Virology 403 (2010) 128–136

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Enhanced human papillomavirus type 8 oncogene expression levels are crucial for skin tumorigenesis in transgenic mice

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ARTICLE INFO

Article history:
Received 22 December 2009
Returned to author for revision 1 March 2010
Accepted 14 April 2010
Available online 7 May 2010

Keywords:
Human papillomavirus
Transgenic mice
Skin tumors
UV-irradiation
Skin wounds

ABSTRACT

Human papillomavirus 8 (HPV8) is involved in skin cancer development in epidermodysplasia verruciformis patients. Transgenic mice expressing HPV8 early genes (HPV8-CER) developed papillomas, dysplasias and squamous cell carcinomas. UVA/B-irradiation and mechanical wounding of HPV8-CER mouse skin led to prompt papilloma induction in about 3 weeks. The aim of this study was to analyze the kinetics and level of transgene expression in response to skin irritations. Transgene expression was already enhanced 1 to 2 days after UVA/B-irradiation or tape-stripping and maintained during papilloma development. The enhanced transgene expression could be assigned to UVB and not to UVA. Papilloma development was thus always paralleled by an increased transgene expression irrespective of the type of skin irritation. A knock-down of E6 mRNA by tattooing HPV8-E6-specific siRNA led to a delay and a lower incidence of papilloma development. This indicates that the early increase of viral oncogene expression is crucial for induction of papillomatosis.

Introduction

Nonmelanoma skin cancer (NMSC), which includes basal cell carcinoma, squamous cell carcinoma (SCC) and Bowen's disease is the most common cancer in fair-skinned populations (30% of all cancers) (Alam and Ratner, 2001; DePinho, 2000; Pfister, 2003). As UV radiation is the main risk factor, NMSC emerges primarily on sun-exposed skin areas (Leiter and Garbe, 2008). A linkage between human papillomaviruses (HPV) and the development of human skin cancer is accepted for the rare inherited disorder epidermodysplasia verruciformis (EV) and the so called EV-HPV, today phylogenetically grouped as beta-HPV (de Villiers et al., 2004; Majewski et al., 1997; Pfister and Ter Schegget, 1997). The clinical picture of EV is distinguished by a life-long occurrence of multiple flat warts and macular lesions with a high risk of developing SCC later in life (Jablonska and Majewski, 1994; Orth, 2006). About 14 different HPV types have been found in benign tumors of EV-patients (Pfister, 2003), but only a few virus types were found in cancers, mainly HPV5 or HPV8, which are therefore considered high-risk types. The same HPV types have been found in actinic keratoses (AK), which are regarded as SCC precursors (Harwood and Proby, 2002; Pfister, 2003). Seroepidemiology supports this correlation, and antibodies against the major HPV8 capsid protein are associated with both cutaneous SCC (Feltkamp et al., 2003; Masini et al., 2003) and precancerous AK (Bouwes Bavinck et al., 2000). Furthermore, the early genes E2, E6 and E7 of HPV8 have been associated with cellular transformation *in vitro* (Akgül et al., 2006, 2007). Together these findings support the idea that EV-HPV types contribute to AK and SCC development in the general population. Infections with beta-HPVs are acquired early in infancy and most people probably carry persistent infections with multiple types (Antonsson et al., 2003; Weissenborn et al., 2009a). In the general population the DNA loads of these HPV types are very low in the skin and in hair bulbs, which represent their probable reservoir (Pfister, 2003; Weissenborn et al., 2009b). EV patients have a strongly enhanced risk for the development of SCC, which may be related to high viral DNA loads, which in turn may result in high oncogene expression levels (Dell'Oste et al., 2009). Different transgenic mouse lineages expressing all early genes of HPV8 (E1/E2/E4/E6/E7, HPV8-CER) (Schaper et al., 2005) or only E2 (Pfefferle et al., 2008) were recently established at our institute. All transgenes are expressed under the control of the human keratin-14 (hK14) promoter, which directs the expression of the transgenes to the stratum basale of the skin, the hair follicle and to a lesser extent to the stratum spinosum. HPV8-CER and -E2 mice spontaneously develop papillomas, dysplasias and in 2% (HPV8-E2) or 6% (HPV8-CER) SCC. UVA/B-irradiation led to a synchronized induction of papilloma development in about 3 weeks (Marcuzzi et al., 2009; Pfefferle et al., 2008). To study a possible role of viral oncogenes in this rapid tumor development, we

Abbreviations: EV, epidermodysplasia verruciformis; HPV, human papillomavirus; SCC, squamous cell carcinoma; UV, ultraviolet.

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doi:10.1016/j.virol.2010.04.013

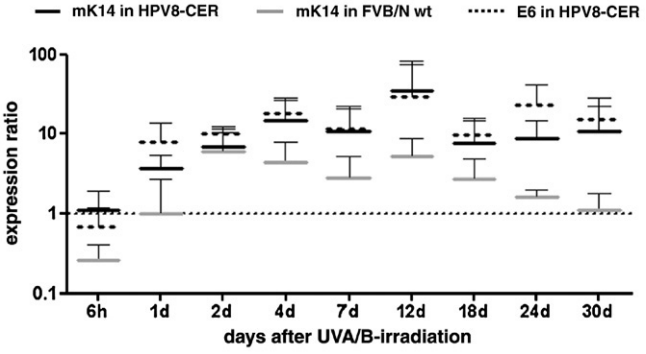
analyzed the kinetics and levels of oncogene expression in these HPV8 mouse lineages following different skin irritations.

Results

Enhanced transgene mRNA expression is induced early after UVA/B-irradiation in HPV8-CER mice

In this study we aimed to determine the impact of HPV8 early gene expression levels on cutaneous papilloma formation in mice. By comparative analyses we found that the levels of hK14-promoter driven transgene expression in HPV8-CER mice were highly correlated with the mK14 mRNA levels after skin irritation ($R=0.85$; $p<0.01$; shown for mK14 and HPV8-E6 in Fig. 1). We therefore chose mK14 expression to compare the response of keratinocytes of FVB/N wt and transgenic mice to skin irritation. In further experiments we ascertained, that neither wounding nor UVA/B-irradiation affected mK14 expression in adjacent skin areas (data not shown).

