also implanted the β-HPV$^+$ SK shown in **Figures 2E–H**, which failed to grow in nude mice probably due to its benign nature. One of the two other AKs was from a patient transplanted in 1995 who had developed three AKs and one SCC after 20 years of transplantation. In this case, we failed to detect any viral protein expression in both xenograft and original tumor as it was the case in all the other skin lesions excised from this KTR (data not shown). The second AK was from the KTR female described above (**Figure 1**). Consistent with the fact that a documented β-HPV reactivation was reported in the female patient as well as in her original skin lesion, in this latter xenograft, we were able to detect in some cells both viral protein E4 expression and the presence of the HPV5 genome by FISH (**Figure 5**). The HPV5 probe was used because this genotype was detected in plucked hair bulbs from the eyebrows (data not shown), which are regarded as representative samples of the virus carriage on a person's skin. Furthermore, one SCC and one BCC were engrafted from the same patient, but they failed to grow in nude mice.

DISCUSSION

In this study, we extend our previous investigation aimed at identifying the presence of active β-HPV infection in skin tumors from KTRs through the detection of viral protein expression, such as E4 and L1 (Borgogna et al., 2014). In this new round of screening, we found positive staining in five tumors that were all removed from two patients who had been both transplanted twice and presented with a long history of immunosuppression (>30 years). The female patient had developed many skin cancers (>15) accompanied by the development of a clinical picture highly resembling that of an EV patient. Consistent with

TABLE 2 | Histological features of the skin tumors grafted into nude mice, timeline of excision and outcome of xenograft establishment.

	No. of tumors (tot: 42)	Xenograft excision after		Xenograft establishment	
		3 months	6 months	Failure	Growth
Squamous cell carcinoma	6	6	0	6	
Basal cell carcinoma	16	9	7	14	2 (BCC)
Keratoacanthoma	1	0	1	1	
Actinic keratoses	14	6	8	11	- 2 (AK) - 1 (SCC+ metastasis)
Seborrheic keratoses	5	3	2	5	

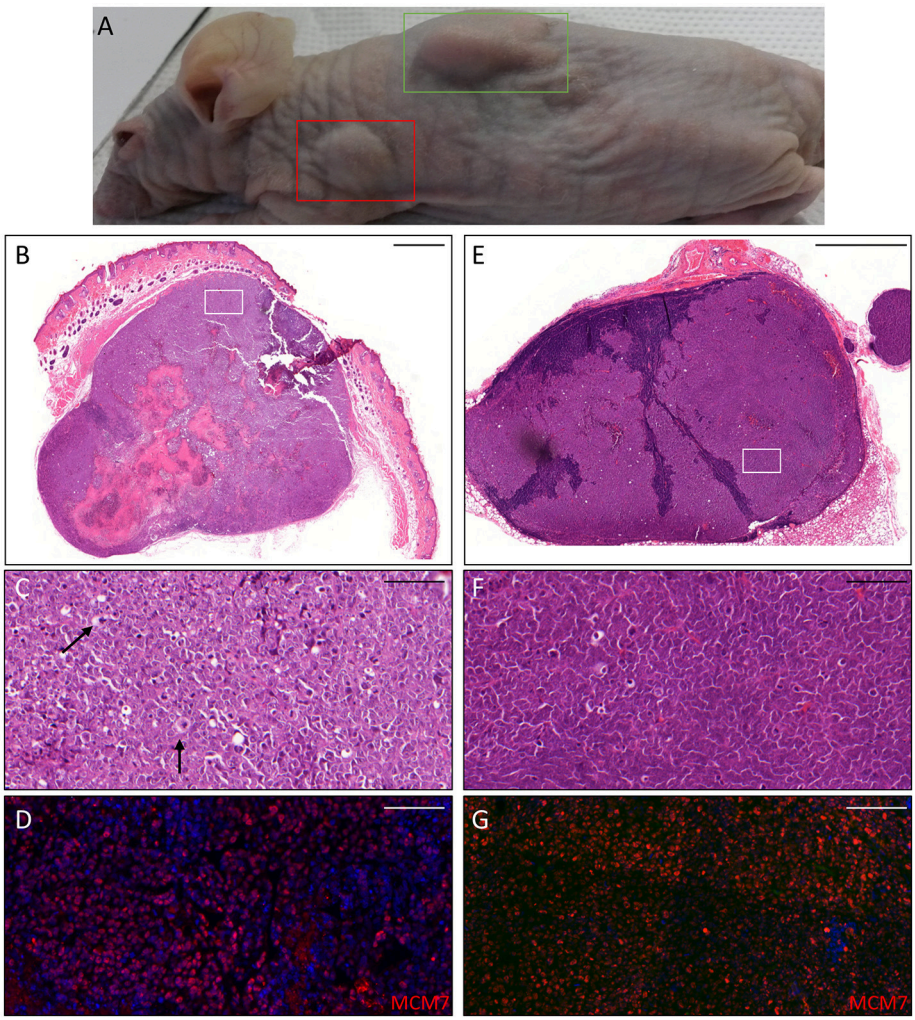

FIGURE 4 | Histopathological analysis of an AK xenograft derived from the male KTR. **(A)** Nude mouse in which the xenograft was diagnosed as SCC (green box) giving rise to a lymph node metastasis (red box). **(B,E)** Histopathological features (H&E staining) of the lesions corresponding to the boxes highlighted in **A** (**B**: SCC; **E**: lymph node metastasis). Scale bars: 1,000 μm. **(C,F)** Magnification of the areas highlighted by the white boxes depicted in **(B,E)**. Scale bar 100 μm. The black arrows indicate cells in mitotic phase. **(D,G)** Immunofluorescence staining for MCM7 (red) of the same tissue section shown in the **(C,F)** respectively. Sections **(D,G)** were counterstained with DAPI (blue) to visualize cell nuclei.

her clinical manifestations revealing diffuse actinic damage on her forehead and dorsum of the hands, β-HPV viral protein expression was observed in three skin tumors, corresponding to two AKs and one KA. Likewise, among the five skin tumors removed from the other male patient, one AK and one SK showed extensive E4 expression.

According to our previous findings, E4 expression was mostly detected at early stages of disease, confirming that virus expansion was taking place in the skin under condition of prolonged immunosuppression, albeit no longer detectable in more advanced stages of disease (Borgogna et al., 2012, 2014; Landini et al., 2014). These infected tissues displayed intraepidermal hyperplasia as demonstrated by histology, enhanced expression of the MCM7 proliferation marker in the suprabasal layers, and ΔNp63 expression. Intriguingly, ΔNp63 protein expression levels were also high in both benign and premalignant lesions as well as in cells that did not express MCM7 and, therefore, were not regarded as actively proliferating.

Overall, given the important role of p63 in the proliferation and maintenance of epidermal stem cells, and a number of *in vitro* and *in vivo* data linking β-HPV infection to ΔNp63 upregulation (Lanfredini et al., 2017; Marthaler et al., 2017), our findings further strengthen the proposed model of β-HPV-induced skin carcinogenesis in an immunosuppressed setting through p63 activation and increased keratinocyte stemness. Consistently, using a novel bitransgenic mouse model where ΔNp63 is overexpressed moderately in the basal layer of stratified epithelia, Devos et al. have recently demonstrated that overexpression of ΔNp63 favors early steps of skin tumorigenesis (Devos et al., 2017). Development of benign lesions, such as papilloma, was indeed accelerated following ΔNp63 overexpression in a dose-dependent manner in a classical two-stage chemical carcinogenesis protocol where an increased number of papilloma was observed.

Emerging evidence clearly indicates that β-HPVs contribute to skin cancer development at early stages and through molecular mechanisms that are different from those reported for high-risk α-types, such as HPV16 and 18 (Howley and Pfister, 2015; Quint et al., 2015). Our group and others have reported that β-HPV can increase stemness properties of keratinocytes in both monolayer cultures of keratinocytes transduced with HPV8 E6 and E7 viral proteins and an HPV8 transgenic mouse model where the skin-specific transcription of the early region of HPV8 is driven by the K14 promoter (Hufbauer et al., 2013; Lanfredini et al., 2017). In all these experimental models, as well as in β-HPV-infected human skin, this increased stemness is always accompanied by ΔNp63 overexpression (Marthaler et al., 2017). Thus, together with previous reports pointing to a possible cooperation between E6 expression and UVB exposure in skin carcinogenesis, our findings provide *in vivo* evidence that β-HPV infection might be a co-factor of early stages of skin cancer progression. This hypothesis is fully compatible with the "hit-and-run" mechanisms of carcinogenesis, with cutaneous β-HPV playing an important role for tumor initiation and progression, but not required for tumor maintenance. Assuming that viral replication is more active at very early stages of carcinogenesis, or when lesions are not yet even clinically evident and rarely removed, it seems reasonable to propose that at later stages of disease, when lesions require surgical excision, β-HPV infection is no longer detectable or otherwise restricted to some residual areas at the edges of the lesion. These assumptions can explain the low percentage of positivity found in our study. However, the observed reactivation of β-HPV infection occurring in the skin of KTRs alongside the demonstration that these viruses can increase keratinocyte stemness and interfere with the DNA damage response argues in favor of a model whereby the cooperation between β-HPV and UVB, which triggers skin carcinogenesis, is no longer required to maintain the malignant state at later stages of neoplastic development (Hufbauer and Akgül, 2017; Lanfredini et al., 2017; Wendel and Wallace, 2017).

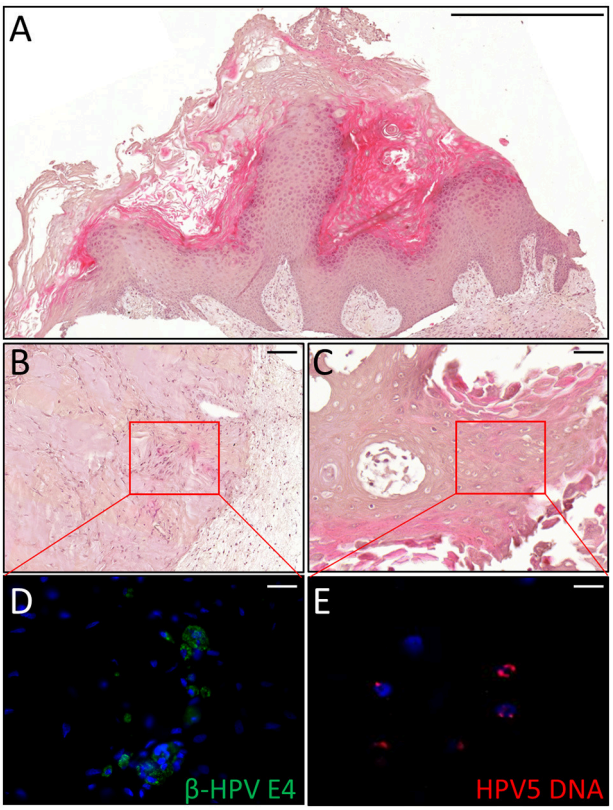

FIGURE 5 | Histopathological analysis of an AK xenograft derived from the female KTR. **(A)** H&E staining of a representative sections of the xenograft. Scale bar 1,000 μm. **(B,C)** H&E staining of two representative areas positive for the viral markers. Scale bars: 100 μm. **(D,E)** Magnification of the areas corresponding to the red squares highlighted in the top pictures and immunostained for β-HPV E4 protein (green, **D**) and HPV5 DNA (red, **E**). Sections **(D,E)** were counterstained with DAPI (blue) to visualize cell nuclei. Scale bars: 10 μm.

Another important aspect of the current study is the optimization and implementation of a unique xenograft model system in nude mice (Patel et al., 2012). Thanks to the creation of a humanized stromal bed in the site of grafting, here we show that it is possible to obtain xenograft growth also from small biopsy samples from relatively benign skin lesions such as AKs. However, the overall success rate of this procedure was very low as only three out of 14 engrafted AKs grew as PDXs. Furthermore, we failed to grow any SCCs while two out of the 16 engrafted BCCs gave rise to a tumor mass with the same histological features of the original tumor. This low rate of success may be due to the low proliferating index of the tumor, especially for the AK, or the size of the engrafted tumor, which in some cases was small and very likely did not include enough cells with high

proliferating capacity. Despite these limitations, the results of this study are extremely exciting because we show for the first time that β-HPV$^+$ and ΔNp63$^+$ intraepidermal hyperplasia can indeed progress to an aggressive SCC, including metastasis. As this SCC developed in 12 weeks in a nude mouse, we cannot rule out that the engrafted AK already harbored small amount of SCC. Nevertheless, cancer cells were originated from the infected epithelium that clinically appeared as field cancerization. Furthermore, the observation that both ΔNp63 and β-HPV are no longer detectable in the SCC suggests that their roles might be more important at premalignant stages where they likely cooperate to create an environment conducive to skin cancer progression.

Overall, the xenografts obtained following our protocol may prove useful not only in following the fate of each lesion but also in increasing the availability of biological samples for molecular investigations, which is generally limited due to surgical removal of premalignant lesions. In this regard, every effort should be made by clinicians involved in the daily management of KTRs to identify patients with clinically evident virus reactivation in the skin, and as such at increased risk of developing cancer.

AUTHOR CONTRIBUTIONS

MG, MD and GP conceived the project. CB and CO designed experiments. CB, CO, SL, FC, RB, and ET performed experiments and collected, analysed data. EZ and PS enrolled patients and collected clinical samples. MG drafted the manuscript with inputs from all authors. CB and MG handled funding, supervision, and coordination. All authors have made final approval for the final version to be submitted.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Cutaneous Papillomaviruses and Non-melanoma Skin Cancer: Causal Agents or Innocent Bystanders?

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There is still controversy in the scientific field about whether certain types of cutaneous human papillomaviruses (HPVs) are causally involved in the development of non-melanoma skin cancer (NMSC). Deciphering the etiological role of cutaneous HPVs requires – besides tissue culture systems – appropriate preclinical models to match the obtained results with clinical data from affected patients. Clear scientific evidence about the etiology and underlying mechanisms involved in NMSC development is fundamental to provide reasonable arguments for public health institutions to classify at least certain cutaneous HPVs as group 1 carcinogens. This in turn would have implications on fundraising institutions and health care decision makers to force – similarly as for anogenital cancer – the implementation of a broad vaccination program against "high-risk" cutaneous HPVs to prevent NMSC as the most frequent cancer worldwide. Precise knowledge of the multi-step progression from normal cells to cancer is a prerequisite to understand the functional and clinical impact of cofactors that affect the individual outcome and the personalized treatment of a disease. This overview summarizes not only recent arguments that favor the acceptance of a viral etiology in NMSC development but also reflects aspects of causality in medicine, the use of empirically meaningful model systems and strategies for prevention.

Keywords: cutaneous papillomaviruses, hit-and-run mechanism, *Mastomys coucha*, causality, skin cancer, animal models, NMSC

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Abbreviations: AK, actinic keratosis; ALK, ALK Receptor Tyrosine Kinase; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3-related protein; BPV1, bovine papillomavirus 1; BRAFi, Inhibitors of the B-Raf Proto-Oncogene; CDKN2A, cyclin-dependent kinase inhibitor 2A; cGMP, current good manufacturing practice; CKIT, KIT proto-oncogene receptor tyrosine kinase; CREBBP, CREB binding protein (CREB, CAMP responsive element binding protein 1); DDR, DNA damage response; E6AP, Ubiquitin Protein Ligase E3A; EGFR, epidermal growth factor receptor; FAT1, FAT atypical cadherin 1; FGFR3, fibroblast growth factor receptor 3; HIPK2, homeodomain-interacting protein kinase 2; HRAS, Harvey rat sarcoma virus oncogene; KA, Keratoacanthoma; KRAS, Kirsten rat sarcoma viral oncogene homolog; MAML1, mastermind like transcriptional coactivator 1; McPV2, *Mastomys coucha* papillomavirus 2; MHC, major histocompatibility complex; MmuPV1, Mus musculus papillomavirus type 1; MnPV, *Mastomys natalensis* papillomavirus; NMSC, non-melanoma skin cancer; OTRs, organ transplant recipients; PIK3CA, Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha; pRb, RB transcriptional corepressor 1; Pten, phosphatase and tensin homolog; SCC, squamous cell carcinoma; TLR, Toll-like receptor; TP53, tumor protein P53 (human); *Trp53*, transformation related protein 53 (mouse).

THE PROBLEM – A SERIOUS AND COMMON DISEASE: NON-MELANOMA SKIN CANCER

Non-melanoma skin cancer is the most frequent cancer in fair-skinned individuals worldwide and its incidence has increased during the last three decades (Welsh et al., 2011; Lomas et al., 2012). There are up to 3 million new cases every year, whereby women below 45 years are even more affected (Al-Dujaili et al., 2017). Based on different UV exposure rates, there are also geographical variations, with the highest incidence of NMSC in Australia (Lomas et al., 2012). Although the metastatic potential and the mortality rates in immunocompetent individuals are low in comparison to malignant melanomas, NMSC has a strong impact on the quality of life of the affected persons and the financial burden on health care systems. Taking the United States as an example, total annual expenses for NMSC medical care amounts to 650 million dollars (Apalla et al., 2017). Hence, there should be a quite obvious public health interest to understand and to prevent this cancer entity.

Non-melanoma skin cancer principally refers to cancer derived from keratinocytes (Small et al., 2016) and can be further divided into basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs), with relative frequencies of 80 and 20%, respectively (Eisemann et al., 2014). While BCCs are not very different in their biological behaviors, SCCs show broad histopathological diversities that are associated with markedly different clinical outcomes. Depending on the immune status of the patient, these may range from indolent tumors with low metastatic capacity to aggressive tumors with high invasive potential. Approximately 97% of invasive SCCs are found in association with malignant progression of an actinic keratosis (AK), considered as a precursor of SCCs. These lesions can display erythematous, scaling, and rough patches. They can either spontaneously regress, persist as a benign AK or progress to an invasive SCC where cells infiltrate the basement membrane into the dermis (reviewed in Yanofsky et al., 2011). TP53 is found to be mutated in dysplastic AKs, indicating that functional loss is a very early event, connected with loss of chromosomes including the cell cycle regulator p16INK4A (Ratushny et al., 2012). To define some general histopathological features, a committee of European dermatologists has recently identified telangiectasia, atrophy, and pigmentation disorders as the most reliable markers for AKs (Dreno et al., 2017).

Other clinically common lesions are keratoacanthomas (KAs) which can form spontaneously but, after a proliferative and subsequent resting phase, have the tendency to regress, apparently due to apoptosis. Histologically, a KA is an exo-/endophytic growing squamous proliferative tumor with a characteristic central keratin plug, surrounded by epidermal lips, forming a relatively well-defined symmetrical structure (Takai, 2017). Of note, approximately 25% of KAs can develop into SCCs (Sanchez Yus et al., 2000). Although still controversially discussed in terms of whether a KA is a variant of SCC, other dermatologists believe it represents a self-resolving benign epidermal lesion (reviewed in Savage and Maize, 2014). This illustrates the current discrepancies between histopathological phenotypes and deep sequencing DNA profiles of respective specimens, since KAs and SCCs are distinct entities at least by their unique molecular gene signatures (Ra et al., 2015). Nonetheless, according to the "World Health Organization Classification of Tumors" as a guideline for pathologists in diagnostics, KAs are still regarded as a well-differentiated SCC (LeBoit et al., 2006).

Another thought-provoking inquiry that has clinical significance in the context of NMSC pathogenesis emerged during the last years, when patients with advanced metastatic melanomas were treated with BRAF inhibitors (BRAFi) (e.g., vemurafenib or dabrafenib) (reviewed in Gibney et al., 2013). The off-target outcome of such drugs results in deleterious dermatological side effects, histopathologically ranging from *verrucal keratosis,* hair follicle changes, plantar hyperkeratosis, KAs and finally cutaneous SCCs (Anforth et al., 2013). Vemurafenib also has a photosensitizing activity (Boussemart et al., 2013), consistent with the finding that most SCCs can be detected at chronically sun-damaged skin regions (Hassel et al., 2015).

ENDOGENOUS AND ENVIRONMENTAL RISK FACTORS FOR NMSC

The Individual Genotype

Cancer in general is a multi-factorial disease and as individual as the patient, a reasonable concept that is culminating in the contemporary discourse of personalized medicine (reviewed in Mavropoulos et al., 2014; Harwood et al., 2016). Referring to this notion, meta-analyses of the current literature have deciphered several risk factors that are either distinct in terms of an inherent genetic predisposition of an individual patient and/or caused by environmental factors (reviewed in Wang et al., 2014; Belbasis et al., 2016). Individual risk factors for NMSC are age, family history, pigmentation, polymorphisms within genes encoding IL10, IL4R, TNF, or TNFR2 (Welsh et al., 2011), several inborn genetic disorders like *Xeroderma pigmentosum* (Black, 2016), the basal cell nevus syndrome (reviewed in Bresler et al., 2016) or the WHIM (Wart, Hypogammaglobulinemia, Infection, and Myelokathexis) syndrome, an autosomal dominant inheritance immune deficiency, harboring mutations in the chemokine receptor CXCR4 (Chow et al., 2010; Meuris et al., 2016). Such patients show a high susceptibility for both cutaneous and mucosal HPVs. Multiple warts can occur on hands and feet, but also genital condylomata acuminata can be found (Leiding and Holland, 2012). In the case of patients suffering from the rare hereditary disease *Epidermodysplasia verruciformis* (EV), mutations within at least two genes of homologous transmembrane channel-like (TMC) proteins, TMC6 (EVER1) and TMC8 (EVER2) were described (Lazarczyk et al., 2009, 2012). These proteins are involved in zinc homeostasis and are not only expressed in keratinocytes, but also in lymphocytes, suggesting additional functions in the immune response (reviewed in Kalinska-Bienias et al., 2016). Due to their interaction with the Zn^{2+} transporter protein ZnT-1, TMC6/8 can further modulate MAP kinases

and in turn AP-1, a transcription factor involved in many signal transduction pathways (reviewed in Lazarczyk and Favre, 2008).

Immune Status

Another important risk factor is iatrogenic immunosuppression as SCCs appear 65–250 times more frequently in OTRs compared to the general population (reviewed in Nindl and Rösl, 2008; Vinzón and Rösl, 2015). Moreover, the frequency of tumor formation correlates with the extent and duration of immunosuppression (reviewed in Euvrard et al., 2003; Hampton, 2005). It is estimated that up to 40% of OTRs will develop skin cancers within the first 10 years of transplantation and up to 80% after 20 years (Rangwala and Tsai, 2011). Although early detection of NMSC evidently improves the overall survival rate, a 20 year follow-up study including more than 85,000 patients showed that OTRs have a much worse prognosis and a higher mortality rate as compared to immunocompetent individuals (Acuna et al., 2016). The exact physiological reason is still unexplained, but it is assumed that tumors are simply more aggressive in an immunosuppressive environment since substances like cyclosporine both promote tumor invasion and favor neovascularization (reviewed in Geissler, 2015). Immunosuppressive drugs apparently also impair the DDR and counteract p53-dependent cellular senescence (Wu et al., 2010; Kuschal et al., 2012). Since SCCs develop mostly within sun-exposed areas, UV exposure is immune compromising (reviewed in Nakad and Schumacher, 2016). Despite the obvious increase of NMSC, population-based studies of skin cancer mortality after organ transplantation and in immunocompetent individuals are still scant (John et al., 2016). This is mainly due to geographic variability of incidence rates and, most importantly, the exclusion of NMSC from central cancer registries records (reviewed in Apalla et al., 2017) as recently noticed in the "*European Code against Cancer: Infection and Cancer*" (Villain et al., 2015). Such an obvious gap in cancer documentation should be filled as exemplified by initiatives such as the "Keratinocyte Carcinoma Consortium" (Madeleine et al., 2017), because NMSC incidence is increasing worldwide and caused by daily and life-long risk factors such as UV exposure and skin infections (Garrett et al., 2016; Prasad and Katiyar, 2017).

Sun Exposure

Another major environmental risk factor that has to be taken into account for the development of NMSC is the cumulative lifelong exposure to UV light, as well as sunburns in youth (Kennedy et al., 2003). UV light is divided into three categories according to its energy (wave length), which are UVA (320–400 nm), UVB (290–320 nm), and UVC (100–290 nm). UVC is almost completely filtered by the atmospheric ozone layer, which also shields up to 90% of UVB radiation. Thus, only UVA and UVB play roles as risk factors. Although not penetrating in the skin as deep as UVA, due to its higher energy output, UVB is more harmful and can damage cells heavily (reviewed in Seebode et al., 2016). UVB has also additional deleterious side-effects on the immune system, for example, by perturbing the function of antigen-presenting cells and in turn increasing a local immunodeficiency (reviewed in Prasad and Katiyar, 2017). Thus, cumulative UV exposure can contribute to skin tumor development by impairing immune surveillance of the skin (Maglennon et al., 2014). UV has also direct effects on the DNA, leading to a covalent joining of adjacent pyrimidines to form cyclobutane pyrimidine dimers (CPDs). Due to the misincorporation of an adenine at the position complementary to the UV DNA damage, CPDs predominantly lead to C→T transitions (≥60%) and rarely to CC→TT transitions (5%), leaving characteristic signatures within the DNA that can be detected in the landscape of SCCs upon whole genome sequencing (reviewed in Brash, 2015).

However, it is still not clear how to functionally rank the significance and frequencies of such mutations since the appearance of NMSC is, like other forms of cancer, the result of a multi-step process in which the temporal/spatial order and the impact on tumor formation cannot retrospectively be defined. Moreover, comparatively little is known about the temporal combinatory linkage of altered gene expression within a cellular network, thus contributing to the individual manifestation of a tumor (Krogan et al., 2015). Notably, deep-sequencing analyses of normal skin sections of persons with different histories of sun exposures even revealed identical UV specific signatures in their genome with exactly the same genes affected as later found in SCCs or BCCs (Martincorena et al., 2015). The reasons why such mutations are tolerated in normal cells suggest a counter-selective environment consisting of stromal, endothelial, and inflammatory components that prevent the outgrowth of clones finally forming a tumor. Considering recent concepts, cancer is not simply a linear route, but a highly flexible and emergent adaptation process of certain cells where clonal expansion is following according to evolutionary principles that limit the predictability of therapeutic outcomes (reviewed in Greaves, 2015; Lipinski et al., 2016; Davis et al., 2017).

Cutaneous Papillomaviruses

Since individuals are "open systems," the most critical exogenous risk factor for NMSC is not only UV exposure, but also infections with papillomaviruses. These target keratinocytes of the skin and mucosa of different vertebrate species, including humans. Up to date more than 200 human papillomavirus (HPV) types are known according to the PaVE database[1] (Van Doorslaer et al., 2017), which are associated with different clinical manifestations. Genital HPVs cause diverse lesions ranging from benign warts (for "low-risk" HPV types) to different malignancies (for "high-risk" types) of which cervical cancer is the most prominent (zur Hausen, 2002; McLaughlin-Drubin et al., 2012).

On the other hand, there is also a wide range of cutaneous HPV types that have been associated with diverse skin diseases (reviewed in Grce and Mravak-Stipetic, 2014). Including more than 100 healthy human volunteers in a broad study of meta-genomic analyses, whole-genome shotgun sequencing revealed an overall HPV prevalence of 69% in the skin, followed by the vagina (42%), the mouth (30%), and the gut (17%) (Ma et al., 2014). Another study even demonstrated that more

[1] https://pave.niaid.nih.gov

than 95% of all viral sequences detected in skin samples belong to the papillomavirus family, mostly to the beta-and gamma genera. Here, also co-infections could be discerned, which leads to speculation about potential temporal inclusion/exclusion interaction mechanisms between different HPV types in the same cell that may modulate the immune response and in turn viral persistence (Bzhalava et al., 2014).

High prevalence of cutaneous HPV can be already detected in skin of infants and young children, indicating that viral exposure occurs very early in life (Antonsson et al., 2003). Recurrent types are HPV2, 7, 27, 57 (alpha genus), HPV23 and 75 (beta genus), HPV4 and 65 (gamma genus) and HPV1 (mu genus) that typically cause only benign lesions such as common warts (*verrucae vulgaris*), plantar warts (*verrucae plantaris*), and flat warts (*verruca plana*) (Jablonska et al., 1997; Cardoso and Calonje, 2011; Bruggink et al., 2012) which usually spontaneously regress (van Haalen et al., 2009). However, they can represent a serious problem for immunocompromised individuals, especially OTRs, who frequently suffer from confluent wart formation all over the body. The prevalence within this population ranged from 48 to 92% in the first 5 years after immunosuppression (Jablonska et al., 1997; Wang et al., 2014). The HIM study ("cutaneous HPV infection in healthy men") reported a different median time of incidence (=newly diagnosed in skin) and duration of persistence of beta and gamma HPV infections ranging between 6–8 and 6–11 months, respectively. Older volunteers showed a significant higher association with prevalent and persistent HPV infections (Hampras et al., 2014). However, merely monitoring the skin for the presence of a viral DNA does not allow any conclusions about an ongoing infection and the exposure of viral antigens to the immune system. Such questions can only be answered when combined with immunological studies to get estimations about the rates of seroconversion and the serodynamics of cutaneous HPV infections (Rahman et al., 2016), an important criteria when preclinical models are used (see below).

"High Risk" Cutaneous HPVs?

The term "infection" indicates the presence of a microbe which can be pathogenic, commensal or opportunistic (reviewed in Casadevall and Pirofski, 2000). Hence, considering the individual complexity of the human skin virome that in turn also represents an interactome with a defined microbial environment (Hannigan et al., 2015), it is a huge challenge of modern biomedicine to dissect such networks in terms of functionality and causality. Indeed, the skin is an open ecosystem, colonized by a broad spectrum of bacteria and viruses whose landscape and impact on local immunity change during their individual life span and by environmental factors (Belkaid and Segre, 2014). We still do not understand how these different constituents dynamically interact with other microorganisms colonizing the skin, what the selective advantage of such a commensalism is, how the virome in turn affects the transcriptome of the skin (Edqvist et al., 2015), form the individual immunophenotype and how these properties vary with respect of individual skin types, under immunosuppression or UV exposure (Virgin, 2014). The categories commensal, pathogenic or opportunistic are helpful notions in the context of tumor viruses (Moore and Chang, 2017). Accordingly, commensal tumor viruses (e.g., EBV or cutaneous HPVs), are almost ubiquitously spread and usually do not cause symptoms. Contrarily, pathogenic tumor viruses are not so frequent and acquired, for instance via sexual intercourse or blood transfusion (e.g., high-risk mucosal types of HPV or Hepatitis B virus, HBV). Due to their ubiquitous prevalence in the general healthy population, there is still skepticism whether cutaneous HPVs are causally involved in NMSC development. However, there are many epidemiological and mechanistic *in vitro/in vivo* data showing increasing evidence that at least some commensal HPVs represent an essential, if not even a decisive factor for this type of skin cancer, especially for SCC (Madeleine et al., 2013; Wang et al., 2014; Chahoud et al., 2016).

