Expression kinetics of viral oncogenes, miRNAs and their targets during papilloma development in human papillomavirus 8 transgenic mice

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1 Introduction

High-risk genital human papillomaviruses (HPV) are known to cause cervical cancer. Whether cutaneous HPV play an active role in the pathogenesis of non-melanoma skin (NMSC) cancer in the general population is currently discussed. At least in immunosuppressed and epidermodysplasia verruciformis (EV) patients, an association between cutaneous HPV and NMSC is accepted. Furthermore, an oncogenic potential for the cutaneous HPV type 8 could be demonstrated in transgenic mice, which are used as a model for HPV8-dependent NMSC development in this study.

1.1 Papillomavirus structure

Papillomaviruses (PV) are small (∅ 55nm), non-enveloped, double stranded DNA viruses. Their icosahedral capsid consists of 72 capsomeres. The DNA is associated with cellular histones building a nucleosome-like structure (Favre *et al*. 1977). HPV constitute a large, heterogeneous group, whose genome size ranges between 7200-8000 base pairs and is typically organized in two functionally distinct regions: a non-coding region (NCR) and a coding region (Figure 1) (Pfister and Fuchs 1994). The coding region comprises at least seven open reading frames (ORF) which are located on one DNA strand. Depending on the expression in the life cycle of the PV the ORF are divided in early (E) and late (L) genes. The early proteins are involved in replication and transcription of the PV genome and in cell transformation. The late proteins L1 and L2 are structural proteins building the capsid. The transcription of the polycistronic mRNAs starts at least from two promoters (Baker 1993). From the early promoter, which is located at the 3'-end of the NCR right before the E6 ORF, mRNAs coding for E1, E2, E5, E6 and E7 are transcribed. The late promoter controls the expression of L1 and L2, but also of E1, E2 and E4 (Stubenrauch *et al*. 1992; Pfister and Fuchs 1994; Stubenrauch and Laimins 1999). The length, function and organization of the ORFs are conserved among different PV types, despite great differences in sequence (Pfister and Fuchs, 1987). Due to alternative splicing a fusion protein named E1^E4 can be produced, which is involved in the release of

infectious virus particles (Doorbar *et al*. 1991). The NCR contains essential cis-regulatory control elements like origin of replication (*ori*), promoters and a keratinocyte-specific enhancer, to control the viral gene expression and replication (Akgül *et al.* 2003). It is located between the L1 and E6 ORF and shows high similarity among closely related PV and greater differences to NCRs of other genera.

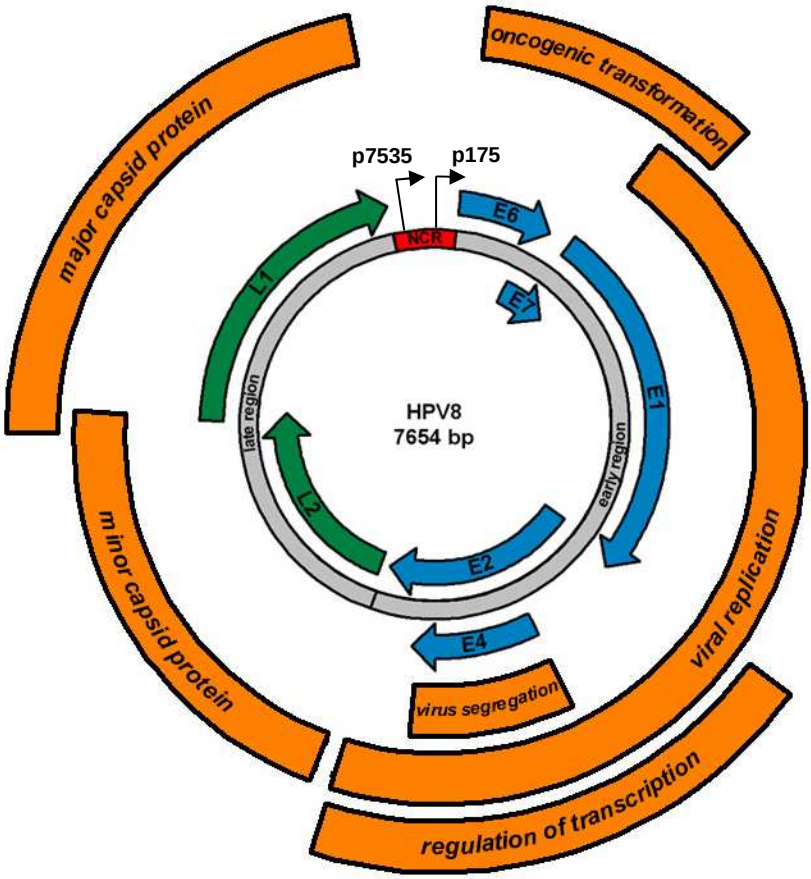


Figure 1: Schematic drawing of the HPV8 genome.
The HPV8 genome is divided into the early region coding for of the early genes (blue arrows), the late region coding for of the late genes (green arrows) and the NCR (red bar). Two promoters reside in the NCR (p175 and p7535). Orange bars define the functions of the encompassed genes.

1.2 HPV taxonomy

PV show high diversity, until now alone in humans more than 100 HPV types are fully sequenced (Bernard 2005). Furthermore 120 partial DNA sequences exist, suggested to represent putative new types (zur Hausen 2002; de Villiers *et al*. 2004). A new PV type is defined by a sequence homology below 90% within the conserved major capsid protein gene L1 compared to all established PV types. Differences in the nucleotide sequences of L1 ORF between 2-10% or less than 2% define a

subtype or variant, respectively (de Villiers *et al*. 2004). HPV are grouped in cutaneous and mucosal or genital HPV types, which are again divided into low-risk and high-risk types according to their oncogenic potential. Furthermore, HPVs can be classified into five genera (Alpha, Beta, Gamma, Mu und Nu). The two largest genera Alpha and Beta comprise 90% of the identified HPV. Genus Alpha mostly consists of the HPV that infect mucosal surfaces, such as the anogenital tract and the oral lining. Amongst these are the high risk types 16, 18 and 33 that have shown a strong association with the development of cervical carcinoma. The genus Beta includes cutaneous HPV types. Most of them were originally detected in the lesions of EV-patients, for example HPV5 and 8.

1.3 HPV life cycle

HPV specifically infect keratinocytes, the predominant cell type in the epithelia (Eckert *et al*. 1997). Given that HPV infect only the epithelia, their entire life cycle, culminating in viral replication and virion shedding, depends upon the host cells' molecular machinery that is ultimately coupled to the differentiation state of keratinocytes. The human skin is composed of the three primary layers subcutis, dermis and epidermis. The epidermis is differentiated into *stratum basale*, *stratum spinosum*, *stratum granulosum* and *stratum corneum* (Figure 2). To establish an infection HPV reach the basal keratinocytes via micro traumata. Following skin injuries, keratinocytes express $\alpha_6\beta_4$ integrin on their surface, a transmembrane glycoprotein, which has been proposed as a candidate receptor for HPV (Evander *et al*. 1997). Another adsorption target is heparin, which is expressed on the surface of the host cell (Joyce *et al*. 1999; Giroglou *et al*. 2001). Following entry into basal epithelial cells by endocytosis (Selinka *et al*. 2002), the genome is transported by a yet unknown mechanism into the nucleus. HPV are established and maintained as episomes in low copy number with the help of the early proteins, mostly E1 and E2. During mitosis of the infected basal cells the viral DNA which is linked to the chromosomes, is distributed between daughter cells (Oliveira *et al.* 2006), some of which will remain in the basal layer, while others will undergo differentiation. Because maturation of HPV is restricted to differentiating cells, the remaining basal cells will not be harmed by virus production (Stubenrauch and Laimins 1999). In the *stratum*

spinosum the expression of regulatory viral proteins increases and the vegetative replication of the viral DNA begins (Figure 2). Besides the viral proteins E1 and E2, cellular replication factors are necessary for this purpose, which are not expressed anymore in the suprabasal cells. Therefore the cell cycle arrest, keeping the keratinocytes locked in the G1-phase, is relieved by the oncoprotein E7 to permit the entry into the S-phase (Banerjee *et al.* 2006). In the uppermost layers of the *stratum spinosum* the expression of the capsid proteins L1 and L2 is induced. This expression is enhanced in the *stratum granulosum* where the maturation of the virions takes place. Finally, the infectious, mature viruses are shed with the squames of the *stratum corneum* (Bryan and Brown 2001). Probably the E4 protein is essential for this process by degrading the cytoskeleton (Doorbar *et al*. 1991).

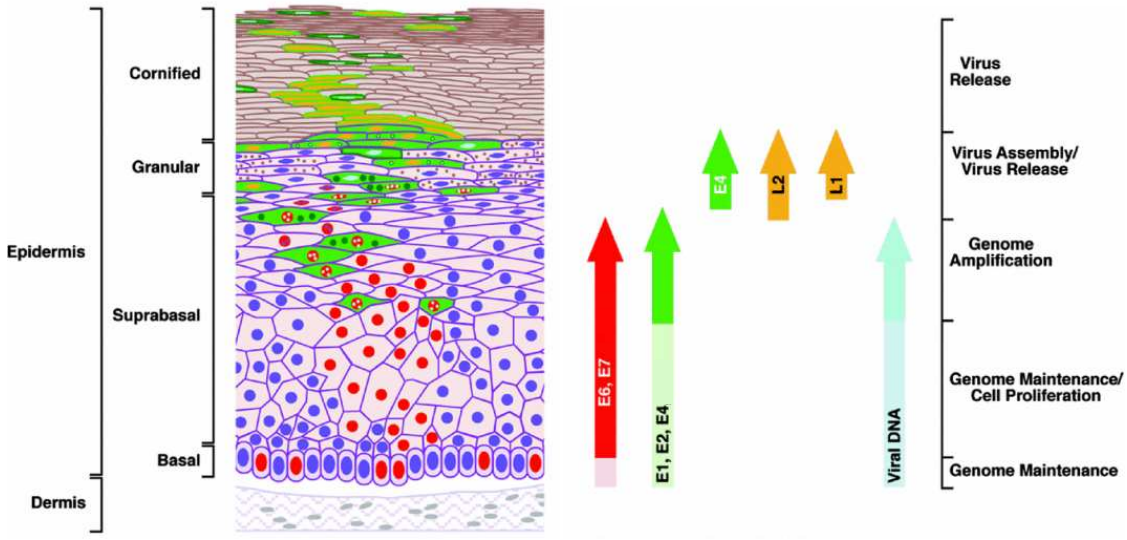


Figure 2: Life cycle of cutaneous HPV and profile of the skin.
The profile shows the different layers of the skin and the maturation of HPV in dependency of skin differentiation (modified from Doorbar 2006).

1.4 Functions of the viral proteins

1.4.1 E2 protein

The 43-48 kDa nuclear phosphoprotein E2 is involved in transcriptional regulation. It functionally acts as a dimer and is organized in three domains, a N-terminal transactivation domain, a central hinge region and a C-terminal DNA binding and dimerization domain. E2 facilitates the binding of the DNA helicase E1 to the viral *ori* thereby unwinding that DNA region. This process is necessary for viral replication (Kuo *et al.* 1994), as it allows access of the cellular DNA polymerase to the viral

genome. With the DNA binding and dimerization domain E2 dimers bind the palindromic motif 5'-ACCG$(N)_4$CGGT-3', which is present throughout the HPV genome, mostly within the NCR, where four copies can be found (Steger *et al.* 1996). By sterical interference, E2 dimers can act either as transactivator or repressor of E6 and E7 transcription, depending on its own concentration (Bouvard *et al*. 1994; Enzenauer *et al*. 1998; Morgan *et al*. 1998). For example high concentrations of E2 block the binding of the cellular activator specific protein 1 (Sp1) and TATA binding protein (TBP) thereby repressing promoter activity (Tan *et al.* 1992; Demeret *et al.* 1994; Dong *et al.* 1994). By reducing the expression of the oncogenes E6 and E7, E2 exerts a negative effect on cell proliferation and arrests the cells in the G2-phase (Frattini *et al.* 1997; Fournier *et al.* 1999). Mice, transgenic for the E2 protein of HPV8, spontaneously develop skin tumors, thereby showing that E2 exhibits transforming potential (Pfefferle *et al.* 2008).

1.4.2 E6 protein

The E6 protein of HPV is a 18 kDa phosphoprotein, which is localized in the nucleus and in non-nuclear membranes (Grossman *et al.* 1989). It contains four Cys-X-X-Cys motifs which form two unusually big zinc finger domains of 29-30 AA, respectively, which are important for protein stability and activity (Smola-Hess and Pfister, 2002). E6 proteins of genital high-risk HPV types carry a PDZ binding motif at the C-terminus with the consensus sequence X-S/T-X-V/L. With this motif they are able to interact with PDZ domains of other proteins, for example hDlg, MAGUKs, MUPP1, hScrib and direct them to ubiquitin-dependent proteolysis (Gardiol *et al.* 1999; Lee *et al.* 2000; Nakagawa and Huibregtse 2000; Thomas *et al.* 2002). One major function of the genital HPV16-E6 protein is the inhibition of apoptosis by inactivation of p53 (Scheffner *et al.* 1993). For this purpose the E6 protein forms a complex with p53 and the cellular ubiquitin ligase E6-associated protein (E6-AP) (Werness *et al.* 1990). This causes a deregulation of the cell cycle control at the G1/S and G2/M check points, an important step for the replication of HPV, because a productive infection cycle is only possible in cells, which are in the S-phase of the cell cycle. However, this cell cycle manipulation can lead to activation of oncogenes or inactivation of tumor suppressors and DNA damages cannot be repaired, which leads to genetic

instability and to malignant transformation of high-risk HPV-infected cells (Thomas and Laimins 1998; Somasundaram 2000; Fehrmann and Laimins 2003). Another important way how E6 proteins of genital HPV contribute to transformation is the activation of the human telomerase reverse transcriptase promoter, which controls the transcription of the catalytic telomerase subunit (Gewin and Galloway 2001). E6 proteins of cutaneous HPV do not interact with p53 or E6-AP and do not degrade p53 (Steger and Pfister 1992; Elbel *et al.* 1997). Nevertheless, the E6 proteins of EV-HPV exhibit oncogenic potential. The E6 protein of HPV5 for example can interfere with the repair of UVB-induced cyclobutane pyrimidine dimers (Giampieri and Storey 2004) and the E6 protein of HPV8 can bind the cellular protein $XRCC_1$ thereby impairing the repair of DNA single strand breaks (Iftner *et al.* 2002). Furthermore E6 proteins of both cutaneous and anogenital HPV are able to target the proapoptotic protein bak for ubiquitin-dependent degradation by assembling E6-AP, thereby inhibiting apoptosis (Jackson and Storey 2000).