The kinetics of mK14 expression were examined over a 30-day period in UVA/B-irradiated HPV8-CER and FVB/N wt mice (Fig. 1). As reported in Marcuzzi et al. (2009) the mRNA levels of the housekeeping genes hypoxanthine phosphoribosyltransferase and β-actin increase after UVA/B-irradiation. This effect did also occur in our experiments and therefore mRNA levels were normalized to the total RNA input of one microgram in all qRT-PCR experiments. Generally, we observed stronger mean levels of mK14 mRNA in HPV8-CER mice than FVB/N wt mice. Induction of mK14 as well as HPV8-E6 expression was already seen 1 day after UVA/B-irradiation in HPV8-CER mice. At all time points between day 2 and day 12 after UVA/B-irradiation enhanced mK14 expression was measured in both mouse strains. In FVB/N wt mice mK14 expression returned to almost basal level along with healing of the irradiated skin. In contrast, mK14 expression as well as HPV8-E6 expression persisted at a high level in HPV8-CER mice at all following time points paralleled by papilloma growth.



samples per point in time	6h	1d	2d	4d	7d	12d	18d	24d	30d
mK14 in HPV8-CER	9	4	4	12	11	17	5	8	5
mK14 in FVB/N wt	3	4	4	6	6	9	3	6	3
HPV8-E6 in HPV8-CER	5	3	3	7	8	13	5	8	5

Fig. 1. Enhanced HPV8-E6 and mK14 expression was induced early after UVA/B-irradiation in HPV8-CER mice. Skin areas (4 cm^2) of shaved HPV8-CER and FVB/N wt mice were UVA/B-irradiated. Skin samples were taken at different time points from irradiated and non-irradiated areas of skin. MK14 and HPV8-E6 mRNA levels were measured in 1 μg total RNA via qRT-PCR. The mean expression ratio of mK14 in HPV8-CER mice (black solid lines), mK14 in FVB/N wt mice (gray lines) and HPV8-E6 in HPV8-CER mice (black dashed lines) is plotted against time. Error bars represent the standard deviation. The number of analyzed samples per point in time from different mice is given in the table below the graph.

Oncogene expression levels correlated with papilloma development in UVA/B-irradiated HPV8-E2 mice

Only a fraction of HPV8-E2 mice showed papillomatosis after UVA/B-irradiation (Pfefferle et al., 2008). To test whether papilloma formation correlates with different oncogene expression level we examined skin samples from three E2 mice taken 12 days after UVA/B-irradiation. The HPV8-E2 mouse, which developed papillomas showed a 7.5-fold enhanced E2 mRNA expression level. The two other mice did not develop papillomas and in these animals the HPV8-E2 expression levels were only 4.1- and 4.7-fold increased.

HPV8-E2 protein expression is increased in HPV8-CER mice soon after UVA/B-irradiation

To study whether the increase of transgene mRNA expression in the skin of HPV8-CER mice is mirrored at the protein level and to characterize the HPV8-oncoprotein positive epidermal layers, immunohistochemical HPV8-E2 staining experiments of UVA/B-irradiated and non-irradiated HPV8-CER skin sections were performed. Untreated HPV8-CER skin revealed a clear but faint HPV8-E2 signal in the epidermis (Fig. 2) whereas in line with RNA data, HPV8 transgene protein expression increased already 1 day after UVA/B-irradiation in the epidermis. Enhanced number of transgene expressing cells emerging from an increased number of epidermal cell layers and a more intense staining were observed. The enhanced HPV8-E2 protein expression was maintained over the whole observation period ending on day 24 after UVA/B-irradiation, when a papilloma with multiple epidermal cell layers and horn pearls had already developed. Keratinocytes of FVB/N wt skin were not stained by the anti-HPV8-E2 serum, proving the specificity of the antibody (Fig. 2, FVB/N 10 days and 24 days after UV-irradiation). Histology of UVA/B-irradiated FVB/N wt mouse skin showed an only slightly thinner epidermis than in HPV8-CER mice until day 7 after UVA/B-irradiation. However, about 10 days after UVA/B-irradiation, the epidermis of control FVB/N wt mice started to return to its normal status with one or two cell layers.

UVB-irradiation alone is sufficient to induce transgene expression in HPV8-CER mice

It was previously shown by Marcuzzi et al. (2009), that 0.36 J/cm^2 UVB-irradiation alone was sufficient to induce papillomatosis in HPV8-CER mice, while 10 J/cm^2 UVA-irradiation did not lead to papilloma development. To assess whether these treatments also differ in their ability to stimulate HPV8 transgene expression, mK14 levels were measured in UVA- and UVB-irradiated HPV8-CER and FVB/N wt mice. In contrast to the confluent papillomatosis induced by UVA/B-irradiation, UVB-irradiation alone induced multiple small papillomas. Twelve days after UVB-irradiation both FVB/N wt and HPV8-CER mice showed an upregulated mK14 expression (Fig. 3), similarly to the data presented in Fig. 1. In contrast, after UVA-irradiation, mK14 expression increased in neither FVB/N wt nor HPV8-CER mice.

Mechanical skin irritation as an inducer of papillomatosis in HPV8-CER mice

Wounding of HPV8-CER mouse skin by punch biopsy elicits the development of a confluent mass of papillomas (Marcuzzi et al., 2009). In this study tape-stripping was chosen as a model for mechanical skin irritation. Tape-stripping ablates cornified keratinocytes (Lademann et al., 2009) and induces multiple small punctate wounds (Fig. 4, 24 h after treatment). Macroscopically the wounds appeared to be healed within 10 days. Starting on about day 14, papillomas began to appear on areas of treated skin, particularly those with the most severe wounds. These papillomas grew larger over the