Historically, the oncogenic potential of beta-type HPV infection (e.g., HPV5 and HPV8) in NMSC has been originally identified in patients suffering from EV, characterized by an increased susceptibility to viral infection (Orth, 2006). The oncogenic and transforming capacity of the EV-HPVs has not only been shown in transgenic mouse models (Schaper et al., 2005; Pfefferle et al., 2008; Viarisio et al., 2011), but also in organotypic raft cultures under *in vitro* conditions (Borgogna et al., 2012; Heuser et al., 2016b; Marthaler et al., 2017). Beta-type papillomaviruses were also detected in NMSCs of non-EV patients, although the viral loads are usually very low. It is assumed that the reservoirs for latent infection are hair follicle stem cells (Hufbauer et al., 2013). Different studies have reported HPV DNA in 30–50% of NMSCs from immunocompetent persons (Harwood et al., 2006), whereas in immunosuppressed patients this figure goes up to 90% (Harwood et al., 2000; Arnold and Hofbauer, 2012). Transcriptionally active beta-type HPVs can be found in premalignant lesions such as AKs (Borgogna et al., 2014) but also in normal skin and plucked eyebrow hairs suggesting a functional correlation between the presence of viral DNA and an increased risk of NMSC (Neale et al., 2013). UV exposure *per se* can also directly stimulate viral transcription of the EV-types HPV5 and HPV8 (Akgül et al., 2005b), while other cutaneous types such as HPV38 E6/E7, for instance, can block UV-induced expression of the toll-like receptor 9 (TLR9), normally responsible for endosomal sensoring of exogenic DNA (Pacini et al., 2017). This shows the complexity of a virus–host interaction, demonstrating several modes of action by different HPV types to interfere with signal transduction pathways of the host cell. Moreover, in contrast to anogenital tumors, there are also SCCs that either completely lack HPV or not all cells are virus-positive (Neale et al., 2013), indicating that viral oncoprotein expression is not necessary to maintain a malignant phenotype (reviewed in Arron et al., 2011; Hufbauer and Akgül, 2017).

SOME REFLECTIONS ABOUT THE "CAUSALITY" OF INFECTIONS

The lack of HPV in cancerous skin lesions is in apparent contradiction to the so-called Henle-Koch's postulates that were traditionally used as criteria for "causality" of an infection

(reviewed in Inglis, 2007). However, one should keep in mind that the conception of causality is a term historically referring to physical properties of the inanimate matter and not to living systems. Physicists deal with reproducible regular events and experimental settings, while cancer research is investigating "anomalous" scenarios, sometimes rare disorders in terms of cancer development which are, as aforementioned, multi-factorial and individually shaped. Hence, the simple detection of an infection or frequent mutations found in genetically non-predisposed persons cannot be considered as "causal," because they are only contingent and the individual clinical outcome is determined by many additional factors (reviewed in Greaves, 2015). More correct in an epistemic sense is the word "etiology," since this term has a more suitable connotation, reflecting more the complexity of an organism and not implying necessarily a direct and quite obvious causal relationship.

Nonetheless, in principle the Henle-Koch's postulates state that an infectious agent should be consistently detected in the respective lesions, it should be isolated and its re-inoculation should induce the same symptoms or clinical manifestations. However, these postulates have their limitations, because they are not considering multi-factorial events in the development of a disease (reviewed in Edwards and Rohwer, 2005; Virgin, 2014; O'Malley, 2016). Moreover, they also do not reflect asymptomatic, latent or persistent infections, all states in which HPV DNA can exist (Sudenga and Shrestha, 2013). Anecdotally, Robert J. Huebner, Chief of the Laboratory of RNA Tumor Viruses (NCI) recognized this inadequacy by referring it as the "The Virologist's Dilemma" (Huebner, 1957). As a consequence, he paved the way for the inclusion and particularly the acceptance of epidemiological data to corroborate the involvement of a virus as a cause for a specific disease (Vonka, 2000).

The progress in molecular technologies and immunological methods (Falkow, 2004) therefore led to a revision of these criteria, by both including epidemiological and molecular data as well as terminologies like "plausibility" (=whether a causal relationship makes biological sense) and "coherence" (=asking whether causality is compatible with present knowledge of the disease), respectively (Fredricks and Relman, 1996). Such an extension avoids an important category of empiricism, namely the notion of "falsification" and the consequential dump of a theory in favor of another (Buchanan et al., 2006). Revised criteria rather allow the inclusion of other scenarios to explain the absence of viral sequences in some SCCs. For instance, considering the importance of cell–cell communication in a tumor microenvironment, it is also conceivable that a small fraction of HPV-positive cancer cells secrete paracrine acting vesicles (e.g., exosomes) that may stimulate abnormal proliferation of surrounding virus-negative cells and in turn tumor growth (Dalla Pozza et al., 2017; Harden and Munger, 2017). However, the strongest arguments for at least an obligatory initial function of certain viruses in cancer development are vaccination strategies where the prevention of tumor formation is the most stringent read-out criteria for the success of a vaccine (Moore and Chang, 2017).

Hence, the question of an etiological or merely an opportunistic role of beta-HPV types in skin cancer development is accomplished in a concept, referred as the "hit-and-run" mechanism of microbe/virus induced carcinogenesis (reviewed in Galloway and McDougall, 1983; Iwasaka et al., 1992; Niller et al., 2011; Hufbauer and Akgül, 2017). Alternatively, the term "indirect carcinogen" was suggested (Mossman et al., 2004), but this semantically points more toward a substance or an irradiation event as environmental carcinogen than to an infectious agent and confuses the notion of prophylactic vaccination to prevent tumor formation induced by an "indirect" carcinogen. However, a "hit-and run" model of gene regulation is not without precedent. As already suggested several decades ago (Schaffner, 1988), recent experiments indeed showed that certain transcription factors (e.g., bZIP1) epigenetically change the histone code after transient binding ("hit") that allows the assembly of transcription complexes to continue gene expression after its dissociation ("run") (reviewed in Varala et al., 2015). Whether certain HPV types also use this mechanism remains to be elucidated.

Nevertheless, numerous seroepidemiological reports support the notion of an association between beta-HPV infection and SCC or its precursors (Andersson et al., 2012; Chahoud et al., 2016), despite the occasional absence of the viral DNA within malignant lesions. Here, another distinct property between mucosal and cutaneous HPV types may account for the occasional loss of viral DNA during skin carcinogenesis, namely the physical state. In contrast to anogenital cancer, cutaneous HPVs do not integrate into the host genome and persist as extrachromosomal elements in a defined copy number. Since HPVs completely depend on the host cell replication machinery, UV-induced DNA damage and the mode of accompanying repair mechanisms may disrupt the maintenance of episomal DNA during viral persistence (reviewed in Doorbar, 2016; Bristol et al., 2017; Wendel and Wallace, 2017).

HOW CAN A "HIT-AND-RUN" MECHANISM BE EXPLAINED?

Accepting tentatively an etiological function of beta-HPV in conjunction with UV exposure in NMSC development, several studies have identified different mechanisms that may reasonably explain a viral contribution to a "hit-and-run" mode of carcinogenesis. Cutaneous HPVs infect and persist as extrachromosomal genomes in basal keratinocytes especially around hair follicles (Weissenborn et al., 2012) which are a reservoir for stem cells with different properties (Jaks et al., 2010). During wound healing (Donati et al., 2017) and skin cancer development in transgenic mice expressing the complete early region of HPV8 (Lanfredini et al., 2017), these cells become activated and start to proliferate.

The main targets of mucosal HPVs are p53 and pRB, which is sufficient to transform the host cell and initiate uncontrolled proliferation (see Doorbar et al., 2012 and references herein), by impairing many downstream pathways such as cell cycle arrest, metabolism, apoptosis, and cross-talks to the immune system

(Harris and Levine, 2005; Rozan and El-Deiry, 2007; Moody and Laimins, 2010). In contrast, cutaneous papillomaviruses developed several alternative strategies to interfere with their host cells: instead of binding E6AP to facilitate p53 degradation as alpha type HPVs, E6 of most other HPV genera, amongst them the cutaneous types HPV1 and HPV8, binds to MAML1 to inhibit NOTCH signaling, a feature also shared by animal PVs (e.g., BPV1, MmuPV1, and MnPV) (Brimer et al., 2012, 2017; Meyers et al., 2017). MAML1 together with the histone acetyltransferases p300 and CREBBP can form a transcription complex that activates this pathway (Rozenblatt-Rosen et al., 2012). Amongst its function as a tumor suppressor, NOTCH drives differentiation of keratinocytes and is therefore found to be frequently mutated in cutaneous SCCs (Pickering et al., 2014; South et al., 2014). An impairment of this pathway either by mutations or by intervention of HPV keeps the cell in a proliferative state and promotes tumorigenesis. HPV5 and HPV8 additionally interfere with host cell differentiation by an E6-mediated degradation of p300, which in turn decreases the expression of keratin 1 and 10 and involucrin (Howie et al., 2011).

Furthermore, HPV38 E6 binds p300 thereby preventing p53 acetylation at lysine 382 and blocking p53-mediated apoptosis (Muench et al., 2010). HPV38 E6 can also increase the expression of the N-truncated isoform of p73, lacking a transactivation domain. ΔNp73 in turn competes with p53 activity that may perturb apoptosis and the eradication of damaged HPV-positive cells (Caldeira et al., 2003). A recent study further showed that by targeting p300, HPV8 E6 maintains a proliferating state of the host cell by downregulating the CCAAT/enhancer-binding protein (C/EBPα) and decreasing the expression of microRNA-203, a repressor of ΔNp63 expression (Marthaler et al., 2017). While p63, the master regulator of epithelial stemness, can induce cell cycle arrest and apoptosis like p53 and p73, ΔNp63 can act in a dominant-negative manner and maintain proliferation (reviewed in Candi et al., 2015). Indeed, certain beta-HPVs target central hubs within the cellular network that control differentiation, senescence as well as apoptosis, which reasonably explains the capacity of these viruses to stimulate proliferation of undifferentiated cells. Such effects on tissue homeostasis are of profound importance in NMSC development, particularly when HPV-infected skin is cumulatively exposed to UV.

UV exposure under physiological circumstances induces p53 stabilization and activation that leads to a cell cycle arrest and DNA repair or – at higher dosages – to apoptosis (reviewed in Zuckerman et al., 2009). Perturbed p53 function in turn leads to a gradual accumulation of genetically altered cells, thereby promoting the development of NMSC (Feltkamp et al., 2008). While high-risk mucosal types directly target p53 itself after DNA damage, cutaneous beta-HPVs affect a plethora of DDR proteins downstream of p53 (White et al., 2014; Wendel and Wallace, 2017). An important protein that links the ATM/ATR pathway to p53-induced apoptosis is the conserved Ser/Thr kinase HIPK2 (Matt and Hofmann, 2016). Upon severe UV damage, HIPK2 forms a complex with p53 and the CBP acetyltransferase leading to HIPK2-mediated p53 phosphorylation at serine 46 and CBP-mediated p53 acetylation at lysine 382 and finally a strong p53 activation with induction of pro-apoptotic gene expression (reviewed in Bitomsky and Hofmann, 2009; Matt and Hofmann, 2016). As shown for one of the most prevalent HPV types found in skin (de Koning et al., 2009), HPV23 E6 can inhibit HIPK2-mediated phosphorylation of p53 in response to UV damage (Muschik et al., 2011). Intriguingly, HIPK2 also controls the number of stem and progenitor cells in the skin (Wei et al., 2007), the reservoir for cutaneous HPVs (reviewed in Egawa et al., 2015). Moreover, HIPK2-deficient mice show an enhanced susceptibility to develop SCCs (Wei et al., 2007) consistent with decreased levels HIPK2 expression in KAs and SCCs compared to AK (Kwon et al., 2015). Additionally, beta-HPVs can abrogate UV-induced pyrimidine dimer excision (Giampieri and Storey, 2004) or target the pro-apoptotic protein Bak for proteolytic degradation (Jackson et al., 2000; Underbrink et al., 2008; Holloway et al., 2015). In any case, the interference with the DDR and pro-apoptotic pathways can promote long-lasting effects on genomic instability and favors the accumulation of damaged cells (reviewed in Wendel and Wallace, 2017).

Moreover, UV-activated EGFR signaling which causes keratinocyte hyperproliferation and hyperplasia of the skin (El-Abaseri et al., 2006) is enhanced by HPV8 E6, thereby contributing to SCC formation (Taute et al., 2017). Furthermore, HPV5 and 8 E7 alter beta-catenin and zona occludens-1 anchor proteins which disturb cellular adherence and tight junctions (Heuser et al., 2016a). Their reorganization imbalances tissue homeostasis and lead to epithelial-mesenchymal transition (EMT) (Polette et al., 2007), also suggesting a role in NMSC development (reviewed in Chen et al., 2016). This is in line with an HPV8 E7-mediated upregulation of metalloproteases that remodel the extracellular matrix (Akgül et al., 2006) to facilitate migration and invasion of HPV8-positive cells (Akgül et al., 2005a). Furthermore, HPV8 E7 upregulates fibronectin, another EMT marker (Lee et al., 2006), by inducing a switch from E-cadherin to N-cadherin expression in suprabasal cell layers of organotypic skin cultures, considered to be a primary event in invasion of carcinoma cells (Heuser et al., 2016b).

As soon as HPV-infected altered squamous cells invade deeper layers and undergo EMT, it is likely that HPV replication, known to be highly dependent on cell differentiation, cannot be maintained (reviewed in Doorbar et al., 2012). Consequently, viral presence is no longer necessary for malignant progression, since the tumor becomes heterogeneous in terms of acquiring additional mutations. Despite different modes of action for mucosal and cutaneous HPVs, the outcome is similar: in the case of cervical carcinoma, HPV is integrated into the host cell genome but maintains the transformed phenotype by continuous E6/E7 expression (reviewed in Doorbar et al., 2012). Conversely, cutaneous HPVs interfere with their host cell apparently in the beginning of the multi-step process of carcinogenesis, finally leading to an intracellular environment counteracting episomal DNA replication. This may explain why lower or no viral loads are detected in SCCs as compared to pre-malignant lesions (Weissenborn et al., 2012). In any case, this is a "dead end" for both mucosal and cutaneous HPVs, since the

permissive cycle is interrupted and no new virus progenies can be formed.

THE IMPORTANCE OF PRECLINICAL MODELS FOR INFECTIOUS AGENTS – SOME GENERAL CONSIDERATIONS

Models in bioscience are indispensable to reduce the complexity of a disease manifested in patients, but of course they also have their inherent limitations (Anisimov et al., 2005; Mazzocchi, 2008; Dougherty and Shmulevich, 2012). Moreover, in contrast to the empirical supposition of a working hypothesis as "right" or "wrong," models can also be tentatively categorized as "relevant" or "irrelevant" for the investigation of a particular scientific question or even a multifactorial disease. Since there is a strong social pressure on basic research to bring reproducible laboratory results into the clinic (Begley and Ellis, 2012), there are indeed many recent initiatives to improve this transfer by validating the relevance and impact of *in vitro* and *in vivo* models with respect of their translational applicability (Denayer et al., 2014; Day et al., 2015; Dolgos et al., 2016; Horvath et al., 2016).

Animal experiments for infectious agents like papillomaviruses are also indispensable (Doorbar, 2016; Christensen et al., 2017) and should reflect as much as possible the molecular and histological key features of a disease as found in patients (reviewed in Greek and Menache, 2013). Nevertheless, besides pragmatic reasons in working with easily managing laboratory animals (e.g., rodents) (reviewed in Hu et al., 2017; Uberoi and Lambert, 2017), there are several other important criteria for validation and predictability to guarantee a successful clinical translation. They can be summarized as follows (Kern, 1982; Greek and Menache, 2013; Denayer et al., 2014):

1. The infectious agents should have the same species specificity, tissue tropism and genome organization as their human etiological counterparts.
2. The natural infection mode should be similar. This is of particular importance with respect of the extent and spread of an infection since it may affect both the time course of seroconversion as well as the pathological outcome of the disease.
3. The infectious agent should accomplish the revised Henle-Koch criteria in terms of etiology (Fredricks and Relman, 1996; Falkow, 2004; Mossman et al., 2004). Here, some restrictions have to be considered, especially in the light of novel insights into the organization of individual human virome that can be changed in a time-dependent manner (Virgin, 2014; Gentile and Micozzi, 2016; Vouga and Greub, 2016).
4. The viral inoculum in an experimental infection should not over stimulate the immune system (Kern, 1982).
5. The animal should be immunocompetent and specific antibodies to the respective agents normally absent before exposure. Although especially inbred rodent systems do not necessarily completely mirror human immunology (reviewed in Mestas and Hughes, 2004), they still allow dissecting virus–host interactions in terms of acute and chronic/persistent infections in correlation with the serological response.
6. Depending on immune surveillance in the case of tumor formation, the same lesions should appear, ideally in the same time frame relative to the median life time of the animals ("face validity").
7. Since cancer is a multi-factorial process, the animal model should also allow the inclusion of additional non-genetic risk factors for a disease (e.g., immunosuppression, UV exposure), leading to an enhanced tumor formation.
8. Having identified a potential infectious agent, subsequent vaccination of animals should prevent the disease. Hence, preclinical models ideally should develop the same clinical manifestations as found in patients, an important read-out criterion for the success of a vaccine to be transferred into the clinic (Moore and Chang, 2017).
9. To be valid as a preclinical model, a scoring system should be applied that includes the careful selection of the animal species, the degree of reflecting a disease, face validity, complexity, and predictability (Denayer et al., 2014).

HOW DO THE CURRENT RODENT MODELS FOR CUTANEOUS HPVS FIT TO THESE CRITERIA?

Transgenic Mice

Transgenic mice are suitable model systems for most research fields and have also contributed enormously to our mechanistic knowledge about mucosal and cutaneous PVs (reviewed in Doorbar et al., 2015; Lambert, 2016; Santos et al., 2017). Current genome editing technologies make them relatively easy to generate and to manipulate (Rocha-Martins et al., 2015). The transgene, regularly containing the open reading frames (ORFs) of E6, E6/E7 or the complete early region of the selected HPV type is typically expressed under control of the keratin 14 promoter which facilitates constitutive oncogenic expression in cycling epithelial cells. The composition of the ORFs and the varying oncogenic potential of cutaneous HPVs significantly influence the outcome of experiments, e.g., the tumor induction. For example, mice harboring the complete early region of HPV8 spontaneously develop papillomatosis, acanthosis and hyperkeratosis, epidermal dysplasia and, to lower frequency, SCC formation (Schaper et al., 2005). Here, overexpression of the early region leads to a clonal expansion of a population of Lrig1$^+$ keratinocyte stem cells in the hair-follicles that is accompanied by a switch of p63 to its ΔNp63 isoform, lacking the N-terminal transactivation domain (Lanfredini et al., 2017). This in turn interferes with the function of p63 and p73 to induce cell cycle arrest and apoptosis. It also impairs the cellular differentiation program and drives proliferation (reviewed in Candi et al., 2015). Due to the involvement of Lrig1$^+$ stem cells in wound healing (Donati et al.,

2017), it is not surprising that development of papillomatosis could be also accelerated after UV exposure, further suggesting a synergistic effect between HPV8 and exogenous factors (Marcuzzi et al., 2009). Furthermore, via targeting of C/EBPβ, HPV8 E7 also suppresses the expression of the CC chemokine ligand 20 (CCL20), which plays a pivotal role in the recruitment of Langerhans cell precursors into the epidermis (Sperling et al., 2012). Hence, considering all these scenarios, the impact of HPV8 on cell differentiation and innate immunity can reasonably explain how a viral infection commits cells to malignant transformation.

The mechanisms by which cutaneous HPVs interfere with their host cell, e.g., by disturbing p53-connected pathways (White et al., 2014) or the DDR to UV, can lead to different outcomes of experiments (Dong et al., 2008; Hufbauer et al., 2011; Viarisio et al., 2011; Holderfield et al., 2014). For instance, as shown recently for HPV49 E6/E7 transgenic mice, these animals are less susceptible to form UV-induced tumors as compared to mice expressing other beta-HPVs, but are prone to chemically induced carcinogenesis of the upper digestive tract (Viarisio et al., 2016). Moreover, in contrast to the HPV8 model where one UV dose was sufficient to induce skin lesions (Marcuzzi et al., 2009), HPV38 E6/E7 transgenic mice require an extended irradiation protocol (Viarisio et al., 2011), mimicking a more natural situation as found in humans after life-long UV exposure (Maglennon et al., 2014). Tumors obtained in the HPV8 model after a single UV dose lack mutations in the *Trp53* and *Notch1* loci, but these were present after cumulative UV exposure in HPV38 mice. Notably, SCCs appeared much earlier in the latter model when UV irradiation and BRAFi treatment were combined. Intriguingly, although no HRAS and KRAS mutations were found in these tumors, the mitogen-activated protein kinase pathway was activated (Viarisio et al., 2017). Monitoring clinical SCC samples obtained from melanoma patients, it seems that HRAS mutations and the presence of HPV is mutually exclusive (Holderfield et al., 2014), a constellation that is reminiscent of transgenic mice expressing HPV38 E6/E7 constitutively.

Considering the concept of a "hit-and-run" mechanism during tumorigenesis, transgenic models are obviously not fully suitable when used in a conventional way by constitutive transgene expression. However, Viarisio et al. (2018) recently were able to overcome this problem by knocking out the HPV38 E6/E7 cassette via Cre-lox recombination as soon as UV-induced lesions appeared. After this, SCCs continued growing even without oncogene expression, clearly demonstrating that viral presence was no longer required to maintain the tumorigenic phenotype. Moreover, these SCCs acquired a large panel of potential "driver" mutations within *Trp53*, *Notch1*, and other genes (Viarisio et al., 2018) that can also be observed in human and murine SCCs (Pickering et al., 2014; Chitsazzadeh et al., 2016).

Although transgenic and knock-out models definitely have their justification when investigating potential principles of an *in vivo* virus–host interaction, concerns may be raised as they only reflect parts of the whole picture and do only partially, if at all, fulfill the above mentioned criteria to guarantee a successful clinical translation. The described examples are mostly typical "gain-of-function" studies, where the viral transgene is integrated and controlled by a heterologous promoter, thereby not mimicking the physiological gene dosage and the physical state of the viral DNA which normally persists extrachromosomally in the host cell. Additionally, the encoded transgenic proteins, provided there is no conditional gene expression, are self-tolerant and do not mirror a proper immune response against the corresponding genuine antigens expressed during a natural infection (Walrath et al., 2010). Moreover, according to quantitative trait locus (QTL) mapping analysis for cancer susceptibility loci, inbred strains of mice tremendously vary in their ability to develop tumors (Fleming et al., 2013). Hence, although being extremely helpful, experiments with transgenic mice have to be complemented with natural infection models to get also insight in immunological aspects, particularly allowing the production of appropriate vaccines to prevent NMSC (Vinzón and Rösl, 2015).

Natural Model Systems

Restricting this overview only to rodent systems, and also considering the above mentioned criteria for a relevant preclinical model, the MmuPV1/ mouse model (reviewed in Hu et al., 2017) is one of the most attractive, since the mouse is not only the best-characterized laboratory animal, but can also be infected by a virus isolated from the same species. Although just discovered in 2011, Xue et al. (2017) recently published a full transcription map of MmuPV1, providing a basis for a further characterization of viral proteins and their function in skin tumor formation. Moreover, first vaccination studies have been carried out in this system (Jiang et al., 2017). However, even with the MmuPV1/mouse model, the setup of a natural infection followed by a complete viral life cycle in a normal and immunocompetent host is unfortunately still not completely fulfilled. In this model, for instance, lesions appear only when infecting mice with high and, in comparison to humans, non-physiological amounts of virus particles at certain sites like the tail and muzzle, while the back skin is relatively resistant (Handisurya et al., 2013, 2014; Wang et al., 2015). This may be attributed to differential expression of MHC molecules, the presence of Langerhans cells and variations in the keratin network (Sundberg et al., 2014; Quigley et al., 2016), supporting the importance of the local immune surveillance (Da Silva et al., 2014) at the region of infection. These differences may also cause inappropriate conditions for virus particle formation that have to be overcome by a high infection rate, since fewer progenies could be detected in back skin than in mucosal tissue, despite equal viral DNA loads in both regions (Cladel et al., 2017). Cutaneous MmuPV1-induced tail papillomas contained high amounts of viral DNA (Uberoi et al., 2016), but no SCCs lacking MmuPV1 DNA are formed in immunocompetent animals that allow to study a "hit-and-run" mechanism as proposed.

Another preclinical model that is used to explore the impact of cutaneous papillomaviruses on skin carcinogenesis is the African multimammate mouse *Mastomys coucha*, formerly taxonomically classified as *Mastomys natalensis* (**Figure 1**; Kruppa et al., 1990). These animals belong to the Muridae family and are naturally infected with MnPV and McPV2, which – like

cutaneous and genital HPVs – infect epidermal and mucosal tissues, respectively (Muller and Gissmann, 1978; Nafz et al., 2008). MnPV can spontaneously induce epithelial lesions of the skin (mainly papillomas and KAs), while McPV2 causes anogenital lesions like condylomata at the anus, vulva, and penis, respectively. Similar to cutaneous HPVs, MnPV and McPV2 persist as episomes without any indication of integration but – depending on the type of lesion – in different copy numbers (Nafz et al., 2008). Considering the natural infection mode, *M. coucha* acquire the virus early after birth, as MnPV DNA is found in the skin of four-week-old animals where at the same time seroconversion is taking place (Schäfer et al., 2011).

Similar to cutaneous HPVs, the MnPV genome lacks an ORF for E5 (de Villiers et al., 2004) while parts of the E6, E1, and L1 genes are phylogenetically related to HPV types associated with EV (Tan et al., 1994). MnPV-induced benign lesions do not spontaneously regress but have the capacity to transform to SCCs (Reinacher et al., 1978; Vinzón et al., 2014). The exo-/endophytic keratinized lesions contain high numbers of episomal virus genomes (**Figures 1B–D**) and – similar to cutaneous HPVs – do not show any sign for integration (Amtmann et al., 1984; Hasche et al., 2017). As the animals are immunocompetent, E2-specific antibodies serve as an early infection marker found already in four-week-old animals, while L1-specific antibodies appear later, correlating with the appearance of skin lesions (Schäfer et al., 2011). Having a virus-free colony of *M. coucha* as control, the animals also served as preclinical model for the development of MnPV-specific vaccination strategies both under normal and immunosuppressed conditions (Vinzón et al., 2014). According to the relevance criteria for animal systems summarized above, *M. coucha* permits the follow-up of the complete infection cycle, starting from primary infection until the development of lesions that are not restricted to a certain body area (Nafz et al., 2007). Transcriptome analysis of skin tumors lead to the identification of various mRNA isoforms of MnPV (Salvermoser et al., 2016). Moreover, *M. coucha*-derived cell lines (Hasche et al., 2016) can be used to test the effects of additional factors on viral transcription and replication, making previous drawbacks compared to mouse systems more and more negligible.

Notably, the *M. coucha* model resembles in many characteristics UV-induced NMSC development in humans. In a large long-term study, the animals were irradiated with UV doses that are comparable to different areas of the world (Hasche et al., 2017). Naturally infected animals developed SCCs significantly more often than virus-free controls. Some of these SCCs were well differentiated and keratinizing (KSCCs), containing high amounts of extrachromosomal and transcriptionally active MnPV genomes as observed previously in spontaneous MnPV-induced tumors (Amtmann et al., 1984; Vinzón et al., 2014), whereas poorly differentiated and non-keratinizing SCCs (nKSCCs) only contained low amounts or even lacked viral DNA. Histologically KSCCs were similar to human AKs and Bowen's disease (Majores and Bierhoff, 2015) which also contain high viral loads whereas SCCs usually lack HPV DNA (Weissenborn et al., 2005). However, irrespective of the tumor type, all tumor-bearing animals developed antibody responses against the viral L1 capsid protein, providing evidence for preceding infections, which again is seen in SCC patients (Andersson et al., 2008). Notably, although showing the same time course of induction, the histology of UV-induced SCCs was profoundly influenced by the dose, with KSCCs mostly developing in the lowest dose group and nKSCCs preferentially under higher doses, pointing to the cumulative effect of UV causing human

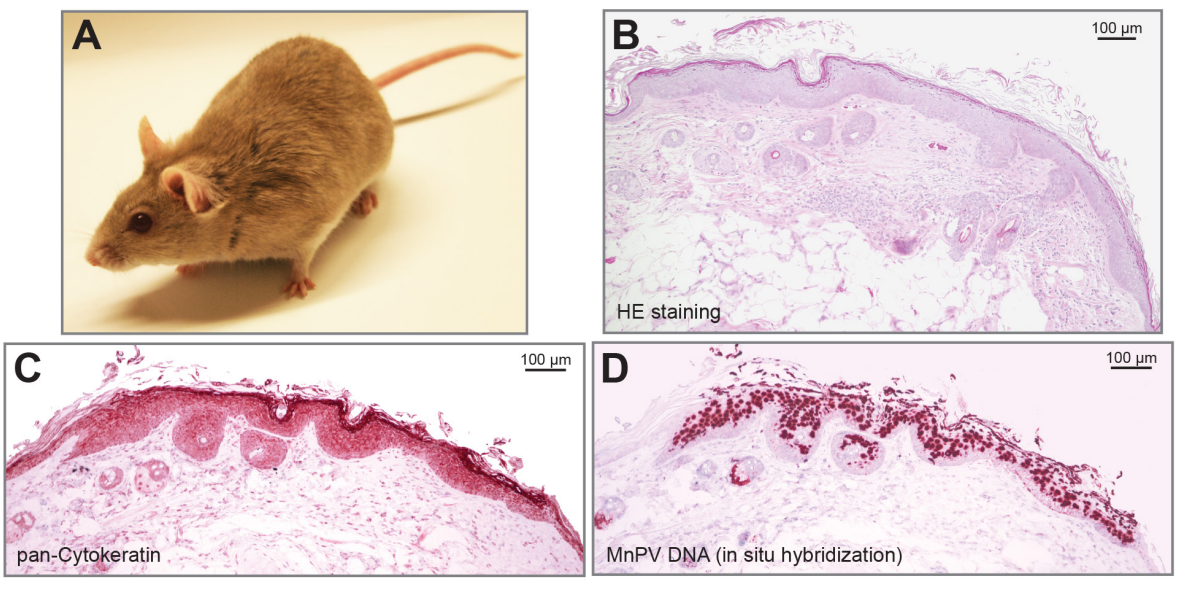

FIGURE 1 | The preclinical animal model *Mastomys coucha* develops MnPV-induced skin lesions. **(A)** A young *Mastomys coucha*. **(B)** HE staining of a dysplastic epithelium at the edge of a spontaneously emerging skin lesion. **(C)** Pan-cytokeratin staining shows the dysplasia of the epithelium. **(D)** *In situ* hybridization reveals squamous cells positive for MnPV DNA.