1.4.3 E7 protein

E7 is a 11 kDa protein with a zinc finger motif. It acts as an oncogene in genital high-risk HPV and is able to immortalize primary foreskin keratinocytes (Halbert *et al.* 1991). The major part of the transforming potential of E7 is due to the binding and induction of ubiquitin-dependent degradation of the tumor suppressor retinoblastoma protein (Rb) (Dyson *et al.* 1989; Berezutskaya and Bagchi 1997). The competitive binding of E7 to Rb and its degradation lead to the segregation of the transcription factor E2F. In the G1-phase, E2F is inactivated in a complex with Rb. After segregation, E2F can induce the expression of genes, which are important for DNA synthesis and cell cycle control. Additionally E7 can bind the inhibitors of cyclin-dependent kinases $p21^{CIP1}$ and $p27^{KIP1}$ and inhibit their functions (Münger *et al.* 2001). Both events direct the cell into the S-phase and enable the viral replication. Most E7 proteins of low-risk HPV and EV-HPV, including HPV8, have a much lower binding affinity for Rb and do not induce its degradation (Iftner *et al.* 1988; Kiyono *et al.* 1989; Schmitt *et al.* 1994). However, in organotypic skin cultures, HPV8-E7 could induce an invasion of keratinocytes into the dermis, implicating a direct involvement of E7 in the oncogenesis of cutaneous HPV-types (Akgül *et al.* 2006).

1.5 Human Papillomaviruses are involved in cervical cancer

HPV is the most common sexually transmitted virus (Garland 2002). Although HPV can persist for years in infected tissue, in most cases an infection with HPV is clinically unapparent. Nevertheless, low-risk HPV types 6 and 11 can induce benign tumors such as warts and condylomas (zur Hausen 2000). High-risk types are widespread within all human populations, particularly HPV16, and an infection with these types result in squamous intraepithelial lesions. In most cases these lesions exhibit a limited growth and regress spontaneously, probably due to a cell-mediated immune response (Shah and Howley 1996). However, in a small percentage the infection persists and promotes malignant progression of epithelial tumors of the anogenital tract. In the course of persisting infections with high-risk HPV types 16 or 18 the normal cell differentiation of the *cervix uteri* is gradually lost, resulting in cervical intraepithelial neoplasia. In 10-30% of these cases an invasive cervical carcinoma develops, worldwide the second most common cancer in women (Einstein and Goldberg 2002). In most carcinomas the HPV DNA is integrated into the host genome, which normally leads to the loss of the E2-ORF and the E2-dependent repression of E6 and E7 expression, thereby promoting carcinogenesis (Turek 1994; Wells *et al.* 2000; Arias-Pulido *et al.* 2006; Gammoh *et al.* 2006). In 1995 the World Health Organization (WHO) declared HPV as the causing factor for cervical cancer, because DNA of mucosal high-risk HPV types could be detected in almost 100% of all cervical cancers (Walboomers *et al*. 1999). Mainly DNA of the high-risk HPV types 16 (44-68%), 18 (10-14%), 31 and 45 (2-8%) was detected in carcinomas (Munoz 2000; Clifford *et al*. 2003). Since 2006 vaccines are available on the basis of virus like particles (VLP), which consist of the major capsid protein L1. Two vaccines are licensed in the EU, a quadrivalent vaccine including L1 VLPs from HPV6, 11, 16 and 18 (Gardasil) and a bivalent vaccine containing L1 VLPs from HPV16 and 18 (Cervarix), which protect from an infection with the respective HPV types.

1.6 Role of HPV in non-melanoma 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. The incidence of NMSC has epidemically increased and now represents approximately 30% of all cancers (DePinho 2000; Alam and Ratner 2001; Pfister 2003). As ultraviolet (UV) radiation is the main risk factor, NMSC emerges primarily on sun-exposed skin areas (Leiter and Garbe 2008). However, other factors than solar exposure, such as genetic background, immunological status and the presence of selected viral infections, may also be involved in determining the induction of NMSC (Stern 1999; Corona *et al*. 2001). Infections with beta-HPV are acquired early in infancy and most people probably carry persistent infections with multiple types (Antonsson *et al.* 2003; Weissenborn *et al.* 2009). A linkage between HPV and the development of human skin cancer is accepted for the rare, autosomal recessive, genetic disorder EV and the so called EV-HPV, today phylogenetically classified as beta-HPV (Majewski *et al.* 1997; Pfister and Ter Schegget 1997; de Villiers *et al.* 2004). The clinical picture of EV, first described in 1922 by Lewandowsky and Lutz, 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). EV-patients are somehow not able to control the HPV infection in the keratinocytes (Majewski *et al.* 1997). The tumors grow locally invasive, but are not metastatic. In contrast to cervical carcinoma the HPV genome is hardly ever integrated into the cellular genome in cutaneous SCC (Orth 2006). A genetic predisposition for EV could be assigned to DNA-loci on the long arm of chromosome 17 and chromosome 2 (Ramoz *et al*. 2000). A mutation in one of the two genes EVER1 and EVER2 located on chromosome 17 is responsible for this condition (Ramoz *et al*. 2002). Although the precise function of these genes is not yet fully understood, they play an important role in regulating the distribution of zinc in the cell nucleus. It could be shown that zinc plays an essential role as cofactor for viral proteins, which is retained by the EVER1/EVER2 complex thereby inhibiting viral growth (Lazarczyk and Favre 2008; Lazarczyk *et al*. 2008). While about 20 different HPV types have been found in benign tumors of EV-patients, HPV5 or 8 were found in 90% of SCC (Pfister 2003), which are therefore considered high-risk types. Furthermore, these beta-HPV-types were found in high DNA copy numbers in SCC of EV-patients (Pfister 2003), and are

actively transcribed (Orth 2005). Although these HPV types were also found in the general population, the DNA loads are very low in the skin and in hair bulbs, which represent their probable reservoir (Pfister 2003; Weissenborn *et al.* 2009). Remarkably, the prevalence of HPV DNA was in SCC lower than in premalignant actinic keratosis (AK) suggesting an association between EV-HPV and the early steps of skin carcinogenesis (Pfister 2003). A high EV-HPV prevalence was also detected in NMSC of immunosuppressed organ transplant recipients. An etiological role for HPV in the development of NMSC in the genral population is a matter of debate, because of the high prevalence of low-level infections in the general population, the absence of high-risk HPV types and the low HPV DNA copy numbers in SCC indicating that not every tumor cell contains an HPV genome (reviewed in Pfister, 2003). However, seroepidemiological data and the extremely high prevalence of EV-HPV DNA in AK substantiated the involvement of HPV in the early development of NMSC of the general population. Furthermore, case-control studies showed that seropositivity, especially against HPV8, constitutes a significant risk for a NMSC diagnose (Feltkamp *et al.* 2003; Masini *et al.* 2003), demonstrating that HPV8 could also be a high-risk type in the general population. Moreover NMSC of immunesuppressed patients revealed a high prevalence of EV-HPV pointing to an important role of the immune system in the prevention of HPV-mediated skin cancer (Berkhout *et al*. 2000; Harwood *et al*. 2000; Pfister 2003).

1.7 HPV transgenic mice

HPV8 transgenic mouse lineages were established at our institute expressing all early genes of HPV8 (E1/E2/E4/E6/E7, HPV8-CER) (Schaper *et al.* 2005) or only E2 (Pfefferle *et al.* 2008) or E6 (Marcuzzi *et al.* 2009). The genetic background of these mice is FVB/N. 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. Almost all HPV8-CER and -E6 mice spontaneously develop papillomas, dysplasias and in 6% SCC. Tumors developed mostly dorsal caudal and earlier in male mice. Therefore, it is speculated that most of the tumors elicited from position fight wounds. UVA/B-irradiation or wounding by taking punch biopsies led to a synchronized

induction of papilloma development within about three weeks in these mice, while the skin of FVB/N wild type (wt) mice healed completely within this time (Marcuzzi 2006). In summary, HPV8-E6 mice perfectly mirror the HPV8-CER phenotype, including rate and prevalence of papillomatosis. The HPV8-E2 mouse strain used in this study showed spontaneous papilloma development only in 8% and UV-induced papillomatosis in 87% of the animals. SCC were detected in 2% in these mice, which showed an abnormally high amount of spindle cells (Pfefferle *et al.* 2008). This generally rare histology is characteristic for very aggressive carcinomas of immunosuppressed organ transplant recipients (Harwood *et al.* 2006). Transgenic CBA/C57B1 mice expressing the early region of HPV1, which causes benign warts, under the control of the K6-promoter showed only transient hyperproliferation (Tinsley *et al.* 1992). FVB/N mice expressing the oncoproteins E6 and E7 of HPV38 under the control of the bovine homologue of the hK10-promoter did not develop spontaneous papillomas during their life of 2 to 2.5 years. Seven to eight weeks after treatment with DMBA and TPA, the classical two step carcinogenesis protocol, these animals developed several papillomas and three of eight mice developed SCC (Dong *et al.* 2005). Moreover hairless SKH-hr1 mice, expressing the oncoproteins E6 and E7 of HPV20 under the control of the K10-promoter, did not develop spontaneous tumors during the observation period of two years (Michel *et al.* 2006). The animals were then irradiated three times a week with UVB (90 to 200 mJ/cm^2) beginning at an age of six weeks and ending at an age of 15 weeks. A slightly higher tendency for papillomatosis was observed in the transgenic animals compared to the control animals. In non-transgenic mice more papilloma regressed and the regression started at an earlier point in time. Altogether two SCCs were detected in HPV20-transgenic mice.

1.8 RNA interference

1.8.1 History of RNA interference

RNA interference (RNAi) was first observed by plant biologists in the late 1980, however, its molecular mechanisms remained unclear until Fire *et al.* (1998) showed in the nematode *Caenorhabditis elegans* that RNAi is an evolutionarily conserved gene-silencing mechanism. Two types of small RNA molecules, microRNA (miRNA) and small interfering RNA (siRNA), are central to RNAi. This sequence-specific posttranscriptional gene-silencing by double-stranded RNA is conserved among different organisms including plants, animals and viruses (Lagos-Quintana *et al.* 2001; Lau *et al.* 2001; Lee and Ambros 2001; Berezikov *et al.* 2006; Ruby *et al.* 2006). Besides regulating the gene expression, this process also plays an important role in the defense against viruses and the mobilization of transposons (Tijsterman *et al.* 2002).

1.8.2 MicroRNA-biogenesis and silencing mechanism

MiRNAs are small (~ 22 nt), noncoding RNA molecules regulating the gene expression (Ambros 2003). Most miRNAs arise from long primary transcripts (pri-miRNA) generated by Pol II polymerase (Figure 3) (Cai *et al.* 2004; Lee *et al.* 2004; Borchert *et al.* 2006). Pri-miRNAs form a stable stem loop, which is processed by the RNase III enzyme Drosha into ~70 nt long hairpin precursor miRNAs (pre-miRNA) (Lee *et al.* 2003; Han *et al.* 2004). Exportin 5 translocates the pre-miRNA into the cytoplasm, where the pre-miRNA is cleaved by the RNase III enzyme Dicer into the mature ~22 nt miRNA:miRNA* duplex (Bohnsack *et al.* 2004; Lund *et al.* 2004; Forstemann *et al.* 2005; Saito *et al.* 2005). Afterwards one of the two miRNA strands is incorporated into the RNA-induced silencing complex (RISC), among others composed of Argonaute proteins and Dicer (Gregory *et al.* 2005; Maniataki and Mourelatos 2005). The incorporated miRNA guides RISC to its target RNA by base pairing interactions. Depending on the degree of sequence complementarity between miRNA and target mRNA, the target mRNA is cleaved or translationally silenced. Imperfect binding leads to inhibition of translation and complete binding to cleavage

of the target mRNA (Ambros 2004; Bartel 2004; Diederichs and Haber 2007). Most miRNA targets are translationally repressed, only one endogenous animal miRNA target (HOXB8) has been reported to be cleaved (Yekta *et al.* 2004). Translationally repressed mRNAs are not actively degraded but can be destabilized due to deadenylation and subsequent decapping (Behm-Ansmant *et al.* 2006). The mechanism of translational repression by miRNAs is unclear. There is evidence that miRNAs block translation initiation but also that miRNAs block translation elongation (Maroney *et al.* 2006; Petersen *et al.* 2006). The complex of Argonaute proteins, miRNA and target mRNA accumulates in processing bodies (P-bodies), known cytoplasmic foci for mRNA degradation (Liu *et al.* 2005; Pillai *et al.* 2005). Several proteins in P-bodies can interact with Argonaute proteins, thereby inhibiting the translation (Liu *et al.* 2005; Behm-Ansmant *et al.* 2006). Although gene silencing typically takes place in the cytoplasm, it has also been reported to occur within the nucleus of human cells. In this case miRNAs induce transcriptional gene silencing through *de novo* DNA methylation of the promoter region or histone modification (Bayne and Allshire 2005; Tan *et al.* 2009). Furthermore, it has been reported that miRNAs can act as cis-regulators to modulate gene expression (Kim *et al.* 2008).