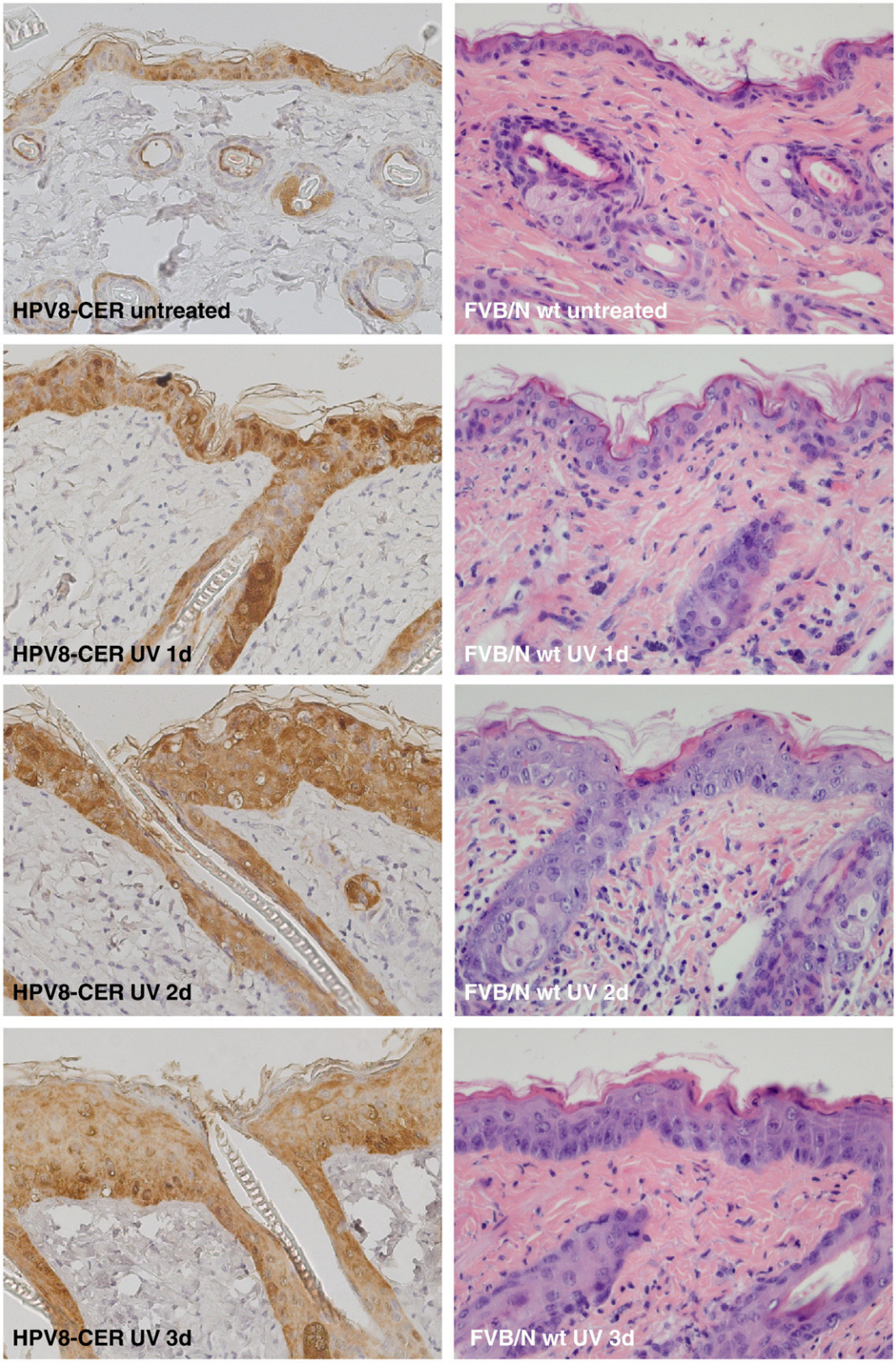


Fig. 2. Immunohistochemical staining for HPV8-E2 in HPV8-CER skin sections revealed rapid enhancement of HPV8-E2 protein expression after UVA/B-irradiation. Skin areas (4 cm^2) of shaved HPV8-CER and FVB/N wt mice were UVA/B-irradiated (10 J/cm^2; 1 J/cm^2). For each point in time skin samples were taken from two mice from the irradiated skin. Skin samples were also taken from two non-irradiated HPV8-CER and FVB/N wt mice. After formalin-fixation and paraffin embedding, samples were cut into 4 µm sections. HPV8-CER sections were stained against HPV8-E2 and counterstained with hematoxylin. FVB/N wt UV 10-day and 24-day sections were also incubated with the anti-HPV8-E2 serum and counterstained with hematoxylin, all other FVB/N wt sections were just stained with H&E. Magnification: 400×.

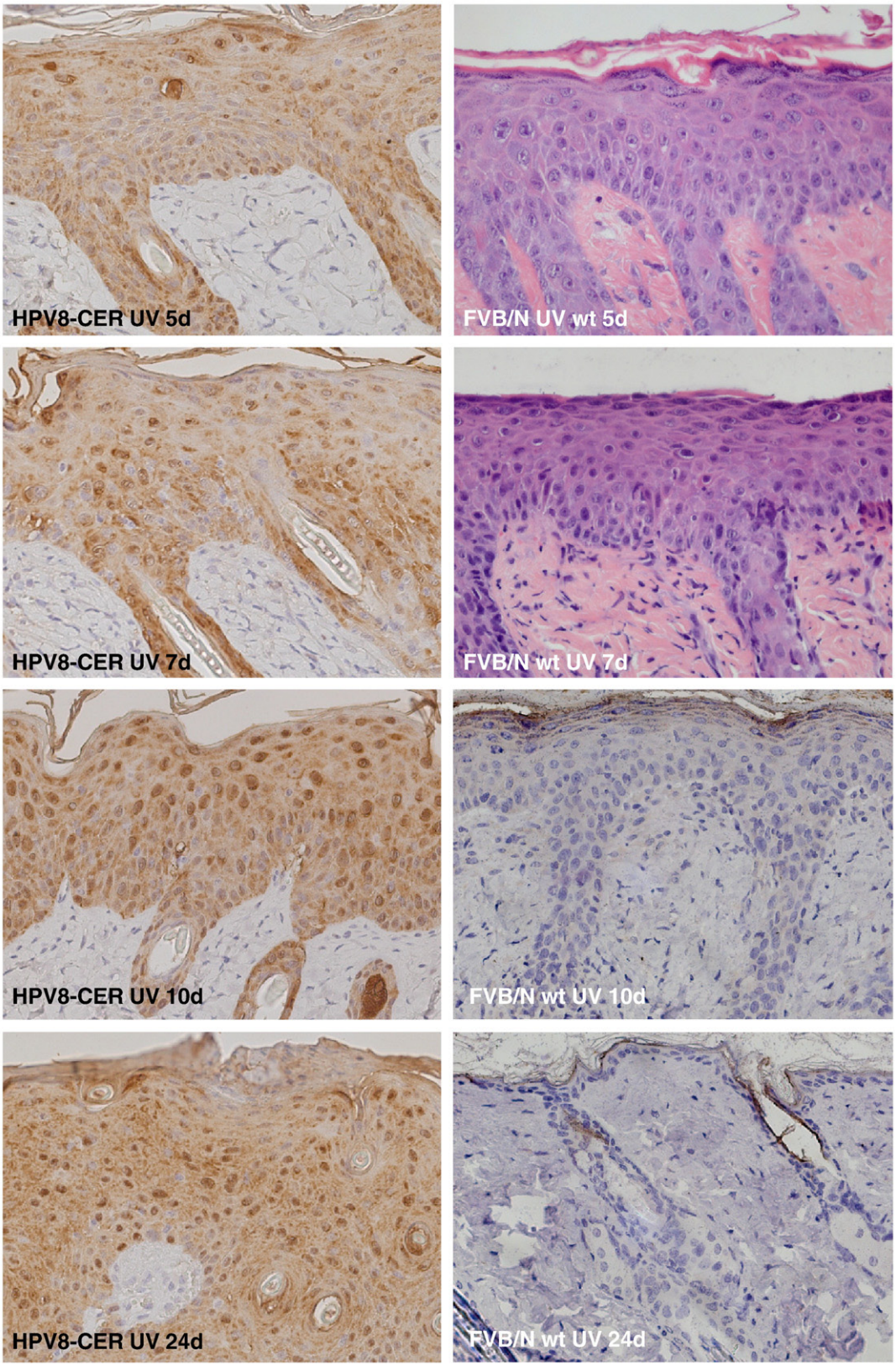


Fig. 2 (*continued*).

following observation period. Beginning on day 19, additional small spike-shaped papillomas were detectable on the treated skin area. The influence of mechanical skin irritation on transgene expression was investigated in three tape-stripped HPV8-CER mice. Two days after tape-stripping, four samples of wounded skin with different grades of injury were taken from each mouse to quantitate HPV8-E6 mRNA