SCCs (Leiter and Garbe, 2008). Consequently, nKSCCs more often harbored p53 mutants, especially at two hot-spots also known from human SCCs (Pickering et al., 2014) and incapable of activating expression of downstream targets. As shown in mice before, a loss of functional p53 leads to less differentiated tumors (Flores et al., 2016; Page et al., 2016) and induces cell migration and degradation of the extracellular matrix (reviewed in Muller et al., 2011). Since MnPV replication depends – like cutaneous HPVs – on cell differentiation (reviewed in Doorbar et al., 2012), this constellation can explain the loss of viral genomes in these tumors by acquiring additional "driver" mutations (e.g., *Trp53*) (**Figure 2**). Moreover, taking into account that more DNA damages have been detected in MnPV-positive skin compared to MnPV-negative skin, this model provided for the first time evidence for a "hit-and-run mechanism" in a natural infection system (Hasche et al., 2017).

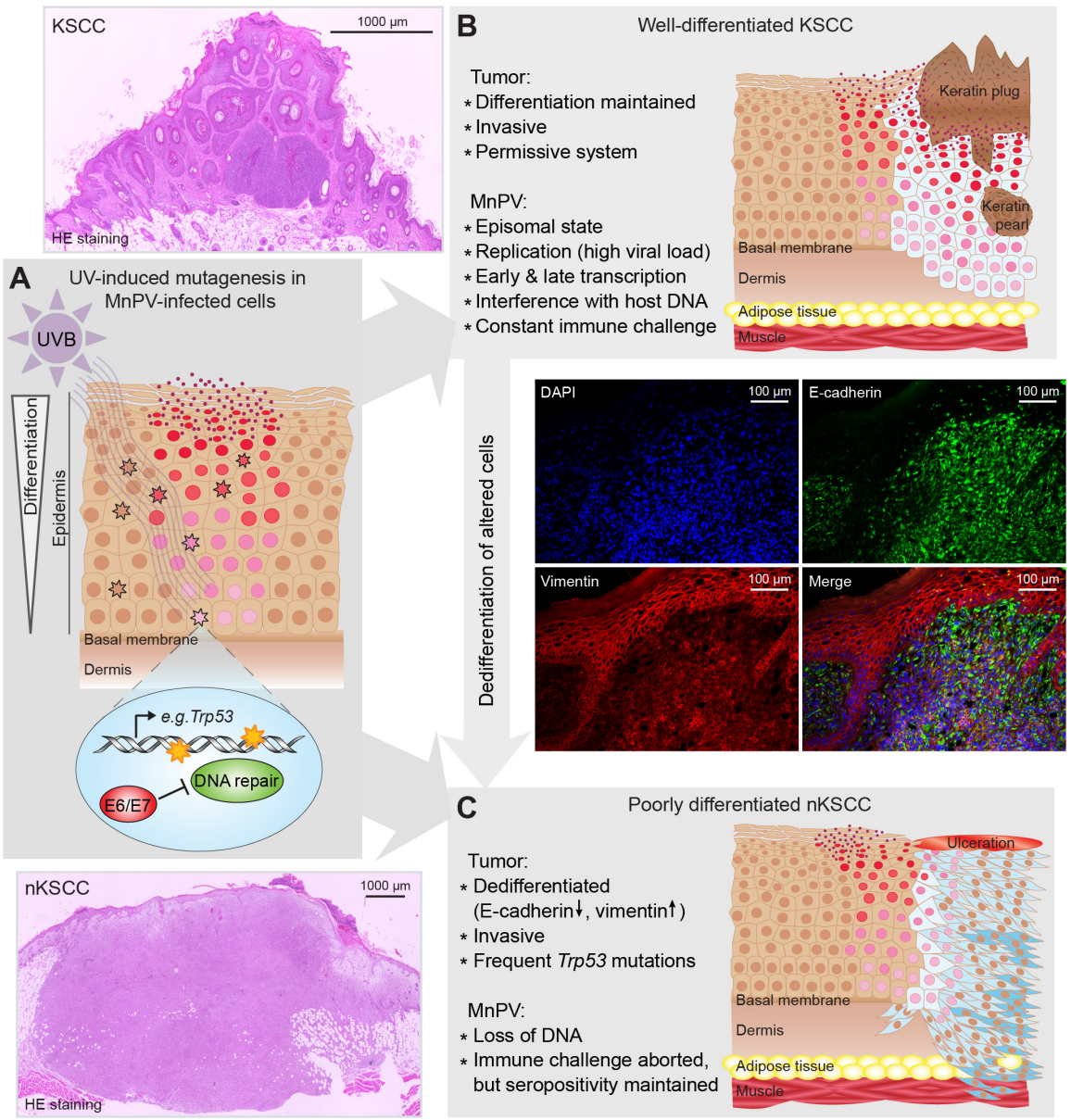

FIGURE 2 | Suggested "hit-and-run" model based on experiments with *Mastomys coucha*. **(A)** Virus replication and virion formation depend on differentiating squamous cells and are favored by UV-induced hyperproliferation. UV induces photoproducts, e.g., in *Trp53*. In uninfected cells, damages are repaired. In infected cells, MnPV reduces chromosomal stability and inhibits DNA repair leading to an accumulation of mutations. **(B)** Altered squamous cells become neoplastic (light blue) and start forming a well-differentiated keratinizing SCC, still representing a permissive system that allows viral replication and formation of virions. **(C)** When neoplastic squamous cells accumulate further mutations (dark blue), a dedifferentiated phenotype (characterized by a switch from E-cadherin to vimentin expression) is acquired, forming a poorly differentiated, non-keratinizing SCC (nKSCC). MnPV cannot replicate in this intracellular environment, resulting in a loss of viral DNA. (Scheme modified from Hasche et al., 2017).

Notably, a recent *in vitro* study showed that MnPV E6 does not interact with E6AP, a prerequisite for p53 degradation, but – like other cutaneous human and animal PVs – interacts with MAML1 to inhibit NOTCH signaling (Brimer et al., 2017). The same was reported for MmuPV1 E6, which also inhibits differentiation of keratinocytes to keep the host cell in a proliferative state (Meyers et al., 2017). However, to examine the effect of UV exposure in the context of a cutaneous PV infection requires more systematic studies, particularly to understand how a single UV dose, shown to have immunosuppressive effects (reviewed in Norval and Halliday, 2011), makes wildtype mice susceptible to MmuPV1-induced tumors that can progress to SCCs (Uberoi et al., 2016), while in other systems more frequent exposure is necessary. This is an important point, especially for immunosuppressed OTRs and the high incidence of NMSC in these patients. However, the fundamental question is still how cutaneous PVs contribute to tumor initiation and what kind of selection mechanisms account for SCC formation and the loss of viral DNA.

CURRENT WORK AND PERSPECTIVES ON VACCINATION AGAINST CUTANEOUS HPVS

The strongest argument for an etiological role of a virus in cancer development is the application of a prophylactic vaccine that prevents tumor formation. In the case of anogenital tumors, three HPV vaccines (Cervarix, Gardasil, and Gardasil 9), consisting of L1 (the major papillomavirus virion protein)-based virus-like particles (VLPs) are currently licensed (Pogoda et al., 2016). They are targeted against two, four, or nine mucosal HPV types (HPV6, 11, 16, 18, 31, 33, 45, 52, and 58), respectively, and – regardless of being very effective – they present limitations, making the development of broad protective second-generation HPV vaccines necessary. Most of the mechanistic studies showed that vaccine-induced neutralizing antibodies are the primary mediators of the elicited response, protecting from a subsequent infection by the targeted HPV types and therefore conferring immunity (Schiller and Lowy, 2012). Since the response has been shown to be largely type-specific and cross-reactivity among different HPV types is almost absent, these vaccines target, at best, the high risk types that cause 90% of the cervical cancer and no cutaneous types. Indeed, there are more than 40 HPV types plausible of being targeted by a vaccine to prevent human diseases: mucosal high-risk HPVs implied in the pathogenesis of cervical and other cancers (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68), additional mucosal types which cause genital warts (HPV6 and 11), several beta HPV types potentially linked to NMSC (HPV5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, 49) and the different alpha (HPV2, 3, 10, 27, 28, 57), gamma (HPV4, 60, 65), mu (HPV1, 63), and nu (HPV41) types that induce different lesions, being a special burden in children and immunocompromised patients. Given the potential benefits of covering so many different HPV types, a broad-protecting prophylactic HPV vaccine is a rational goal for second-generation vaccine development.

In this context, the papillomavirus minor capsid protein L2 contains a region with a major cross-neutralizing epitope that has been the main immunogen for second-generation vaccines (Gambhira et al., 2007). The goal of developing an L2-based vaccine is to generate a single- or oligo-valent antigen with a distinctively larger spectrum of protection against genital and cutaneous HPVs than the current formulations (Pouyanfard and Muller, 2017). Given that L2-derived linear peptides induce antibody levels which are several orders of magnitude lower that those induced by L1 VLP vaccines, different strategies had been explored in order to achieve a successful immune response. Among these, good responses were found by formation of L2 concatemers (Jagu et al., 2009), conjugation to a T-helper epitope and a TLR ligand (Alphs et al., 2008), display on structures like bacteriophages (Tumban et al., 2011) or on adeno-associated viruses (Nieto et al., 2012), chimeric HPV L2 peptide/L1-VLPs (Schellenbacher et al., 2009), integration into the thioredoxin active site (Rubio et al., 2009) or lipidation and fusion to FcγR-targeting scaffold (Zhang et al., 2016). These approaches have shown elicitation of neutralizing antibodies with a broad range of cross-neutralization, setting the proof-of-principle for the application of this approach.

Several vaccine formulations, either L1- or L2-based, have shown the development of antibodies neutralizing to PV cutaneous types (summarized in **Table 1**), either by targeting them directly or as a cross-protection. In particular, two studies have shown the potential of these vaccines to effectively prevent skin lesions in preclinical models. When *M. coucha* was used as a model, a VLP-based vaccine against a cutaneous papillomavirus could prevent spontaneous skin tumor formation both under normal and immunosuppressed conditions (Vinzón et al., 2014). As mentioned, MnPV-infected *M. coucha* resemble many characteristics of cutaneous HPVs and the human situation, like the natural and persistent infection early in life (Schäfer et al., 2011). Assessing the efficacy of a VLP-based vaccine on either previously or newly established infections, a long-lasting response could be shown, characterized by the induction of neutralizing antibodies that confer protection against both benign and malignant skin tumors even in immunosuppressed animals (Vinzón et al., 2014). Another novel finding of this study was that protection involves the maintenance of a low viral load in the skin by an antibody-dependent prevention of virus spread and that it is effective in preventing tumor formation even in individuals already infected at the time of vaccination. These results offer evidence that VLPs elicit an effective immune response in the skin even under immunosuppressed conditions, irrespective of the infection status at the time of vaccination, which sets the basis for a future clinical implementation of a vaccine against cutaneous HPV-induced tumors to prevent SCCs, especially in OTRs.

DNA vaccines were also explored in the context of cutaneous HPV infection. A recent study (Marcuzzi et al., 2014) showed that a vaccine consisting of HPV8 E6 DNA can generate a specific cellular response in roughly half of the vaccinated animals and partially prevent papilloma formation in those which developed HPV8-E6-specific T cell immunity as demonstrated by ELISPOT. Regarding L2 vaccines, a simple HPV16-L2 DNA

TABLE 1 | Preclinical studies showing reactivity of HPV vaccines to cutaneous types/challenge.

Immunogen scaffold/type	PV immunogen	Cutaneous HPV types neutralized by the vaccine *in vitro*	HPV types neutralized by cutaneous challenge	Projected vaccine development	Reference
VLPs	L1 from HPV 5, 8, and 92	HPV 5	ND	Unknown	Handisurya et al., 2009
Capsomers/VLPs	L1 from HPV 2, 27, and 57	HPV 2, 27, and 57	ND	Unknown	Senger et al., 2010
VLPs	L1 from MnPV	MnPV	MnPV	No	Vinzón et al., 2014
Lipopeptide (P25-P2C-HPV)	HPV16 L2(17–36)	HPV 5, BPV 1	HPV 16 and 45	Unknown	Alphs et al., 2008
L2 concatemers	Fusion of the L2(11-88) region of HPV types 6, 16, 18, 31, 39, 51, 56, and 73	HPV 3, 5, 8, 23, 27, 38, 57, 76	ND	Unknown	Kwak et al., 2014
Lipidated L2-repeat fusioned to an anti-hFcγRI scFv	HPV16 L2(17–36)	HPV 2 and 5	ND	Unknown	Zhang et al., 2016
Chimeric VLPs	HPV16 L2(17–36)	HPV 2, 3, 5, 27, 76	ND	Being produced under cGMP for clinical trials by the NCI PREVENT program (Shi et al., 2001).	Schellenbacher et al., 2009, 2013
Chimeric VLPs	HPV17 L2(17–36)	HPV 5, 20, 24, 38 and 96	HPV 5	Unknown	Huber et al., 2017
Chimeric VLPs	HPV 58 L2(16–37)	HPV 2, 5, 27 and 57	ND	Unknown	Chen et al., 2017
Fusion to bacterial flagellin	L2(11–200), L2(11–88) and/or L2(17–38) of HPV 6, 16, 18, 31, 39 and 52	ND	HPV 6, 16, 18, 31, 39, 52 and CRPV	Unknown	Kalnin et al., 2014, 2017
Pyrococcus furiosus thioredoxin with L2 octamer	L2(20–38) from HPV 6, 16, 18, 31, 33, 35, 51, and 59	HPV 3, 4, 5, 10, 38, 63, 76, 92, 95, and 96	ND	Close to cGMP production for a planned human trial (Pouyanfard and Muller, 2017).	Pouyanfard et al., 2017; Spagnoli et al., 2017
PP7/MS2 bacteriophage VLPs	HPV16 L2(20–29), (17–31), (14–40) and (14–65)	ND	HPV5	Being developed by the company Agilvax with DMID/NIAID/NIH support (Pouyanfard and Muller, 2017).	Tumban et al., 2012
Naked-DNA, fusion to calreticulin	MmuPV1 E6 and E7 and L2(11–200)	ND	ND	Being produced under cGMP for clinical trials by the NCI PREVENT program (Shi et al., 2001).	Jiang et al., 2017

vaccine showed no induction of neutralizing antibodies and therefore no cross-reactivity potential (Hitzeroth et al., 2009). In contrast, another vaccine composed of HPV16-L2/E6/E7 fused to human calreticulin (hCRT) as a strategy to enhance MHC class I presentation, induced both E6/E7-specific T-cell responses and L2-specific neutralizing antibodies, showing both therapeutic and prophylactic potential (Kim et al., 2008). hCRT-E6E7L2 conferred partial protection in an *in vivo* neutralization assay (Jiang et al., 2017), but its cross-neutralization activity has not been yet reported. Furthermore, when exploring the vaccine in an immunosuppressed setting, hCRT-E6E7L2 vaccination maintained cellular immune reactivity in $CD4^+$ T cell-depleted mice but lost the ability to induce a humoral response (Peng et al., 2014). hCRT-E6E7L2 is currently produced under cGMP for clinical trials by the NCI PREVENT program (Schellenbacher et al., 2017).

The recent development in the MmuPV1 model allowed vaccination studies also in this animal system (Jiang et al., 2017). An hCRT-MmuPV1E6E7L2 DNA vaccine induced strong mE6 and mE7 $CD8^+$ T cell responses and anti-L2 antibodies. In addition to the vaccine-induced antibody titers, also a robust anti-L1 response could be detected regardless of the papilloma status of the animals, probably due to the inoculum used for the experimental infection. Remarkably, persistent papillomas disappeared within 2 months after treatment and the virus could no longer be detected. Given the design of the vaccine, the importance of neutralizing anti-L2 antibodies cannot be dissected, as the anti-L2 response is concomitant to the E6 and E7 responses.

Therefore, the effectivity of pure L2 vaccines in preventing skin tumors is yet to be studied in a complete preclinical model. One important issue to be answered is whether L2 vaccination

can elicit enough neutralizing antibodies to confer protection against tumor formation, especially against non-cognate HPV types, since L2 responses are known to be lower than their L1 counterparts. In that context, even when sera display titers below detection limit by *in vitro* neutralization assays and *in vivo* neutralization assays after passive transfer, vaccinated animals still show complete protection against infection, which points to the fact that very low titers are indeed sufficient to confer immunity (Kalnin et al., 2017).

CONCLUSION

Current data strongly support an involvement of cutaneous HPVs in the pathogenesis of NMSC. It is clear that a vaccine targeting a broader spectrum, including mucosal and cutaneous HPVs would be beneficial for certain patients, especially those who are immunosuppressed. Since cutaneous HPV infections are acquired very early in life, vaccination in infancy seems to be most appropriate. The other option would be the vaccination of immunocompromised individuals, for example patients awaiting organ transplantation. By eliminating one of the main risk factors (HPV infection), the incidence of NMSC should be reduced. Clinical development of vaccines that could achieve this goal is currently being pursued.

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All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Characterization of a Merkel Cell Polyomavirus-Positive Merkel Cell Carcinoma Cell Line CVG-1

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Merkel cell polyomavirus (MCV) plays a causal role in ∼80% of Merkel cell carcinomas (MCC). MCV is clonally integrated into the MCC tumor genome, which results in persistent expression of large T (LT) and small T (sT) antigen oncoproteins encoded by the early locus. In MCV-positive MCC tumors, LT is truncated by premature stop codons or deletions that lead to loss of the C-terminal origin binding (OBD) and helicase domains important for replication. The N-terminal Rb binding domain remains intact. MCV-positive cell lines derived from MCC explants have been valuable tools to study the molecular mechanism of MCV-induced Merkel cell carcinogenesis. Although all cell lines have integrated MCV and express truncated LT antigens, the molecular sizes of the LT proteins differ between cell lines. The copy number of integrated viral genome also varies across cell lines, leading to significantly different levels of viral protein expression. Nevertheless, these cell lines share phenotypic similarities in cell morphology, growth characteristics, and neuroendocrine marker expression. Several low-passage MCV-positive MCC cell lines have been established since the identification of MCV. We describe a new MCV-positive MCV cell line, CVG-1, with features distinct from previously reported cell lines. CVG-1 tumor cells grow in more discohesive clusters in loose round cell suspension, and individual cells show dramatic size heterogeneity. It is the first cell line to encode an MCV sT polymorphism resulting in a unique leucine (L) to proline (P) substitution mutation at amino acid 144. CVG-1 possesses a LT truncation pattern near identical to that of MKL-1 cells differing by the last two C-terminal amino acids and also shows an LT protein expression level similar to MKL-1. Viral T antigen knockdown reveals that, like other MCV-positive MCC cell lines, CVG-1 requires T antigen expression for cell proliferation.

Keywords: Merkel cell polyomavirus, MCV, Merkel cell carcinoma, MCC, cell line

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INTRODUCTION

Merkel cell carcinoma (MCC) is an aggressive skin cancer associated with immunosuppression and ultraviolet exposure (Lemos and Nghiem, 2007; Heath et al., 2008). The incidence of MCC is approximately 1,500 cases per year in the United States, which has increased threefold over the past two decades. The discovery of Merkel cell polyomavirus (MCV) in 2008 from MCC lead to a more precise understanding of the pathogenesis of these tumors (Feng et al., 2008).

Merkel cell polyomavirus was discovered by digital transcriptome sequencing of MCC and was found to be clonally integrated in 70–80% of these tumors. While the viral integration sites in the tumor genome are random (Feng et al., 2008; Sastre-Garau et al., 2009; Martel-Jantin et al., 2012), MCC tumor cells express the viral T antigen early gene. The MCV early locus is essential for virus replication and encodes four viral proteins: (1) large T (LT), (2) small T (sT), and (3) 57kT, which are expressed by alternative splicing mechanisms (Shuda et al., 2008), and (4) alternate frame of the large T open reading frame (ALTO) produced through alternative translation initiation (Carter et al., 2013). The MCV LT protein directly replicates the viral genome through its carboxyl (C)-terminal DNA origin binding domain (OBD) and DNA helicase activities, while sT enhances viral DNA replication by stabilizing LT (Kwun et al., 2009, 2013). The functions of 57kT and ALTO in the viral lifecycle have not been determined. Viral sequence analyses in MCC consistently show that tumor-derived LT antigens contain mutations that prematurely truncate the C-terminal helicase domain, while preserving the N-terminal tumor suppressor targeting domains for Rb-binding and DnaJ chaperone-binding functions (Grundhoff and Fischer, 2015; Wendzicki et al., 2015; DeCaprio, 2017). Most MCC tissues, if not all, express the truncated LT and intact sT proteins, whereas 57kT and ALTO have never been detected in tumor tissues (Shuda et al., 2009; Rodig et al., 2012).

These viral features in MCC tissues – clonal integration, LT helicase ablation, and T antigen expression - are also observed in the MCV-positive MCC cell lines. Since the first description of the MCV-positive MKL-1 cell line (Rosen et al., 1987; Shuda et al., 2008), 16 additional cell lines, including MKL-2, WaGa, BroLi, LoKe (Houben et al., 2010, 2012), MCCL-3, MCCL-11, MCCL-12 (Fischer et al., 2010), PeTa, AIDo, WoWe, HeRo, KaRi (Houben et al., 2013), MS-1 (Guastafierro et al., 2013), UM-MCC13, UM-MCC29, and UM-MCC565 (Verhaegen et al., 2014) have been established. The complete viral sequence has been only determined in nine MCC cell lines. The use of MCV-positive cell lines in T antigen knockdown studies revealed that T antigen expression is essential for cell proliferation in most MCV-positive MCC cell lines, consistent with the oncogenic role of T antigens in Merkel cell carcinogenesis (Houben et al., 2010).

Both MCC and small-cell lung carcinoma (SCLC) are neuroendocrine tumors, and MCC cell lines share cytological and histochemical similarities (Carney et al., 1985; Leonard et al., 1993). According to SCLC cell line classification, MCC cell lines can be divided into two groups: (1) classic and (2) variant, as defined by their immunohistochemical expression of markers including neuron-specific enolase and chromogranin A. In addition, cell lines can be classified further into four subtypes (type I-IV) based on growth morphology, colony shape, and aggregation (Leonard et al., 1995). Interestingly, with rare exceptions (Fischer et al., 2010), most MCV-positive MCC cell lines identified to date conform to the classic type with high neuroendocrine marker expression and exhibit type III morphology in which cells grow in loosely aggregated clusters. Gene expression analyses in MCV-positive MCC cell lines demonstrated that classic virus-positive cell lines such as MKL-1 and WaGa mirror the transcriptome profile of MCC tumors (Daily et al., 2015).

Despite their similarities, MCV-positive MCC cell lines show variability in morphology, growth characteristics, and tumorigenicity. Mouse xenograft studies using four MCV-positive MCC cell lines have demonstrated significant differences in the latency required for grafted tumor cell lines to grow (Dresang et al., 2013). In the same study, Dresang et al. also showed that response to the survivin inhibitor YM155 varies between cell lines (Dresang et al., 2013). While individual cancer cell lines possess unique host genetic mutations, phenotypic differences seen in the cell lines may be dependent, in part, on viral factors including the copy number of integrated viral genome and viral T antigen expression levels (Houben et al., 2010; Guastafierro et al., 2013). Further, each MCC tumor and MCV-positive cell line contains unique LT truncation mutations that produce LT proteins of different sizes.

Here, we describe the characteristics of a novel early passage MCV-positive MCC cell line, CVG-1, derived from a metastatic MCC explant and that exhibits LT truncation pattern and integrated viral copy number similar to that of the previously established MKL-1. While we found a unique leucine (L) to proline (P) missense mutation in the CVG-1-derived sT, this substitution mutation did not affect the transformation activity of CVG-1 sT in rodent fibroblasts. In fact, CVG-1 cell proliferation shows oncogenic addiction to viral T antigen expression similar to other MCV-positive MCC cell lines. Despite these similarities in virological features, a striking difference in tumor cell morphology was seen in CVG-1. Thus, establishment of CVG-1 not only extends the repertoire of MCV-positive cell lines but also serves as a tool to investigate host cell factors that contribute to the variations in growth morphology and tumorigenicity observed in other MCC cell lines.

MATERIALS AND METHODS

Tissues and Cell Lines

Metastatic MCC to the left cervical lymph nodes was surgically resected from a 70-year-old female with a previous history of MCC. After surgical resection, the tissue [Collaborative Human Tissue Network (CHTN)] was placed in the RPMI1640 medium. All specimens were tested under University of Pittsburgh Institutional Review Board-approved guidelines. Patient informed consent was obtained by the participating CHTN hospitals in accordance with the Declaration of Helsinki. Tumor tissue was minced to disaggregate tumor cells. To adapt cells to cell culture, cells were initially cultured in the RPMI medium containing 20% heat-inactivated fetal bovine serum (FBS), 0.01% penicillin/streptomycin, 0.01% fungizone, 0.01% insulin–transferrin–selenium, 50 μM bathocuproine disulfate, and 1 mM L-cysteine. After 9 days, cells were transferred into RPMI medium containing 10% FBS and non-essential amino acids (NEAA, Mediatech). CVG-1 cells of passage <10 were cryopreserved in 10% DMSO in FBS. The experimental analyses in this study were carried out using cells at low passage (<40).

Other MCC cell lines (MKL-1, MKL-2, and MS-1) were cultured in the RPMI medium supplemented with 10% FBS and NEAA.

Immunohistochemistry

Formalin-fixed paraffin-embedded tumor tissue was stained according to a previously published protocol (Shuda et al., 2009). Briefly, slides were deparaffinized in xylene and rehydrated through a series of ethanol solutions. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 min. Epitope retrieval was performed using 1 mM EDTA buffer, pH 8.0, at 125°C for 3 min and 90°C for 15 s in an antigen retrieval chamber (Decloaking Chamber, Biocare Medical). Following blocking with serum-free Protein Block (Dako), MCV large T antigen was immunostained with mouse monoclonal antibody CM2B4 (1 μg/ml) diluted in antibody buffer (1% BSA, 0.1% gelatin, 0.5% Triton-X, 0.05% sodium azide in PBS, pH 7.4) for 1 h at room temperature. After extensive rinsing in 1X tris-buffered saline solution (TBS), sections were incubated with mouse Envision Polymer (Dako) for 30 min at room temperature, reacted with diaminobenzidine (Dako), and counterstained with hematoxylin (Dako).

Immunoblotting

Cells were lysed in RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 0.1% SDS, 0.5% Triton X, 0.5% sodium deoxycholate, pH 7.0) containing proteinase inhibitor cocktail (Roche). Lysates were sonicated to shear genomic DNA, and protein concentration was determined by the colorimetric DC-Protein Assay kit (Bio-Rad). After protein lysates were mixed with Laemmli buffer and heat-denatured, 30 and 50 μg of total protein were resolved in 12% SDS-polyacrylamide gel for LT and sT detection, respectively. MCV LT protein was detected by CM2B4 (Shuda et al., 2009). MCV sT was detected by CM5E1 (Shuda et al., 2011; **Figure 2C**) or by CM8E6 (Kwun et al., 2009; **Figures 3B,C**).

MCV Genomic Sequencing

Genomic DNA was extracted from CVG-1 cells using standard phenol-chloroform extraction methods. The MCV DNA in CVG-1 cells was then amplified using polymerase chain reaction (PCR) primer sets (Contig1 to Contig13 primers) as described previously (Feng et al., 2008). PCR reactions in this study were performed by Q5 Hot Start High-FIdelity DNA polymerase (NEB). Sanger sequencing was performed by MCLAB (San Francisco, CA, United States).

Viral Copy Number Analysis and Quantitative Reverse Transcription (RT)-Polymerase Chain Reaction (PCR)

Subcellular fractionation was performed to isolate nuclei from MCC cells prior to genomic DNA extraction by Proteinase K/SDS buffer (10 mM Tris/HCl pH 8.0, 100 mM NaCl, 25 mM EDTA, 0.5% SDS, 0.1 mg/mL Proteinase K). After overnight incubation at 50°C, 100 ng/mL of RNaseA was added to the lysates and incubated for 1 h at 37°C. Extracted DNA was further purified by phenol-chloroform extraction and ethanol precipitation prior to quantitative PCR (qPCR). The obtained genomic DNA was dissolved in water and quantified by UV-Vis spectrophotometry. qPCR was performed to obtain absolute quantification using the aforementioned total T antigen primer set. In order to determine the absolute copy number, a dilution series of plasmid MCV-HF was used as a reference template DNA (1 ng MCV-HF plasmid is equivalent to 1.13×10^8 molecules).