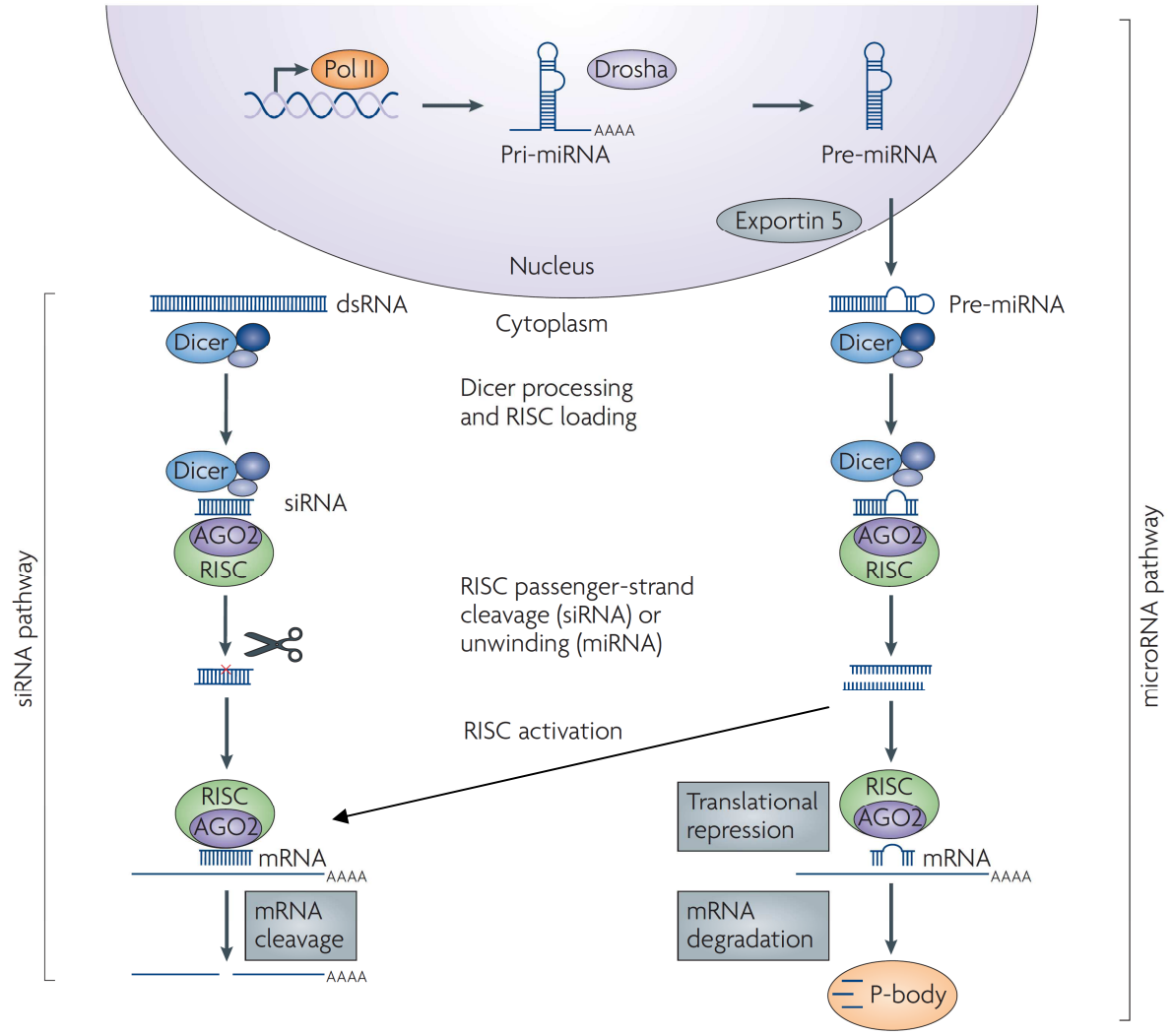


Figure 3: Mechanism of RNAi.
RNAi pathways are guided by small RNAs that include siRNAs and miRNAs. The diagram describes the siRNA pathway (left side) and the miRNA pathway (right side) (modified from de Fourgerolles *et al.* 2007). The miRNA pathway is described in detail below. The pri-miRNA generated by Pol II or Pol III polymerase can contain several miRNA sequences and are up to several kilobases long. Like mRNAs, Pol II-transcribed pri-miRNAs contain 5' cap structure, are polyadenylated and may be spliced (Bracht *et al.* 2004; Cai *et al.* 2004). MiRNAs are located either within the introns or exons of protein-coding genes (70%) or in intergenic areas (30%) (Rodriguez *et al.* 2004; Kim and Kim 2007). The requirement for the recognition and subsequent processing of the pri-miRNA is the ability to generate a stable stem loop of at least 30 bp. In the nucleus this pri-miRNA stem loop is processed by a protein complex called the Microprocessor of which the core proteins are the RNase III enzyme Drosha and the double-stranded RNA-binding domain (dsRBD) protein DGCR8/Pasha into ~ 70 nt long hairpin pre-miRNA (Lee *et al.* 2003; Han *et al.* 2004). The generated 2 nt 3' overhang is recognized by Exportin 5 which translocates the pre-miRNA into the cytoplasm by a Ran-GTP-dependant mechanism (Bohnsack *et al.* 2004; Lund *et al.* 2004). Next, the pre-miRNA is cleaved by the RNase III enzyme Dicer together with the dsRBD proteins TRBP/Loquacious into the mature ~22 nt miRNA:miRNA* duplex (Forstemann *et al.* 2005; Saito *et al.* 2005). Afterwards TRBP recruits the Argonaute protein Ago2 and together with Dicer they build a trimeric complex that initiates the assembly of RISC (Gregory *et al.* 2005; Maniataki and Mourelatos 2005). The miRNA strand with the thermodynamically lower stability of base pairing at its 5'-end is incorporated into RISC, while the miRNA* is usually degraded (Schwarz *et al.* 2003). The incorporated miRNA guides RISC to its target RNA by base pairing interactions, in this process the complementarity between the nucleotides 2 to 8 from the 5'-end of the guide strand (seed region) and the target mRNA is critical (Parker *et al.* 2006). Depending on the sequence complementarity between miRNA and target mRNA the target mRNA is either translationally repressed or degraded.

1.8.3 MiRNA functions

MiRNAs exhibit highly conserved sequences among different organisms indicating that they are under strong evolutionary pressure and have functions in essential processes (Pasquinelli *et al*. 2000; Ambros 2004). In fact, they play an important role in embryogenesis and cell differentiation but also by regulating the cell cycle and apoptosis (Harfe 2005; Carleton *et al*. 2007). The expression is strongly cell- and tissue-dependent (Zhao and Srivastava 2007). For the human genome 721 miRNA sequences are listed in the Sanger mirBase: release 14.0, which are predicted to regulate about 30 % of all human genes (Lewis *et al*. 2005). Half of these human miRNAs are located inside or near fragile sites of the chromosomes and in regions, which are associated with cancer (Calin *et al*. 2004), suggesting that miRNA dysregulations have substantial impact on carcinogenic processes. Several studies already reported that miRNAs are involved in the primary steps of carcinogenesis and its progression (Calin *et al*. 2002; Croce and Calin 2005; Iorio *et al*. 2005; Volinia *et al*. 2006). Some of these miRNAs are known to target oncogenes or tumor suppressors (Table 1). Interestingly, in preliminary experiments the HPV8-CER mouse also showed deregulated miRNA expressions (Reinartz 2007).

MicroRNA	Confirmed targets	Reference
miRNA-let-7a	RAS, c-myc	(Johnson *et al.* 2005; Sampson *et al.* 2007)
miRNA-15a **miRNA-16-1**	Bcl-2	(Cimmino *et al.* 2005)
miRNA-17-92 cluster	E2F1, Bim, PTEN	(reviewed in Garzon *et al.* 2009)
miRNA-21	PTEN, PDCD4, TPM1	(Meng *et al.* 2007; Zhu *et al.* 2007; Frankel *et al.* 2008)
miRNA-34a,b,c	CDK4, CDK6, cyclinE2, E2F3	(reviewed in Garzon *et al.* 2009)
miRNA-106a	Rb	(Volinia *et al.* 2006)
miRNA-155	KGF	(Pottier *et al.* 2009)

miRNA-203	ΔNp63,	(Yi *et al.* 2008)
miRNA-206	MET	(Yan *et al.* 2009)
miRNA-218	LAMB3	(Martinez *et al.* 2008)
miRNA-372,373	LATS2	(Voorhoeve *et al.* 2007)

Table 1: MiRNAs and their respective known targets.

1.8.4 Small interfering RNA biogenesis and silencing mechanism

Endogenous siRNAs derive from double stranded RNAs (dsRNAs) produced by transposable elements, complementary annealed transcripts or replicating viruses. Similar to the miRNA biogenesis the dsRNAs are processed by Dicer into siRNA duplexes of 19-25 bp (Figure 3) (Bernstein *et al.* 2001). These duplexes are incorporated into RISC, whereupon an ATP-dependent helicase unwinds the duplex, enabling one of the two strands to independently identify its target mRNA (Kisielow *et al.* 2002). In contrast to miRNAs, siRNAs bind their target mRNA with perfect complementarity inducing RISC to cleave the mRNA strand between the nucleotides that are complementary to nucleotides 10 and 11 of the guide strand relative to the 5′-end (Caudy *et al.* 2003). The cleavage products are released and degraded. This mechanism of gene regulation has provided a powerful new tool for biological research and drug discovery by using synthetic siRNA duplexes.

1.9 Aim of the study

The transgenic mouse line HPV8-CER, expressing all early genes of HPV8, shows spontaneous papilloma development, predominantly on scratched skin areas. Furthermore a single UVA/B-dose leads to papilloma development within three weeks in these mice, while the skin of FVB/N wt mice heals completely within this time.

In order to clarify the role of skin wounding and its influence on HPV expression in the early stages of tumor development in this mouse model, the kinetics and levels of transgene expression were analyzed in response to exogenous skin irritations, including UVA/B-irradiation and mechanical wounding. To narrow down the time period during which the HPV8-E6 oncogene expression is necessary for transformation in HPV8-CER mice, HPV8-E6 specific siRNA was topically applied on mouse skin. Based on the initial observation, that HPV8-CER mice showed deregulated expressions of cellular miRNAs (Reinartz 2007), the expression kinetics of selected miRNAs and their respective targets were determined after UVA/B-irradiation, to check for a correlation with HPV oncogene expression.

2 Material

2.1 Bacterial strain

- ***Epicurian coli* ® XL1-Blue**
  genotype: recA1, end A1, gyr96, thi-1, hsdR17, supE44, relA1, lac[F'proAB lacqZΔAM15 Tn10(Tetr)] (Stratagene, La Jolla, USA)

2.2 Eukaryotic cells

- **HaCaT**
  A spontaneously immortalized, aneuploid, human, keratinocyte cell line derived from human skin, which kept the characteristics of differentiation. It shows a transforming phenotype, but is not tumorigenic. Contains mutated p53 (Boukamp *et al.* 1988).
- **HaCaT-pLXSN8-E6**
  HaCaT cell line retrovirally transduced with pLXSN8-E6, expresses HPV8-E6 (present work)
- **PM1**
  A premalignant keratinocyte cell line, contains wt p53
- **PM1-pLXSN**
  PM1 cell line, retrovirally transduced with the empty vector pLXSN (present work)
- **PM1-pLXSN8-CER**
  PM1 cell line, retrovirally transduced with pLXSN8-CER, expresses the early genes of HPV8 (present work)

2.3 Nucleic acids

2.3.1 Synthetic oligonucleotides

The HPLC purified oligonucleotides were ordered from TIB MOLBIOL (Berlin), except for the oligo-dT_{23}-primer, which was purchased from Sigma-Aldrich (Steinheim). Numbers define the position of the oligonucleotides in the respective gene.

2.3.1.1 Oligonucleotides for quantitative real-time PCR (mRNA)

Name	Sequence 5'→3'	Position
β-actin (human) forward	TCCCTGGAGAAGAGCTACGA	787 - 806 (NM_001101)[1]
β-actin (human) forward	AGCACTGTGTTGGCGTACAG	961 - 980 (NM_001101)[1]
β-actin (murine) forward	CCAGAGCAAGAGAGGTATCCTGAC T_{ann} 55℃	253 - 276 (NM_007393)[1]
β-actin (murine) reverse	CATTGTAGAAGGTGTGGTGCCAG T_{ann} 55℃	334 - 356 (NM_007393)[1]
B2m (murine) forward	ATTCACCCCCACTGAGACTG T_{ann} 50℃	318 - 337 (NM_009735)[1] RTPrimerDB[2] ID: 2866
B2m (murine) reverse	TGCTATTTCTTTCTGCGTGC T_{ann} 50℃	491 - 510 (NM_009735)[1] RTPrimerDB[2] ID: 2866
HPRT1 (human) forward	TGACACTGGCAAAACAATGCA T_{ann} 55℃	577 - 598 (NM_000194)[1] RTPrimerDB[2] ID: 5
HPRT1 (human) reverse	GGTCCTTTTCACCAGCAAGCT T_{ann} 55℃	650 - 671 (NM_000194)[1] RTPrimerDB[2] ID: 5

HPRT1 (murine) forward	CCTAAGATGAGCGCAAGTTGAA T_{ann} 55℃	798 - 820 (NM_013556) RTPrimerDB[2] ID: 45
HPRT1 (murine) reverse	CCACAGGACTAGAACACCTGCTAA T_{ann} 55℃	860 - 884 (NM_013556)[1] RTPrimerDB[2] ID: 45
HPV8E2 forward	AACAGCCACAACAAACCG T_{ann} 50℃	3410 - 3427 (M12737)[1]
HPV8E2 reverse	AGGACCTGGACCTGGATACG T_{ann} 50℃	3567 - 3586 (M12737)[1]
HPV8E6 forward	GCAACGTTTGAATTTA T_{ann} 50℃	433 - 448 (M12737)[1]
HPV8E6 reverse	CATGATACAAATGCTTAC T_{ann} 50℃	638 - 655 (M12737)[1]
HPV8E7 forward	CCTGAAGTGTTACCAGTTGACCTGC T_{ann} 50℃	710 - 734 (M12737)[1]
HPV8E7 reverse	CAGTTGCGTTGACAAAAAGACG T_{ann} 50℃	848 - 869 (M12737)[1]
K14 (murine) forward	TCCAGAGATGTGACCTCC T_{ann} 50℃	1403 - 1420 (NM_016958)
K14 (murine) reverse	CCACCTTGCCATCGT T_{ann} 50℃	1458 - 1472 (NM_016958)[1]