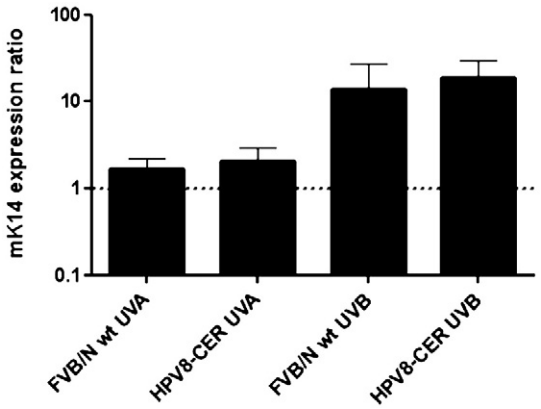


Fig. 3. UVB-irradiation alone led to enhanced mK14 expression in HPV8-CER and FVB/N wt mice, while UVA-irradiation did not. Skin areas (4 cm^2) of three shaved HPV8-CER and three shaved FVB/N wt mice were either UVA-irradiated (10 J/cm^2) or UVB-irradiated (0.36 J/cm^2). Skin samples were taken 12 days after UVA- or UVB-irradiation from the irradiated and non-irradiated skin. MK14 mRNA expression levels were measured in one microgram total RNA via qRT-PCR. Bars represent the mean mK14 expression ratio. Error bars represent the standard deviation.

expression (Fig. 5). Mouse A and B sustained moderate to severe injuries and showed enhanced HPV8-E6 expression, while mouse C had only minor wounds and minimal if any elevation of HPV8-E6 expression. Overall, samples with a macroscopically visible wound (A1, A2, A3, B1, B2) showed enhanced (1.5- to 2.9-fold) HPV8-E6 expression, while samples with no visible or only tiny wounds (A4, B3, B4, C1, C2, C3, C4) showed normal or slightly enhanced (0.8- to 1.5-fold) HPV8-E6 expression.

Knock-down of HPV8-E6 during tumor induction inhibits papilloma development

Wounding of the mouse skin with a tattoo machine can also induce papilloma development within 2 to 3 weeks (Fig. 6A). This opened up the possibility to test whether tattooing of HPV8-E6 specific siRNAs into HPV8-CER mouse skin could reduce or even inhibit papillomatosis by knocking-down HPV8-E6 expression. To characterize the HPV8-E6 knock-down kinetics, RNA was isolated at different points in time from HPV8-E6 expressing HaCaT cells transfected with four siRNAs. As shown in supplementary figure 1c, HPV8-E6 mRNA was knocked-down to 10% at 24 h and 30 h post transfection of siRNA-1 duplex. The HPV8-E6 mRNA levels began to rise again 48 h after transfection. Ten HPV8-CER mice were tattooed with water or siRNA-1 duplex/Lipofectamin 2000 on two separate, shaved, and depilated skin areas each, thereby inducing 40 wounds. Images of tattooed skin were taken at different points in time. The wounds caused by tattooing water or siRNA-1 duplex/Lipofectamin 2000 (Fig. 6A) started to heal and the skin seemed to be restored around day 10 after treatment. On day 18 after treatment, papilloma development started in the water tattooed areas, while only a small cicatricial spot could be observed in the siRNA-1 tattooed skin. On day 28, papilloma development was also visible in some siRNA-1 tattooed areas, but it was remarkably slowed down and reduced. Overall, papilloma development in skin areas wounded by tattooing siRNA-1 was delayed by about 10 days when compared to water treated areas (Fig. 6B). On day 42, about half of the siRNA-1 tattooed areas still showed no papillomatosis (11 out of 20 wounds), whereas only 5 out of 20 water-tattooed skin areas were devoid of papillomatosis. The emerged papillomas in the siRNA-1 tattooed area remained small and locally restricted in contrast to the pronounced papillomas in the water treated areas, which overgrew the former wound areas (Fig. 6A).

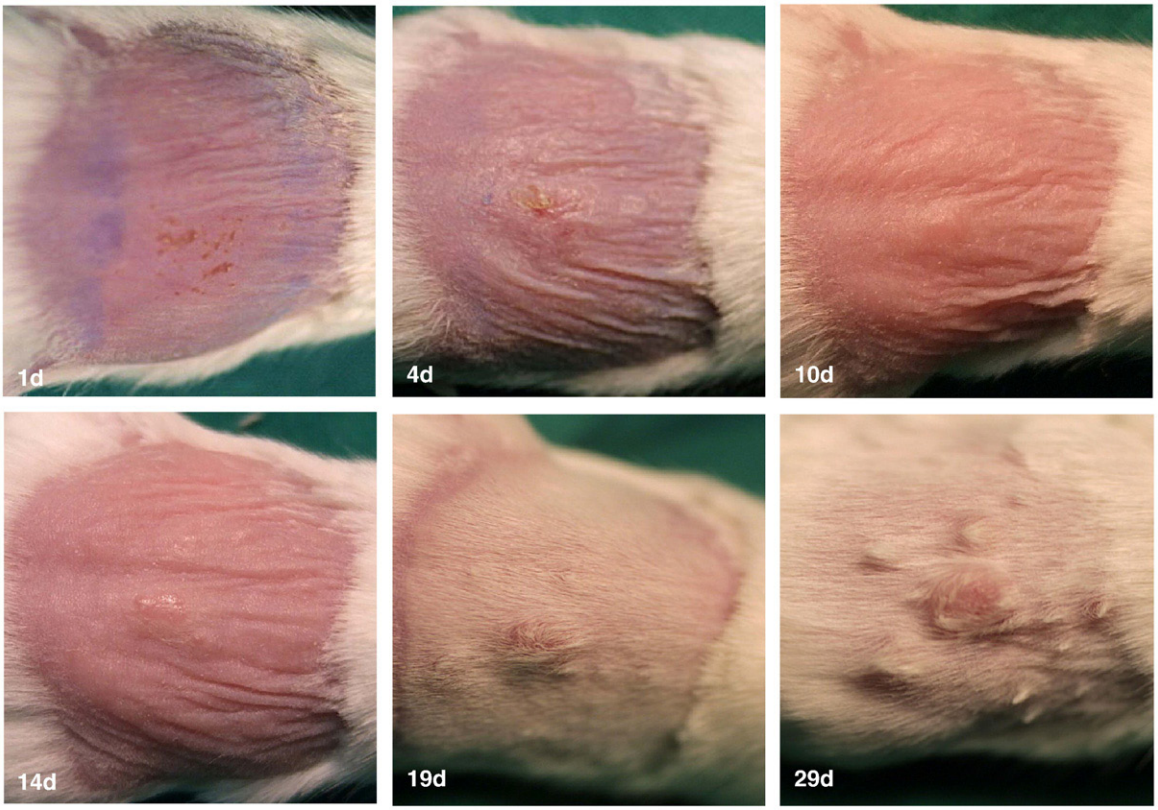


Fig. 4. Induction of papilloma growth by mechanical skin irritation. HPV8-CER mice were shaved and treated with depilatory cream on a 4 cm^2 sized skin area and then treated by attaching and detaching 15 times a new piece of tape. Pictures show treated skin areas at different points in time. A papillomatous area is first visible on day 14 after treatment and starting from day 19 additional small spike-shaped papillomas were detectable on the treated skin area.