Total cellular RNA was extracted and purified from MCV-positive MCC cells using Purezol (Bio-Rad) per manufacturer's instructions, treated with RNase-free DNaseI (NEB), and extracted again with Purezol reagent prior to cDNA synthesis. For cDNA synthesis, 500 ng of total RNA was subjected to first strand synthesis using the iScript cDNA synthesis kit (Bio-Rad). Subsequently, real-time PCR was performed using PowerUP SYBR Green Master mix to quantify total T antigen mRNA (sense: 5′-GCT CCT AAT TGT TAT GGC AAC AT-3′ and antisense: 5′-CAA CAT CCC TCT GAT GAA AGC-3′), sT mRNA (sense: 5′-TCC TTG GGA AGA ATA TGG AAC T-3′ and antisense: 5′-GCG AGA CAA CTT ACA GCT AAT AC-3′), and 18S rRNA (sense: 5′-GGA CAC GGA CAG GAT TGA CA-3′ and antisense: 5′-ACC CAC GGA ATC GAG AAA GA-3′). Real-time PCR reactions were triplicated for each sample and performed on the QuantiStudio 3 system (Applied Biosystems). The determined threshold cycle (Ct) values were used to calculate the mRNA abundance relative to CVG-1 using the delta-delta Ct method. The Ct values of 18S rRNA were used as reference.

Transformation Assays

Rat-1 cell transformation assays were performed as described previously (Shuda et al., 2011). For doxycycline-inducible transgene expression, pLenti TRE.MCS EF.Puro-2A-rTet was modified from TLCV2 by removing the U6 promoter-stuffer fragment by *Kpn*I and *EcoR*I digestion, followed by displacing Cas9-2A-eGFP fragment with double strand DNA linker containing unique restriction sites using *Age*I and *Nhe*I restriction sites. TLCV2 was a gift from Adam Karpf (Addgene #87360). Then, codon-optimized wild-type MCV sT and the amino acid substitution mutants including L114P, LSD, as well as L142A were inserted into pLenti TRE.MCS EF.Puro-2A-rTet by unique *Age*I and *Sbf*I restriction sites. For lentivirus production, 293FT cells (Invitrogen) were transfected with pLenti TRE MCV sT, psPax2, and pMD2.G using Lipofectamine 2000 (Invitrogen). The medium was changed at 12 h after transfection, and the lentivirus produced in the culture supernatant was harvested at 72 h after transfection.

For the foci formation assay, Rat-1 cells were seeded in 6-well plate and infected with 150 μL of lentiviruses encoding multiple MCV sT variants. At day 3 post-infection, fresh growth medium with or without 0.5 μg/mL of doxycycline was added. Rat-1 cells were cultured for 11 days, and cell culture medium was refreshed every 3 days to maintain transgene expression. Cells were stained by 0.5% crystal violet to visualize transformation-associated foci. For soft agar colony formation assays, Rat-1 cells transduced with the aforementioned lentivirus constructs were treated with puromycin (3 μg/mL). After puromycin-resistant cells were selected, 1.0 mL (1.25×10^4) of the cells containing 0.3% noble agar (Sigma) was overlaid on the 2 mL of growth medium containing 0.6% agar. Cells were cultured for 2 weeks

to observe colony formation in the soft agar. Colony numbers were counted under a microscope. The data is presented as mean ± standard deviation (SD) ($N = 3$).

shRNA Knockdown of the Viral T Antigen and Cell Proliferation Assays

A modified version of the enhanced 7SK Pol III promoter (e7SK) was used as described previously (Haraguchi et al., 2016). In order to express short-hairpin (sh) RNA under the strong e7SK promoter, we synthesized a DNA fragment of the e7SK promoter (gBlock, IDT) and inserted it into the pENTR1A vector (Addgene plasmid #17398) to generate the pENTR e7SK-Pro construct using *Dra*I and *Eco*RV restriction sites. Oligonucleotides used to generate previously described shpanT (5′-CCG GAA GAG AGG CTC TCT GCA AGC TCT CGA GAG CTT GCA GAG AGC CTC TCT TTT TTG-3′ and 5′-AAT TCA AAA AAG AGA GGC TCT CTG CAA GCT CTC GAG AGC TTG CAG AGA GCC TCT CTT-3′) or control shRNA (5′-CCG GCC TAA GGT TAA GTC GCC CTC GCT CGA GCG AGG GCG ACT TAA CCT TAG GTT TTT G-3′ and 5′-AAT TCA AAA ACC TAA GGT TAA GTC GCC CTC GCT CGA GCG AGG GCG ACT TAA CCT TAG G-3′) (Houben et al., 2010) were annealed and inserted into pENTR e7SK-Pro using *Age*I and *Eco*RI restriction sites to generate pENTR1A-e7SK-shpanT and pENTR1A-e7SK-shCtrl, respectively. These entry vectors were inserted into the pLenti Dest-puro lentiviral vector using LR-clonase II (Invitrogen) to produce pLenti e7SK-shpanT-puro and pLenti e7SK-shCtrl-puro. pLenti Dest-puro was constructed from pMuLE Lenti Dest-Neo (Addgene plasmid #62178) by replacing the neomycin phosphotransferase gene with the puromycin N-acetyltransferase gene containing *Sbf*I and *Xho*I restriction sites. Lentivirus production was performed as described in transformation assays section. pENTR1A no ccdB (w48-1) and pLenti Dest Neo were a gift from Eric Campeau and Paul Kaufman and from Ian Frew, respectively.

For cell proliferation assays, CVG-1 cells were transduced with e7SK-shpanT-puro and pLenti e7SK-shCtrl-puro lentiviruses in the presence of 1 μg/mL polybrene for 2 days and selected for 4 days by puromycin treatment (1 μg/mL) as previously described (Houben et al., 2010). At day 6 post-transduction, 2.5×0^4 cells were seeded in a 96-well plate (day 0). Cell proliferation was measured using WST-8 (Wako) at days 1, 3, 5, 7, 9, and 11. OD values were normalized by values from day 1. The WST-8 formazan product was measured at 440 nm with a reference filter at 600 nm using a Synergy 2^{TM} Multi-Mode Microplate Reader (BioTek).

RESULTS

Establishment of the MCV-Positive MCC CVG-1 Cell Line

A small piece of the metastatic MCC tissue used to establish MCC cell line CVG-1, was formalin-fixed and embedded in paraffin for hematoxylin/eosin (H&E) staining and immunohistochemistry. MCC tumor cell generally exhibit cytological features of monomorphic small round cells with high nuclear to cytoplasmic ratio. However, H&E staining of CVG-1 showed a heterogeneous cell population accentuated by large tumor cells (**Figure 1A**, Top panel). Immunohistochemistry using the MCV LT antibody (CM2B4) showed strong nuclear staining in both large and small tumor cells (**Figure 1A**, Bottom panel) confirming the presence of virus in each tumor cell.

Similar to the morphologic features of the parental MCC tumor, the CVG-1 cell line also displayed tumor cell size variation, having a mixture of large and small round cells (**Figure 1B**). We attempted to single-cell subclone large and small CVG-1 cells to demonstrate that both cells are LT-positive MCC but were unsuccessful. Nevertheless, since MCC tumor tissues are uniformly positive for LT protein, both large and small cells are likely to be MCV infected. CVG-1 cells grow in loose clusters in culture, whereas most MCC cells including MKL-1, MKL-2,

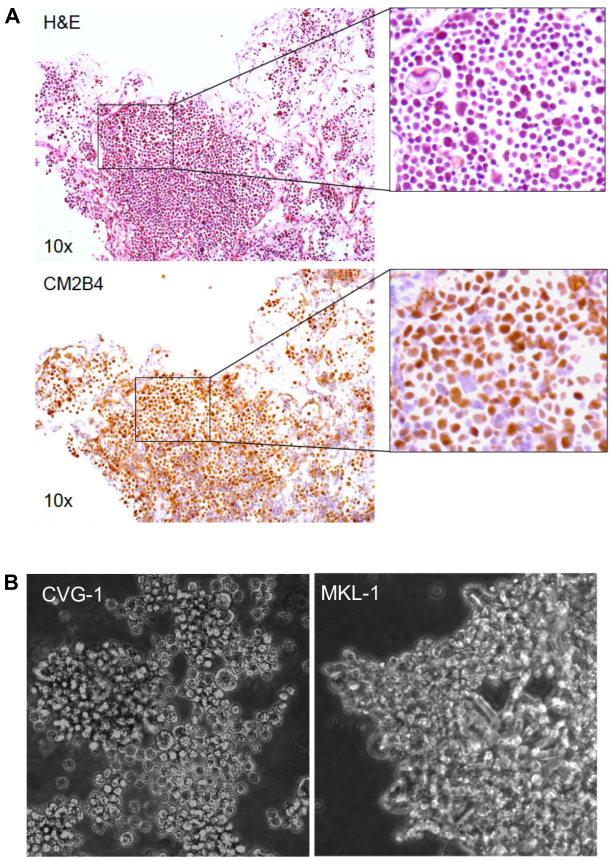

FIGURE 1 | Establishment of the Merkel cell polyomavirus (MCV)-positive CVG-1 cell line from metastatic Merkel cell carcinomas (MCC). **(A)** Hematoxylin/eosin (H&E) staining (top) and MCV large T (LT) immunohistochemistry (bottom) of the metastatic MCC tissue that the CVG-1 cell line was established from. MCV LT-positive MCC tumor cells (brown nuclei) demonstrate significant variation in cell size (inset). **(B)** Morphology of the CVG-1 cell line under phase contrast microscopy. CVG-1 exhibits cell size variation and a small round appearance resembling lymphoid cell lines, as opposed to other MCV-positive MCC cell lines, such as MKL-1, which usually grow in loosely aggregated clusters.

and MS-1 grow in large, tightly cohesive aggregates (Guastafierro et al., 2013; **Figure 1B**). CVG-1 cells make contact with each other, but their contact involves only a small segment of the periphery of each cell.

MCV Copy Number and Viral T Antigen Gene Expression in CVG-1

To determine the sequence of the MCV genome in CVG-1 cells, we amplified the viral DNA from CVG-1 using 13 pairs of contiguous primer sets that span the entire region of the MCV genome (Feng et al., 2008). The expected DNA fragments were successfully amplified with all primer sets used, indicating that the whole viral genome is present in CVG-1 cells. Next, the viral genome copy number was determined by qPCR using the previously characterized MKL-1, MKL-2, and MS-1 cell lines for comparison. CVG-1 contains the highest copy number of integrated MCV genome similar to MKL-1 (approximately 7 copies per cell), whereas MS-1 and MKL-2 contained relatively low copy numbers of MCV genome (4 copies and 2 copies per cell, respectively) (**Table 1**). Although genome copy numbers are similar between CVG-1 and MKL-1, analyses using qRT-PCR demonstrated that total T antigen mRNA and sT mRNA expression levels approximately 2.5-fold higher in CVG-1 than in MKL-1 (**Figure 2A**). MKL-2 expressed the lowest levels of T antigen mRNAs, consistent with previous results (Guastafierro et al., 2013; **Figure 2A**).

Viral LT Truncation Mutation in CVG-1

Merkel cell carcinoma tissue-derived MCV harbors mutations that prematurely truncate the DNA helicase domain of LT (Shuda et al., 2008). These truncation mutations have also been identified in MCV-positive MCC cell lines (Guastafierro et al., 2013). Thus, we sought to map the LT truncation mutations in CVG-1. MCV DNA was amplified by the contiguous primers from genomic DNA extracted from CVG-1 cells, and the PCR products were subjected to direct sequencing (GenBank accession no. MH136801) (Feng et al., 2008). CVG-1 LT terminated at nucleotide position 1617 as a result of an A–T transversion mutation, generating a premature termination codon. In the DNA sequencing, while the CVG-1 cell contains ∼3.6 copies of integrated viral genomes per haploid cell (**Table 1**), there was no presence of multiple peaks/mixed signals, suggesting that all the copies of integrated MCV possess the identical truncation mutation. On the other hand, MKL-1 LT terminates at nucleotide position 1618 due to a 46-bp deletion that causes a frameshift. This frameshift mutation results in the addition of two unrelated amino acids prior to the termination codon. As their coding regions suggest, both CVG-1 and MKL-1 LTs are composed of 330 amino acids (**Figure 2B**), and the only difference between these was found to be two amino acids at their C-terminal ends—Ser-Asn for CVG-1 and Lys-Leu for MKL-1. As observed in the MKL-1 LT, the premature truncation mutation found in the CVG-1 LT resulted in the complete deletion of the OBD and helicase domains without the affecting Rb-binding domain (**Figure 2B**). The CVG-1 and MKL-1 cell lines are the first pair of cell lines that express the same length LT protein out of the nine MCV-positive MCC cell lines characterized to date. Next, we determined that the LT genetic truncation in CVG-1 has the same migration pattern as MKL-1 in SDS–PAGE (**Figure 2C**). Consistent with the LT truncation pattern observed in the MKL-1 cell line, the CVG-1 LT migrated at 50 kDa. The mobility of CVG-1 LT, however, was marginally slower than that of MKL-1. Furthermore, LT levels in CVG-1 were slightly lower than those in MKL-1, whereas the T antigen mRNA levels in MKL-1 were higher than those in MKL-1 cells (**Figure 2A**).

CVG-1 contains a unique missense mutation in the sT coding sequence (nucleotide position 536) that generates a Leu(L)-to-Pro(P) substitution at the amino acid position 114 of the sT protein. In the nine MCV-positive MCC cell lines in which the viral sequence has been defined, only one amino acid substitution has been found in three cell lines (MKL-2, PeTa, and WoWe), all at position 20 of the sT protein (Ala (A) to Ser(S)). This position corresponds to a common exon1 of the T antigen gene, and thus, both LT and sT proteins are affected by the A20S substitution. Thus, L114P is a unique MCV sT amino acid substitution found in CVG-1. In contrast to LT, sT protein expression is relatively comparable across all the cell lines tested, irrespective of differences in mRNA expression (**Figure 2C**). These results suggest that LT and sT protein expression may be regulated at the post-transcriptional level.

While no precise correlation was observed between T antigen mRNA and protein expression, MKL-1 and CVG-1 cells harboring higher copy numbers of the integrated MCV genome (**Table 1**) tend to express higher LT protein than MKL-2 and MS-1 cell lines, which contain lower copy numbers of viral genome (**Figure 2C**). We also confirmed protein expression of MCC marker CK20 by immunoblot. Consistent with previous results (Houben et al., 2010), all cell lines including CVG-1 express similar levels of CK20 except for MKL-1 which expresses significantly lower levels of CK20.

TABLE 1 | MCV copy numbers in MCV-positive MCC cell lines.

Cell line	Absolute copy number/ng nuclear DNA	Copy number per cell (*N*)*
CVG-1	1007.6 ± 30.1	3.63 ± 0.11
MKL-1	1014.6 ± 72.6	3.65 ± 0.26
MKL-2	280.2 ± 17.0	1.01 ± 0.06
MS-1	553.6 ± 18.3	1.99 ± 0.07

**Merkel cell polyomavirus (MCV) copy number per genome was calculated based on the assumption that 100 ng of nuclear DNA from merkel cell carcinomas (MCC) cells contains 27,777 copies of single human genome (3.6 pg per haploid cell).*

Transforming Activity of CVG-1 sT With L114P Missense Mutation

Merkel cell polyomavirus sT is the major MCV oncoprotein that causes loss of contact inhibition and confers anchorage-independent growth capabilities in immortalized rodent fibroblasts (Shuda et al., 2011). This viral oncoprotein is a well-conserved protein across different isolates. In CVG-1 cells, we found a unique mutation that gives rise to a L114P amino acid substitution in the sT specific region. To date, this is the

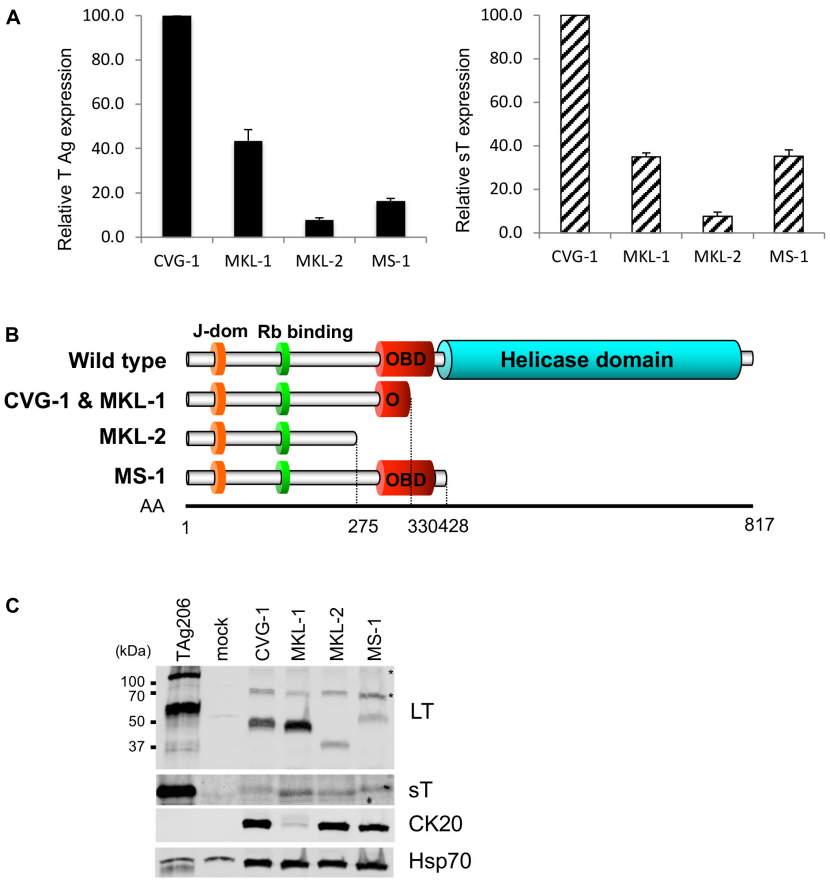

FIGURE 2 | Expression of truncated MCV LT and small T (sT) antigens in CVG-1. **(A)** qRT-PCR analysis of T antigen transcripts in CVG-1 and three previously described MCV-positive MCC cell lines. The levels of total T antigen transcripts and sT transcript expressed in MCC cell lines were analyzed by the delta-delta Ct method to compare abundance of total T antigen (Left) and small T antigen transcripts (Right) relative to CVG-1 cells. Ct values from 18S rRNA amplification were utilized as an internal control. **(B)** Schematic representation of the LT truncation mutation in CVG-1, showing the ablation of the entire helicase domain. **(C)** Immunoblotting analysis of MCV LT and sT protein expression. MCV LT and sT protein expression was detected by CM2B4 and CM5E1 antibodies, respectively. CM2B4 detects full length LT (~110 kDa) and multiply spliced 57kT (~60 kDa) proteins in a positive control prepared from 293FT cells transfected with genomic MCV T antigen expression vector (TAg206), but not in a mock-transfected negative control. Cytokeratin 20 (CK20) expression was also detected as a marker of MCC. Hsp70 was used as a loading control. Asterisks indicate non-specific reactivities of CM2B4 antibody.

second substitution mutation identified in the sT protein in MCV-positive MCC cell lines. In a previous study, we found that the large T stabilization domain (LSD), a domain critical for the transformation activity of sT, is localized to amino acids 91–95 of the sT protein and is part of a disordered loop structure based on the predicted MCV sT structure (Kwun et al., 2013, 2015). Because the L114P mutation is in close proximity to LSD and proline is an amino acid that introduces sharp kinks in the polypeptide backbone, we wondered whether this newly identified sT mutation affects CVG-1 sT transformation activity. We used a previously described rodent cell transformation assay (Shuda et al., 2011). Rat-1 cells were transduced with lentiviruses expressing wild type sT and various sT mutants in the presence of doxycycline (500 ng/mL). Induction of L114P sT mutant expression in cells resulted in dense foci formation similar to that observed in cells expressing wild-type MCV sT and L142A PP2A-binding mutant, whereas cells expressing the non-transforming LSD mutant LSD_{A91-95} did not form dense foci (**Figure 3A**, Top). In parallel with the foci formation assay, we performed a soft agar colony formation assay and observed anchorage-independent growth in cells expressing the L114P sT mutant (**Figure 3A**, Bottom). Colony size and numbers were comparable between L114P and the other transforming MCV sT variants tested, suggesting that the L114P mutation does not affect the transforming activity of MCV sT. Comparable sT protein expression in lentivirus-transduced cells was confirmed by immunoblotting (**Figure 3B**).

CVG-1 Requires Viral T Antigen for Cell Proliferation

While most MCV-positive MCC cell lines established to date require viral T antigen oncogene expression for proliferation, T antigen expression is dispensable only in one MCV-positive MCC cell line, LoKe (Houben et al., 2012). We examined whether CVG-1 also requires T antigen expression by using shRNA panT1

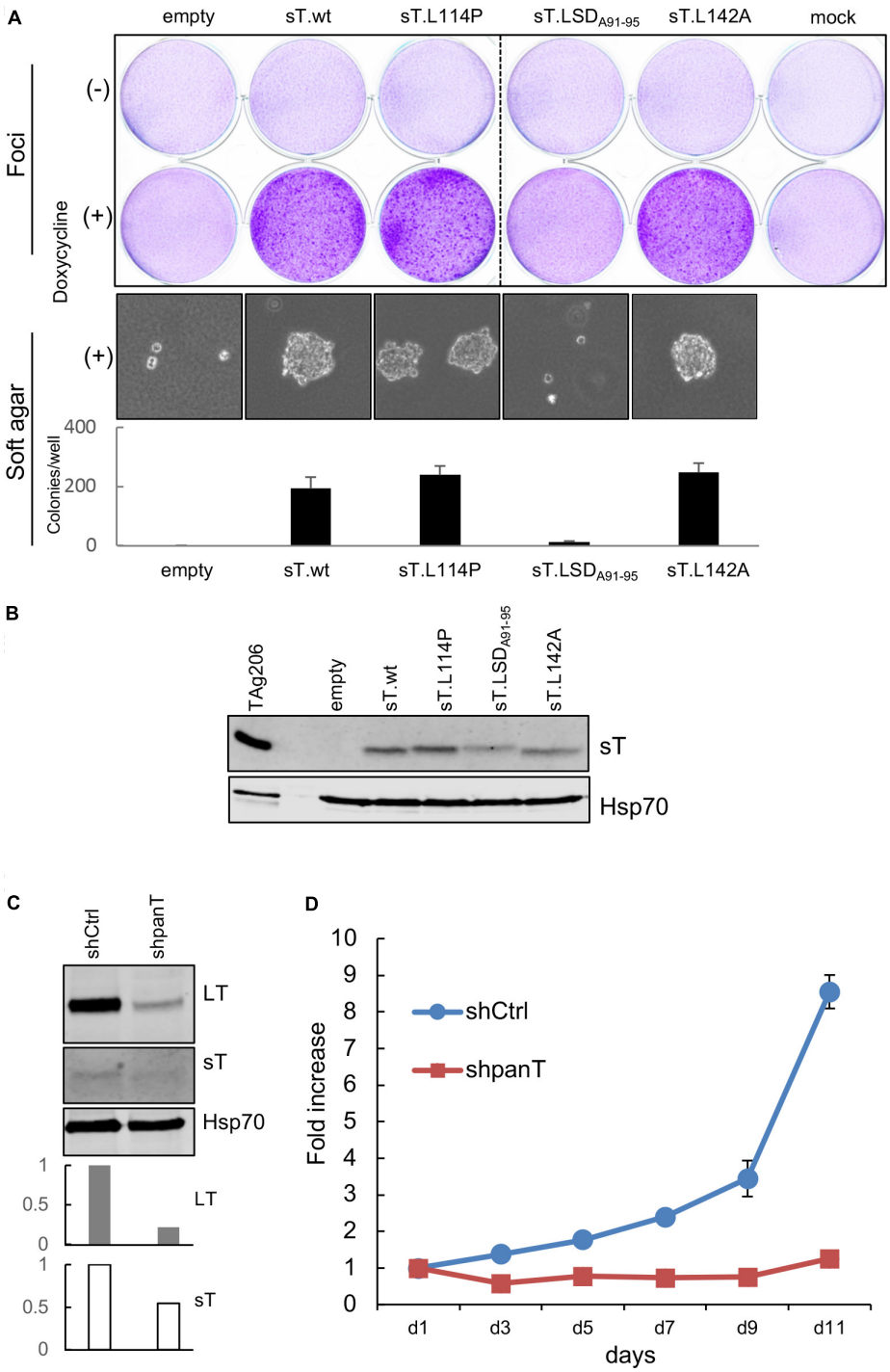

FIGURE 3 | CVG-1 encodes transforming MCV sT and requires viral T antigen expression for cell proliferation. **(A)** Transformation activities of MCV sT L114P substitution mutant expressed in CVG-1. Foci-formation assay was performed in Rat-1 cells transduced with doxycycline-inducible lentiviral vectors that encode MCV sT and various substitution mutants in the presence or absence of 0.5 μg/mL doxycycline (Top). Soft agar colony formation assay was also performed in the presence of 0.5 μg/mL doxycycline (Bottom). The graph shows the average ± standard deviation (SD) of the number of soft agar colonies counted in triplicate wells. **(B)** Immunoblotting analysis confirming MCV sT protein expression in Rat-1 cells. A positive control lysates used in **Figure 2B** was loaded in the first lane. sT was detected by the CM8E6 antibody. Hsp70 was used as a loading control. **(C)** CVG-1 requires MCV T antigen expression for cell proliferation. CVG-1 cells were transduced with shRNA lentiviral vector targeting both LT and sT (shpanT) and control shRNA (shCtrl). After transduced cells were selected by puromycin, knockdown activity was confirmed by immunoblotting for LT, sT, and Hsp70 proteins. Graphs demonstrate knockdown activities of sT relative to shCtrl quantified for LT and sT proteins by LI-COR immunoblotting. T antigen protein expression was normalized by the Hsp70 protein expression. **(D)** CVG-1 cell proliferation requires viral T antigen expression. After knockdown was confirmed, cells were seeded in a 96-well plate, and the proliferation was assessed using the WST-8 assay.

(shpanT) targeting common T antigen exon1 and control shRNA (shCtrl) (Shuda et al., 2011). After shRNA-transduced CVG-1 cells were selected by puromycin, knockdown was examined by quantitative LI-COR immunoblotting. Expression of LT and sT proteins in CVG-1 cells was reduced by 79% and by 46%, respectively, by knockdown with shpanT, as compared to cells transduced with shCtrl (**Figure 3C**). In addition, cell proliferation assays performed with WST-8 showed that T antigen knockdown by shpanT completely ablated the cell proliferation activity of CVG-1 cells (**Figure 3D**). Thus, similar to most MCV-positive cell lines, tumorigenicity of CVG-1 also depends on the viral T antigen expression.

DISCUSSION

Here we report the establishment of a new, early passage MCV-positive MCC cell line CVG-1. As seen in other MCV-positive MCC cell lines MKL-1(Shuda et al., 2008) and MCCL12 (Fischer et al., 2010), CVG-1 harbors multiple copies of the integrated MCV genome with a uniform LT truncation mutation, which may have resulted from the multiplication of a mutated single genome by rolling circle DNA replication (Starrett et al., 2017). Unlike most other MCV-positive MCC cell lines that grow in clusters, CVG-1 cells remain more disaggregated, and trypsin digestion was not required to isolate single cells. The CVG-1 cell line uniquely shows remarkable cell-size variation including large cells, even though MCC is defined as a small cell neoplasia. While we still do not know the specific cause of the size variation in CVG-1 cells, many type of cancers display morphological, proliferative, and functional heterogeneity, which arise from genetic mutations or cancer cell differentiation seen in cancer initiating cells or cancer stem cells. The former could be caused by the MCV sT antigen, which induces aneuploidy by compromising cell cycle checkpoint functions (Kwun et al., 2017). SCLC, another neuroendocrine cancer composed of cells capable of differentiating into neuronal and endocrine lineages, displays comparable morphologic heterogeneity and a gene expression signature similar to MCC (Salcido et al., 2010; Codony-Servat et al., 2016). Thus, self-renewal and cancer cell differentiation may also contribute to MCC tumorigenicity and give rise to morphological heterogeneity.

While the morphology of CVG-1 was unique among previously reported MCV-positive MCC cell lines, CVG-1 tumorigenicity is still dependent on MCV T antigen expression as in other MCV-positive cell lines. Out of seven cell lines tested for T antigen knockdown, only the LoKe cell line does not require T antigen expression for growth (Houben et al., 2012). Characterization of LoKe demonstrated that tumor cells harbor an Rb gene deletion that compensates for the oncogenic function of MCV LT expression in MCC (Houben et al., 2012; Hesbacher et al., 2016). However, it has been shown that Rb gene deletions/mutations are rare in MCV-positive MCC (Gonzalez-Vela et al., 2017; Harms et al., 2017). Thus, it remains likely that most MCV-positive MCC cell lines including CVG-1 require MCV T antigen expression to maintain their tumorigenicity.

The cell of origin in MCC remains unknown. It has been thought to be the epidermal Merkel cells based on the similarities in ultrastructure and gene expression between normal Merkel cells and MCC (Tang and Toker, 1978; Green et al., 1984; Battifora and Silva, 1986). However, it has recently been proposed that the cellular origin of MCV-positive cells may not be the Merkel cells (Zur Hausen et al., 2013; Sauer et al., 2017; Sunshine et al., 2018). Liu et al. (2016) have shown that MCV can infect dermal fibroblasts *in vitro*, but infection of Merkel cells has never been confirmed. Merkel cells are epidermal cells localized in the basal layer of the epidermis. While Merkel cells originate in the epidermis (Van Keymeulen et al., 2009), cutaneous MCCs typically involve only the dermis, and MCCs that develop in the epidermis are rare (<10%) (Smith et al., 1993). Recently, Verhaegen et al. (2017) reported transgenic mice that develop MCC-like tumors by expressing MCV sT and a Merkel cell lineage factor Atoh1 under the keratin 5 (KRT5) promoter, the activity of which is restricted to the basal layer of the epidermis (Ramirez et al., 1994). Interestingly, the pathogenesis of MCC-like tumors in this mouse model was restricted to epidermal layers, consistent with MCC-*in situ* or Merkel cell hyperplasia (McFalls et al., 2017). These data suggest the posibility that most MCV-positive dermal MCCs may originate from non-Merkel cells while MCC-*in situ*, which is confined to the epidermis, may arise from Merkel cells (Ferringer et al., 2005).