[1] Accession number, [2] primer ID from the primer databas RTPrimerDB

2.3.1.2 Oligonucleotides for quantitative real-time PCR (miRNA)

Name	Sequence 5'→3'	Position
mmu-let-7a	TGAGGTAGTAGGTTGTATAGT	MIMAT0000521[1]
mmu-miR-7	TGGAAGACTAGTGATTTTGTT	MIMAT0000677[1]
mmu-miR-15a	TAGCAGCACATAATGGTTTG	MIMAT0000526[1]
mmu-miR-16	TAGCAGCACGTAAATATTGG	MIMAT0000527[1]

mmu-miR-17-5p	CAAAGTGCTTACAGTGCAGGTAG	MIMAT0000649[1]
mmu-miR-19a/b	TGTGCAAATCYATGCAAAACT	MIMAT0000651/[1] MIMAT0000513[1]
mmu-miR-20b	CAAAGTGCTCATAGTGCAGGTA	MIMAT0003187[1]
mmu-miR-21	TAGCTTATCAGACTGATGTTGA	MIMAT0000530[1]
mmu-miR-34a	TGGCAGTGTCTTAGCTGGTT	MIMAT0000542[1]
mmu-miR-92	TATTGCACTTGTCCCG	MIMAT0000539[1]
mmu-miR-106a	CAAAGTGCTAACAGTGCAGGTA	MIMAT0000385[1]
mmu-miR-127	TCGGATCCGTCTGAGCTT	MIMAT0000139[1]
mmu-miR-133a*	GCTGGTAAAATGGAACCAA	MIMAT0003473[1]
mmu-miR-133a/b	TTGGTCCCCTTCAACCAGC	MIMAT0000145/[1] MIMAT0000769[1]
mmu-miR-145	AATGGCGCCACTAGGGTTGTGCA	MIMAT0000157[1]
mmu-miR-155	TTAATGCTAATTGTGATAGG	MIMAT0000165[1]
mmu-miR-200a/141	TAACACTGTCTGGTAACGATG	MIMAT0000519/[1] MIMAT0000153[1]
mmu-miR-206	TGGAATGTAAGGAAGTGTGTGG	MIMAT0000239[1]
mmu-miR-207	GCTTCTCCTGGCTCTCCTCCCTC	MIMAT0000240[1]
mmu-miR-218	TTGTGCTTGATCTAACCATGT	MIMAT0000663[1]
mmu-miR-224	TAAGTCACTAGTGGTTCCGTTTA	MIMAT0000671[1]
mmu-miR-290	CTCAAACTATGGGGGCACTTTTT	MIMAT0000366[1]
mmu-miR-346	TGTCTGCCCGAGTGCCTGCCTCT	MIMAT0000597[1]
mmu-miR-347-5p	ATATAATACAACCTGCTAAGT	MIMAT0003727[1]
mmu-miR-467a	ATATACATACACACACCTACAC	MIMAT0003409[1]
mmu-miR-467b	ATATACATACACACACCAACAC	MIMAT0005448[1]
mmu-miR-468	TATGACTGATGTGCGTGTGTCTG	MIMAT0002109[1]
mmu-miR-652	AATGGCGCCACTAGGGTTGTGCA	MIMAT0003711[1]
mmu-miR-680	GGGCATCTGCTGACATGGGGG	MIMAT0003457[1]
mmu-miR-698	CGTCCCCGCTCGGCGGGGTCC	MIMAT0003488[1]
mmu-miR-721	CAGTGCAATTAAAAGGGGGAA	MIMAT0003515[1]
miRNA reverse	GCGAGCACAGAATTAATACGAC adapted from Shi et al. (2005)	

[1] Accession number, T_{ann} was for all oligonucleotides 60℃

2.3.1.3 Oligonucleotides for sequencing

Name	Sequence 5'→3'	Position
pJETseq	GGAGCAGGTTCCATTCATTG	387 – 406 in pJET1/blunt

2.3.1.4 Oligonucleotides for reverse transcription

Name	Sequence 5'→3'
Oligo-dT$_{23}$-Primer	$(T)_{23}V$
Random Nonamers	$(N)_9$
miRNA adaptor	GCGAGCACAGAATTAATACGACTCACTATAGG$(T)_{12}$VN adapted from Shi and Chiang (2005)

V = A, C, G; N = A, C, G, T

2.3.1.5 Oligonucleotides for in situ hybridization probes

Name	Sequence 5'→3'
as-mmu-miR-21	+TC+AA+CA+TC+AG+TC+TG+AT+AA+GC+TA
as-mmu-miR-106a	+CT+AC+CT+GC+AC+TG+TT+AG+CA+CT+TT+G
as-mmu-miR-155	+ACC+CCT+ATC+ACA+ATT+AGC+ATT+AA
as-mmu-miR-206	+CCA+CAC+ACT+TCC+TTA+CAT+TCC+A

+ = locked nucleic acid (LNA)

2.3.2 siRNA duplex

The HPLC purified siRNA duplex were either ordered from Qiagen (Hilden) or Biospring (Frankfurt am Main). Numbers define the position of the siRNA in the HPV8 genome (M12737)

Name	Sequence 5'→3'	Target
siRNA-HPV8E6-1 sense	CCGCAACGUUUGAAUUUAAdTdT	HPV8-E6 431-449

siRNA-HPV8E6-1 antisense	UUAAAUUCAAACGUUGCGGdTdT	
siRNA-HPV8E6-2 sense	GGAGUUUGCAGGCUUUGUAdTdT	HPV8-E6 622-640
siRNA-HPV8E6-2 antisense	UACAAAGCCUGCAAACUCCdTdT	
siRNA-HPV8E6-3 sense	AGGCAGAGAUAUUGAAUUAdTdT	HPV8-E6 474-492
siRNA-HPV8E6-3 antisense	UAAUUCAAUAUCUCUGCCUdAdG	
siRNA-HPV8E6-4 sense	GGAUAUCAUAGAGAAAUUAdTdT	HPV8-E6 552-570
siRNA-HPV8E6-4 antisense	UAAUUUCUCUAUGAUAUCCdAdA	
siRNA-control-fluorescein sense	UUCUCCGAACGUGUCACGUdTdT	non targeting, linked with a fluorescein molecule
siRNA-control-fluorescein antisense	UUCUCCGAACGUGUCACGUdTdT	

2.3.3 Cloning vectors

- **pJET1/Blunt** (3,1 kb) blunt-end cloning vector from GeneJET™ PCR Cloning Kit (Fermentas, St. Leon-Rot). PCR products were ligated into this vector to prepare serial dilutions to generate a standard curve in qPCR experiments.

2.3.4 Retroviral expression vectors

- **pLXSN** (5.9Kb) a retroviral expression vector which can be transfected into a packaging cell line. These cells secrete recombinant retroviruses into the supernatant, which can be used to transduce cells. The gene of interest is expressed under the control of the 5'-LTR promoter. Contains neomycin resistance gene (Clontech, Heidelberg).

2.3.5 Recombinant plasmids

- **pJET1-hHPRT1** (3,2 kb) human HPRT1 PCR product cloned into pJET1/Blunt to prepare serial dilutions to generate a standard curve in qPCR experiments (present work).
- **pJET1-mHPRT1** (3,2 kb) murine HPRT1 PCR product cloned into pJET1/Blunt to prepare serial dilutions to generate a standard curve in qPCR experiments (present work).
- **pJET1-hβ-actin** (3,3 kb) human β-actin PCR product cloned into pJET1/Blunt to prepare serial dilutions to generate a standard curve in qPCR experiments (present work).
- **pJET1-mβ-actin** (3,2 kb) murine β-actin PCR product cloned into pJET1/Blunt to prepare serial dilutions to generate a standard curve in qPCR experiments (present work).
- **pJET1-mK14** (3,2 kb) murine K14 PCR product cloned into pJET1/Blunt to prepare serial dilutions to generate a standard curve in qPCR experiments (present work).
- **pLXSN-8E6** (6,4 kb) the early gene E6 of HPV8 was amplified by PCR and cloned into *Eco*RI/*Bam*HI-digested retroviral vector pLXSN. Recombinant retroviruses can be produced when transfected into pT67 cells (Akgül *et al.* 2007).
- **pLXSN-CER** (11 kb) the complete early coding region of HPV8 (nt. 1 - 5111) was cloned by inserting the *Hpa*I/*Bam*HI fragment of HPV8 into the retroviral vector pLXSN. Recombinant retroviruses can be produced when transfected into pT67 cells (Akgül *et al.* 2007).

2.3.6 DNA markers and loading dyes

- 6X DNA Loading Dye (Fermentas, St. Leon-Rot)
- 6X Orange DNA Loading Dye (Fermentas, St. Leon-Rot)
- GeneRuler™ 100bp DNA ladder (Fermentas, St. Leon-Rot)
- GeneRuler™ 1kb DNA ladder (Fermentas, St. Leon-Rot)
- GeneRuler™ DNA Ladder, Low Range (Fermentas, St. Leon-Rot)

2.3.7 Miscellaneous nucleic acids

- Adenosine 5'-triphosphate (Fermentas, St. Leon-Rot)
- Deoxynucleotide triphosphates (Fermentas, St. Leon-Rot)
- Single stranded salmon sperm DNA (Sigma-Aldrich, Steinheim)

2.4 DNA preparation

- DIG Oligonucleotide 3´-End Labeling Kit" (Roche Diagnostics, Mannheim)
- DirectPCR Lysis Reagent (Tail) (PEQLAB, Erlangen)
- GeneJET™ Plasmid Miniprep Kit (Fermentas, St. Leon-Rot)
- GeneJET™ PCR Cloning Kit (Fermentas, St. Leon-Rot)
- PureLink™ HiPure Plasmid Filter Maxiprep Kit (Invitrogen, Karlsruhe)
- QIAprep 8 Miniprep Kit (Qiagen, Hilden)
- QIAquick Gel Extraction Kit (Qiagen, Hilden)
- QIAquick PCR Purification Kit (Qiagen, Hilden)
- QIAprep Spin Miniprep Kit (Qiagen, Hilden)

2.5 Transfection reagents

- HiPerFect Transfection Reagent (Qiagen, Hilden)
- In vivo-jetPEI (PEQLAB, Erlangen)
- Lipofectamine 2000 (Invitrogen, Karlsruhe)
- MIKA-Nuclehicle-Kit (MIKA Pharma GmbH, Bad Oeyenhausen)

2.6 RNA preparation

- DNase I, RNase-free (Fermentas, St. Leon-Rot)
- miRNeasy Mini Kit (Qiagen, Hilden)

- Omniscript RT Kit (Qiagen, Hilden)
- peqGOLD TriFast (PEQLAB, Erlangen)
- Poly(A) Polymerase (Ambion, Austin, USA)
- QIAshredder (Qiagen, Hilden)
- RiboLock™ RNase Inhibitor (Fermentas, St. Leon-Rot)
- RNase-Free DNase Set (Qiagen, Hilden)

2.7 Proteins

2.7.1 Enzymes

Restriction enzymes were purchased from the following companies:
New England BioLabs (Frankfurt am Main) and Fermentas (St. Leon-Rot)

- Calf Intestine Alkaline Phosohatase (CIAP) (Fermentas, St. Leon-Rot)
- Platinum® *Taq* DNA Polymerase (Invitrogen, Karlsruhe)
- Proteinase K (PEQLAB, Erlangen)
- T4-DNA Ligase (Fermentas, St. Leon-Rot)
- *Taq* DNA Polymerase (Fermentas, St. Leon-Rot)

2.7.2 Antibodies

- **α-BIM (Ab-65):** a polyclonal rabbit antibody produced against synthesized non-phosphopeptide derived from mouse BIM around the phosphorylation site of serine 65 (P-A-S^{p}-P-G), detects human, mouse and rat BIM
  (Source BioScience AUTOGEN, Calne, UK)
- **anti-digoxigenin-POD, Fab fragments:** Fab fragments of an anti-digoxigenin antibody from sheep, conjugated with POD, detects digoxigenin
  (Roche Diagnostics, Mannheim)
- **α-KGF (C-19):** a goat polyclonal antibody raised against a peptide mapping at the C-terminus of KGF of human origin, detects precursor and mature KGF and, to a lesser extent, FGF-10 of human, mouse and rat origin
  (Santa Cruz Biotechnology, Santa Cruz, USA)

- **α-guinea-pig-POD:** POD conjugated antibody, detects IgG from guinea pig
  (personal communication: Janet Brandsma)
- **α-HPV8-E2:** a guinea pig antibody raised against a 6x-His-tagged HPV8-E2 protein, generated in bacteria and purified on a nickel column
  (personal communication: Janet Brandsma)
- **α-MET (Ab-1003):** a polyclonal rabbit antibody produced against synthesized non-phosphopeptide derived from human MET around the phosphorylation site of tyrosine 1003 (V-D-Y^p-R-A), detects human, mouse and rat MET
  (Source BioScience AUTOGEN, Calne, UK)
- **α-PDCD4 (EPR3432):** a monoclonal rabbit antibody produced against a synthetic peptide corresponding to residues near the C-terminus of human PDCD4, detects human and mouse PDCD4
  (Epitomics, Burlingame, USA)
- **α-PTEN (Ab-370):** a polyclonal rabbit antibody produced against synthesized non-phosphopeptide derived from human PTEN around the phosphorylation site of serine 370 (D-V-S^p-D-N), detects human, mouse and rat PTEN
  (Source BioScience AUTOGEN, Calne, UK)
- **α-Rb (C-15):** a rabbit polyclonal antibody raised against a peptide mapping at the C-terminus of Rb of human origin, detects human, mouse and rat Rb p110
  (Santa Cruz Biotechnology, Santa Cruz, USA)