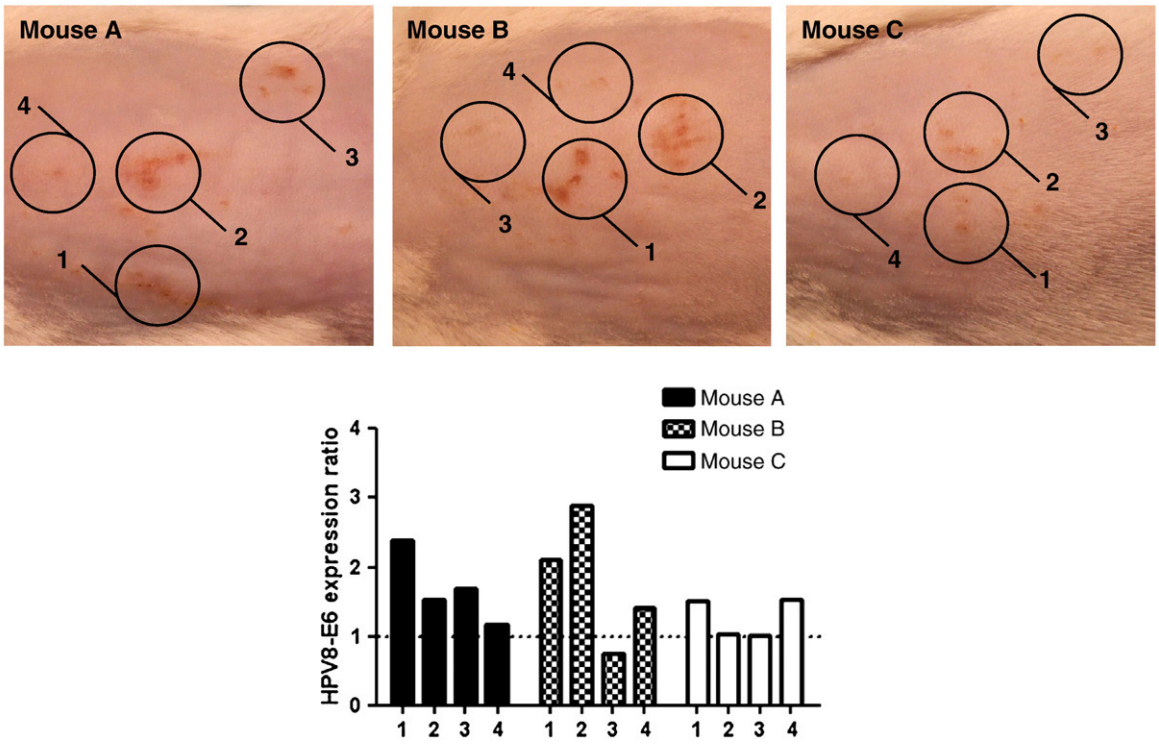


Fig. 5. Severe mechanical skin irritation enhances HPV8-E6 expression early after treatment. Three HPV8-CER mice were shaved and treated with depilatory cream on a 4 cm^2 sized skin area and then treated by attaching and detaching 15 times a new piece of tape. Two days after treatment four skin samples were taken from each mouse as indicated by the circles in the pictures. Additionally one area of untreated skin was taken from each mouse. HPV8-E6 mRNA expression levels were measured in one microgram total RNA via qRT-PCR. Bars represent the HPV8-E6 expression ratio of a single skin sample.

Discussion

We recently showed the oncogenic potential of HPV8 in the skin of transgenic mouse lines (Marcuzzi et al., 2009; Pfefferle et al., 2008; Schaper et al., 2005). However, little is known about the triggers of tumorous skin growth. In this study, we investigated the kinetics of HPV8 gene expression in transgenic mouse models in response to exogenous skin irritations. Furthermore, we investigated the relationship between increased expression of the viral oncogenes and the induction of tumor development. It is known that UV-irradiation, the main risk factor for SCC development (Leiter and Garbe, 2008), can induce the natural promoters of the HPV5 and 8 oncogenes (Akgül et al., 2005), as well as the cellular K14 promoter (Kinouchi et al., 2002). Therefore, we first used UVA/B-irradiation to enhance the hK14-promoter driven viral oncogene expression in HPV8-CER mice. As expected, the endogenous mK14 promoter was induced in parallel just as in non-transgenic FVB/N wt mice. HPV8-E6 mRNA levels rose already 1 day after irradiation and reached a maximum after two to 4 days. High levels of HPV8-E6 and mK14 mRNAs persisted in the transgenics accompanied by papilloma development. In contrast, in the FVB/N wt mice, the level of mK14 mRNA gradually returned to normal when the UVA/B-induced hyperplasia started to resolve. Immunohistological staining for HPV8-E2 protein in sections from UVA/B-irradiated HPV8-CER skin confirmed increased transgene expression already 1 day after induction. In the HPV8-CER mice, UVB-irradiation alone induced multiple small papillomas whereas UVA plus UVB elicited confluent papillomas in the whole irradiated skin area. However, the HPV8 transgene levels in papillomas elicited by either UVB or UVA/UVB irradiation were similar. In contrast, UVA-irradiation alone enhanced neither mK14 expression nor papilloma development. Thus, in our mouse models UVB is the main cause of enhanced transgene expression and tumor growth. Additional stimuli may further promote papilloma development, and our data indicate that UVA has a synergistic effect on UVB-induced papilloma development (Marcuzzi et al., 2009). This effect is not mediated by enhancing transgene expression, but possibly by causing oxidative stress (Burke and Wei, 2009).

Papillomas also developed spontaneously in non-irradiated HPV8-CER mice, but at lower frequency and slower rates and mostly dorsal caudal, despite a constitutive transgene expression in the entire mouse epidermis (Marcuzzi et al., 2009; Pfefferle et al., 2008; Schaper et al., 2005). Since male mice fight more than female mice, we speculated that fight wounds might enhance tumor development. This assumption is supported by the present observation that skin regeneration processes induced by tape-stripping are sufficient to induce papilloma development in HPV8-CER mice. In contrast to the area wide papillomatous growth elicited by UVA/B-irradiation, tape-stripping elicited only small, discrete papillomas. As the K14-promoter is known to be activated in hyperplastic skin (Coussens et al., 1996; Rossiter et al., 2001; Schaper et al., 2005; Sethi and Palefsky, 2004) it was conceivable to find enhanced HPV8-E6 expression levels in tape-stripped skin areas. The E6 expression levels in tape-stripped skin areas were overall less pronounced than those observed in UVA/B-irradiated areas. This may point to a smaller number of stimulated keratinocytes in tape-stripped skin in contrast to UVA/B-irradiated areas for which the stimulation of the majority of keratinocytes may be expected with the associated enhanced risk for tumor growth. The severity of skin wounding by tape-stripping appeared to correlate with enhanced transgene expression 2 days after treatment and with the probability of papilloma induction. In concordance with our data an induction of the viral promoter was also detected in papillomas of UV-irradiated rabbits latently infected with the cottontail rabbit papillomavirus, whereas no induction of the viral promoter was observed in UV-irradiated, papilloma-free, but CRPV-DNA positive skin (Zhang et al., 1999).