Since an animal model that mimics dermal MCC carcinogenesis has not been developed, MCC cell lines are useful tools to study the cellular origin of MCC. It has been shown that SV40 T antigen and human papilloma virus E6/E7 oncoproteins can reversibly transform primary human hepatocytes and human pancreatic duct epithelial cells without affecting normal diploid status (Kobayashi et al., 2000; Inagawa et al., 2014). The MCV-positive MCCs usually contain fewer genetic mutations and sustain normal karyotypes when compared to virus negative MCCs (Harms et al., 2017). Thus, some MCC cell lines may preserve normal genetic components that allow tumor cells to redifferentiate into untransformed, post-mitotic state cells with inhibition of T antigen expression. While most MCV-positive MCC cell lines become arrested after T antigen knockdown, a portion of cells commit non-apoptotic cell death as seen in MKL-1 (Houben et al., 2010). In early-passage cell lines like CVG-1 and MS-1 cells, however, many cells remain viable after T antigen knockdown and are arrested in G0/G1 (unpublished observation). Further molecular and cellular analyses in these early passage cell lines may lead to the identification of host genetic or functional features that represent the cellular origin of MCC.

Studies using MCC cell lines have revealed critical oncogenic pathways regulated by sT and LT. A recent study demonstrated that MCV sT binds to L-Myc and the EP400 histone acetyltransferase complex to activate L-Myc-mediated gene expression in MCC cells critical for MCC cell proliferation (Cheng et al., 2017). MCV LT expression in MCC activates the genes downstream of the E2F transcription factor by inhibiting the function of Rb through its LxCxE Rb-binding domain (Hesbacher et al., 2016). MCV-positive MCC is a unique cancer

that has a gene expression signature similar to neuroendocrine Merkel cells. Because MCV T antigens alone are not sufficient to transform normal human fibroblasts (Cheng et al., 2017), MCC-specific oncogenic factors that are amplified in MCC such as L-Myc, may also play important roles in MCV-induced MCC carcinogenesis (Paulson et al., 2009; Cheng et al., 2017). Thus, MCC cell lines are essential tools to study the interplay between viral T antigens and MCC-specific host cell factors.

CONCLUSION

We established a new, early passage MCV-positive MCC cell line CVG-1 from a patient with metastatic MCC. CVG-1 displays different morphologic features from other MCV-positive MCC cell lines, but nevertheless requires MCV T antigen for cell proliferation. While CVG-1 sT antigen contains a unique missense mutation, the mutant sT demonstrated similar transformation activity to prototypic sT in rodent cells. CVG-1 shows similarities to MKL-1 in viral copy numbers and LT truncation patterns. Further analyses of CVG-1 and MKL-1 may lead to the identification of critical host factors beyond the viral T antigen that contribute to the variations observed in MCC cell lines.

AUTHOR CONTRIBUTIONS

CV, YC, and MS established the CVG-1 cell line. MS conceived and designed the experiments. YA performed the qPCR experiments. MS performed the sequencing experiments. YA, AH, and MS performed the immunoblot and cell transformation experiments. TT performed the immunohistochemistry. MS, YA, and YC analyzed the data. MS, YC, CV, and YA wrote the paper.

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The Human Polyomavirus Middle and Alternative T-Antigens; Thoughts on Roles and Relevance to Cancer

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Approximately 15–20% of human cancer is related to infection, which renders them potentially preventable by antimicrobial or antiviral therapy. Human polyomaviruses (PyVs) are relevant in this regard, as illustrated by the involvement of Merkel cell polyomavirus (MCPyV) in the development of Merkel cell carcinoma. The polyomavirus Small and Large tumor antigen (ST and LT) have been extensively studied with respect to their role in oncogenesis. Recently it was shown that a number of human PyVs, including MCPyV and the trichodysplasia spinulosa polyomavirus (TSPyV), express additional T-antigens called Middle T (MT) and alternative T (ALT). ALT is encoded by ORF5, also known as the alternative T open reading frame (ALTO), which also encodes the second exon of MT, and overlaps out-of-frame with the second exon of LT. Previously, MT was considered unique for oncogenic rodent polyomaviruses, and ALT was still unknown. In this mini-review, we want to point out there are important reasons to explore the involvement of MT and ALT in human cellular transformation. First, just like their rodent equivalents, MT and ALT probably disrupt cellular pathways that control signaling and proliferation. Second, expression of the MT and ALT-encoding ORF5/ALTO characterizes a monophyletic polyomavirus clade that includes human and animal PyVs with known oncogenic potential. And third, ORF5/ALTO is subject to strong positive selection aimed specifically at a short linear motif within MT and ALT that overlaps completely with the RB-binding motif in LT. The latter suggests tight interplay between these T-antigens with possible consequences for cell transformation.

Keywords: polyomavirus, T-antigens, oncogenesis, cell-signaling, evolution

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BACKGROUND

An established causal relationship between a certain cancer and a particular infection provides ample opportunities to study oncogenesis in detail, and identify unique targets for cancer prevention and treatment. The best example in this regard is probably cervical cancer and human papillomavirus (HPV) infection. The E6 and E7 oncoproteins encoded by high risk HPV types are directly involved in tumorigenesis and maintenance of the transformed state (McLaughlin-Drubin and Munger, 2009). Such knowledge has contributed directly to development of preventive HPV vaccines that reduce the incidence of HPV-related (pre)malignancies (Harper and DeMars, 2017).

The rapidly expanding group of human polyomaviruses (PyVs) is also relevant in this regard. For one human PyV, the Merkel cell polyomavirus (MCPyV), causal involvement in the development of Merkel cell carcinoma (MCC) has been established (Feng et al., 2008;

Shuda et al., 2008; Houben et al., 2010). Consequently, MCPyV has been defined as a group 2A carcinogenic biological agent (Bouvard et al., 2012). The polyomavirus Small and Large T-antigens (ST and LT) that resemble HPV E6 and E7 in several ways, are the usual suspects when it comes to cellular transformation, as they are known to deregulate cellular pathways controlling the cell cycle, DNA repair, and apoptosis. The focus of this mini-review, however, will be on two other T-antigens, Middle T (MT) and Alternative T (ALT), both encoded by the Alternate T-antigen open reading frame (ALTO), also known as ORF5, depending on the polyomavirus in which the open reading frame was recognized (Carter et al., 2013; van der Meijden et al., 2013b; Lauber et al., 2015). ORF5/ALTO is expressed by MCPyV (Carter et al., 2013) and by the trichodysplasia spinulosa polyomavirus (TSPyV) that causes dysplasia of human hair inner root sheath cells and follicular spine formation of the skin (van der Meijden et al., 2010, 2015). While for MCPyV ALTO only ALT expression has been demonstrated, TSPyV ORF5 was shown to express both MT and ALT. This mini-review will address what is currently known about the human PyV MT and ALT products and speculate about their role in human cellular transformation.

THE POLYOMAVIRUS T-ANTIGENS, ESPECIALLY THE ORF5/ALTO-ENCODED MT AND ALT PRODUCTS

Because the human PyVs cause rare diseases observed only in specific immunocompromised patient groups, they are less known. Nevertheless they have striking similarities with HPV, including inactivation of the tumorsuppressor proteins p53 and RB through the T-antigens (DeCaprio and Garcea, 2013). Until recently human PyVs were believed to express only two major T-antigens, ST and LT, next to some smaller splice products thereof (Gjoerup and Chang, 2010). Rodent PyVs, including some oncogenic ones (Gross, 1953; Eddy et al., 1962; Girardi et al., 1962), are known to express an additional T-antigen called MT (Ito et al., 1977; Courtneidge et al., 1991; Fluck and Schaffhausen, 2009; Schaffhausen and Roberts, 2009).

Until the discovery of MCPyV in 2008, the T-antigens were merely seen as viral oncoproteins useful to study oncogenesis *in vitro* and in animal models, for instance the murine polyomavirus (MPyV) in the mouse mammary tumor virus (MMTV) breast cancer model (Fluck and Schaffhausen, 2009). This situation changed considerably with the identification of MCPyV in human MCCs (Feng et al., 2008). MCC is a rare but aggressive skin tumor of neuroendocrine origin, although early B-cells have also been proposed as cells of origin (Sauer et al., 2017). The majority of MCCs harbor clonally integrated MCPyV genome copies that express ST and preliminary truncated versions of LT (Feng et al., 2008; Shuda et al., 2008, 2009; Houben et al., 2010). Studies have shown that MCPyV-positive MCC tumor cell growth depends on the interaction of LT with RB (Houben et al., 2012), while ST promotes cell proliferation by deregulation of the mTOR signaling pathway via inactivation of 4E-BP1 (Shuda et al., 2011; Velasquez et al., 2016). The contribution of ALT and MT to development of MCC or any other human tumor type, respectively, is not known at the moment. Below, we summarize some recent findings that should prompt the interest in MT and ALT as potential viral oncoproteins that merit further study.

ONCOGENIC POLYOMAVIRUSES EXPRESSING ORF5/ALTO PHYLOGENETICALLY CLUSTER TOGETHER

Recently, the Polyomaviridae Study Group of the International Committee on Taxonomy of Viruses (ICTV) (Polyomaviridae Study Group of the International Committee on Taxonomy of Viruses et al., 2016; Moens et al., 2017a) established a new phylogeny-based taxonomy based on conserved regions in LT. This resulted in the demarcation of four genera (*alpha–delta*) within the polyomavirus family (**Figure 1**, right part), and the recognition of 13 human PyV *species* found among three genera (*alpha*, *beta,* and *delta)*. The *alpha* genus contains many (if not all) known naturally oncogenic PyVs, underlined in **Figure 1**, including MCPyV (*Human polyomavirus 5*), the raccoon polyomavirus (RacPyV, *Procyon lotor polyomavirus 1*) (Dela Cruz et al., 2013), MPyV (*Mus musculus polyomavirus 1*) and hamster PyV (*Mesocricetus auratus polyomavirus 1*) (Gross, 1953; Graffi et al., 1969). The dysplasia-inducing TSPyV (*Human polyomavirus 8*) also belongs to the *alpha* genus (van der Meijden et al., 2010; Kazem et al., 2012). The BK and JC polyomaviruses (*Human polyomavirus 1 and 2*), which are sometimes implied in bladder and colon cancer, respectively (Dalianis and Hirsch, 2013), do not belong to the alpha genus (**Figure 1**, in blue).

As far as reported, *alpha* PyVs, including three additional human PyVs so far without attributable disease (HPyV9, HPyV12, and New Jersey PyV), are prevalent in the general population, with a seroprevalence up to 80% (Kean et al., 2009; Nicol et al., 2013; van der Meijden et al., 2013a; Gossai et al., 2016). In our laboratory and elsewhere it was shown that only *alphapolyomaviruses* contain a full length ORF5/ALTO open reading frame (Carter et al., 2013; Lauber et al., 2015; van der Meijden et al., 2015; Moens et al., 2017b) (**Figures 1**, **2A**, arrow). The ORF5/ALTO-like shorter open reading frame in PyVs from other genera contains a premature stop codon and does not encode the hydrophobic C-terminus. Full length ORF5/ALTO was previously recognized only in the (oncogenic) hamster and murine PyVs. It is also found in RacPyV causing brain tumors in raccoons (Brostoff et al., 2014).

ALTERNATIVE EXPRESSION OF ORF5/ALTO INVOLVES LT TRANSCRIPTION

The location and context of ORF5/ALTO in the PyV genome is shown in **Figures 2A,B**. ORF5/ALTO overlaps entirely (out

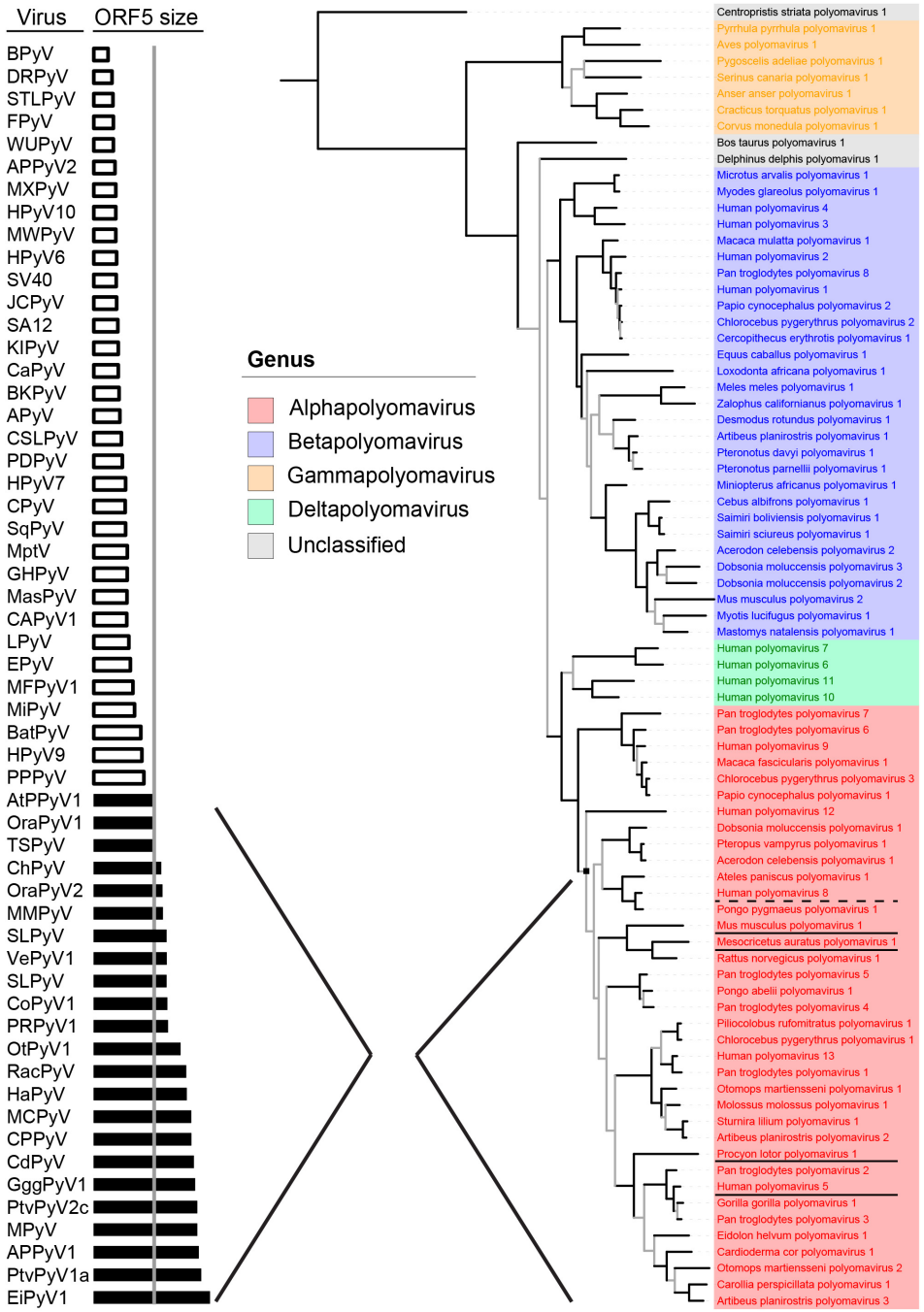

FIGURE 1 | Evolutionary conservation of Middle T and ALT (ORF5) Bayesian phylogeny based on conserved regions in LT, as used by the Study Group of the ICTV for the demarcation of genera (adapted from Moens et al., 2017b) is shown on the right. On the left, polyomaviruses are ordered by ORF5 size. Shown in black bars are intact versions of ORF5, containing the characteristic disordered domains, SLIMs, proline-rich areas and a C-terminal transmembrane domain, that define the major clade (node) of the *alphapolyomaviruses* shown in red on the right. *Alphapolyomaviruses* with known oncogenic or dysplastic properties are respectively underlined or with dashed line.

of frame) with the second exon of LT, which is encoded by ORF2. As shown by us for TSPyV (van der Meijden et al., 2015), ORF5/ALTO is expressed in two different ways to encode MT or ALT. MT is expressed through alternative splicing resulting in a transcript that combines exon 1, which largely overlaps with ST and the first exon of LT, with exon 2 encoded by ORF5/ALTO. Next to this regular expression pattern seen in many more PyVs and HPVs, ORF5/ALTO can be expressed also on its own to encode ALT. For this purpose, the major T-transcript encoding LT is used (van der Meijden et al., 2015). *In vitro*

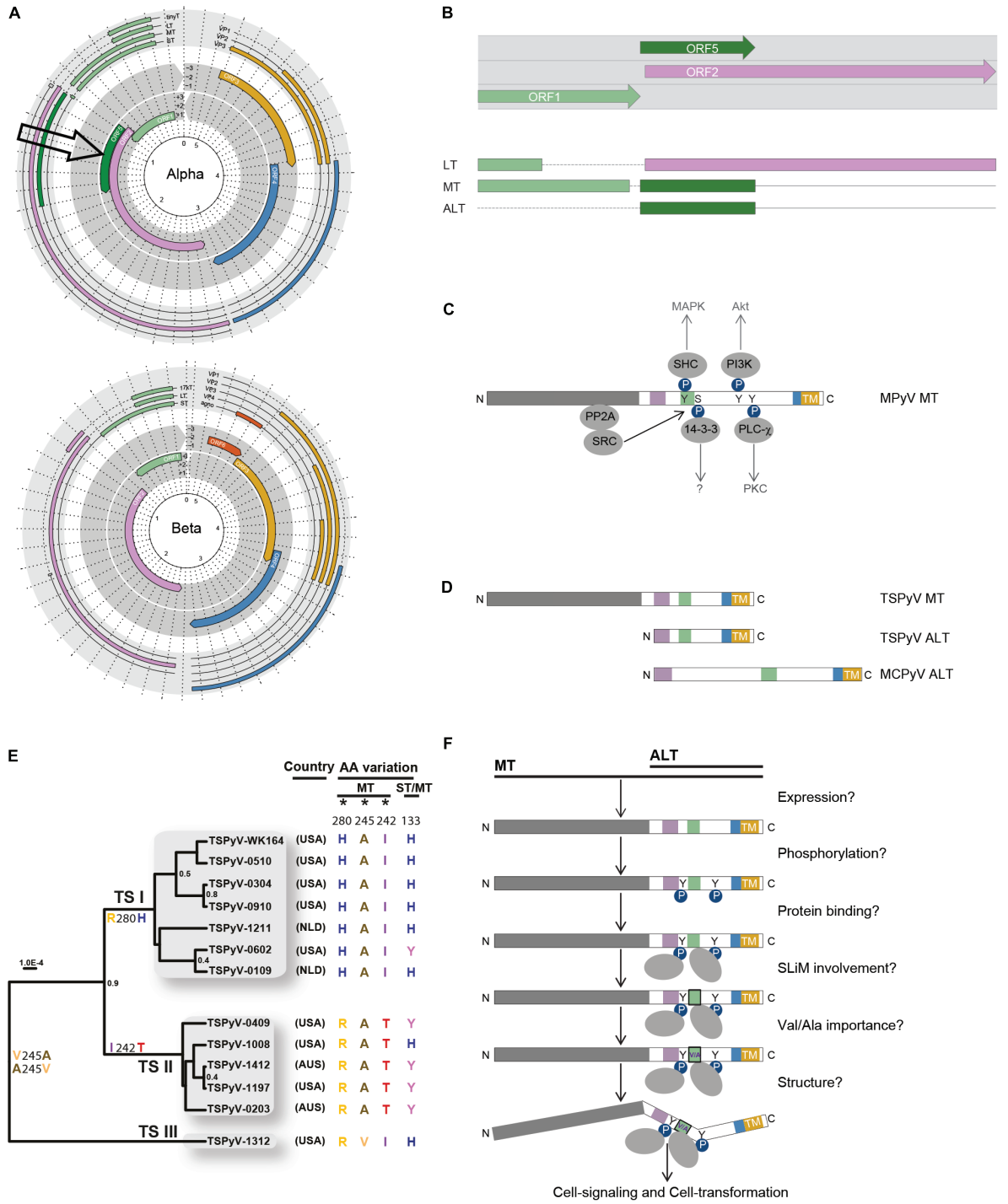

FIGURE 2 | Expression, evolution, and putative role of ORF5 and MT/ALT in cell-signaling and transformation. **(A)** Genomes of an *alpha* and *betapolyomavirus* are shown (adapted from Moens et al., 2017b). Notice the presence of ORF5 in the *alphapolyomavirus* genome (arrow). **(B)** ORF5 encodes the second exon of MT and ALT, and overlaps with the second exon of LT. The colors of the (spliced) T-antigens correspond to the encoding ORF. **(C)** Phosphorylation of mouse polyomavirus (MPyV) MT by Src tyrosine kinase interacting with PP2A-bound by the N-terminal part of MT shown in gray. Phosphorylated MT induces docking and activation of members of the signal transduction pathway, SHK, PI3K, 14-3-3, and PLC (Dilworth, 2002). The colored blocks represent SLiMs present in the intrinsically disordered region (IRD) shown in white (Lauber et al., 2015). The C-terminal transmembrane domain (TM) is shown in yellow. Phosphorylation (P-spheres) of tyrosines (Y) and protein interactions (gray ovals) are depicted. **(D)** Schematic representation of human MT and ALT expressed by TSPyV and MCPyV (colors and shapes as explained in **C**). **(E)** TSPyV phylogeny adapted with permission from Kazem et al. (2016) showing three lineages of TSPyV defined by predominant non-synonymous substitutions in MT/ALT during evolution. **(F)** Schematic overview of HPyV MT/ALT related issues regarding its state, structure and role (colors and shapes as explained in **C**).

at least, the latter route seems to generate the most abundant product, as much more ALT than MT protein is detected (van der Meijden et al., 2015). For MCPyV thus far only ALT expression from ORF5/ALTO was shown. How the major start codon used by the LT transcript is avoided and whether ALT expression involves an internal ribosome entry site (IRES) and the use of a downstream alternative start codon has not been resolved yet.

It is challenging to speculate why ORF5/ALTO is expressed in different ways, from different transcripts. We are not aware of any other viral product that is encoded like this. Possibly ORF5/ALTO expression, either as MT or as ALT, is of crucial importance to the virus life cycle and therefore needs to be ensured at all times, for example during different stages of the host cell cycle or in differentiating host cells. Alternatively, alternating ALTO expression might play a role in regulating LT expression, as there might be competition between ALT and LT for the same transcript. Further studies are needed to explore this possible regulatory process, which might impact on the transforming potential of LT as well. In the case of MCPyV, there might be a correlation with the truncation of MCPyV LT observed in MCC cells, which might call for ALT expression, for instance to supplement a necessary factor normally provided by LT. It should be noticed, however, that in MCC also ALT is often truncated (Carter et al., 2013). Whether the capacity to express both MT and ALT (e.g., TSPyV) opposed to only ALT (e.g., MCPyV) correlates with the capacity to integrate in the host genome as shown for MCPyV is unknown at the moment.

THE ORF5/ALTO ENCODED MT AND ALT PRODUCTS ARE LARGELY INTRINSICALLY DISORDERED AND LIKELY INVOLVED IN CELL-SIGNALING

The shared C-terminal part of MT and ALT encoded by ORF5/ALTO contains several small conserved regions called short linear motifs (SLiMs, colored blocks in **Figures 2C,D**) within larger intrinsically disordered regions (IDRs) (Lauber et al., 2015). IDR and SLiM-containing unstructured proteins are frequently encoded by overlapping ORFs and involved in numerous biologic processes related to disease, including cancer (van der Lee et al., 2014; Xue et al., 2014). As expected for intrinsically disordered proteins, comparison of the shared MT and ALT encoding sequences between different *alphapolyomaviruses* reveals little homology.

From murine studies we know that the second part of MT is proline-rich, contains phosphotyrosine and phosphoserine residues and harbors a transmembrane domain at the very C-terminal end (**Figure 2C**) (Dilworth, 2002; Cheng et al., 2009; Fluck and Schaffhausen, 2009). Functionally, MT acts as an engaged transmembrane growth factor receptor (Dilworth, 2002; Fluck and Schaffhausen, 2009). Upon membrane and PP2A binding, MT interacts with Src tyrosine kinase, which results in phosphorylation of MT and subsequent activation of important phosphokinases, such as PI3K and MAPK involved in cell-signaling (**Figure 2C**) (Dilworth, 2002). Furthermore, the Hippo pathway effectors Taz and Yap, bind to MPyV MT and thereby support Src activation and cellular transformation by MT (Rouleau et al., 2016; Shanzer et al., 2017).

Expression of a full-length ORF5/ALTO (**Figure 1**, black bars), containing the characteristic disordered domains, the SLIMs, the proline-rich areas, the phosphoamino acid residues and the transmembrane domain, defines the vast majority of PyVs within the *alpha* genus (**Figure 1**), including MCPyV and TSPyV. Whether human PyV MT and ALT also show established rodent MT-like properties and are potentially involved in cellular transformation is not known yet. From sequence comparisons showing that the involved motifs are present, for example PP2A in MT, this seems likely. In co-immunoprecipitation experiments and with mutational analysis it was shown that TSPyV MT interacts with PP2A, which involves activation of the MAPK signaling pathway (Wu et al., 2017), as described for MPyV MT (Dilworth, 2002; Cheng et al., 2009; Fluck and Schaffhausen, 2009). We have recently obtained preliminary data showing that MCPyV ALT indeed is phosphorylated.

For MCPyV it was shown that an intact transmembrane domain is required for the subcellular distribution pattern of ALT, comparable to discrete subcellular membrane binding observed for MPyV MT (Zhou et al., 2011; Carter et al., 2013). MPyV MT mutant studies showed that membrane association is required for MT-induced transformation (Novak and Griffin, 1981; Carmichael et al., 1982).

EVOLUTIONARY CONSERVATION OF ORF5/ALTO IS TIGHTLY LINKED TO THE LT RB-BINDING MOTIF

As illustrated above, ORF5/ALTO expression seems important for specific human PyVs, including MCPyV and TSPyV. This relevance is underscored by two recent studies that looked into the evolutionary conservation of ORF5/ALTO among PyVs, especially members of the *alpha* genus (Carter et al., 2013; Lauber et al., 2015). Especially the latter study showed that expression of a full-length ORF5/ALTO (**Figure 1**, black bars), containing the characteristic disordered domains, the SLIMs, the proline-rich areas and the transmembrane domain, defines the vast majority of PyVs within the *alpha* genus (**Figure 1**).

Single nucleotide polymorphism and evolutionary analyses of TSPyV revealed that ORF5/ALTO is the predominant site of non-synonymous substitutions during PyV evolution (**Figure 2E**) (Kazem et al., 2016), subject to strong positive selection (Lauber et al., 2015). Analysis of the entire PyV family showed this pressure is focused on a single amino acid position (MT245/ALT44 for TSPyV, **Figure 2E**) located within a conserved SLiM (green block in **Figures 2C,D**) allowing only two SLiM variants. The latter is highly unusual among conserved residues and results in binary toggling between

Valine and Alanine. Particular interesting in this regard is the information that this restriction is imposed by the highly conserved RB-binding motif (LXCXE) within LT, which completely overlaps with the relevant SLiM (Lauber et al., 2015).

The impact of this finding might seem trivial, since no preference for either a Valine or an Alanine residue is observed among oncogenic PyVs or on the (human) *alphapolyomaviruses* as a whole. However, a dynamic analysis among PyV species taking into account the binary exchange rate of the second codon position corresponding to either Valine–Alanine clearly showed that codon-constrained Valine–Alanine (COCO-VA) toggling is significantly accelerated in ORF5-containing (*alpha)polyomaviruses* compared to ORF5-less viruses (Lauber et al., 2015).

Comparison of 13 TSPyV genomes showed one divergent isolate with a Valine instead of an Alanine residue at position 245 (TSPyV-1312, **Figure 2E**) (Kazem et al., 2016). Adaptation within one species is not observed for any other PyV. For instance in MCPyV, an Alanine residue at position 245 is present in all GenBank-deposited genome isolates regardless of expression of full-length or truncated ALT.

The finding of two overlapping, extremely conserved motifs (the MT/ALT SLiM and the LT RB-binding motif), again suggests that the functional roles of MT/ALT and LT are mutually connected. Since the highly conserved RB-binding motif is also present in the E7 gene of HPV (McLaughlin-Drubin and Munger, 2009), and in the E1A gene of adenoviruses (Munger et al., 2004), we looked for a comparable situation in these related virus families. However, in both of these virus families the RB-binding motif is located in a non-overlapping coding region of the genome, highlighting the unique constellation of the ORF5/ALTO-expressing PyVs.

In general, the presence of two overlapping conserved motifs is rare. More often the organization of functional motifs is such that critical functional residues in one protein overlap with highly mutable regions of the other protein, like observed in the overlapping E2 and E4 genes, and the tat and rev genes of HPV and HIV1, respectively (Hughes and Hughes, 2005; Narechania et al., 2005; Fernandes et al., 2016).

CONCLUSION, DISCUSSION, AND PERSPECTIVE

So far, searches into the role of polyomaviruses in human cancer have focused on the traditional T-antigens LT and ST. Since expression of full length ORF5/ALTO characterizes members of the 'oncogenic' *alphapolyomavirus* genus, we consider it important to study the transforming properties of MT and ALT as well. Next to straightforward approaches investigating involvement of MT and ALT in human cell-signaling and cell cycle regulation through phosphorylation (**Figure 2F**), the relevance of the identified MT/ALT SLiM and Valine–Alanine variants on cell-signaling and cell cycle regulation merits further study. Since SLiMs are involved in (transient) protein interactions and folding of disordered proteins (Davey et al., 2012) (COCO-VA) SLiM variants might impact on the structure, protein-binding and role of MT and ALT (**Figure 2F**). These investigations should take into account the role of the overlapping, possibly competing or supplementing, LT product with known transforming properties that contains the MT/ALT SLiM-overlapping RB-binding motif. Altogether, we believe studying MT and ALT in conjunction with LT will provide valuable insight in the biology of PyV, which will further reveal the involvement of PyV infection in human cancer and possibly provide new targets for cancer prevention and treatment.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Detection of Merkel Cell Polyomavirus in Seborrheic Keratosis

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Seborrheic keratosis (SK) is the most common benign cutaneous neoplasm. A subset shows increased p16 expression. Since SK shares several features with verruca vulgaris, e.g., increased p16 expression, human papillomaviruses (HPV) have been suggested as possible causal agents. However, a relevant association could not be established between HPV and SK. In the present study we aimed to investigate the presence of Merkel cell polyomavirus (MCPyV) in relation to p16 expression in SK. P16 expression was investigated using immunohistochemistry (IHC). Presence of MCPyV was assessed in 23 formalin-fixed paraffin-embedded tissue samples of SK by molecular techniques (i.e., PCR and FISH) and IHC. 16/23 SK showed strong to moderate p16 expression. 6/23 of SK were MCPyV positive by PCR which was confirmed by FISH. Of interest, two samples with strong FISH signals also showed MCPyV expression as tested by IHC. Samples with weaker signal intensity were negative in IHC. P16 expression was not associated with the presence of MCPyV. Concluding, the detection of MCPyV DNA by PCR and FISH in SK reflects the widespread prevalence of MCPyV in the skin. However, low detection rates exclude MCPyV as a major pathogenic factor in SK, most likely representing a coincidental infection. P16 IHC does not appear as useful adjunctive surrogate marker for the presence of MCPyV in SK.