2.8 Staining reagents

- AEC (Sigma-Aldrich, Steinheim)
- DAPI (Merck, Darmstadt)
- DePeX (Serva Electrophoresis, Heidelberg)
- Eosin Y solution (AppliChem GmbH, Darmstadt)
- Hematoxylin solution (Sigma-Aldrich, Steinheim)
- Kaiser´s glycerol gelatine (Merck, Darmstadt)
- Liquid DAB Concentrated Substrate Pack (BioGenex, San Ramon, USA)
- TSA Biotin System (PerkinElmer, Waltham, USA)
- VECTASTAIN Elite ABC Kit (Universal) (Vector Laboratories, Burlingame, USA)
- VECTASTAIN Elite ABC Kit (Goat IgG) (Vector Laboratories, Burlingame, USA)

2.9 Buffers and solutions

- **AEC stock solution**

Dimethylformamide	5 ml
AEC	1 pellet

- **AEC working solution**

50 mM Na-acetat buffer (pH 5,2)	1ml
AEC stock solution	67 µl
H_2O_2 30 %	2µl

- **Ethidium bromide bath**

Ethidium bromide	0,5 µg/ml in H_2O

- **Sodium phosphate buffer**

NaH_2PO_4	0,5 M
Na_2HPO_4	0,5 M

- **PBS (Phosphate Buffered Saline)**

NaCl	137 mM
KCl	2,7 mM
Na_2HPO_4	4,3 mM
KH_2PO_4	1,4 mM

- **Prehybridization-mix**

Formamide	500 µl
20 X SSC	100 µl
Sodium phosphate buffer	100 µl
10 % SDS	50 µl
0,5 M EDTA	2 µl
Salmon sperm DNA	10 µg
H_2O	245 µl

- **20x SSC (pH 7,0)**

NaCl	3 M
Na-Citrate	0,3 M
H_2O	add 1 l

- **50x TAE buffer (pH 8,0)**

Tris-Acetat	2 M
EDTA	50 mM

- **TE-buffer (pH 8,0)**

Tris	10 mM
EDTA	1 mM

2.10 Chemicals

All chemicals used in this study were purchased from Applichem (Darmstadt), Invitrogen (Karlsruhe), Merck (Darmstadt), Roth (Karlsruhe) and Sigma-Aldrich (Steinheim) and complied the reagent grade “p.a.”.

- Acetone (Roth, Karlsruhe)
- Agarose (Sigma-Aldrich, Steinheim)
- Ampicillin (Sigma-Aldrich, Steinheim)
- β-Mercaptoethanol (AppliChem, Darmstadt)
- Chloroform (Roth, Karlsruhe)
- Dimethylformamide (Fluka, Buchs, Switzerland)
- DMSO (AppliChem GmbH,Darmstadt)
- Ethidium bromide (Roth, Karlsruhe)
- Fetal calf serum (FCS) (Invitrogen, Karlsruhe)
- Formamide (Fluka, Buchs, Switzerland)
- Formaldehyd (Merck AG, Darmstadt)
- Glucose (Roth, Karlsruhe)
- Glycin (Roth, Karlsruhe)
- Glycogen (Sigma-Aldrich, Steinheim)
- Isoamylalkohol (Roth, Karlsruhe)
- Mixer Mill 301 (Retsch, Haan)
- Polyethylene glycol (Sigma-Aldrich, Steinheim)
- Pepsin (Sigma-Aldrich, Steinheim)
- Phenol (Roth, Karlsruhe)
- RNALater (Qiagen, Hilden)
- Sybr Green I (Sigma-Aldrich, Steinheim)

Plastic goods were purchased from Eppendorf (Hamburg), Falcon BD (Heidelberg), Greiner (Solingen), Sarstedt (Nümbrecht) and TPP (Trasadingen, Switzerland)

2.11 Media

2.11.1 Media for cultivation of bacteria

- **LB-medium** (Luria-Bertani-medium) pH 7,2 (Difco, Detroit, USA)

Bacto tryptone	10 g/l
Bacto yeast-extract	5 g/l
NaCl	8 g/l
H_2O	add 1 l

autoclaved for 20 min at 121°C

Medium was cooled down to 50°C before ampicillin was added in a final concentration of 100 µg/ml (optional)

- **LB Agar** pH 7,2 (Difco, Detroit, USA)

Bacto tryptone	10 g/l
Bacto yeast-xtract	5 g/l
NaCl	8 g/l
Bacto agar	15 g/l
H_2O	add 1 l

autoclaved for 20 min at 121°C

Agar was cooled down to 50°C before ampicillin was added in a final concentration of 100 µg/ml (optional) and poured into petri dishes

- **TSB-medium** (Transformation and Storage Medium)

PEG (MW=3350)	30 mM
DMSO	5 %
$MgCl_2$	10 mM
$MgSO_4$	10 mM
LB-medium	add 1 l

2.11.2 Media for cultivation of eukaryotic cells

- **Dulbecco´s modified Eagle´s medium** (DMEM) (Invitrogen, Karlsruhe)
  with Glutamax™, 4500 mg/l glucose
  without sodium pyruvate

FCS	10 %	(Invitrogen, Karlsruhe)
Penicillin	100 units/ml	(Invitrogen, Karlsruhe)
Streptomycin	100 µg/ml	(Invitrogen, Karlsruhe)

- **Freezing medium**

DMSO	10%	(Invitrogen, Karlsruhe)
FCS	90%	(Invitrogen, Karlsruhe)

- **PBS** (Invitrogen, Karlsruhe)
- **Trypsin/EDTA** (Invitrogen, Karlsruhe)

2.11.3 Antibiotics for cell culture

- **G418-Sulfat**
  An aminoglycoside antibiotic that blocks polypeptide synthesis by inhibiting the elongation step in both prokaryotic and eukaryotic cells. Resistance to G418 is conferred by the neomycin gene. (PAA, Pasching, Austria)

2.12 Miscellaneous

- Axiophot microscope (Zeiss, Göttingen)
- 5-Liner needles (Bortech, Wuppertal)
- Cryotome Leica CM3000 (Leica, Wetzlar)
- Depilatory cream (Balea, Karlsruhe)
- Fluorescence microscope Leica DMI 6000 B (Leica, Wetzlar)
- LightCycler System (Roche Diagnostics, Mannheim)
- Punch biopsy tool (pfm, Köln)

- Steel balls (Retsch, Haan)
- T3-Thermocycler (Biometra, Göttingen)
- Tape (Tesa, Hamburg)
- Tattoo machine Rotary 12000PL (Bortech, Wuppertal)
- Tissue-Tek O.C.T. Compound (Sakura Finetek, Heppenheim)

2.13 Mice

Line	Description	Reference
FVB/N wt	wildtype, breeding	Charles River, Germany, Sulzfeld
FVB/N HPV8-CER	transgenic for the early genes of HPV8	Schaper *et al*., 2005
FVB/N HPV8-E2	transgenic for HPV8-E2	Pfefferle *et al*., 2008
FVB/N HPV8-E6	transgenic for HPV8-E6	Marcuzzi *et al*., 2009

3 Methods

3.1 Bacterial culture

3.1.1 Production of competent bacteria for transformation

Transformation competent *E.coli* XL1 Blue were produced as described in the protocol from Chung and Miller (1988). 100 ml LB-medium was inoculated with 2,5 ml of a fresh bacterial culture and incubated at 37℃ under agitation until an OD_{600} of 0,5 was reached. Bacteria were put on ice for 10 min followed by centrifugation for 10 min at 3000 g at 4℃. The pellet was resuspended in 5 ml ice-cold TSB-medium and put again on ice for 10 min. The now competent bacteria were aliquoted in prechilled tubes, shock frozen in liquid nitrogen and stored at -80℃. The efficiency of the competent bacteria was tested by transforming 100 µl bacteria with 1 ng plasmid. With the help of the number of formed colonies the efficiency of the bacteria could be calculated when transformed with 1 µg DNA. Normally the efficiency was between $5x10^3$ and $3x10^6$ colonies per microgram DNA.

3.1.2 Transformation of competent bacteria

Transformation of competent *E.coli* XL1 bacteria was done by pipetting DNA (ca. 100 ng) to 100 µl bacteria and incubating on ice for 30 min. Afterwards bacteria were subjected to heat shock at 42℃ for 45 seconds, then shifted back on ice for 3 min. Subsequently 100 µl LB-medium with 20 mM glucose was added and after 1 h at 37℃ under agitation, bacteria were plated onto ampicillin LB-agar plates and incubated over night at 37℃.

3.1.3 Culturing bacteria for plasmid isolation

All plasmids in this study contain an ampicillin resistance gene. Therefore selection of bacteria transformed with a plasmid could be accomplished by adding 100 µg/ml

ampicillin to LB-medium and agar plates. For analytic plasmid isolation 5 ml LB-medium was inoculated with bacteria in a 13 ml PE-tube (Sarstedt, Nümbrecht) and was put into a rotation incubator overnight at 37℃ under agitation. For preparative plasmid isolation bacterial cultures were grown in 200 ml LB-medium in an Erlenmeyer flask and were put into a rotation incubator at 37℃ under agitation overnight.

3.1.4 Bacterial glycerol stock

From a dense bacterial culture 800 µl were mixed with 200 µl glycerol and was stored at -80℃.

3.2 Cell culture

All cell lines listed here were adherent cell lines, cultivated at 37℃, 6 % CO_2 and 80-90% humidity with DMEM containing 10 % FCS, 100 units/ml penicillin and 100 µg/ml streptomycin. All operations with these cell lines were done under a laminar flow.

3.2.1 Cultivation of cell lines

Cells were passaged constantly when they reached a density of about 90 % at most, by washing with PBS following Trypsin/EDTA treatment. As soon as the cells detached from the bottom of the flask they were resuspended in fresh medium and seeded at the desired density in a new tissue culture flask.

3.2.2 Freezing of cell lines

For long term storage cells were grown in 150 cm^2 tissue culture flask until they reached a density of about 90%. Then the cells were washed with PBS and detached with 5 ml Trypsin/EDTA and mixed with 9 ml of the appropriate cell culture medium. After centrifugation at 300 g for 5 min the pellet was resuspended in 4,5 ml freezing

medium, aliquoted into 1,5 ml in freezing vials (Nunc, Langenselbold), and stored at -80℃.

3.2.3 Cell counting using a hemocytometer

The cell count of a cell suspension was determined by a hemocytometer with a cover slip attached. The hemocytometer is divided in nine main squares which form a bigger square. It has a depth of 0,1 mm and each main square has an area of 1 mm^2. An aliquot of a well mixed suspension was pipetted to the edge of the cover slip and was sucked under the cover slip due to capillary power. Cells were counted in the four squares of the corners via microscope. The mean value of the four squares multiplied with 10^4 equals the cell count in one ml of the cell suspension.

3.2.4 Transfection of cells with Lipofectamine 2000

SiRNA transfections were done with “Lipofectamine 2000” (Invitrogen, Karlsruhe). For this purpose 3 x 10^4 cells were seeded in a 24-well and incubated overnight at 37℃. The following day cells were washed once with PBS and fresh medium without antibiotics was added. Unless otherwise noted 30 picomole siRNA were added to 50 µl medium without antibiotics and FCS and 1,5 µl “Lipofectamine 2000” was added to another 50 µl medium without antibiotics and FCS. After 5 min incubation at RT the diluted “Lipofectamine 2000” was added to the diluted siRNA and incubated for 20 min at RT. The siRNA-Lipofectamine 2000 complexes were then added to the well containing cells.

3.2.5 Creation of the stable cell lines HaCaT-pLXSN8-E6, PM1-pLXSN and PM1-pLXSN8-CER via transduction

One day before transduction 2 x 10^5 HaCaT and PM1 cells were seeded in a cell culture dish (6 cm). The next day cells were incubated with 2 ml DMEM without additives but with 5 µg/ml polybrene (Sigma-Aldrich, Steinheim) for 10 min at 37℃. Two ml retroviral supernatant pLXSN8-E6, pLXSN or pLXSN8-CER (virus particles

provided by Dr. Baki Akgül) with 2 ml DMEM without additives but with 5 µg/ml polybrene (Sigma) was added to the cell culture dish. The cells were centrifuged at 300 g for 1h at RT, washed twice with PBS and finally incubated with 2 ml normal cultivation media for 48 h at 37℃. Since pLXSN enc odes the neomycin resistance gene, selection of transduced cells was performed by adding G418 (PAA, Pasching, Austria) to the media in a final concentration of 500 µg/ml. As a negative control HaCaT and PM1 cells without transduction were also incubated with media containing G418 (PAA) in a final concentration of 500 µg/ml. After 48 h cells reached a confluence of about 80 % and were splitted in a 75 cm^2 tissue flask. Selection media was exchanged with normal cultivation media when all control cells were dead, usually after one week. Transcription of stably transduced DNA was verified by qRT-PCR (3.3.8).

3.3 DNA methods

3.3.1 DNA standard methods

The methods mentioned below were performed according to the protocols from Sambrook *et al.* (1989)

- DNA digestion by restriction nucleases
- Dephosphorylation of 5‘-end DNA fragments by alkaline phosphatase
- Ligation of DNA by T4-Ligase
- Phenol/Chloroform extraction of DNA
- Ethanol precipitation of DNA

3.3.2 Determination of DNA concentration

DNA concentration and degree of purity was determined spectrophotometrically at a wavelength of 260 nm and 280 nm using a NanoDrop®ND-1000 UV/Vis spectral photometer (PEQLAB, Erlangen).