The reduced penetrance of tumor development after the less efficient induction of transgene expression following abrasive skin irritations compared to UVA/B-irradiation points to a dose-response

relationship. This notion is supported by the findings in HPV8-E2 transgenic mice. Both different basic levels of HPV8-E2 mRNA in three HPV8-E2 transgenic strains (Pfefferle et al., 2008) and different levels of UVA/B-induced E2 mRNA (this paper) turned out to influence incidence and rate of tumor development. As no papilloma growth was observed in tape-stripped HPV8-CER mice and UV-irradiated HPV8-E2 mice with only moderate induction of viral mRNA, a certain threshold of oncogene expression seems to exist, which has to be crossed for acute transformation of keratinocytes.

A direct link between elevated viral gene expression and tumor formation could be demonstrated by siRNA-mediated HPV8-E6 mRNA knock-down, which clearly reduced papilloma development. Residual papilloma development in siRNA treated animals may be explained by an inconsistent delivery of the siRNA. As demonstrated in cell culture experiments, the efficient HPV8-E6 knock-down lasted about 4 days after transfection, with a maximal effect around 30 h after siRNA transfection. Similar kinetics were reported for siRNAs injected into mice, where the knock-down decreased after a maximum on day 1 or 2 (Layzer et al., 2004; Nishina et al., 2008). Assuming that the knock-down also lasts for only a few days in the skin of HPV8-CER mice, the initial rise of HPV8-E6 expression seems to have a decisive impact on the early steps in papilloma development. The maintenance of high levels of oncogene mRNA is likely to be due to the ongoing papilloma growth.

The enhanced expression of viral oncogenes after irritation of mouse skin may mirror the conversion of a latent beta-HPV infection into an activated infection in man following sunburn or immunosuppression. The crucial importance of a shortly enhanced viral oncogene expression over a certain threshold for the initiation of tumor growth in HPV8 transgenic mice may indicate that such a transient induction of the viral promoter also elevates the risk for tumor development in man. Cellular processes affected by solar exposure or wounding will certainly be relevant for perpetuation and progression of lesions together with ongoing viral activities.

Methods

Mice, skin irritation and sample collection

Mice used here include transgenic hemizygous mouse lineages either expressing all early genes of HPV8 (E1/E2/E4/E6/E7, HPV8-CER) or -E2 separately under the control of the hK14 promoter, backcrossed into the FVB/N background and FVB/N wild-type (wt) mice (Pfefferle et al., 2008; Schaper et al., 2005). Mice were anesthetized with ketamine-hydrochloride (Sigma, Deisenhofen, Germany) and xylazine-hydrochloride (Sigma, Deisenhofen, Germany) and shaved with an electric shaver (Wella, Karlsruhe, Germany) before dorsal caudal skin irritation at an age of 6–12 weeks.

For UV-experiments HPV8-CER and FVB/N wt mice were either irradiated with 10 J/cm^2 UVA and 1 J/cm^2 UVB or with 10 J/cm^2 UVA alone or 036 J/cm^2 UVB alone on a 4 cm^2 sized area as reported before (Marcuzzi et al., 2009; Pfefferle et al., 2008). Treated skin areas were macroscopically observed daily and skin samples for RNA analysis were removed from anesthetized or euthanized mice with a 4 mm skin punch biopsy tool (PFM, Cologne, Germany) 6 hours, 1 days, 2 days, 4 days, 7 days, 12 days, 18 days, 24 days or 30 days after UVA/B-irradiation and 12 days after UVA or UVB-irradiation and stored at -20 °C in RNALater according to protocol (Qiagen, Hilden, Germany). Negative control skin samples were taken from untreated skin at the end of the respective observation interval. For immunohistochemical experiments skin samples were collected 0 days, 1 days, 2 days, 3 days, 5 days, 7 days, 10 days and 24 days after UVA/B-irradiation from HPV8-CER and FVB/N wt mice, formalin-fixed and embedded in paraffin. For tape-stripping experiments HPV8-CER mice were treated with depilatory cream (Balea, Karlsruhe, Germany) on a 4 cm^2 sized area after shaving. These mice were further treated by attaching and detaching 15 times a new piece of tape (Tesa, Hamburg, Germany) in the depilated skin. Four samples of the irritated skin and one sample of non-irritated skin from each mouse were taken 2 days after treatment and stored at -20 °C in RNALater (Qiagen). Tattooing was performed using a “Rotary 12000PL” tattoo machine with 5-Liner needles (Bortech, Wuppertal, Germany) run by 17.5 V (DC POWER SUPPLY, DF 1730 SB3A, Bortech, Wuppertal, Germany) thereby inducing 725 pinholes per second. Ten microliter transfection-mix, composed of 1 µl siRNA-1 duplex (100 µM), 1 µl Lipofectamin 2000 (Invitrogen, Karlsruhe, Germany) and 8 µl water or 10 µl water alone were delivered to a dorsal area of shaved and depilated HPV8-CER mouse skin followed by tattooing a skin stripe of 1 cm length three times for 5 s each. The tattoo machine was adjusted to allow the tattoo needles to penetrate the skin 1-2 mm. The siRNA used in this study was designed and manufactured by Qiagen (Hilden, Germany) and had the following sequence: siRNA-1 sense: CCGCAACGUUUGAAUUUAAdTdT, siRNA-1 antisense: UUAAAUUCAAACGUUGCGGdTdT.