Keywords: seborrheic keratosis, Merkel cell polyomavirus, MCPyV, p16, fluorescence *in situ* hybridization, FISH, immunohistochemistry, IHC

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INTRODUCTION

Seborrheic keratosis (SK) is the most frequent benign human skin proliferation, with increasing incidence in the elderly. The preferential localization is on the chest, interscapular region, waistline, and forehead (Yeatman et al., 1997; Kyriakis et al., 2012). SK shares several clinical and histopathological features with verruca vulgaris and condyloma acuminatum including increased p16 expression which is a tumor-suppressor protein and cyclin-dependent kinase (cdk) inhibitor (Chazal et al., 2002; Hodges and Smoller, 2002; Nakamura and Nishioka, 2003; Genders et al., 2017). Assessment of p16 protein expression by immunohistochemistry (IHC) is frequently used as a surrogate marker for human papillomavirus (HPV) infection in genital and oropharyngeal cancers (Dehn et al., 2007; Shelton et al., 2017). HPV infection has been suggested as a possible causative agent in SK, however a higher prevalence of HPV was only detected in subgroups of genital SK in comparison to non-genital SK, thereby excluding HPV to play a major pathogenic role (Zhu et al., 1991, 1992; Gushi et al., 2003; Nakamura and Nishioka, 2003; Tardío et al., 2012). Limited

data is available concerning a possible role of p16 expression in SK and association with Merkel cell polyomavirus (MCPyV; Andres et al., 2010; Mertz et al., 2010). Similar to HPV, MCPyV is composed of a circular double-stranded DNA genome, possesses an icosahedral capsid symmetry, a homologous LxCxE motive in the encoded Retinoblastoma (Rb) binding site (Felsani et al., 2006; Shuda et al., 2008), and has a similar genome size (approximately 5,600 bp for MCPyV and 8,000 bp for HPV). In high risk HPV infection the Rb protein is inactivated resulting in cell cycle progression. As a consequence p16 expression is upregulated. Approximately 80% of Merkel cell carcinomas (MCC), a rare highly aggressive neuroendocrine skin cancer, are associated with MCPyV infection (Feng et al., 2008; Kassem et al., 2008). Interestingly, MCC and SK share several risk factors, such as long-term sun exposure, older age, and immunosuppression. Based on these findings we investigated p16 IHC expression in SK and tested for the presence of MCPyV using IHC and molecular methods (FISH and PCR).

MATERIALS AND METHODS

Patients and Tissues

Formalin-fixed and paraffin-embedded (FFPE) tissues of 23 skin specimens were included in this study. All respective samples had been excised for diagnostic and therapeutic reasons and were obtained from the Maastricht Pathology Tissue Collection (MPTC). All use of tissue and patient data was in agreement with the Dutch Code of Conduct for Observational Research with Personal Data (2004) and Tissue (Federatie van Medisch Wetenschappelijke Verenigingen, FMWV, https://www.federa.org/) with written informed consent from all subjects in accordance with the Declaration of Helsinki. The protocol was approved by the Maastricht Ethic Committee (MEC) group. Diagnoses were previously defined by histology in routine diagnostics and have been reviewed by 3 experienced dermatopathologists (VW, AzH, LMH). The patient group consisted of 9 men and 13 women (ages 34–79; mean 58.8). Further details of the clinicopathologic parameters are included in **Table 1**. Serial sections of the specimens were used for hematoxylin and eosin (H.E.) staining, IHC, fluorescence *in situ* hybridization (FISH) and DNA isolation. In addition 16 non-neoplastic skin specimens originating from patients undergoing plastic surgery were tested (ages 18–63 year; mean 41.4 year).

Immunohistochemistry (IHC)

The following antibodies and dilutions were used in this study: anti-LT MCPyV (clone: CM2B4, dilution 1:50, Santa Cruz, Inc.), anti-p16 (clone: JCS, dilution 1:400, Santa Cruz, Inc.). Immunohistochemical stainings were conducted on a Dako Autostainer Link 48 using the EnVision FLEX Visualization Kit K8008 DAKO according to standard diagnostic routine protocols and manufacturers' instructions. P16 expression was correlated with the results obtained by MCPyV FISH and PCR data as described earlier (Hopman et al., 2005; Mertz et al., 2013; Haugg et al., 2014). P16 expression was evaluated by 4 experienced investigators (AzH, VW, DR, LH) with − as negative, + as moderate and ++ as strong positive score (**Figure 1**).

DNA Extraction

Five consecutive 10 μm thick sections were cut from each FFPE tissue. After deparaffinization, the tissues were lysed with proteinase K overnight (56°C) until complete tissue lysis, and DNA was extracted using a DNA Isolation QIAamp minikit (Qiagen, Hilden, Germany). Purified DNA was measured in a spectrophotometer (Nanodrop, 2000; Thermo Scientific). DNA quality and integrity were assessed by a specimen control size (SCS) ladder (**Table 1**) as described earlier (van Dongen et al., 2003). Inadequate samples were excluded from further study.

Polymerase Chain Reaction (PCR)

PCR was performed with 150 ng of genomic DNA using the AmpliTaq Gold (Roche) DNA polymerase in a final volume of 50 μl. For MCPyV detection we used the VP1 and M1M2 primer sets and PCR conditions as published earlier (Feng et al., 2008; Kassem et al., 2008). Negative controls with water instead of patient samples were included in each amplification series.

Sequence Analysis

PCR products were submitted to automated nucleotide sequencing in an ABI 3130XL genetic analyser (ABI). DNA sequences were compared and analyzed with the reference sequences of the National Center for Biotechnology Information (NCBI) Entrez Nucleotide database gb EU375803.1 (MCC isolate 350) and gb EU375804.1 (MCC isolate 339) using the NCBI Blast program. Multiple sequence alignments were performed with Clustal W2 (EMBL-EBI-2015).

MCPyV Fluorescence *in situ* Hybridization (FISH) Probe

FISH was optimized and performed as described earlier (Hopman et al., 2005; Haugg et al., 2011, 2014). In brief, full length (5104 bp) MCPyV DNA was cloned into a StrataClone PCR Cloning Vector (pSC-A-amp/kan; Stratagene, Santa Clara, CA). The Plasmid DNA Purification Kit NucleoBond® PC 2000 (Macherey-Nagel, Dueren, Germany) was used to extract MCPyV plasmid DNA and sequenced using T7 and T3 primers. Labeling of the DNA was performed by standard nick translation with Biotin-Nick Translation Mix (Roche, Mannheim, Germany) containing biotin (Bio)-16-dUTPs. The final concentration of the labeled DNA was 2 ng/μl in 50% formamide, 20% dextran sulfate, 2x SSC pH 7.0, 50x excess carrier DNA from salmon sperm (Sigma Chemical, St. Louis, MO) and 50x tRNA from S. cerevisiae (Sigma Chemical, St. Louis, MO).

Detection of MCPyV by FISH MCPyV

Deparaffinized 3 μm thick sections were pretreated for 20 min with 0.2 M HCl, incubated with 1 M NaSCN for 30 min at 80°C, washed in dH_2O and 2x SCC and digested with 1 mg/ml pepsin (2,500–3,500 U/mg, Sigma Chemical, St. Louis, MO) in 0.14 M NaCl solution, pH2. The biotin labeled full length MCPyV DNA probe was added to the samples at a concentration of 5 ng/μl followed by denaturation of DNA (5 min, 80°C) and hybridization overnight (37°C, humid chamber, Thermobrite, Abbott, IL). Unbound MCPyV DNA probe was stringently washed away in 2x SSC, pH7

TABLE 1 | Summary of clinicopathological data, immunohistochemical staining and molecular results.

Lab ID	Age	G	Localization	P16 IHC	SCS	PCR MCPyV M1M2	PCR MCPyV VP1	FISH MCPyV	IHC MCPyV
VS1.1	55	f	Breast	+	+	+	+	+	−
VS2.2	79	f	Back	++	+	−	−	NA	−
VS4.1	58	f	Head	++	+	−	−	−	−
VS5.1	49	f	Back	−	+	+	+	+	−
VS6.1	70	f	Back	−	+	−	−	−	−
VS8.1	52	f	Head	++	+	+	−	+	−
VS10.1	65	f	Breast	+	+	+	−	+	−
VS11.1	56	m	Head	+	+	+	−	++	+
VS12.1	34	f	Axilla	−	+	−	−	−	−
VS13.1	59	f	Head	++	+	+	−	++	+
VS14.1	58	m	NA	−	+	−	−	NA	NA
VS15.2	59	m	Back	+	+	−	−	NA	NA
VS16.2	68	f	Head	+	+	−	−	NA	NA
VS17.2	47	f	Head	++	+	−	−	NA	NA
VS19.2	49	f	Head	−	+	−	−	NA	NA
VS20.2	73	m	Back	−	+	−	−	NA	NA
VS25.2	60	f	Breast	+	+	−	−	NA	NA
VS30.2	73	m	Back	+	+	−	−	NA	NA
VS37.2	58	f	Breast	++	+	−	−	NA	NA
VS38.2	73	m	Back	++	+	−	−	NA	NA
VS53.2	58	m	Head	++	+	−	−	NA	NA
VS54.2	54	m	Back	−	+	−	−	NA	NA
VS56.2	45	m	Back	+	+	−	−	NA	NA
Total: 23 samples	Mean: 58.8 year, SD 10.6 year	f ($n = 14$, 60.9%) m ($n = 9$, 39.1%)		++ 8/23 (34.8%) + 8/23 (34.8%) − 7/23 (30.4%)		6/23 26.1%	2/23 8.7%	6/23 26.1%	2/23 8.7%

f, female; FISH, fluorescence in situ hybridization using full length MCPyV as probe with ++, strong, +, moderate, and −, negative nuclear signal pattern; G, gender; p16 IHC, immunohistochemistry using p16 with ++, strong; +; moderate; −, negative staining pattern; Lab ID, laboratory identification; m, Male; M1M2 PCR, PCR amplifying the M1M2 PCR product (178 bp); NA, not applicable; SCS ladder, specimen control size ladder; SD, standard deviation; VP1, PCR amplifying the viral capsid protein 1 (VP1) gen (315 bp); y, years.

at 70°C for 2 min. Bound probe was detected by sequential incubation in a combination of fluorescein isothiocyanate (FITC) biotinylated avidin (AvFITC; 1:500; Vector, Brunswig Chemie, Amsterdam, The Netherlands) and biotin conjugated goat antiavidin (BioGaA; 1:100; Vector). Prior to incubation aspecific binding sites were blocked with Boehringer Blocking reagent. Cell nuclei were counterstained and coverslipped with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; 0.2 μg/ml, Vectashield, Vector Laboratories, CA). Samples were visualized using a DM 5000B fluorescence microscope (Leica, Wetzlar, Germany) coupled to an online digital camera (Leica DC 300 Fx) for independent evaluation of FISH signals by 3 investigators (AzH, DR, LH) according to criteria described earlier (Hafkamp et al., 2008; Haugg et al., 2014).

Statistics

Data analysis was performed by SPSS statistical software (SPSS for windows, release 23.0; SPSS Inc., Chicago, IL, USA). Dichotomous variables were compared using the Pearson's chi-square test or Fisher's exact test as appropriate. The Spearman's rank correlation was used in nonparametric data to study the associations between different variables. A two-sided *P-value* less than 0.05 was considered statistically significant.

RESULTS

P16 Expression in SK

In total 16/23 (69.6%) of SK samples showed increased p16 expression in comparison to normal adjacent perilesional skin in all 16 samples. 8/23 (34.8%) showed strong (++) nuclear as well as cytoplasmatic p16 expression of the keratinocytes patchy scattered throughout the lesions. 8/23 (34.8%) showed a moderate (+) patchy p16 staining profile. 7/23 (30.4%) samples were negative for p16 expression (**Figure 1** and **Table 1**).

MCPyV Detection in SK by PCR, FISH, and IHC

PCR directed against diverse regions of the viral genome revealed that 6/23 (26.1%) of SK were MCPyV positive, including the viral M1M2 PCR product and in two cases the VP1 PCR product (**Table 1**). Sequence analyses of the PCR amplicons identified all PCR products as MCPyV DNA sequences. In the non-neoplastic

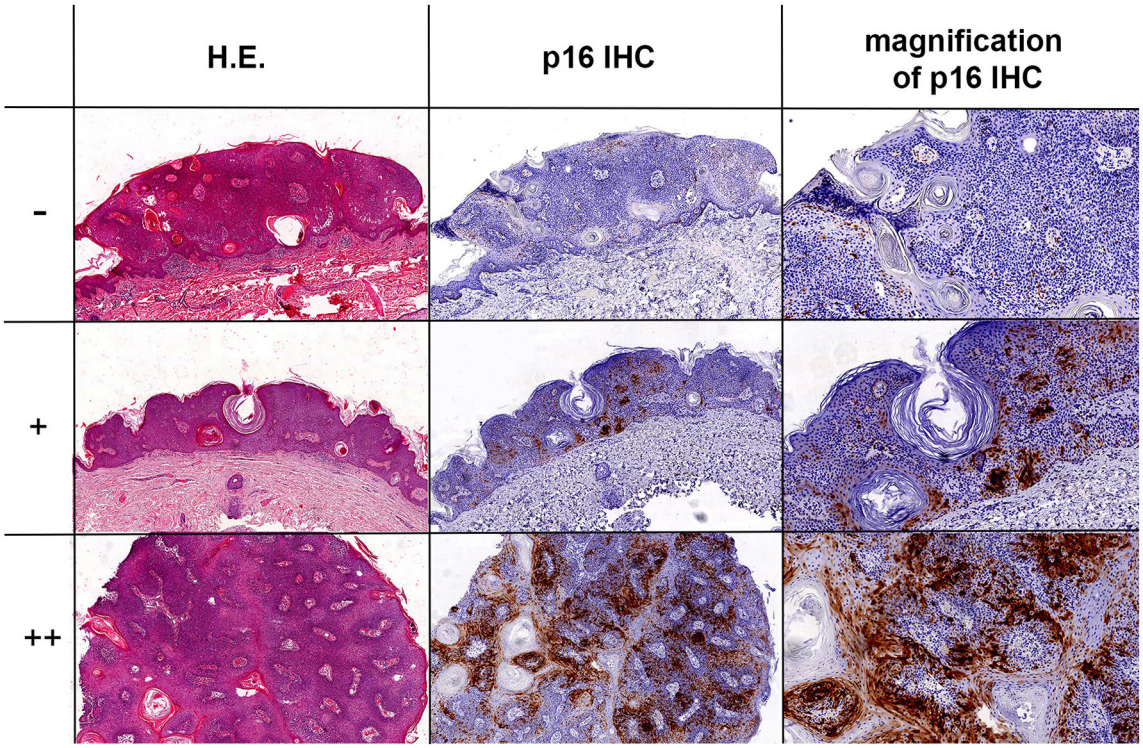

FIGURE 1 | P16 IHC expression in SK. Three representative SK samples with H.E. staining and p16 IHC. The top images show a negative (–) score, center images a moderate (+) positive score, and bottom images a strong (++) positive score with nuclear and cytoplasmic staining for p16 in SK. The magnification of all pictures is 10x, with exception of the pictures in the very right row (top and center plot magnification: 20x; bottom plot magnification: 40x).

skin specimens the presence of MCPyV PCR products was detected in 3/16 (18.8%) cases (data not shown). Results from PCR analyses could be confirmed by MCPyV FISH revealing nuclear hybridization with a punctual signal pattern in 6/23 (26.1%) of SK (**Figure 2A** and **Table 1**). In sample 11 and 13 there was a strong (++) nuclear signal pattern with FISH analyses. In sample 1, 5, 8, and 10 FISH signal intensity was moderate (+) and presence of MCPyV could be confirmed on PCR level, but was not detected by IHC. By IHC sample 11 and 13 (2/23, 8.7%) showed nuclear positivity for MCPyV large T (LT) antigen. The nuclear staining was seen in keratinocytes of SK and partly in surrounding intracapillary blood cells that resembled lymphocytes (**Figure 2B**). Molecular analyses with PCR and FISH were in good agreement (MCPyV M1M2 PCR vs. FISH with $p = 0.03$ and MCPyV VP1 PCR vs. FISH with $p = 0.6$). Importantly, specific nuclear hybridization signals of MCPyV were in several samples not only restricted to intralesional keratinocytes, but also seen in the adjacent preexistent skin. Thoroughly tested MCPyV-PCR negative cases subjected to FISH analyses and IHC staining remained negative for MCPyV.

No Correlation of p16 Expression with MCPyV in SK

P16 expression was neither associated with presence of MCPyV on the DNA level ($p = 0.69$ for the M1M2 PCR product, $p = 0.34$ for the VP1 PCR product) nor on the translational level ($p = 0.91$). Furthermore there was no significant correlation of p16 expression with clinicopathological data (i.e., age, gender or localization of the lesions, **Table 1**).

DISCUSSION

In this study, we systematically evaluated p16 expression and the presence of MCPyV in SK and normal skin on the DNA and on the translational level by IHC. We were able to correlate molecular PCR results with the presence of viral DNA at a single cell level visualized by FISH analyses.

In our study 16/23 (69.6%) of SK showed a patchy moderate to strong p16 expression, and 7/23 (30.4%) of SK were negative for p16 in IHC. These findings are consistent with previous observations reporting 65% of SK with a patchy p16 expression profile and 35% of SK to be negative for p16 (Harvey et al., 2013). In another study 75% of benign keratotic lesions (including 11 SK and 5 verruca vulgaris) showed p16 staining including two SK lesions with intense p16 staining profile (Genders et al., 2017). Since the discovery of p16 in 1993 (Serrano et al., 1993), there has been an ongoing, controversial discussion concerning the meaning of increased p16 expression, which acts as a cyclin dependent kinase (CDK) inhibitor and specifically blocks CDK4 and CDK6. This blockade leads to decreased phosphorylation of Rb protein with subsequent arrest in the G1 phase of the cell cycle (Sharpless and DePinho, 1999). In general three major

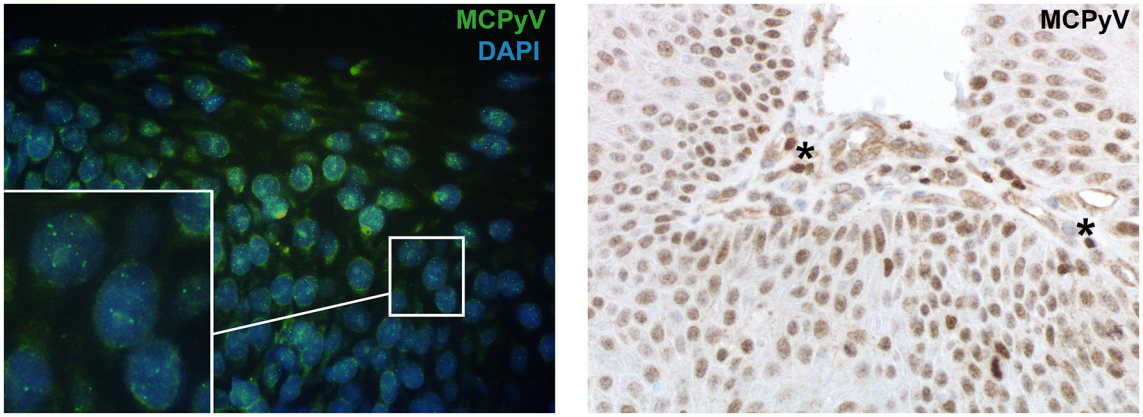

FIGURE 2 | Presence of MCPyV detected by FISH and IHC in SK. **(A)** MCPyV DNA sequence nuclear hybridization signals visualized by FISH in the keratinocytes of the lesion (green); DAPI staining depicts the nuclei of the keratinocytes (blue) with a magnification of 63x. The white square shows a high magnification of nuclei to better illustrate the punctuate hybridization pattern. **(B)** IHC staining directed against MCPyV-LT antigen shows nuclear signals in the keratinocytes as well as in intracapillary blood cells, resembling lymphocytes (see *) with a magnification of 40x.

interpretational lines are followed in case of increased p16 expression in human epithelial cells: (i) p16 and its role as tumor progression marker (Nilsson et al., 2004; Conscience et al., 2006; Bagazgoitia et al., 2010; Harvey et al., 2013), (ii) p16 expression as virus surrogate marker in HPV associated lesions (Klaes et al., 2002; Nilsson et al., 2004; Horn et al., 2008; Schache et al., 2011), and (iii) p16 as a marker for cell senescence (Pavey et al., 1999, 2001; Nakamura and Nishioka, 2003; Vun et al., 2006; Liu et al., 2009; Donati et al., 2013; Shelton et al., 2017). In SK enhanced p16 expression has been mainly attributed to cell senescence and UV-exposure related photo-aging (Pavey et al., 1999, 2001; Nakamura and Nishioka, 2003; Liu et al., 2009; Donati et al., 2013). In the background of cutaneous lesions increased p16 expression has also been associated with malignant progression in actinic keratosis and Bowen's disease (Hodges and Smoller, 2002; Nilsson et al., 2004; Conscience et al., 2006; Bagazgoitia et al., 2010; Harvey et al., 2013). However, this interpretational approach seems not to account for increased p16 expression in SK, in which malignant transformation remains an extremely rare event (Vun et al., 2006; Rajabi et al., 2012; Conic et al., 2017). In the background of virus infection p16 IHC is a well-established surrogate marker for the diagnosis of HPV associated squamous cell neoplasms of the female genital tract and the oropharynx and the nasopharynx (Klaes et al., 2002; Horn et al., 2008; Schache et al., 2011; Vent et al., 2013). In line with this we hypothesized that the increased p16 in SK is possibly associated with a MCPyV infection in SK. However, in this study p16 expression did not correlate with presence of MCPyV.

To date there are only few data available on the prevalence of MCPyV in SK. Sample sizes of SK in other studies included at maximum 12 patients (Andres et al., 2010). Although the present study contains the largest sample number of SK ($n = 23$) and normal skin ($n = 16$) testing for the presence of MCPyV, the study number remains small and might represent a limitation. Designing this study, we aimed for a sample size of $n = 20$ for both groups. This number is statistically sufficient to screen if there is increased presence of MCPyV in relation to normal skin and if this correlates with p16 expression. A positive correlation was not identified such that the sample size has not been enlarged. Since normal skin by definition does not constitute an indication for a diagnostic procedure the number $n = 20$ could unfortunately not be sustained completely ($n = 16$).

Andres et al. reported an overall presence of MCPyV in 6% of sun-exposed non-MCC lesions. Interestingly both MCPyV positive non-MCC samples belonged to the SK-group (2 of 12 samples, 17%) while all lentigo maligna melanoma and basal cell carcinomas were negative for MCPyV (Andres et al., 2010). Concerning SK, this is in line with the findings in this study detecting the presence of MCPyV in 6 out of 23 samples (26.1%). Other reported data about presence of MCPyV in SK represent a case report of one patient with 3 SK samples. The patient was immunosuppressed with a MCPyV positive MCC. Mertz et al. found MCPyV sequences in all of this patients common warts (4/4), half of his carcinoma *in situ* lesions (3/6) and two SK (2/3; Mertz et al., 2010). Depending on the detection method, we found MCPyV in 8.7% of samples via IHC and in 26.1% by molecular analyses by PCR and FISH in SK. We could not demonstrate an increased prevalence of MCPyV in SK compared to the tested normal non-neoplastic skin samples ($n = 16$), thereby most likely excluding MCPyV from playing a major pathogenic role in SK.

In this study MCPyV PCR sequences were detected in 3/16 (18.8%) of normal skin samples. Data from the literature show high variability of MCPyV in normal skin with percentages between 0% (Garneski et al., 2009; Kassem et al., 2009; Mangana et al., 2010) and 78% (Loyo et al., 2010) with most data ranging from 17 to 24% (Wieland et al., 2009; Foulongne et al., 2010; Mertz et al., 2010). The differences in the reported prevalence between the studies may be caused by heterogeneity of the study population (immune status, age-, and gender distribution), as well as heterogeneity in performed methods for detection of viral load (i.e., primer selection, viral-DNA copy number, etc. Dworkin et al., 2009; Garneski et al., 2009; Wieland et al., 2012).

In case of PCR as screening method, multiple factors may account for the variable MCPyV detection rates including tissue quality, quantity and fixation, DNA extraction method, PCR technique, choice of viral gene targets, and primer selection. A further limitation is the relatively low number of investigated samples from studies which have addressed normal, non-neoplastic, skin samples.

In line with other studies, the used MCPyV primer sets yielded partially heterogeneous results. Differences in detection frequency might be caused by changes due to the viral integration process (Andres et al., 2010; Kassem et al., 2010). In this study we were able to correlate molecular results from PCR to a single cell level and visualize MCPyV nucleic acids with FISH, thereby studying infected cells within the histological context. The two samples with strong punctual nuclear signal pattern in FISH analyses also showed IHC MCPyV expression on the translational level. PCR positive samples with weak signal intensity in FISH analyses were negative in IHC. This observation is in line with findings of several other studies which detected MCPyV on the IHC level to a lesser extent than by PCR (Katano et al., 2009; Shuda et al., 2009; Mertz et al., 2010; Ly et al., 2012). Discrepancies in MCPyV detection rates may be attributed to the enhanced sensitivity of PCR and FISH to detect low viral loads in contrast to IHC. Although unlikely, another reason for the lower detection rate with IHC might be a putative mutational loss of the antibody epitope. Indeed, the discrepancy in PCR and FISH compared to IHC is partly because PCR can target other viral components such as structural proteins VP1, VP2, and VP3, small T antigen and large T (LT) antigen, whereas the IHC derived from clone CM2B4 only targets the MCPyV LT antigen. Finally, although PCR effectively detects viral DNA, it does not distinguish incidental presence of virus from a causal infection. Comparatively, MCPyV FISH enables direct visualization of MCPyV DNA on a single cell level, while IHC allows the direct visualization of nuclear LT antigen expression only in the setting of relatively high viral load which may be more indicative of a causal infection (Ly et al., 2012; Haugg et al., 2014). Summarizing these findings indicate that MCPyV infection with low viral amplification rate (i.e., viral load) yields positive in FISH and PCR analyses but negative in IHC results.

Molecular analyses with FISH and observations in IHC revealed that in several samples MCPyV was not only restricted to intralesional keratinocytes and adjacent skin but also present in some intracapillary blood cells with a lymphocytic morphology. These observations indicate MCPyV to be ubiquitously present in diverse human tissue independently of malignant or benign histological status (Feng et al., 2008; Shuda et al., 2008).

The interpretation of the IHC stain for p16 may have been affected by pigmentation in the SKs. Therefore, substitution of the brownish dye for red as secondary antibody was used in strongly pigmented lesions to rule out the possibility of false-positive interpretations.

CONCLUSION

The frequent detection of the MCPyV genome by PCR and FISH reflects ubiquitous spread of the virus. However, the low rate of MCPyV detection by IHC most likely excludes a major pathogenic association of MCPyV in SK development, similar to the lack of evidence for a role of MCPyV in other non-melanoma skin neoplasm. The sporadic detection most likely represents a coincidental infection with a ubiquitous virus (Loyo et al., 2010; Wieland et al., 2012; Mertz et al., 2013; Peretti et al., 2014). Our data indicate that p16 IHC is unlikely to be a helpful adjunctive biomarker in the detection of MCPyV infection.

AUTHOR CONTRIBUTIONS

Designed the experiments: AzH, VW, and LH. Conceived and supervised the study: AzH, AMH, and VW. Performed the experiments: LH and DR. Contributed reagents, materials: E-JS and AMH. Data analyzed: LH, DR, AzH, and VW. Wrote the manuscript: LH and AzH. All authors read and approved the final manuscript.

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The Role of Human Papillomaviruses and Polyomaviruses in BRAF-Inhibitor Induced Cutaneous Squamous Cell Carcinoma and Benign Squamoproliferative Lesions

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Background: Human papillomavirus (HPV) has long been proposed as a cofactor in the pathogenesis of cutaneous squamous cell carcinoma (cSCC). More recently, the striking clinico-pathological features of cSCCs that complicate treatment of metastatic melanoma with inhibitors targeting BRAF mutations (BRAFi) has prompted speculation concerning a pathogenic role for oncogenic viruses. Here, we investigate HPV and human polyomaviruses (HPyV) and correlate with clinical, histologic, and genetic features in BRAFi-associated cSCC.

Materials and Methods: Patients receiving BRAFi treatment were recruited at Barts Health NHS Trust. HPV DNA was detected in microdissected frozen samples using reverse line probe technology and degenerate and nested PCR. HPV immunohistochemistry was performed in a subset of samples. Quantitative PCR was performed to determine the presence and viral load of HPyVs with affinity for the skin (HPyV6, HPyV7, HPyV9, MCPyV, and TSPyV). These data were correlated with previous genetic mutational analysis of H, K and NRAS, NOTCH1/2, TP53, CDKN2A, CARD11, CREBBP, TGFBR1/2. Chromosomal aberrations were profiled using single nucleotide polymorphism (SNP) arrays.