3.3.3 Plasmid preparation

Plasmid isolation for analytic and preparative was done using kits from Qiagen (Hilden) “QIAprep Spin Miniprep Kit, QIAprep 8 Miniprep Kit”, Invitrogen (Karlsruhe) “PureLink™ HiPure Plasmid Filter Maxiprep Kit” and Fermentas (St. Leon-Rot) “GeneJET™ Plasmid Miniprep Kit” according to the manufacturer’s protocol. These kits base upon the extraction method from Birnboim and Doly (1979) and the property of DNA to bind on anion exchanger columns.

3.3.4 Agarose gel electrophoresis

Agarose gels were prepared by dissolving 0,5 g – 2,5 g of agarose (Sigma-Aldrich, Steinheim) in 100 ml of 1X TAE buffer, boiled and poured into a gel slide. 6x (Orange) DNA Loading Dye (Fermentas, St. Leon-Rot) was added to the DNA samples to a final concentration of 1x. Samples and DNA markers were loaded and electrophoresed in 1X TAE buffer. DNA was visualized by UV light.

3.3.5 Isolation of DNA from agarose gels

Following electrophoretic separation, the desired DNA band was cut out from the agarose gel with a scalpel under long-wave UV (365 nm). DNA was isolated from agarose gel with “QIAEX II Gel Extraction Kit” (Qiagen, Hilden) as described in the manufacturer’s protocol.

3.3.6 Isolation of genomic DNA from mouse tails for genotyping

After marking the mouse ear, 5 mm of the mouse tail was cut off. Genomic DNA was isolated from this piece of tail with the “DirectPCR Lysis Reagent (Tail)” (PEQLAB, Erlangen) and “Proteinase K” (PEQLAB, Erlangen) according to the manufacturer´s protocol. The isolated genomic DNA was tested for the presence of the transgene by

PCR (3.3.7). In HPV8-CER mice the DNA was tested for HPV8-E6. In HPV8-E2 and -E6 mice the DNA was tested for the respective transgene.

3.3.7 Polymerase chain reaction (PCR)

Amplification of defined DNA fragments was done by the PCR method (Mullis and Faloona 1987). The reaction was performed using 50 - 100 ng DNA as template in a total volume of 20 µl containing 1 unit *Taq* DNA Polymerase and the associated buffer with $(NH_4)_2SO_4$ (Fermentas, St. Leon-Rot), 1 mM $MgCl_2$, 5 % DMSO (Applichem, Darmstadt) 0.5 µM forward and backward primer each and 0.2 mM deoxynucleotide triphosphates each (Fermentas, St. Leon-Rot). The reaction was carried out in a thermocycler. The cycling protocol conditions were 3 min at 94°C, followed by 30 cycles of 30 second at 94°C, 30 seconds at T_{ann} and 60 seconds at 72°C. A final extension step was carried out at 72° C for 5 min, before samples were cooled at 4°C.

3.3.8 Quantitative real-time polymerase chain reaction (qRT-PCR)

mRNA and miRNA levels were measured by qRT-PCR using the LightCycler System (Roche Diagnostics, Mannheim). Two microliters of a 1:10 dilution of cDNA (generated as described in 3.4.5 or 3.4.6) were used in a total volume of 20 µl containing 1.25 units Platinum *Taq* Polymerase and the associated buffer (Invitrogen, Karlsruhe), 4 mM $MgCl_2$, 1.6 µl of a 1:1000 dilution of SybrGreen I (Sigma-Aldrich, Steinheim), 5 % DMSO (Applichem, Darmstadt) 0.5 µM forward and backward primer each, 500 ng/µl non-acetylated bovine serum albumin (Fermentas, St. Leon-Rot) and 0.2 mM deoxynucleotide triphosphates each (Fermentas, St. Leon-Rot). Samples were analyzed in duplicate together with a cDNA or plasmid dilution series of the plasmids described in 2.3.5, which was used to generate a standard curve. The cycling protocol conditions were 60 seconds at 95°C, followed by 40 cycles of 1 second at 95°C (20°C/s), 5 seconds at $T_{ann.}$ (20°C/s), and 15 seconds at 72°C (20°C/s). Fluorescence was measured once per cycle at the end of the elongation step. Subsequently a melting curve protocol was carried out consisting of 15 seconds

at 95℃, followed by 20 seconds at 70℃ (20℃/s) and 1 second at 95℃ (0,2℃/s). Fluorescence was measured continuously during heating to 95℃. Finally samples were cooled down to 40℃ for 10 seconds. All mRNA levels were normalized to the total RNA input used for reverse transcription, HPRT1 or β-actin. All miRNA levels were normalized to miRNA-127.

3.3.9 DNA Sequencing

DNA sequencing was performed in the CMMC (Center for Molecular Medicine, University of Cologne) by an automated method (Sanger et al. 1977) with fluorescence-labeled oligonucleotides. For this purpose 3 - 10 pmol sequencing oligonucleotides (2.3.1.3) and 250 - 350 ng DNA were used in the sequencing reaction. Sequence analysis was carried out with the program “Chromas Lite” (Technelysium Pty Ltd, Tewantin, Australia).

3.3.10 Oligonucleotide labeling for in situ hybridization

Labeling of oligonucleotides for in situ hybridization probes was done with “DIG Oligonucleotide 3´-End Labeling Kit” (Roche Diagnostics, Mannheim). For this purpose 100 pmol in situ hybridization oligonucleotide (2.3.1.5) was adjusted with RNase-free H_2O to a total volume of 10 µl. The labeling reaction was carried out as described in the manufacturer´s protocol.

3.4 RNA methods

3.4.1 Total RNA Isolation

Total RNA isolation from cells or mouse skin was done by using either “miRNeasy Mini Kit” (Qiagen, Hilden) or “peqGOLD TriFast” (PEQLAB, Erlangen) as described in the manufacturer´s protocol, respectively.
Cells were plated in 6- or 24-well plates for RNA Isolation. At the desired point in time the medium was aspirated and cells were taken up in “lysis buffer” (Qiagen) or

“peqGOLD TriFast” (PEQLAB). When using “miRNeasy Mini Kit” (Qiagen) cells were further homogenized by “QIAshredder” (Qiagen, Hilden) and DNA digestion was performed on column using “RNase-Free DNase Set” (Qiagen, Hilden). When using “peqGOLD TriFast” (PEQLAB), DNA digestion was done after completion of RNA isolation (3.4.3).

For RNA isolation from mouse skin, biopsy samples were transferred from RNALater (Qiagen, Hilden) into a tube containing a steel ball and “lysis buffer” (Qiagen) or “peqGOLD TriFast” (PEQLAB). Afterwards biopsies were homogenized in a swing mill (Mixer Mill 301, Retsch, Haan) at 30 Hz for 3 min. For full homogenization of the mouse skin, tubes were turned around and shaken for another 3 min at 30 Hz. When using “miRNeasy Mini Kit” (Qiagen) DNA digestion was performed on column using RNase-Free DNase Set (Qiagen). When using “peqGOLD TriFast” (PEQLAB), DNA digestion was done after completion of RNA isolation (3.4.3). For all preparations RNA was dissolved in 30-50 µl RNase-free H_2O.

3.4.2 Determination of RNA concentration

RNA concentration and degree of purity was determined spectrophotometrically at a wavelength of 260 nm and 280 nm using a NanoDrop®ND-1000 UV/Vis spectral photometer (PEQLAB, Erlangen).

3.4.3 DNA digestion

When using “peqGOLD TriFast” (PEQLAB, Erlangen), DNA digestion was done with “DNase I, RNase-free” (Fermentas, St. Leon-Rot). One microgram total RNA (100 ng total RNA if RNA was isolated from cells in a 24-well plate) was treated with 1 unit DNase I, 1 µl 10X reaction buffer with $MgCl_2$ and adjusted to a total volume of 10 µl with RNase-free H_2O. DNase digestion was completed after 30 min at 37℃ and was stopped by adding 1 µl 25 mM EDTA and incubating at 65℃ for 15 min.

3.4.4 Polyadenylation of miRNAs

Polyadenylation of miRNAs was adapted from Shi and Chiang (2005). Total RNA was isolated as described in 3.4.1 and DNA was digested during isolation or as described in 3.4.3. One microgram of DNA-free RNA was polyadenylated in a total volume of 20 µl containing 2 units Poly(A) Polymerase (Ambion, Austin, USA), 4 µl of the associated buffer, 2.5 mM $MnCl_2$ and 1 mM ATP (Fermentas, St. Leon-Rot) for 1 h at 37℃. The enzyme was inactivated at 75℃ for 1 0 min and buffer was exchanged by ethanol precipitation.

3.4.5 Reverse transcription of mRNAs

Total RNA was isolated as described in 3.4.1 and DNA was digested during isolation or as described in 3.4.3. One microgram of DNA-free RNA (100 ng total RNA if RNA was isolated from cells of a 24-well plate) was reverse transcribed using “Omniscript RT Kit” (Qiagen, Hilden) in a total volume of 20 µl with 10 µM random nonamers (TIB MOLBIOL, Berlin), 1 µM oligo-dT_{23}-primer (Sigma-Aldrich, Steinheim) and 10 units RiboLock™ RNase Inhibitor (Fermentas, St. Leon-Rot). The reaction was carried out at 37℃ for 1 h, afterwards reverse transcriptase w as inactivated at 95℃ for 10 min.

3.4.6 Reverse transcription of miRNAs

Reverse transcription of miRNAs was adapted from Shi and Chiang (2005). Purified polyadenylated DNA-free RNA (3.4.4) was reverse transcribed as described in 3.4.5, but instead of random nonamers and oligo-dT_{23}-primer 1.5 µM miRNA adaptor was used and the reaction was carried out for 2 h at 37℃.

3.4.7 In situ hybridization (ISH)

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). Sections were then incubated in 0.5% SDS in PBS for 15 min at RT, following two washes with dest. H_2O. Further incubation steps were performed in a humid chamber to prevent drying-out. Permeabilization of tissue was reached by treating the sections with 0,2% pepsin (Sigma-Aldrich, Steinheim) in 0.2 M HCl for 15 min at 37℃ and stopped by incubating two times in 0,1 M Glycine (Roth, Karlsruhe) in PBS for 3 mines at RT. After two washes with PBS, DNA digestion was performed with 50 units/ml DNase (Fermentas, St. Leon-Rot) in the associated buffer for 1 h at 37℃, following a shor t wash step with PBS. Endogenous peroxidases were inactivated by incubation in 3% H_2O_2 in Methanol for 20 min. Afterwards sections were washed two times with dest. H_2O and two times with 2X SSC, 3 min each. The prehybridization-mix was heated for two min. at 90℃ and put on the slides, which then were put on a heat plate at 90℃ for 2 min. After 1-2 h incubation at RT the prehybridization-mix was exchanged with 90℃ preheated prehybridization-mix with 5 pmol digoxigenin-labeled probe (3.3.10)3.3.10. The slides were put on a heat plate at 90℃ for 2 min and incu bated overnight at RT. Next day slides were washed two times with 2X SSC for 15 min at 37℃, one time with 1X SSC for 15 min at 37℃ and one time with 0.5X SSC for 1 5 min at 37℃. Detection of digoxigenin-labeled probes was done with anti-digoxigenin-POD Fab fragments (Roche Diagnostics, Mannheim) in a 1:100 dilution and TSA Biotin System (PerkinElmer, Waltham, USA) according to the manufacturer's protocol. Afterwards AEC staining was performed by pipetting AEC working solution on the slides. Sections were counterstained with hematoxilin (Sigma-Aldrich, Steinheim) and embedded with Kaiser´s glycerol gelatine (Merck, Darmstadt).

3.4.8 MiRNA microarray

Five microgram total RNA (3.4.1) from healthy skin of one FVB/N wt and one HPV8-CER mouse was analyzed in a comparative µParaflo microarray by LC Sciences (Houston, USA) based on the Sanger mirBase: release 8.2.

3.5 Protein methods

3.5.1 Immunohistochemistry (IHC)

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 20 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.
HPV8-E2 staining: After blocking unspecific antigen sites with 20% FCS in PBS for 1 h, samples were incubated with primary antibody α-HPV8-E2 in 2 FCS in PBS for 90 min followed by three washes with PBS. Incubation with secondary antibody α-guinea-pig-POD diluted in 2% FCS in PBS was done for 1 h, following three washes with PBS.
KGF, MET, PDCD4, PTEN, and Rb staining: Blocking of unspecific antigen sites was done with 50% horse serum in PBS for 30 min, for α-KGF antibody 50% rabbit serum in PBS was used. Incubation of primary antibody was done in 2% horse serum for 90 min, α-KGF antibody was diluted in 2% rabbit serum in PBS, followed by three washes with PBS. Detection of primary antibody was done with the “VECTASTAIN Elite ABC Kit (Universal)” (Vector Laboratories, Burlingame, USA), for α-KGF antibody “VECTASTAIN Elite ABC Kit (Goat IgG)” (Vector Laboratories) was used as described in the manufacturer’s protocol.
DAB staining was performed with the “Liquid DAB Concentrated Substrate Pack” (BioGenex, San Ramon, USA). Sections were counterstained with hematoxilin and dehydrated with an ascending alcohol series (70%, 90%, 100%; 3 min each). After a final incubation in xylene for 5 min sections were embedded with mounting medium “DePeX” (Serva Electrophoresis, Heidelberg).

3.6 Experimental operations in mice

Generally, mice were anesthetized with Ketamine-hydrochloride (Sigma-Aldrich, Steinheim) and Xylazine-hydrochloride (Sigma-Aldrich, Steinheim) and shaved with an electric shaver (Wella, Karlsruhe) before any treatment.

3.6.1 UV-irradiation of mouse skin

Dorsal caudal UV-irradiation was performed at an age of 6-12 weeks. 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 0,36 J/cm^2 UVB alone on a 4 cm^2 sized area as reported before (Pfefferle et al. 2008; Marcuzzi et al. 2009).