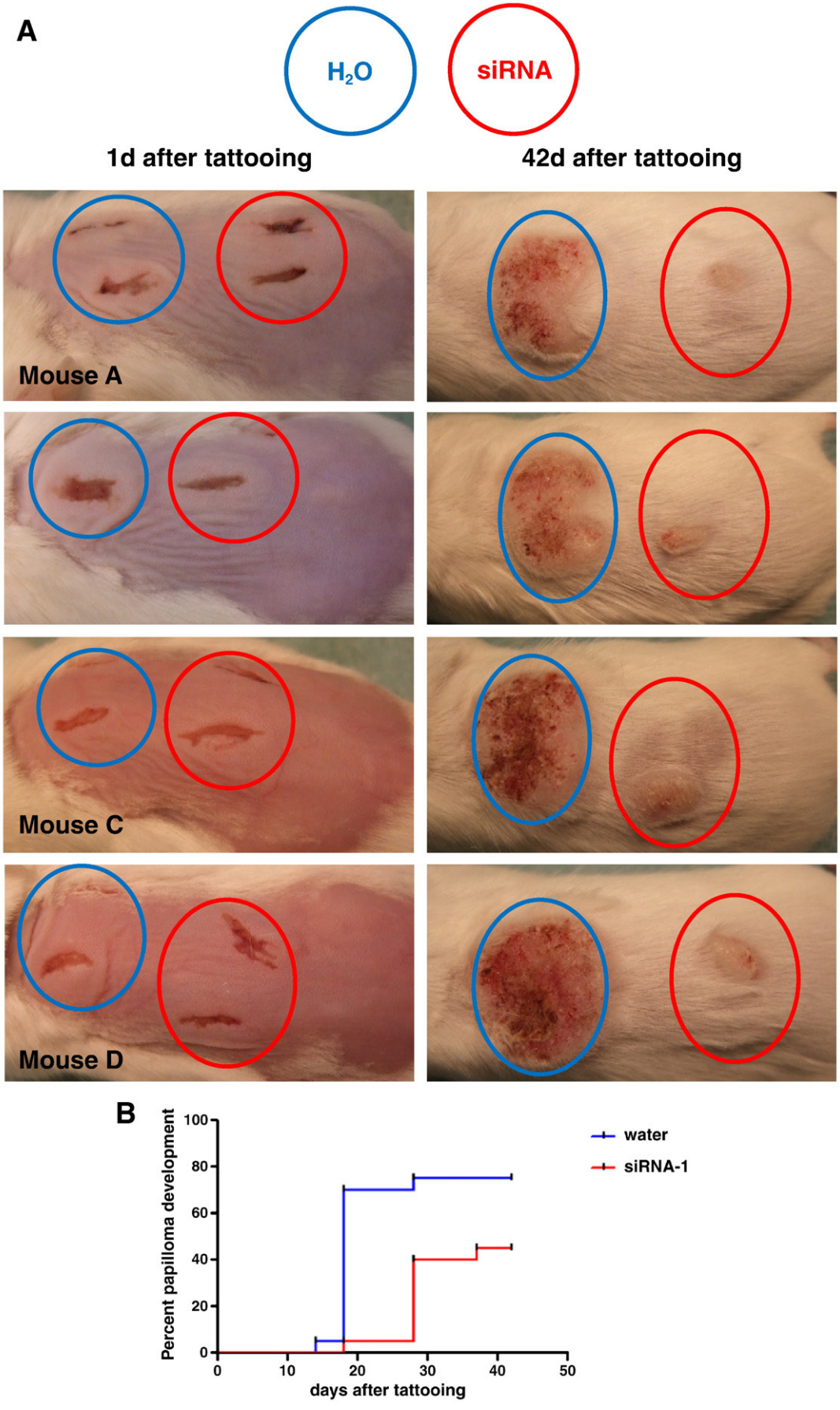
RNA isolation and reverse transcription

RNA from skin samples was isolated with RNeasy kit and DNase digestion was performed on column using RNase-free DNase according to the manufacturer's instructions (Qiagen, Hilden, Germany). One microgram of RNA was reverse transcribed using Omniscript kit (Qiagen, Hilden, Germany) with 1 µM oligo-dT$_{23}$-primer (Sigma, Deisenhofen, Germany), 10 µM Random Nonamers (TIB MOLBIOL, Berlin, Germany) and 10 units RNase Inhibitor (Fermentas, St. Leon-Rot, Germany).

Real-time-PCR

Murine K14 (mK14) and transgene levels were measured by real-time PCR (qRT-PCR) using the LightCycler System (Roche, Mannheim, Germany). Two microlitres of a 1:10 dilution of cDNA were used in a total volume of 20 µl containing 1.25 units Platinum *Taq* Polymerase and the associated buffer (Invitrogen, Karlsruhe, Germany), 4 mM $MgCl_2$, 1.6 µl of a 1:1000 dilution of SybrGreen (Sigma, Deisenhofen, Germany), 5% DMSO, 0.5 µM forward and backward primer each, 500 ng/µl non-acetylated bovine serum albumin (Fermentas, St. Leon-Rot, Germany) and 0.2 mM dNTP each. Samples were analyzed in duplicate together with a cDNA dilution series, which was used to generate a standard curve. Mean values were used for the calculation of expression ratios from untreated to treated skin using Pfaffl's equation (Pfaffl, 2001). RNA levels were normalized to the RNA input of one microgram. 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 $T_{ann.}$ (20 °C/s), and 15 s at 72 °C (20 °C/s). Fluorescence was measured once per cycle at the end of the elongation step. The primers used in this study had the following sequences, mK14fw: 5′-tccagagatgtgacctcc-3′, mK14bw: 5′-ccaccttgccatcgt-3′, HPV8E6fw: 5′-gcaacgtttgaattta-3′, HPV8E6rev: 5′-catgatacaaatgcttac-3′. Primers for HPV8-E2 were taken from Schaper et al. (2005).

Fig. 6. Tattooing HPV8-E6 specific siRNA slowed down papillomatosis in HPV8-CER mice. Ten HPV8-CER mice were shaved and depilated. Ten microliter each of transfection-mix (siRNA-1 duplex/Lipofectamin 2000) were tattooed into two separate areas of the mouse skin. As control 10 µl water were tattooed into two separate skin areas of the same mouse (A) Tattooed skin areas of four mice on days 1 and 42 after tattooing. (B) Kaplan–Meier curves show the percentage of papilloma development in 20 water-tattooed and 20 siRNA-1-tattooed HPV8-CER skin areas plotted against time.


A
H2O
siRNA
1d after tattooing
42d after tattooing
Mouse A
Mouse C
Mouse D
B
Percent papilloma development
100
80
60
40
20
0
0
10
20
30
40
50
days after tattooing
water
siRNA-1

Immunohistochemistry

Deparaffinization of 4 µm thick tissue sections was done by incubation in xylene two times for 5 min. Samples were hydrated through a descending alcohol series (100%, 90%, 70%; 5 min each) and endogenous peroxidases were inactivated by incubation in 3% H_2O_2 in methanol for 10 min. Antigen unmasking was performed by boiling the tissue sections in 10 mM citric buffer in a pressure cooker for 4 min in a microwave. Further incubation steps were performed in a humid chamber to prevent drying-out. After blocking unspecific antigen sites with PBS/20% FCS for 1 h, samples were incubated with a primary guinea pig antibody against HPV8-E2 in PBS/2% FCS for 90 min followed by three washes with PBS. The antibody was raised against a 6x-His-tagged HPV8-E2 protein, generated in bacteria and purified on a nickel column. Incubation with a secondary antibody (anti-guinea pig conjugated with horseradish peroxidase) diluted in PBS/2% FCS was done for 1 h. Following three washes with PBS DAB staining was performed with the Liquid DAB Concentrated Substrate Pack (BioGenex, San Ramon, USA). Sections were counterstained with hematoxylin and dehydrated with an ascending alcohol series and embedded with mounting medium.

Acknowledgments

This study was supported by the Mildred Scheel Stiftung für Krebsforschung/Deutsche Krebshilfe (grant no.: 107162).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2010.04.013.

References

Akgül, B., Lemme, W., Garcia-Escudero, R., Storey, A., Pfister, H.J., 2005. UV-B irradiation stimulates the promoter activity of the high-risk, cutaneous human papillomavirus 5 and 8 in primary keratinocytes. Arch. Virol. 150 (1), 145–151.

Akgül, B., Cooke, J.C., Storey, A., 2006. HPV-associated skin disease. J. Pathol. 208 (2), 165–175.

Akgül, B., Ghali, L., Davies, D., Pfister, H., Leigh, I.M., Storey, A., 2007. HPV8 early genes modulate differentiation and cell cycle of primary human adult keratinocytes. Exp. Dermatol. 16 (7), 590–599.

Alam, M., Ratner, D., 2001. Cutaneous squamous-cell carcinoma. N. Engl. J. Med. 344 (13), 975–983.

Antonsson, A., Karanfilovska, S., Lindqvist, P.G., Hansson, B.G., 2003. General acquisition of human papillomavirus infections of skin occurs in early infancy. J. Clin. Microbiol. 41 (6), 2509–2514.