Results: Forty-five skin lesions from seven patients treated with single agent vemurafenib in 2012–2013 were analyzed: 12 cSCC, 19 viral warts (VW), 2 actinic keratosis (AK), 5 verrucous keratosis/other squamoproliferative (VK/SP) lesions, one melanocytic lesion and 6 normal skin samples. Significant histologic features of viral infection were seen in 10/12 (83%) cSCC. HPV DNA was detected in 18/19 (95%) VW/SP, 9/12 (75%) cSCC, 4/5 (80%) SP, and 3/6 (50%) normal skin samples and in 1/12 cases assessed by immunohistochemistry. HPyV was co-detected in 22/30 (73%) of samples, usually at low viral load, with MCPyV and HPyV7 the most common. SNP

arrays confirmed low levels of chromosomal abnormality and there was no significant correlation between HPV or HPyV detection and individual gene mutations or overall mutational burden.

Conclusion: Despite supportive clinicopathologic evidence, the role for HPV and HPyV infection in the pathogenesis of BRAFi-induced squamoproliferative lesions remains uncertain. Synergistic oncogenic mechanisms are plausible although speculative. Nonetheless, with the prospect of a significant increase in the adjuvant use of these drugs, further research is justified and may provide insight into the pathogenesis of other BRAFi-associated malignancies.

Keywords: human polyomaviruses, human papillomaviruses, cutaneous squamous cell carcinomas, BRAF inhibitors, melanoma

INTRODUCTION

Human papillomaviruses (HPV), particularly those of the beta genus (beta-PV), have long been proposed as cofactors with ultraviolet radiation in the pathogenesis of cutaneous squamous cell carcinoma (cSCC), especially those associated with the rare genodermatosis, epidermodysplasia verruciformis and with immune suppression (Wang et al., 2014; Howley and Pfister, 2015; Quint et al., 2015; Harwood et al., 2017). Over the past decade, 13 human polyomaviruses (HPyV) have been identified and classified (Calvignac-Spencer et al., 2016) and those with affinity for the skin include HPyV6, HPyV7, HPyV9, the trichodysplasia spinulosa-associated polyomavirus (TSPyV), the Merkel cell polyomavirus (MCPyV) (DeCaprio and Garcea, 2013; Feltkamp et al., 2013; van der Meijden et al., 2010, 2013; Nguyen et al., 2017) and the Lyon IARC polyomavirus (LIPyV) (Gheit et al., 2017). MCPyV was the first to be associated with malignancy – the aggressive cutaneous neuroendocrine cancer Merkel cell carcinoma (Feng et al., 2008; Shuda et al., 2008) – and has also been investigated in the context of cSCC (Dworkin et al., 2009; Scola et al., 2012). More recently, the clinical and histologic features of cSCCs and other squamoproliferative lesions that complicate treatment for metastatic melanoma with oral small molecule *BRAF* inhibitors (BRAFi) have raised the possibility of significant viral involvement in their pathogenesis (Boussemart et al., 2013). Both HPV and HPyV have been investigated, but studies have to date provided conflicting evidence for their role. With the prospect of future widespread adjuvant use of these drugs (Long et al., 2017; Maio et al., 2018), a more detailed understanding of the pathogenesis of BRAFi-induced cSCC (BRAFi-SCC) remains important.

Oncogenic mutations in the oncoprotein *BRAF*, which encodes the growth signal transduction serine/threonine protein kinase B-Raf, are found in approximately 50% of melanomas and result in constitutive activation of the RAS/mitogen-activated protein kinase (MAPK) pathway. The most common BRAF mutation results in a substitution of a valine (V) residue to glutamic acid (E) at amino acid position 600 (V600E) (Long et al., 2011). This locks the kinase into the active conformation and results in melanocyte hyperproliferation. BRAF inhibitors have been developed that exploit this mutation and competitively bind to the active conformation of the kinase (Zhang et al., 2009; Ribas and Flaherty, 2011) Vemurafenib and dabrafenib are two such selective small molecule inhibitors of oncogenic *BRAF* and are associated with high response rates and improved progression-free survival and overall survival compared with chemotherapy in patients with BRAFV600 mutated melanoma (Chapman et al., 2017). However, responses are generally temporary, with a median time to relapse of approximately 6 months. Vemurafenib entered routine clinical use in 2011/2012 and has been associated with various cutaneous adverse effects. These include rashes, photosensitivity, hyperkeratosis and development of *de novo* squamoproliferative lesions in 16–26.7% of patients, ranging from benign VW and squamous papillomas/verrucous keratoses (VK) to keratoacanthomas (KA) and cSCC (Flaherty et al., 2010; Sosman et al., 2012; Anforth et al., 2013; Blank et al., 2017; Chapman et al., 2017). The median time to presentation for BRAFi-SCC is 8–12 weeks and it is argued that this rapid timeframe points to pre-existing mutations being given a selective advantage due to BRAFi treatment, rather than mutations arising *de novo* due to therapy. Consistent with this mechanism, there is evidence for paradoxical hyperactivation of the MAPK pathway in cells with wild-type BRAF but mutated RAS through allosteric and catalytic mechanisms that relieve the auto-inhibition of wild-type RAF kinase (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010). Indeed, many of these BRAFi features overlap with the cutaneous manifestations of RASopathies – genetic diseases such as cardiofaciocutaneous and Costello syndromes characterized by activating germ line mutations in RAS (Rinderknecht et al., 2013; Sfecci et al., 2017). Also consistent with this, 18–60% of BRAFi-cSCC have somatic mutations in HRAS or KRAS, which is significantly higher than in sporadic cSCC (Oberholzer et al., 2012; Su et al., 2012; South et al., 2014). Nevertheless, many BRAFi-cSCC are RAS wild type, and RAS mutations have been also been detected in benign epithelial skin lesions (South et al., 2014; Hassel et al., 2015), suggesting that accelerated oncogenesis of RAS-mutated cells is not the only aetiologic mechanism and that additional cofactors may be involved. Attention has focused on infectious agents, particularly oncogenic viruses. HPV has been the main candidate given its previous proposed role in EV and immune suppression-associated cSCCs, coupled with evidence in BRAFi-cSCC of clinical and histological patterns of viral wart-like features and overexpression of p16 (Boussemart

et al., 2013). Human polyomaviruses, particularly MCPyV, have also been investigated but, to date, the available evidence for both HPV and HPyV remains inconclusive (Anforth et al., 2012; Chu et al., 2012; Boussemart et al., 2013; Ganzenmueller et al., 2013; Falchook et al., 2013, 2016; Ko et al., 2013; Frouin et al., 2014; Holderfield et al., 2014; Schrama et al., 2014; Cohen et al., 2015; Dika et al., 2015; Viarisio et al., 2017a,b).

Single agent BRAFi therapy has generally been replaced by combination BRAFi and MEK inhibition (MEKi): phase III studies demonstrated improved clinical outcomes and significantly delayed resistance compared with BRAFi alone and BRAFi-MEKi combination therapy (vemurafenib/cobimetanib and dabrafenib/trametinib) is now the standard of care for BRAF mutated metastatic melanoma (Flaherty et al., 2012; Larkin et al., 2014). Combination therapy also results in decreased incidence of BRAFi-SCC to around 4%, which may be due to the fact that MEKi bypasses the point of paradoxical RAF activation (Dummer et al., 2012). Although rates of BRAFi-SCC are consequently reduced with combination therapy, these drugs are now being introduced as adjuvant treatment in high-risk, non-metastatic primary melanoma (stages IIC–IIIA–IIIB–IIIC). In the recent BRIM-8 study of adjuvant vemurafenib in BRAF-mutated melanoma, 16% of patients treated with adjuvant vemurafenib had BRAFi-cSCC or KA (Maio et al., 2018). This incidence is considerably lower with BRAFi-MEKi combination adjuvant therapy (Long et al., 2017), but with approval for adjuvant treatment comes the prospect of a huge increase in the numbers of patients receiving these agents in the near future. The need to better understand the pathogenesis of their associated cSCC therefore remains important.

In this study we have examined HPV and HPyV status in a series of benign and malignant BRAFi-associated skin lesions and correlated these data with key clinical, histologic and genetic parameters in order to further investigate the contribution of these viruses to the pathogenesis of BRAFi-induced skin tumors.

MATERIALS AND METHODS

Patients and Samples

Patients were recruited from the melanoma clinic at Barts Health NHS Trust. Punch biopsies were taken after surgical excision of lesions or from bisected shave biopsies. They were immediately snap-frozen in liquid nitrogen and stored at −80°C. The remainder of the tissue was sent for formalin fixation and histologic diagnosis. In order to enrich for tumor cell populations, fresh-frozen samples were laser-capture microdissected using the Zeiss Palm Microbeam microscope (Zeiss, Cambridge, United Kingdom). Depending on sample size and purity, as estimated from a reference hematoxylin and eosin slide, between 30 and 60 sections of 8 mm thickness were cut onto 1.0 mm PEN membrane slides (Zeiss), stained in 0.05% acid fuchsin (Acros Organics, Morris Plains, NJ, United States) in distilled water and 0.05% toluidine blue O (Acros Organics, Morris Plains, NJ, United States) in 70% ethanol, and microdissected, with tumor cells collected into 180 μl ATL buffer (Qiagen, Crawley, United Kingdom). All sections were cut using a fresh microtome to prevent cross-contamination. DNA extraction was performed using the QIAamp DNA micro kit (Qiagen, Crawley, United Kingdom) according to the manufacturer's instructions. To provide a source of germline DNA, paired venous blood samples were obtained concomitantly with lesional tissue and stored at −80°C before DNA extraction using the QIAamp DNA blood mini kit (Qiagen, Crawley, United Kingdom). The quality of the extracted DNA was assessed by β-globin reference gene PCR.

Histopathology

Histology sections were prepared from formalin-fixed paraffin-embedded tissue and stained with hematoxylin and eosin under standard conditions. All diagnoses were confirmed after review by an experienced dermatopathologist (HR). Samples were scored as having features consistent with viral infection if koilocytosis was observed in conjunction with at least three of the following five features: acanthosis, hypergranulosis, parakeratosis, hyperkeratosis, and typical papillomatous architecture. Consensus scoring of viral features was reached with two additional observers (KP, CH).

HPV Detection and Genotyping

Beta, gamma, alpha, mu, nu, and novel HPV types were detected using a comprehensive panel of HPV detection and typing methodologies. The presence of beta-HPV was investigated using RHA kit skin (beta) HPV detection system (de Koning et al., 2006) according to the manufacturer's instructions (Diassay, Rijswijk, The Netherlands). In addition, the RHA kit HPV SPF10-LiPA25 (Labo Bio-medical products BV, Rijswijk, The Netherlands) was used to detect the presence of 25 high- and low-risk mucosotropic alpha-HPVs, according to the manufacturer's instructions. Degenerate nested PCR protocols were used to investigate the presence of cutaneous alpha-HPV and mu and nu genera (Harwood et al., 1999) and the gamma genus (Forslund et al., 1999; Antonsson et al., 2000).

Polyomavirus Detection and Genotyping

In lesional samples for which sufficient DNA was available, quantitative PCR was performed as previously described (van der Meijden et al., 2014, 2017), to determine the presence and load of HPyV9, MCPyV, and TSPyV. For HPyV6 and HPyV7 quantitative PCR the following primers and probes located in VP1 were used: 5′-GTAGGGTATGCTGGTAAC-3′ (HPyV6 sense), 5′-CAGGAATTGTCTAAACATCATATC-3′ (HPyV6 antisense), 5′-CTCTCCTCTGTCTGAAGTGAACTC-TAA-3′ (HPyV6 probe), 5′-GTGCTGATATGGTTGGAA-3′ (HPyV7 sense), 5′-TCTGCAGTGGACTCTAAA-3′ (HPyV7 antisense), 5′-AGCCTGTACTGTTCTCTGGTTACT-3′ (HPyV7 probe). Input cell equivalents were determined by normalization to beta-globin.

All DNA extractions and PCRs for both HPV and HPyV were performed using standard operating procedures designed to reduce the possibility of contamination (Harwood et al., 1999). DNA extraction, water and buffer PCR controls were used to exclude contamination and these were consistently negative.

Immunohistochemistry

Immunofluorescence analysis was carried out using polyclonal antibodies raised against the E4 proteins of HPV 5, 8, and 23 (beta-PV types), or the E4 protein of HPV2 and 57 (alpha-PV types) using the protocols previously described (Griffin and Doorbar, 2016). In the double staining experiments, a monoclonal antibody (8H3) prepared against the HPV 8 E4 protein was used in place of the beta-PV E4 polyclonal antibodies. Polyclonal and monoclonal antibodies were prepared against GST-E4 fusion proteins (Doorbar et al., 1997; Borgogna et al., 2012). Techniques for the overlay of fluorescence staining patterns onto the hematoxylin and eosin sections have been described previously (Griffin et al., 2015). All tissue sections were formalin fixed prior to staining. Sections were counterstained with DAPI to visualize cell nuclei.

SNP Array Analysis of Gross Chromosomal Aberrations

Cutaneous squamous cell carcinoma and paired venous blood DNA samples were subjected to the GeneChip Genome-Wide Human SNP Array 6.0 assay (Affymetrix Inc., Santa Clara, CA, United States) according to the manufacturer's protocol. Processing was performed as previously described (Teh et al., 2005) using the Genome Oriented Laboratory File (GOLF) system for the analysis and display of single nucleotide polymorphism (SNP) signal data. Copy number profiles of vemurafenib-associated cSCC were compared with those observed in a previous study of sporadic cSCC (Purdie et al., 2009).

Genetic Mutational Analysis

Targeted genetic analysis of all samples was undertaken using 454 pyrosequencing performed using the GS Junior system (Roche/454 Life Sciences, Branford, CT, United States) and Fluidigm (Fluidigm Corporation, San Francisco, CA, United States) PCR amplicon libraries as template. In addition to *H, K,* and *NRAS,* we also analyzed the genes *NOTCH1, NOTCH2, TP53, CDKN2A, CARD11, CREBBP, TGFBR1,* and *2,* all of which our previous research has implicated in the pathogenesis of cSCC (Brown et al., 2004; South et al., 2014; Cammareri et al., 2016; Watt et al., 2015, 2016). Primers were designed and validated by Fluidigm (Fluidigm Corporation, San Francisco, CA, United States) as per recommended guidelines for Roche Titanium sequencing (Roche, Mannheim, Germany). Variant detection required a minimum of four supporting reads and a minimum variant allele frequency threshold of 0.1. Detailed genetic analysis of these samples has previously been reported (South et al., 2014; Cammareri et al., 2016).

Ethical Approval

This study was carried out in accordance with the recommendations of East London and City Health Authority local ethics committee. The protocol was approved by the East London and City Health Authority local ethics committee. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

RESULTS

Patients

A total of seven patients with 45 skin lesions were recruited (**Figures 1**, **2** and **Table 1**). They included 4 men (mean age 61.25 years, range 35–87 years) and 3 women (mean age 56.3 years, range 33–82 years). All patients had metastatic melanoma with V600E BRAF mutations. All were treated in 2012–2013 with single agent vemurafenib. For all patients, samples were collected at first presentation with skin lesions after starting vemurafenib. The mean time to either disease progression (n = 3 patients) or death from melanoma whilst receiving vemurafenib (n = 4 patients) was 7.7 months (range 3–17 months).

Samples (Table 1)

Samples consisted of 12 SCC, 19 VW, 2 actinic keratoses (AK), one melanocytic lesion and 6 non-lesional skin samples (3 normal skin and 3 normal skin perilesional to cSCC), 4 verrucous keratoses (VK – squamous papillomas, some with viral features but without evidence of dysplasia) and one squamoproliferative (SP) lesion with viral features and dysplasia but no clear invasion (SP). Lesions were from both chronically sun-exposed (SE) and non-chronically sun-exposed (NSE) sites. SE sites included head and neck, arms, and lower legs in females. NSE included all other sun-protected sites. Lesions from SE sites included 6/12 (50%) cSCCs, 5/5 VKs, 9/19 (47%) VWs, and 1/2 AKs. The normal skin samples comprised 2 SE, one NSE and 3 perilesional to SCCs on SE sites.

The mean time to development of biopsy-proven lesions after initiation of vemurafenib was 8.75 weeks for AK (range 6–11.25), 9.7 weeks for VK/SP (range 8–12), 9.9 weeks for VW (range 6–13), and 11.25 weeks for cSCC (range 8–16 weeks). For 2 patients, additional samples were collected at more than one further time point: for patient 1, samples were collected at 9, 13, 14, 18, and 34 weeks; for patient 2, at 9 and 28 weeks.

Histopathology

Significant histological features of viral infection (i.e., koilocytosis and at least three of acanthosis, hypergranulosis, parakeratosis, hyperkeratosis or typical papillomatous architecture) were seen in all VW and AKs, 10/12 (83%) SCC, but no normal skin or VK samples (**Figure 3** and **Table 1**).

Detection of Human Papillomavirus and Human Polyomaviruses

Human Papillomavirus Detection

Human papillomavirus DNA was detected in 18/19 (95%) VW, 9/12 (75%) SCC, 2/2 AK, 4/5 (80%) VK/SPs, and 3/6 (50%) normal skin samples (**Table 1**). HPV positivity was significantly higher in VW compared to normal skin (Fisher's exact test: p = 0.0312,) but not in cSCC (Fisher's exact test: p = 0.344). Multiple HPV types were detected in 28/37 (76%) of HPV positive samples, with a median number of 4 in VW and 3 in SCC compared with 0.5 in normal skin (two-sided Mann–Whitney U-test: p = 0.007 for VW and 0.13 for SCC).

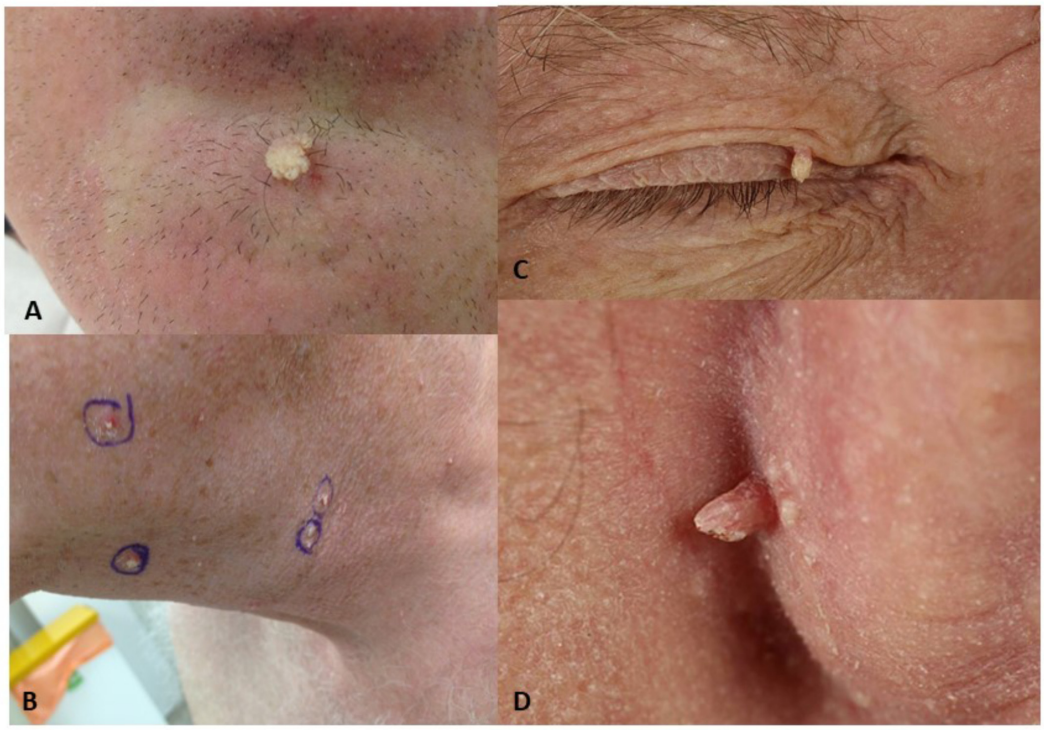

FIGURE 1 | Viral-wart like lesions developing after BRAFi exposure. **(A)** Lesion V10, viral wart on chin of patient 2; **(B)** Viral warts on the neck and chin of patient 1; **(C)** Viral wart on the eyelid of patient 5; **(D)** Lesion V22, post-auricular viral wart in patient 3.

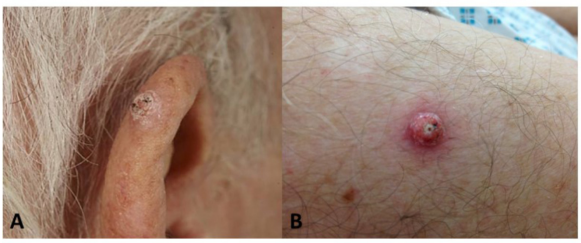

FIGURE 2 | Cutaneous squamous cell carcinomas developing after BRAFi exposure. **(A)** Well-differentiated SCC on the ear of patient 1; **(B)** Lesion V4, well-differentiated SCC on the arm of patient 1.

Cutaneous beta-PV were detected in all HPV positive lesions, with HPV-8, 12, 24, 36, and 92 the most frequent types. Alpha genus HPV types (alpha-PV) were found VW only (4/19, 21%) and were mucocutaneous alpha-PVs (HPV28 and HPV57), but not low or high-risk mucosal alpha-PVs. One cSCC contained a gamma HPV type. Although the RHA detection methodology used for beta and alpha-PV detection is not strictly quantitative, band intensity provides a surrogate read-out for predicted viral load. Analysis of these data suggested that the majority of HPV positive samples were likely to be associated with low viral load: the bands identified were faint or very faint in all cases, with the exception of samples V10, V18, V20, V29, V44 in which strongly positive bands were obtained for HPV types 57, 12, and 80. Four of these five lesions were VWs.

In all patients except one, multiple lesions were analyzed. Clear patterns of HPV carriage for each individual emerged. For example, beta-PVs 8, 12, 24, 36, 92, 93, and 111 were detected in patient 1 across both benign and malignant lesions at different body sites; this patient's normal, non-sun exposed skin sample also harbored HPV92 in addition to HPV5. Patient 2 had a spectrum of HPV types (HPV8, 12, 76, 80, and 57) that were concordant in VW at two separate body sites. Three other patients with more than one HPV positive lesion also had similar individual repertoires of HPV types across at least 2 lesions (patient 3, HPVs 22, 107; patient 4, HPV9; patient 7, HPV80). In the case of patient 6, all samples were negative, including two normal skin samples.

Only a minority of the 36 lesions with significant viral features on histological assessment had high levels of HPV DNA indicative of active infection. However, the 9 samples with no histological evidence of viral change (one cSCC, 2 VKs and 6 normal/perilesional skin samples) had significantly fewer HPV types detected compared to lesions with histological evidence of viral change (2-tailed Mann–Whitney *U*-test: $p = 0.0114$).

Immunohistochemistry was used to further investigate HPV DNA detection in a subset of 12 lesions. Although all had been HPV positive with multiple types detected, HPV protein expression was detected in only a single sample, a viral wart that had been strongly positive for both HPV57 and HPV80:

TABLE 1 | Clinical, histologic, viral, and genetic features of the seven patients and 45 lesions analyzed.

Pt	Sex/ age (years)	Lesion code	Weeks on BRAFi[1] (weeks)	Body site	SE/ NSE[2]	Diagnosis[3]	Viral features[4]	Mutations[5]	Beta-PV[6]	Alpha-PV	Gamma-PV	IHC[7]	HPyV6[8]	HPyV7[8]	HPyV9[8]	TSPyV[8]	MCPyV[8]
1	M/67	V1	9	Scalp	SE	VW	Y	NOTCH1, CREBBP	8, 9, 36, 38, 111	28		ND	7.5×10^{-6}	3.6×10^{-2}	Neg	Neg	1.7×10^{-2}
		V2	9	Suprapubic	NSE	V K	Y	NOTCH1, HRAS, CARD11	8, 24, 36, 92, 111			ND	ND	ND	ND	ND	ND
		V3	9	Post-auricular	SE	VW	Y	TGFBR2, CDKN2a, CARD11, NOTCH2, CREBBP	8, 36			ND	ND	ND	ND	ND	ND
		V4	9	Arm	SE	SCC	Y	TGFBR1, NOTCH1, HRAS	8, 12, 24, 36, 111, FA51			ND	Neg	1.7×10^{-4}	Neg	Neg	4.6×10^{-2}
		V5	9	Inner Thigh	NSE	VW	Y	NOTCH1, NOTCH2, HRAS, CREBBP	8, 12, 36, 111			ND	2.9×10^{-4}	Neg	Neg	Neg	2.8×10^{-2}
		V6	9	Lower Back	NSE	SCC	Y	TP53, HRAS	12, 36, 92			ND	5.12×10^{-5}	1.3×10^{-1}	Neg	Neg	3.9×10^{-3}
		V7	9	Shoulder	NSE	SCC	Y	NOTCH1, TP53, TGFBR1, CREBBP	15, 24, 36, 76, 92, 107, FA84			Neg	Neg	3.9×10^{-5}	Neg	Neg	1.8×10^{-2}
		V8	9	Posterior neck	SE	VK	Y	NOTCH1, CARD11, TGFBR1	8, 24, 36, 93			Neg	4.6×10^{-5}	4.8×10^{-4}	Neg	Neg	2.3×10^{-1}
		V9	9	Cheek	SE	VW	Y	HRAS, TP53*, NOTCH1,* NOTCH2*	8, 12, 24, 36, 76, 92 93	28		ND	ND	ND	ND	ND	ND
		V11	13	Scalp	SE	VW	Y	NOTCH1, TGFBR2, CARD11, CREBBP	8,23, 36, 92, 93			Neg	Neg	2.2×10^{-4}	Neg	Neg	1×10^{-1}
		V12	13	Abdomen	NSE	N-NSE	N	NOTCH1	5, 92			ND	ND	ND	ND	ND	ND
		V13	13	Lower back	NSE	SCC	Y	Nil	8, 24, 92, 107, 111, FA14			Neg	3.9×10^{-5}	2.4×10^{-4}	Neg	Neg	5.9×10^{-3}
		V14	13	Popliteal Fossa	NSE	VW	Y	NOTCH1, CREBBP	12, 24, 36			ND	Neg	1.3×10^{-2}	Neg	Neg	1.7×10^{-3}
		V15	13	Post-auricular	SE	VW	Y	CARD11	93			ND	Neg	1.6×10^{-3}	Neg	Neg	5.6×10^{-2}
		V16	13	Abdomen	NSE	VW	Y	TP53, HRAS	8, 36, 92, 93			ND	Neg	$6, \times 10^{-4}$	Neg	Neg	9.9×10^{-3}

(Continued)

TABLE 1 | Continued

Pt	Sex/ age (years)	Lesion code	Weeks on BRAFi[1] (weeks)	Body site	SE/ NSE[2]	Diagnosis[3]	Viral features[4]	Mutations[5]	Beta-PV[6]	Alpha-PV	Gamma-PV	IHC[7]	HPyV6[8]	HPyV7[8]	HPyV9[8]	TSPyV[8]	MCPyV[8]
		V17	13	Abdomen	NSE	VW	Y	NOTCH1, CDKN2a	8, 36, 92, 113			ND	Neg	1.4×10^{-1}	Neg	Neg	6.2×10^{-4}
		V18	14	Chest	NSE	VW	Y	NOTCH1, HRAS, KRAS	8**, 12, 24, 76, 92**, 111			Neg	1.12×10^{-5}	5, $\times 10^{-5}$	Neg	Neg	5.8×10^{-3}
		V19	14	Groin	NSE	SCC	Y	HRAS, NOTCH1	8, 12, 24, 36, 92			Neg	3.93×10^{-6}	1.4×10^{-4}	Neg	Neg	3.6×10^{-3}
		V20	14	Chest	SE	SCC	Y	Nil	8, 12, 24, 92**, 93			Neg	1.29×10^{-5}	2.4×10^{-4}	Neg	Neg	1.2×10^{-2}
		V25	18	Arm	SE	N-P V27	N	NOTCH1	Neg			ND	ND	ND	ND	ND	ND
		V26	18	Abdomen	NSE	VW	Y	CARD11, NOTCH1, HRAS, CREBBP	8, 92			Neg	Neg	5.9×10^{-5}	Neg	Neg	5.3×10^{-3}
		V27	18	Upper arm	SE	SCC	Y	HRAS, CARD11, CREBBP, NOTCH1 NOTCH2* KRAS* TP53*	Novel			Neg	Neg	Neg	Neg	Neg	Neg
		V28	18	Lower abdomen	NSE	SCC	Y	NOTCH2, NOTCH1*	Neg			Neg	ND	ND	ND	ND	ND
		V31	34	Cheek	SE	VK	N	Nil	Novel			ND	3.66×10^{-6}	2.4×10^{-4}	Neg	Neg	7.6×10^{-3}
		V32	34	Upper Arm	SE	VW	Y	NOTCH1, NOTCH2, HRAS, CREBBP, TP53*	24**, 36, 92, 93			ND	ND	ND	ND	ND	ND
2	M/35	V10	13	Chin	SE	VW	Y	Nil	8, 12, 80**, 76	57**		57, beta	ND	ND	ND	ND	ND
		V29	28	Scapula	NSE	VW	Y	NOTCH2, CREBBP	12**, 8, 20, 23, 36,76, 80, 92, 96	57		ND	ND	D	ND	ND	ND
3	M/56	V21	8	Neck	SE	VK	N	HRAS, CARD11, NOTCH1 * NOTCH2*	22, 115, 150			ND	3.73×10^{-5}	6.4×10^{-5}	Neg	Neg	Neg
		V22	14	Post-auricular	SE	VW	Y	TGFBR1, HRAS NOTCH2*	22, 107			ND	ND	ND	ND	ND	ND
		V23	14	Upper arm	SE	SCC	Y	CREBBP, CARD11, TGFBR1, NOTCH2*	2, 14D, 22, 38, 107, 115			Neg	Neg	Neg	Neg	Neg	1.3×10^{-4}
		V24	14	Upper Arm	SE	N-P to V23	N	NOTCH1	Novel			ND	ND	ND	ND	ND	ND

(Continued)

TABLE 1 | Continued

Pt	Sex/ age (years)	Lesion code	Weeks on BRAFi[1] (weeks)	Body site	SE/ NSE[2]	Diagnosis[3]	Viral features[4]	Mutations[5]	Beta-PV[6]	Alpha-PV	Gamma -PV	IHC[7]	HPyV6[8]	HPyV7[8]	HPyV9[8]	TSPyV[8]	MCPyV[8]
4	F/33	V30	16	Abdomen	NSE	SCC	N	TP53, HRAS, NOTCH1* NOTCH2*	Neg		FA49	ND	Neg	Neg	Neg	Neg	Neg
5	M/87	V33	6	Ear	SE	VW	Y	NOTCH1, TP53	9			ND	Neg	Neg	Neg	Neg	Neg
		V34	6	Chin	SE	AK	Y	Nil	9, 38			ND	Neg	1.5×10^{-5}	Neg	Neg	Neg
6	F/82	V35	12	Neck	SE	SCC	Y	TGFBR1, TGFBR2, NOTCH1, TP53, KRAS, NOTCH2, CARD11, CREBBP	Neg			ND	Neg	Neg	Neg	Neg	Neg
		V36	12	Neck	SE	N-P V35	N	Nil	Neg			ND	ND	ND	ND	ND	ND
		V37	12	Neck	SE	N -SE	N	NOTCH1, CARD11	Neg			ND	ND	ND	ND	ND	ND
		V38	12	Shoulder	NSE	VW	Y	TGFBR1, TGFBR2, TP53, KRAS, NRAS, CARD11, CREBBP	Neg			ND	ND	ND	ND	ND	ND
		V39	12	Lower Leg	SE	SP	Y	TGFBR1, NOTCH1, NOTCH2„ TP53, CREBBP	Neg			ND	Neg	Neg	Neg	Neg	1.5×10^{-3}
		V40	12	Arm	SE	SCC	Y	TGFBR2, CREBBP	Neg			ND	Neg	Neg	4×10^{-4}	Neg	2.9×10^{-3}
7	F/54	V41	11.5	Neck	SE	N-SE	N	NOTCH2, CREBBP	Novel			ND	ND	ND	ND	ND	ND
		V42	11.5	Back	NSE	VW	Y	TP53	80			ND	Neg	Neg	Neg	Neg	Neg
		V43	11.5	Back	NSE	M		NOTCH1, NOTCH2, NRAS, CARD11, CREBBP	80			ND	Neg	Neg	Neg	Neg	9×10^{-4}
		V44	11.5	Back	NSE	VW	Y	CARD11	80**			ND	Neg	Neg	Neg	Neg	Neg
		V45	11.5	Back	NSE	AK	Y	NOTCH1, TP53	80			ND	Neg	Neg	Neg	Neg	1.4×10^{-4}

*[1]Weeks on BRAFi: number of weeks on vemurafenib at the time lesion was removed. [2]SE/NSE: Sun exposed or non-sun exposed body site. [3]Diagnosis: SCC – well differentiated SCC; V, viral wart; VK, verrucous keratosis; AK, actinic keratosis; N-P, normal skin perilesional to; N-SE, normal skin sun exposed; N-NSE – normal skin non sun exposed; M, benign melanocytic; SP, squamoproliferative lesion with viral features and dysplasia but no clear evidence of invasion. [4]Viral features: Y, yes; N, no: yes if koilocytosis was observed in conjunction with at least 3 of the following 5 features: acanthosis, hypergranulosis, parakeratosis, hyperkeratosis and typical papillomatous architecture. [5]Mutations: these have previously been published (South, 2014; Cammareri, 2016). * indicates lesion bisected and results represent total mutations found across both portions. [6]Beta-PV: beta HPV types. Beta-PV types associated with strong bands on the RH assay are recorded as **; neg, negative. [7] IHC; immunohistochemistry – performed on 12 selected lesions. [8]HPyV6, -7, -9, TSPyV and MCPyV: neg, negative; N/D, not done; values recorded are viral copies per cell.*

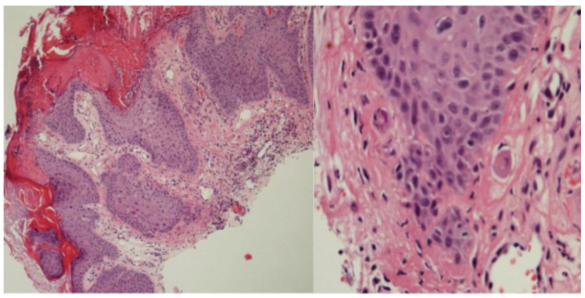

FIGURE 3 | Histology of lesion V4: Micro invasion in an SCC from patient 1 with features of viral infection in overlying and adjacent epidermis.

both HPV57 and beta-PV were detected, but were expressed in spatially distinct cells within the lesion (**Figure 4**).