3.6.2 Mechanical irritation of mouse skin (tape-stripping, tattooing)

After shaving, mouse skin was treated with depilatory cream (Balea, Karlsruhe) on a 4 cm^2 sized area at an age of 6-12 weeks. For tape stripping experiments mice were treated by attaching and detaching 15 times a new piece of tape (Tesa, Hamburg) in the depilated area. For tattooing experiments an area of mouse skin was laid on top of another, in this way both dermis matched each other. 3 lines of about 1 cm length were tattooed on top of each other in the depilated area, each in 5 seconds, thereby penetrating both skin layers. Tattooing was performed using a “Rotary 12000PL” tattoo machine with 5-Liner needles (Bortech, Wuppertal). The machine was run by 17,5 V, resulting in 500 pinholes per second.

3.6.3 Taking skin biopsies

Mouse skin samples were either taken by punch biopsies (4 mm diameter, pfm, Köln) or by scissors. For RNA-analysis samples were stored at -20℃ in RNALater (Qiagen, Hilden) according to the manufacturer’s protocol. For IHC and ISH experiments mouse skin samples were formalin-fixed and embedded in paraffin. For

experiments with siRNA-control-fluorescein, samples were embedded into Tissue-Tek O.C.T. Compound (Sakura Finetek, Heppenheim) and stored at –20°C.

3.6.4 Sectioning mouse skin samples with a cryotome

Mouse skin samples embedded into Tissue-Tek O.C.T. Compound (Sakura Finetek, Heppenheim) were cut with a Leica CM3000 cryotome (Leica, Wetzlar) into 5 µm thick sections and transferred on a slide. Coverslips were mounted on the slides with Kaiser´s glycerol gelatine (Merck, Darmstadt).

3.6.5 Paraffin embedding and sectioning of mouse skin samples

Formalin-fixed mouse skin samples were embedded and cut into 4 µm thick sections at the dermatology of the University Hospital of Cologne.

3.6.6 Cell nucleus staining of mouse skin sections with DAPI

Cell nucleus staining of mouse skin sections was performed with DAPI (Merck, Darmstadt) as described in the manufacturer’s protocol.

4 Results

4.1 Papilloma growth and HPV8 oncogene expression in HPV8-CER mice

4.1.1 Papilloma development is induced after UVA/B-irradiation

The simulation of sunburn by a single UVA/B-dose (10 J/cm^2 UVA and 1 J/cm^2 UVB) led to papilloma formation within three weeks in HPV8-CER mice, while the skin of FVB/N wt mice healed completely within this time period (Marcuzzi 2006). In order to analyze the influence of UVA/B-irradiation on transgene expression levels during papilloma development, kinetics of oncogene expression were determined after UVA/B-irradiation of HPV8-CER mice.

Figure 4 shows typical alterations of the skin of an HPV8-CER mouse over a 30 day period after UVA/B-irriadiation. Six hours after irradiation only a slight redness was visible, which was distinct four days after irradiation indicating sunburn. The irradiated area swelled and became hairless on day seven after irradiation. Papilloma development was first detectable on day twelve. The papillomas grew larger over the following observation period.

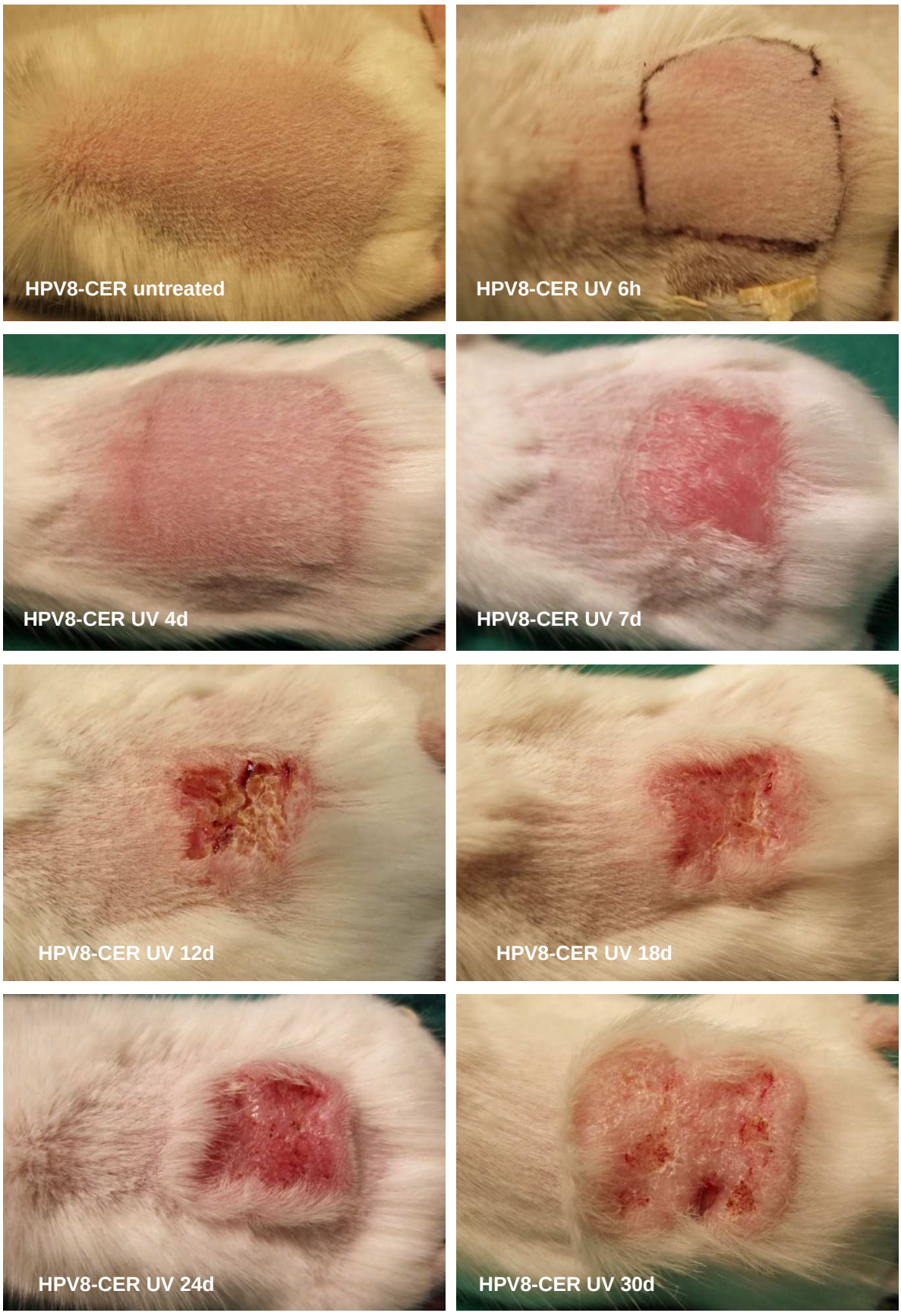
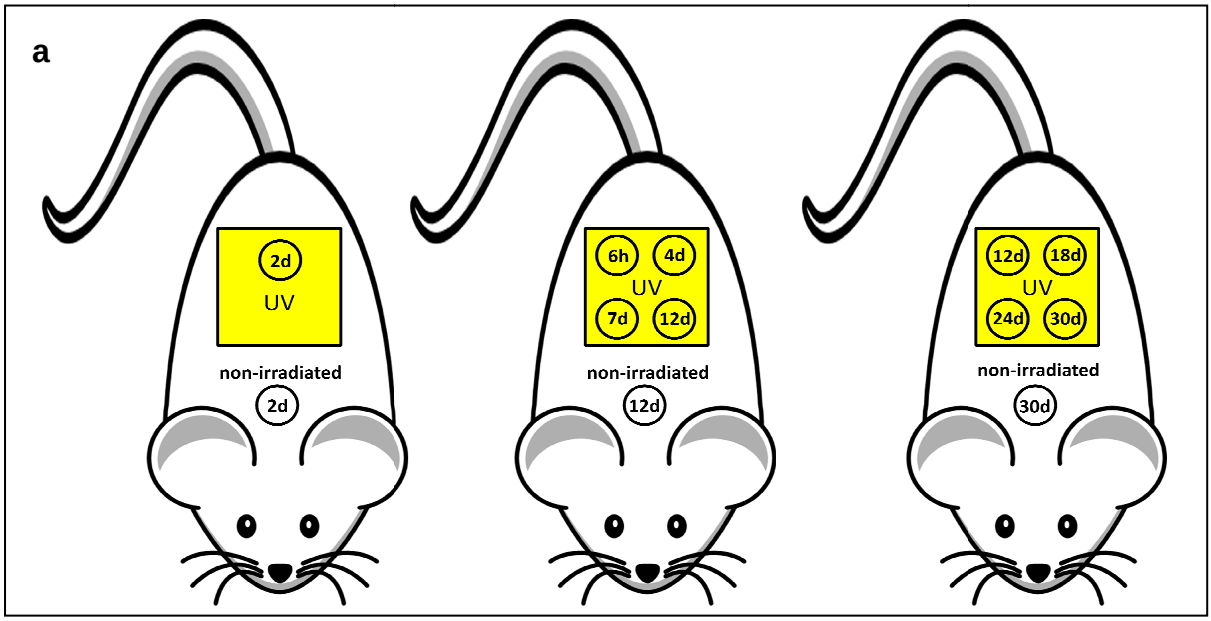
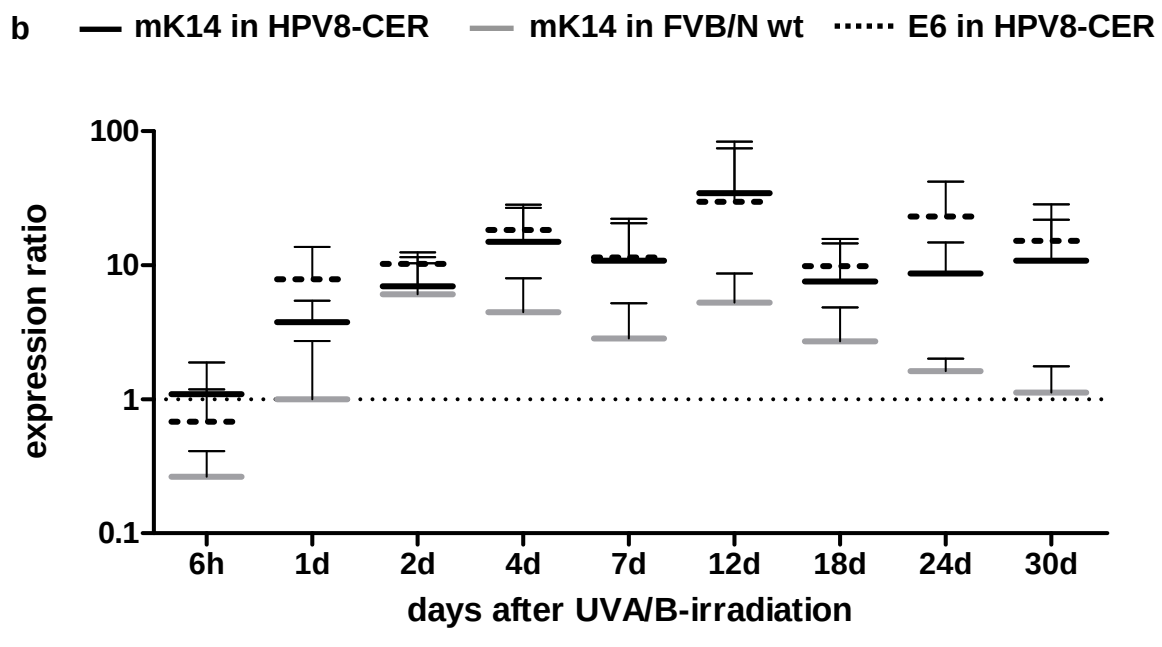


Figure 4: Development of papillomas in an HPV8-CER mouse after UVA/B-irradiation
The back of the mouse was shaved and irradiated with 10 J/cm^2 UVA and 1 J/cm^2 UVB on a 4 cm^2 sized skin area. Pictures show treated skin areas at different points in time after UVA/B-irradiation.

4.1.2 Enhanced HPV8 oncogene mRNA expression was induced early after UVA/B irradiation in HPV8-CER mice

To quantify HPV8 oncogene mRNA levels by qRT-PCR during papilloma development, skin punch biopsies were taken at different points in time over a 30 day period after UVA/B-irradiation of HPV8-CER mice. Furthermore, mK14 levels were determined to compare the status of keratinocytes in transgenic and FVB/N wt skin after irradiation. As the mRNA levels of the housekeeping genes HPRT1 and β-actin also increased after UVA/B-irradiation (Marcuzzi *et al.* 2009) mRNA levels were normalized to the total RNA input of one microgram used for reverse transcription. Two different skin punch protocols were carried out. Skin punch biopsies were either taken at a single point in time (Figure 5a; left mouse, e.g. 2d) or at four different points in time from the same mouse out of the irradiated area (Figure 5a; middle mouse 6h, 4d, 7d, 12d; right mouse 12d, 18d, 24d, 30d). Additionally, skin punch biopsies were taken at the end of the respective time period out of the non-irradiated area. In prevenient experiments it was ascertained that neither skin punching nor UVA/B-irradiation had an effect on mK14 and HPV8-E6 expression in adjacent skin areas. As described earlier, the expression of HPV8 transgenes is controlled by the hK14-promoter. It was therefore not unexpected to discover by comparative analyses that hK14-promoter driven transgene expression was highly correlated with mK14 mRNA levels (R=0,85; p<0,01; shown for mK14 and HPV8-E6, Figure 5b). Therefore mK14 represents a surrogate marker for transgene expression in HPV8-CER mice.





c

Skin 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

Figure 5: Perpetuation of enhanced mK14 and HPV8-E6 expression in HPV8-CER mice after UVA/B-irradiation.
(a) Diagram of the two different skin punch protocols. Skin punch biopsies were either taken at a single point in time (left mouse, e.g. 2d) or at four different points in time from the same mouse out of the irradiated area (yellow box) (middle mouse 6h, 4d, 7d, 12d; right mouse 12d, 18d, 24d, 30d). Additionally, skin punch biopsies were taken at the end of the respective time period out of the non-irradiated area **(b)** The backs of shaved HPV8-CER and FVB/N wt mice were irradiated with 10 J/cm^2 UVA and 1 J/cm^2 UVB on a 4 cm^2 sized area. Skin samples were taken at different points in time after UVA/B-irradiation from irradiated and non-irradiated skin areas. HPV8-E6 and mK14 mRNA expression levels were measured in one microgram of reverse transcribed total cellular RNA by qRT-PCR. The mRNA level in irradiated skin was normalized to the level of the respective mRNA in untreated skin of the same mouse. 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. **(c)** Table shows number of analyzed samples per point in time from different mice.