Bouwes Bavinck, J.N., Stark, S., Petridis, A.K., Marugg, M.E., Ter Schegget, J., Westendorp, R.G., Fuchs, P.G., Vermeer, B.J., Pfister, H., 2000. The presence of antibodies against virus-like particles of epidermodysplasia verruciformis-associated humanpapillomavirus type 8 in patients with actinic keratoses. Br. J. Dermatol. 142 (1), 103–109.

Burke, K., Wei, H., 2009. Synergistic damage by UVA radiation and pollutants. Toxicol. Ind. Health 25 (4–5), 219–224.

Coussens, L.M., Hanahan, D., Arbeit, J.M., 1996. Genetic predisposition and parameters of malignant progression in K14-HPV16 transgenic mice. Am. J. Pathol. 149 (6), 1899–1917.

de Villiers, E.M., Fauquet, C., Broker, T.R., Bernard, H.U., zur Hausen, H., 2004. Classification of papillomaviruses. Virology 324 (1), 17–27.

Dell'Oste, V., Azzimonti, B., De Andrea, M., Mondini, M., Zavattaro, E., Leigheb, G., Weissenborn, S.J., Pfister, H., Michael, K.M., Waterboer, T., Pawlita, M., Amantea, A., Landolfo, S., Gariglio, M., 2009. High beta-HPV DNA loads and strong seroreactivity are present in epidermodysplasia verruciformis. J. Invest. Dermatol. 129 (4), 1026–1034.

DePinho, R.A., 2000. The age of cancer. Nature 408 (6809), 248–254.

Feltkamp, M.C., Broer, R., di Summa, F.M., Struijk, L., van der Meijden, E., Verlaan, B.P., Westendorp, R.G., ter Schegget, J., Spaan, W.J., Bouwes Bavinck, J.N., 2003. Seroreactivity to epidermodysplasia verruciformis-related human papillomavirus types is associated with nonmelanoma skin cancer. Cancer Res. 63 (10), 2695–2700.

Harwood, C.A., Proby, C.M., 2002. Human papillomaviruses and non-melanoma skin cancer. Curr. Opin. Infect. Dis. 15 (2), 101–114.

Jablonska, S., Majewski, S., 1994. Epidermodysplasia verruciformis: immunological and clinical aspects. Curr. Top. Microbiol. Immunol. 186, 157–175.

Kinouchi, M., Takahashi, H., Itoh, Y., Ishida-Yamamoto, A., Iizuka, H., 2002. Ultraviolet B irradiation increases keratin 5 and keratin 14 expression through epidermal growth factor receptor of SV40-transformed human keratinocytes. Arch. Dermatol. Res. 293 (12), 634–641.

Lademann, J., Jacobi, U., Surber, C., Weigmann, H.J., Fluhr, J.W., 2009. The tape stripping procedure—evaluation of some critical parameters. Eur. J. Pharm. Biopharm. 72 (2), 317–323.

Layzer, J.M., McCaffrey, A.P., Tanner, A.K., Huang, Z., Kay, M.A., Sullenger, B.A., 2004. In vivo activity of nuclease-resistant siRNAs. RNA 10 (5), 766–771.

Leiter, U., Garbe, C., 2008. Epidemiology of melanoma and nonmelanoma skin cancer–the role of sunlight. Adv. Exp. Med. Biol. 624, 89–103.

Majewski, S., Jablonska, S., Orth, G., 1997. Epidermodysplasia verruciformis. Immunological and nonimmunological surveillance mechanisms: role in tumor progression. Clin. Dermatol. 15 (3), 321–334.

Marcuzzi, G.P., Hufbauer, M., Kasper, H.U., Weissenborn, S.J., Smola, S., Pfister, H., 2009. Spontaneous tumor development in human papillomavirus type 8 E6 transgenic mice and rapid induction by UV-light exposure and wounding. J. Gen. Virol.

Masini, C., Fuchs, P.G., Gabrielli, F., Stark, S., Sera, F., Ploner, M., Melchi, C.F., Primavera, G., Pirchio, G., Picconi, O., Petasecca, P., Cattaruzza, M.S., Pfister, H.J., Abeni, D., 2003. Evidence for the association of human papillomavirus infection and cutaneous squamous cell carcinoma in immunocompetent individuals. Arch. Dermatol. 139 (7), 890–894.

Nishina, K., Unno, T., Uno, Y., Kubodera, T., Kanouchi, T., Mizusawa, H., Yokota, T., 2008. Efficient in vivo delivery of siRNA to the liver by conjugation of alpha-tocopherol. Mol. Ther. 16 (4), 734–740.

Orth, G., 2006. Genetics of epidermodysplasia verruciformis: Insights into host defense against papillomaviruses. Semin. Immunol. 18 (6), 362–374.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29 (9), e45.

Pfefferle, R., Marcuzzi, G.P., Akgul, B., Kasper, H.U., Schulze, F., Haase, I., Wickenhauser, C., Pfister, H., 2008. The human papillomavirus type 8 E2 protein induces skin tumors in transgenic mice. J. Invest. Dermatol. 128 (9), 2310–2315.

Pfister, H., 2003. Chapter 8: human papillomavirus and skin cancer. J. Natl. Cancer Inst. Monogr. (31), 52–56.

Pfister, H., Ter Schegget, J., 1997. Role of HPV in cutaneous premalignant and malignant tumors. Clin. Dermatol. 15 (3), 335–347.

Rossiter, H., Beissert, S., Mayer, C., Schon, M.P., Wienrich, B.G., Tschachler, E., Kupper, T.S., 2001. Targeted expression of bcl-2 to murine basal epidermal keratinocytes results in paradoxical retardation of ultraviolet- and chemical-induced tumorigenesis. Cancer Res. 61 (9), 3619–3626.

Schaper, I.D., Marcuzzi, G.P., Weissenborn, S.J., Kasper, H.U., Dries, V., Smyth, N., Fuchs, P., Pfister, H., 2005. Development of skin tumors in mice transgenic for early genes of human papillomavirus type 8. Cancer Res. 65 (4), 1394–1400.

Sethi, N., Palefsky, J., 2004. Transcriptional profiling of dysplastic lesions in K14-HPV16 transgenic mice using laser microdissection. FASEB J. 18 (11), 1243–1245.

Weissenborn, S.J., De Koning, M.N., Wieland, U., Quint, W.G., Pfister, H.J., 2009a. Intrafamilial transmission and family-specific spectra of cutaneous betapapillomaviruses. J. Virol. 83 (2), 811–816.

Weissenborn, S.J., Neale, R., de Koning, M.N., Waterboer, T., Abeni, D., Bouwes Bavinck, J.N., Wieland, U., Pfister, H.J., 2009b. Prevalence and multiplicity of cutaneous beta papilloma viruses in plucked hairs depend on cellular DNA input. J. Virol. Methods 161 (2), 280–283.

Zhang, P., Nouri, M., Brandsma, J.L., Iftner, T., Steinberg, B.M., 1999. Induction of E6/E7 expression in cottontail rabbit papillomavirus latency following UV activation. Virology 263 (2), 388–394.