Human Polyomavirus Detection

Thirty lesional samples from 5 individuals were tested for the presence of HPyV (VW, n = 12; SCC, n = 11; VK/SP, n = 4; AK, n = 2; melanocytic, n = 1). Normal skin was not examined. The majority of samples were positive for at least one HPyV, albeit at low levels and independent of the presence or absence of significant histological viral features (**Table 1**). Individual HPyV positivity ranged from 0% positivity for TSPyV; 3.3% for HPyV9 (SCC, n = 1); 33% for HPyV6 (VW, n = 3, 25%; SCC, n = 4, 36%; VK, n = 3, 36%); 60% for HPyV7 (VW, n = 8, 67%; SCC, n = 6, 54%; VK, n = 3, 25%; AK, n = 1, 50%) to 73% positivity for MCPyV (VW, n = 9, 75%; SCC, n = 8, 73%; VK, n = 3, 75%; AK, n = 1, 50%; melanocytic, n = 1, 100%). For all viruses, the difference in positivity between BRAFi-cSCC and VW was not significant (Fisher's exact test: p = 1 for HPyV9, HPyV6, MCPyV; p = 0.68 for HPyV7). Viral loads were generally less than one copy per thousand cells. The exceptions were four HPyV7-positive benign samples with viral loads ranging from 1 copy per 100 cells to one copy per seven cells and eight MCPyV-positive samples (two cSCC with viral loads of 2–5 copies per 100 cells and six benign lesions with viral loads from 1 copy per 100 cells to 1 copy per 4 cells).

Co-detection of HPV and HPyV

At least one HPV type and one HPyV type were co-detected in 22/30 (73%) of lesions. There was no significant correlation between specific HPV and HPyV types. However, it was noteworthy that lesions from patient 6, which were all negative for HPV, were also largely negative for HPyV, despite having significant histological features of viral infection. Similarly, the cSCC for patient 4 was negative for all beta-PV and HPyV types tested.

Virus Status and Chromosomal Changes

In order to examine gross chromosomal aberrations in vemurafenib-associated sSCC, we analyzed six well-differentiated cSCC from patient 1 using SNP array analysis to determine the signal values in tumor and paired non-tumor DNA at 250,000 SNPs throughout the genome. A comparison of tumor: non-tumor signal value ratios from vemurafenib-associated cSCC plotted according to chromosomal position with those from sporadic well-differentiated SCC analyzed in a previous study (Purdie et al., 2009) revealed that the patterns of gross chromosomal aberrations were significantly different: none of these 6 cSCC had gross chromosomal aberrations and there were significantly fewer chromosomal changes compared with sporadic well differentiated SCC (**Figure 5**). There were no clear correlations with virus status identified.

Virus Status and Association With Specific Genetic Mutations

We have reported gene mutations identified by targeted sequencing in these 45 samples for *H, K* and *NRAS, NOTCH1 and 2, TP53, CDKN2A, CARD11, CREBBP, TGFBR1/2* and this has previously been presented in detail (South et al., 2014; Cammareri et al., 2016). In the current study we analyzed the association between these mutations and the presence of HPV and HPyV. HRAS mutations were identified in 5/12 (42%) SCC and 5/19 (31%) VW. Mutation did not correlate with sun-exposed sites or lesion type and no mutations were detected in normal skin samples (**Table 1**). There was no significant difference between HRAS mutated vs. HRAS wild type lesions and HPV or HPyV status. The same was true for virus status and mutations in each of the other genes examined. Although it was noteworthy that v35 – the most highly mutated SCC – was negative for all viruses tested, there was no evidence of a significant correlation between overall mutational burden and virus status.

Virus Status and Clinical Response to Vemurafenib

There was no clear evidence of a significant association between virus status of lesions tested and prognosis in terms of disease progression and death from melanoma (data not shown).

DISCUSSION

We report HPV and HPyV analysis of 45 benign and malignant BRAFi-induced skin lesions from 7 individuals, including BRAFi-SCC and correlate this with clinical, histologic and genetic features. A high proportion of BRAFi-cSCCs had histologic viral wart-like features on histology, consistent with virus-driven processes, and the majority were positive for beta-PV, HPyV7 and MCPyV, which were co-detected in 73% of lesions tested. HPyV6 was found in one third of cases, but HPyV9 and TSPyV were rarely detected. As expected from previous studies of normal skin and hair follicles (Harwood et al., 2004; Bouwes Bavinck et al., 2010, 2017; Proby et al., 2011), normal skin samples also harbored beta-PV, but the HPV burden of individual types detected was significantly fewer than in lesional skin. Histologic evidence of virus infection appeared to correlate with HPV burden. However, viral loads were low in the majority of lesions and validation by immunohistochemistry for HPV was negative in all but one of 12 cases. Gross chromosomal changes characterized by SNP arrays in BRAFi-cSCC indicated that these tumors have

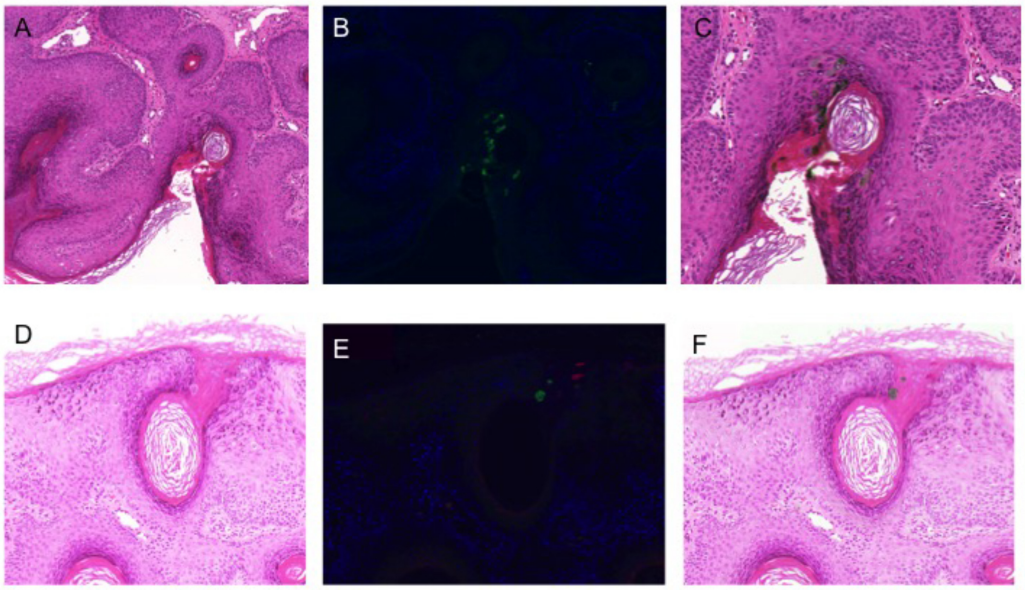

FIGURE 4 | Human papillomavirus immunohistochemistry. **(A,D)** Hematoxylin and eosin stained sections of 2 different areas of lesion V10 (strongly PCR positive for beta-PV types 8, 12, 76, 80, and alpha-PV 57); **(B,E)** double staining immunofluorescence with antibodies to the E4 protein of HPV8 (green) and HPV2/57 (red); **(C,F)** H&E stained sections overlaid with the images from immunofluorescence microscopy to identify cells with productive HPV infection.

significantly fewer chromosomal aberrations than non-BRAFi-cSCC, providing a further indication that additional cofactors may be involved. However, we were unable to establish clear correlations between the presence of either HPV or HPyV and specific genetic mutations or total mutational burden.

Human Papillomaviruses and BRAFi-cSCC

Human papillomaviruses has been investigated as a potential viral carcinogen in BRAFi-cSCC since these drugs were first approved in 2011/2012: the rapid onset of skin lesions, their clinical morphology and viral wart-like histology all point to a possible role for HPV (Boussemart et al., 2013). Initial studies designed to detect alpha-PV infection by immunohistochemistry reported negative results (Anforth et al., 2012; Chu et al., 2012; Ko et al., 2013). A surrogate for alpha-PV infection in mucosal sites is p16 immunoreactivity and in two studies the majority of BRAFi-cSCC were found to strongly express p16 (Anforth et al., 2012; Boussemart et al., 2013), but this is not a consistent finding (Frouin et al., 2014). Both approaches are less sensitive than PCR-based detection methodologies that have been used in other studies, although the latter are often limited by the use of formalin-fixed paraffin-embedded tissue (FFPE) and/or PCR primers detecting a limited range of HPV types. Using an alpha-PV specific PCR/line probe assay method, Dika et al failed to detect alpha-PV in 9 FFPE VK samples (Dika et al., 2015). Using the same assay together with a degenerate PCR methodology (FAP59/64) capable of detecting cutaneous HPV types, HPV was not detected in 8 BRAFi-cSCC/KA FFPE samples (Frouin et al., 2014). Holderfield et al used the FPA 59/64 primers and additional degenerate primers (CP65/CP70 and CP66/CP69) and found 2/13 (15%) FFPE BRAFi-cSCC (Holderfield et al., 2014). Schrama et al. (2014) used E1 primers originally tested for alpha-PV types but theoretically capable of detecting any HPV type; in FFPE samples from 14 cSCC, 3 KA and one acanthoma, all were HPV positive, although the specific types detected were not reported. Subsequently, Cohen et al. (2015) used the most comprehensive degenerate PCR-based methodology in 69 FFPE BRAFi-cSCC and found all samples to be positive with predominantly beta-PV types of which HPV-17, HPV-38 and HPV-111 were the most common. Falchook et al. (2016) found 6/12 (50%) FFPE BRAFi-cSCCs to be positive, almost exclusively with beta-PV types including 12, 17, 24, 47, 124 and novel types but, as in our study, HPV positivity was not significantly different when compared to normal skin. However, we found a significant difference in HPV burden between lesional and normal skin in terms of the numbers of HPV types detected and it is possible that the total burden of HPV types as well as the specific types detected is relevant to potential pathogenic processes.

Against the background of these previous studies, our HPV detection methodology has notable strengths, although the data are still limited by small sample size. We have used a more comprehensive and sensitive approach to detecting HPV from alpha, beta, mu, nu, gamma genera with RHA and degenerate PCR/sequencing. We have also used fresh frozen tissue samples in order to reduce the likelihood of false negative results, which may occur with use of FFPE-derived DNA. Laser capture micro dissection allowed enrichment for lesional tissue and reduces the possibility of contamination by virus carriage in non-lesional tissue. Neither strategy for optimizing viral detection has been used in these previous studies. In addition, we used

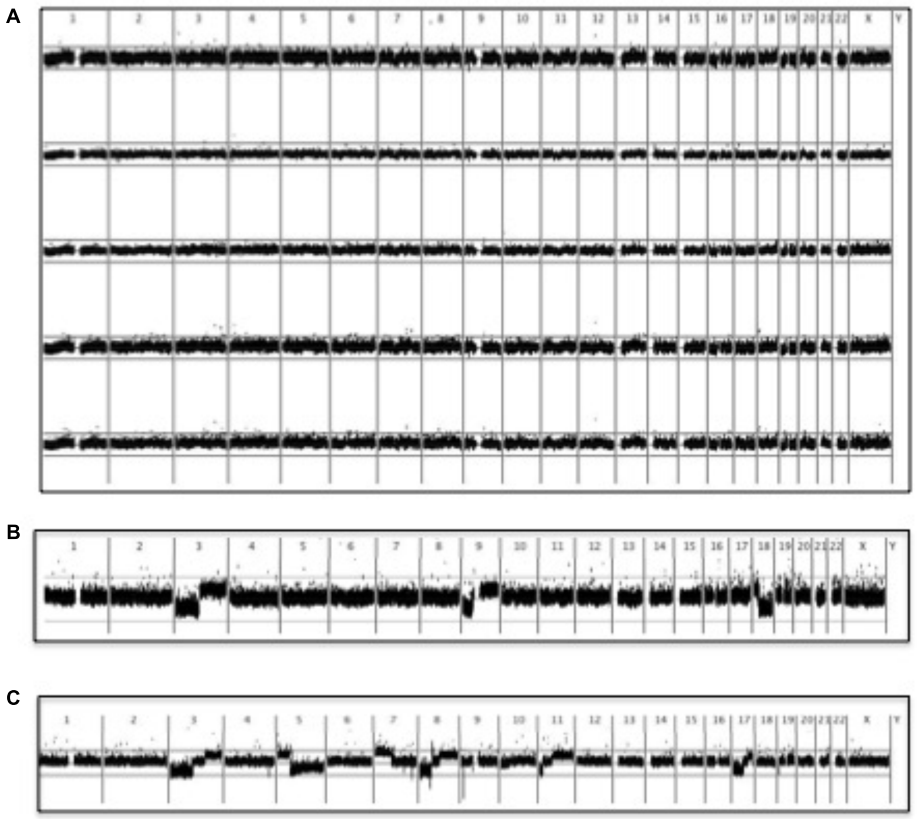

FIGURE 5 | SNP microarray analysis of vemurafenib-associated cSCC. BRAFi-SCC did not display the gross chromosomal aberrations typically observed in sporadic cSCC (Purdie et al., 2009). A running average of 2 consecutive tumor: non-tumor signal value ratios is plotted on a log2 scale according to chromosomal position. Upper line represents log2(2) and lower represents log2(0.5). **(A)** Copy number profiles of 5 vemurafenib-associated cSCC **(B)** Comparison with copy number profile from a sporadic cSCC showing 3p and 9p loss: these changes were characteristic of well-differentiated cSCC (Purdie et al., 2009). Although chromosome 18 loss is also seen in this particular tumor, chromosome 18 aberrations were not as common as 3p and 9p changes in other sporadic well-differentiated cSCC (Purdie et al., 2009). **(C)** More extensive allelic imbalance in a sporadic moderately-differentiated cSCC (Purdie et al., 2009).

immunohistochemistry to try and understand the nature and significance of multiple HPV detection using a double staining technique not used in previous studies of BRAFi lesions.

Our study was not designed to address the functional significance of HPV detection in BRAFi-associated squamoproliferative skin lesions. To date, this has been attempted in only a few other studies. Ganzenmueller et al. (2013) used next-generation sequencing to look for viral transcripts indicative of active HPV infection: none were identified in 4 BRAFi-associated VK. Although this small study arguably calls into question a role for HPV, it is important to note that BRAFi-cSCC were not examined and the presence of very low abundance transcripts cannot be entirely excluded. In a compelling experimental approach, exposure to vemurafenib in a transgenic murine model (K14-HPV16 mice) of alpha-PV-driven cSCC was associated with an upregulation of the MAPK pathway and an increase in cSCC incidence from 22 to 70%. More than half of these tumors were *RAS* wild type, suggesting that vemurafenib and HPV may be cooperating to promote tumorigenesis in both the presence and absence of RAS mutations (Holderfield et al., 2014). Data from a more recent beta-PV transgenic mouse model provides evidence of significant synergism between beta-PV, UV, and BRAFi (Viarisio et al., 2017a). The K14-HPV38 E6/E7 transgenic mouse expresses the beta-PV HPV38 E6 andE7 oncogenes in the basal layer of the epidermis under the control of the cytokeratin K14 promoter and requires UV exposure for AK and cSCC development (Viarisio et al., 2011). Vemurafenib increased the number and size of UV-induced cSCC. All BRAFi-treated transgenic mice developed cSCC by 34 weeks in contrast to none of the wild-type mice and only one-third of untreated transgenic mice. Ras mutations were not detected, but MAPK upregulation was evident in HPV38 E6/E7 over-expressing keratinocytes (Viarisio et al., 2017a).

The mechanisms responsible for the synergism between HPV and BRAFi are speculative. However, experimental data indicate that upregulation of the MAPK pathway enhances alpha-PV replication, stability, and infectivity (Wang et al., 2009; Bowser et al., 2011). If this is also the case for other HPV types, then it is possible that BRAFi-induced paradoxical upregulation of MAPK in the keratinocytes of normal skin – which is likely to harbor beta-PV HPV (Harwood et al., 2004; Bouwes Bavinck et al., 2010, 2017; Proby et al., 2011) – also leads

to enhanced replication and stability of these beta-PV. The resulting increased beta-PV viral load in normal keratinocytes may drive not only benign squamoproliferative lesions associated with BRAFi, but may also enhance synergism between the oncogenic effects of beta-PV and UV, independent of RAS status, which ultimately leads to increased carcinogenesis in the K14-HPV38-E6/7 transgenic murine model (Viarisio et al., 2017a). Consistent with this, experimental evidence increasingly points to a "hit and run" role for beta-PV in skin (Tommasino, 2017). In contrast to high-risk alpha-PV which are required for both initiation and maintenance of a malignant phenotype in mucosal carcinogenesis, the hit-and-run hypothesis proposes that beta-PVs are required only at an early stage of carcinogenesis, with beta-PV E6 and E7 oncogenes facilitating accumulation of UV-induced DNA mutations in the host genome by means of multiple mechanisms that, for example, target DNA repair and apoptosis leading to inactivation of cellular tumor suppressor proteins or activation of oncoproteins (Connolly et al., 2014; Howley and Pfister, 2015; Quint et al., 2015; Tommasino, 2017; Viarisio et al., 2017b). This is the "hit," which ultimately leads to cellular transformation (Viarisio et al., 2017b). Viral oncogene expression is subsequently not required for maintenance of a malignant phenotype, rendering the viral genome dispensible and without consequence if lost from an established cancer – the "run." This would explain the observations that beta-PV DNA loads are always significantly less than one viral genome copy per cell in cSCC and are generally higher in precancerous AK (Weissenborn et al., 2005), that viral transciptomes are absent from BRAFi and non-BRAFi-cSCC (Arron et al., 2011; Ganzenmueller et al., 2013), and are supported by together with findings in the recent murine K14-HPV38E6/7 transgenic models (Viarisio et al., 2018). However, although biologically plausible, this hypothesis remains to be definitively confirmed in human studies.

Such a hit-and-run role for beta-PV may also potentiate the deleterious effects of cSCC cofactors such as immune suppression and contribute to the higher susceptibility to cSCC of immune suppressed individuals such as solid organ transplant recipients (Harwood et al., 2017). We and others have previously shown that beta-PV carriage is significantly more common in the normal skin and hair follicles of immune suppressed individuals (Weissenborn et al., 2012) and, particularly in the presence of concordant beta-PV seropositivity, is associated with cSCC risk in both OTR (Proby et al., 2011; Bouwes Bavinck et al., 2017) and immunocompetent individuals (Bouwes Bavinck et al., 2010; Chahoud et al., 2016). Although detailed comparison of virus status in BRAFi- and non-BRAFi-associated cSCC might provide insight into the effects of BRAFi on the biological activity of beta-PV in skin, the small numbers of published studies in BRAFi-cSCC and the wide variations in HPV analyses used in these studies limit the power of such an analysis.

Human Polyomaviruses and BRAFi-cSCC

We detected HPyV6 in one third of all 30 lesions tested, HPyV7 in 60% and MCPyV in 73%, with no significant differences seen between benign and malignant lesions. In most cases the loads for all viruses were low. In our series HPyV9 was negative in all cases and TSPyV positive in just one cSCC. Our data for MCPyV are very similar to the 72% positivity reported in a series of 18 FFPE BRAFi-cSCCs (Schrama et al., 2014) and 75% of 12 BRAFi-cSCC (Falchook et al., 2016). In contrast, Cohen et al detected MCPyV in only 22% of 58 FFPE benign and malignant FFPE lesions (Cohen et al., 2015) and a fourth study found MCPyV in only two of 19 VKs and none of 7 cSCCs (Frouin et al., 2014). Of the few studies to examine other HPyVs, Schrama et al. (2014) found HPyV6 and 7 in all FFPE samples (14 cSCC, 3 KA, and one acanthoma), generally at low levels, although HPyV6 was present at high level and detectable by IHC in one cSCC. In contrast, in FFPE samples of 19 VKs, 1KA and 7 cSCC, Frouin et al. (2014) found no HPyV6 positivity and HPyV7 in one VK only. Once again, in all previous studies samples were FFPE rather than frozen samples and this, together with the tumor enrichment and PCR approaches used in our study, may account for some of these differences in HPyV detection.

We did not test normal skin samples, but MCPyV, HPyV6 and 7 are well-established members of the normal skin virome, with HPyV9 and TSPyV significantly less common (Schowalter et al., 2010; Foulongne et al., 2012; Kazem et al., 2012; Wieland et al., 2014). MCPyV has previously been detected in 40–62% of skin swabs from normal individuals (Wieland et al., 2009; Schowalter et al., 2010) and HPyV6 and 7 have been detected in 14 and 11%, respectively (Schowalter et al., 2010). In comparison, HPyV9 and TSPyV were found in skin swabs from only 1/111 (0.9%) and 6/249 (2%) healthy individuals, respectively (Sauvage et al., 2011; Kazem et al., 2012). A previous study analyzed MCPyV in 9 FFPE normal skin biopsies from patients with BRAFi-associated proliferative skin lesions and found all to be negative, whereas 5/9 (56%) were positive for HPV of predominantly beta-PV types (Falchook et al., 2016). A case report by the same authors also failed to detect MCPyV in a normal skin of a patient with BRAFi-cSCC (Falchook et al., 2013). Other HPyV have not been analyzed in normal skin from BRAFi-exposed individuals. These data provide a possible signal that HPyV6 and 7 are overrepresented in BRAFi-cSCC, with our findings of 36 and 54% positivity, respectively. However, as normal skin is frequently positive for MCPyV, our findings in BRAFi-cSCC are less convincing. However, this interpretation is speculative and needs to be confirmed in future studies that specifically include matched normal skin samples from BRAFi-exposed individuals.

To date, there have been no functional studies specifically addressing the role of HPyVs in BRAFi-cSCC. The oncogenic potential of MCPyV is well established in MCC (Church and Nghiem, 2015; Paulson et al., 2017) and it is plausible that the large and small T-antigen oncoproteins may be relevant in BRAFi-cSCC. To date, none of the 12 other HPyVs have been implicated in causing cancer (Church and Nghiem, 2015). However, recent *in vitro* studies have indicated that MCPyV, HPyV6, and TSPyV can all induce cellular MAPK pathways (Wu et al., 2016, 2017a,b). It is therefore, plausible that such activity may act synergistically with BRAFi-induced MAPK upregulation and contribute to driving squamoproliferative lesions.

Co-detection of HPV and HPyV

Human papillomavirus and HPyV were co-detected in the majority of virus positive lesions, usually at low copy number. There were no clear associations of specific types, although beta-PVs and MCPyV were most commonly co-detected, as previously reported (Falchook et al., 2013, 2016; Cohen et al., 2015). Our interpretation of these data is limited by the fact we have only examined normal skin for HPVs and not for HPyVs. However, co-detection of these potentially oncogenic viruses in BRAFi-cSCC remains an important observation. Although it is not possible from these data to conclude whether one virus type is biologically more relevant than another in either driving the virus features seen histologically, the potential for their interaction in playing an oncogenic role merits further functional investigation. In particular, it is plausible that the ability of HPyV to upregulate MAPK and the effects of MAPK upregulation on HPV replication, infectivity and stability may act synergistically in enhancing the oncogenic potential of both in contributing to the pathogenesis of BRAFi-associated squamoproliferative lesions.

Virus Status and Genetic Alterations

We have previously used SNP array analysis to show that UV-associated well-differentiated cSCC have significantly different patterns of chromosomal aberrations compared with moderately and poorly differentiated cSCC (Purdie et al., 2009). Although BRAFi-cSCCs are histologically similar to well- differentiated sporadic cSCC, we have shown that they do not display the characteristic gross chromosomal aberrations typically associated with well-differentiated cSCC. This possibly reflects the shorter time course and more prominent role for MAPK upregulation induced by BRAFi, rather than through lifetime accumulated UVR-induced DNA damage, as seen in sporadic UV-associated cSCC (Lambert et al., 2014). Arguably, however, it also provides circumstantial evidence for alternative etiological agents such as oncogenic viruses.

At the individual gene level, as previously reported, we found a significantly higher level of HRAS mutations in BRAFi-cSCC compared with non-BRAFi-cSCC (South et al., 2014). In the current study, we specifically sought a possible association with virus status in HRAS mutated compared with HRAS wild type lesions. This may provide insights into the mechanisms underlying carcinogenesis in BRAFi-cSCC that are additional to mutant HRAS-associated upregulation of the MAPK pathway and, for example, related to viral oncogenes. However, no clear correlation emerged between virus and HRAS status. We also specifically looked for evidence of an inverse association between the detection of virus and mutational burden in terms of the numbers of mutated genes, as is seen in MCC (Harms et al., 2015; Wong et al., 2015; Goh et al., 2016; Becker et al., 2017; Starrett et al., 2017; Carter et al., 2018). Once again, no clear association emerged, although this study may have been underpowered to detect such an association.

CONCLUSION

Despite the suggestive clinical and histologic evidence, a compelling experimental murine model and genetic evidence that HRAS mutations are absent in a significant proportion of BRAFi-induced squamoproliferative skin lesions, the contribution of HPVs and HPyVs to the development of these lesions suggested by results from previous studies remains inconclusive. The data presented here provide further circumstantial evidence for a possible role for HPV and HPyV. They also point to possible synergistic interactions between these potentially oncogenic skin viruses. Given the major increase predicted in adjuvant use of these agents in the near future, further research into the role of these and possibly other existing or novel members of the human skin virome is justified and may provide insights into the pathogenesis of other BRAF-induced skin disorders and malignancies.

AUTHOR CONTRIBUTIONS

CH, KP, CP, and IL conceived and designed the study. CH, KP, HR, EM, MF, JD, HG, AS, GI, IL, and CP developed the methodology. CH, KP, HR, EM, MF, JD, HG, MS, GI, and AS acquired the data. CH, KP, HR, EM, MF, JD, HG, MS, AS, GI, and CP analyzed and interpreted the data. CH, KP, JD, MF, HR, IL, and CP wrote, reviewed, and revised the manuscript. CH, CP, and IL supervised the study.

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