Generally, a stronger mean mK14 expression in HPV8-CER mice than in FVB/N wt mice after UVA/B-irradiation was observed (Figure 5b). Induction of mK14 expression as well as HPV8-E6 was already seen one day after UV-irradiation in HPV8-CER mice. Between day two and day twelve after UVA/B-irradiation enhanced mK14 expression was measured in both mouse strains. In FVB/N wt mice mK14 expression returned to an almost basal level along with healing of the irradiated skin during the following observation period. In contrast, mK14 expression as well as HPV8-E6 expression persisted on a high level in HPV8-CER mice at all following points in time.

4.1.3 Expression ratio of HPV8-E2, -E6 and -E7 in HPV8-CER mice

The HPV8 oncogenes E2, E6 and E7 are all under the control of the hK14-promoter in HPV8-CER mice. Therefore it was speculated that these genes are equally transcribed. To check this hypothesis for further examinations, the transcript levels of HPV8-E2, -E6, and -E7 were measured in non-lesional skin samples from six HPV8-CER mice by qRT-PCR and were correlated with each other.

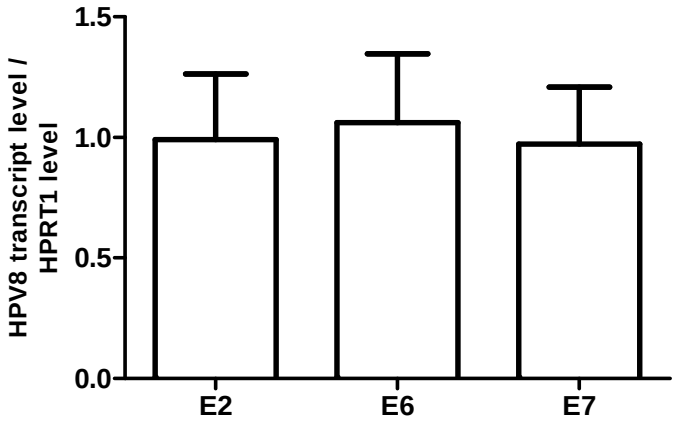


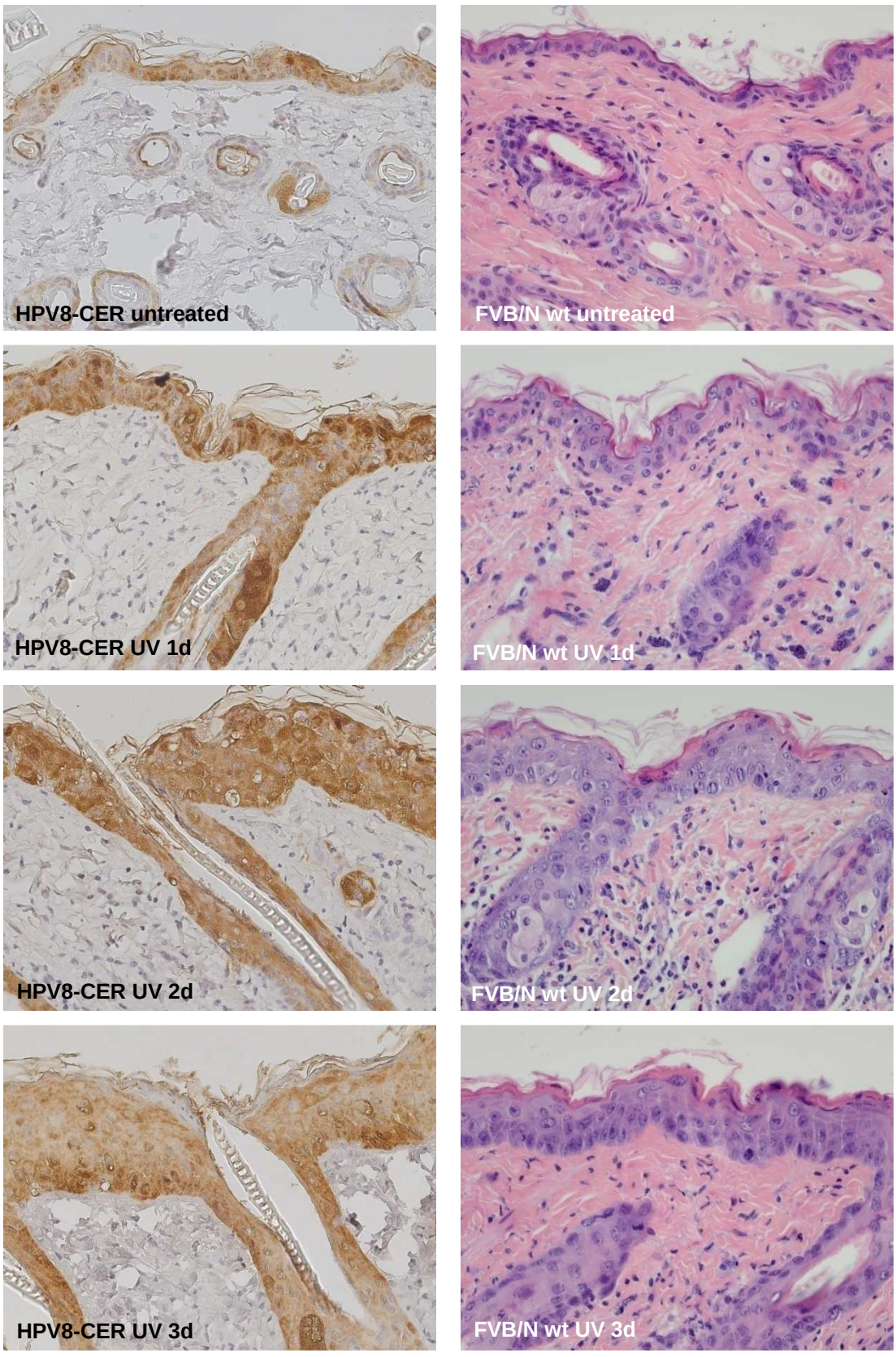
Figure 6: HPV8-E2, -E6 and -E7 are equally expressed in HPV8-CER mice.
HPV8-E2, -E6 and -E7 mRNA levels were measured in one microgram of reverse transcribed RNA from non-lesionsl skin samples of six HPV8-CER mice by qRT-PCR and normalized to the mRNA level of HPRT1.

All three transgenes (E2=0.99; E6=1.06; E7=0.97) showed nearly the exact same transcript level in the skin of HPV8-CER mice (Figure 6). Therefore it can be assumed that all three transgenes are equally expressed from the hK14-promoter. This means that the expression of any of these three transgenes mirrors the expression of the others.

4.1.4 Papilloma growth was paralleled by enhanced HPV8 protein levels

To study, whether the increase of transgene mRNA levels in the skin of HPV8-CER mice is also reflected at the protein level and to characterize the HPV8-oncoprotein positive epidermal layers, sections of UVA/B-irradiated and non-irradiated HPV8-CER skin were immunohistochemically stained for HPV8-E2. As reference for changes in normal mouse skin, the histology of UVA/B-irradiated FVB/N wt skin was also documented by HE-staining. The antibody against HPV8-E2 showed no cross-reactivity on FVB/N wt sections (data not shown).

Untreated HPV8-CER skin revealed a clear but faint HPV8-E2 signal in the epidermis (Figure 7, left column) whereas, in line with RNA data, E2 protein expression increased already one day after UVA/B-irradiation. Both an increased number of transgene expressing cells and more intense staining were observed. In the progress of papilloma development (UV 2d – UV 10d) more epidermal cell layers emerged, all being HPV8-E2 protein positive. The enhanced 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. Histology of UVA/B-irradiated FVB/N wt mouse skin showed an only slightly thinner epidermis than HPV8-CER mouse skin until day seven after UVA/B-irradiation (Figure 7, right column). However, about ten days after irritation, the epidermis of control FVB/N wt mice started to regress to its normal status with one or two cell layers. In conclusion these results demonstrate that enhanced expression levels of HPV8 oncoprotein are paralleled by papillomatosis in HPV8-CER mice.


HPV8-CER untreated
FVB/N wt untreated
HPV8-CER UV 1d
FVB/N wt UV 1d
HPV8-CER UV 2d
FVB/N wt UV 2d
HPV8-CER UV 3d
FVB/N wt UV 3d

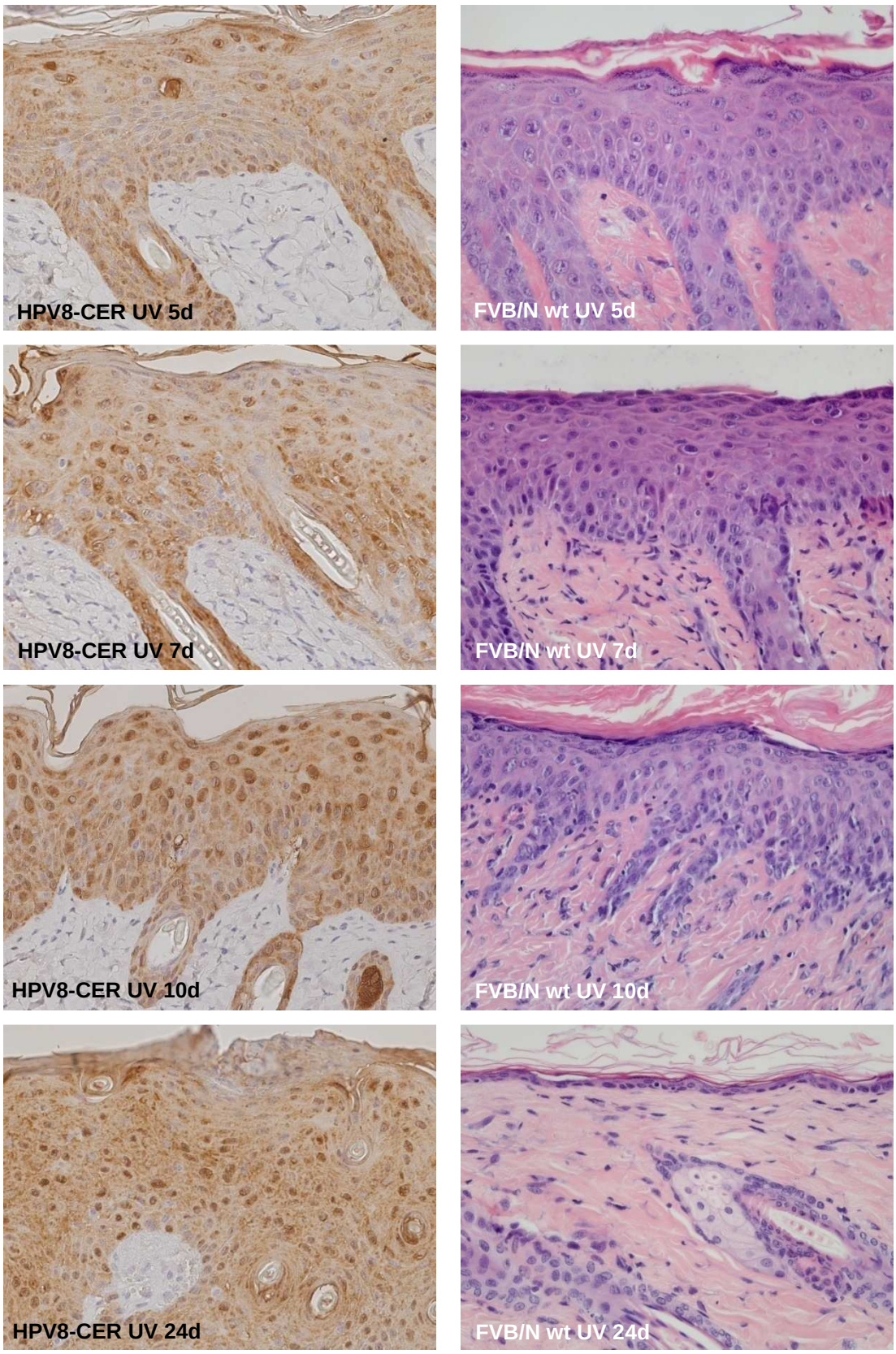


Figure 7: Immunohistochemical staining for HPV8-E2 in HPV8-CER skin sections revealed enhanced HPV8-E2 protein expression after UVA/B-irradiation.
The backs (4 cm^2) of shaved HPV8-CER and FVB/N wt mice were irradiated with 10 J/cm^2 UVA and 1 J/cm^2 UVB. For each point in time biopsies were taken out of the irradiated skin of two mice. Skin biopsies were also taken from two non-irradiated HPV8-CER and FVB/N wt mice. Formalin-fixed and paraffin embedded samples were cut into sections. HPV8-CER sections were stained against HPV8-E2 and counterstained with hematoxylin. FVB/N wt sections were H&E stained. Magnification: 400x.

4.1.5 UVA/B-irradiated HPV8-E2 and -E6 mice with enhanced transgene mRNA expression developed papillomas

As demonstrated at our institute before, HPV8-E6 mice perfectly mirror the HPV8-CER mouse phenotype, including rate and prevalence of spontaneous and UVA/B-induced papillomatosis (Marcuzzi 2006). Therefore, persistently enhanced expression levels of mK14 and accordingly of transgenes were expected for HPV8-E6 mice. In contrast, for HPV8-E2 mice, which present a lower papilloma penetrance (Pfefferle *et al.* 2008), perpetuation of enhanced mK14 expression was only expected in some cases. To test this, skin punch biopsies from three HPV8-E2 and three -E6 mice were taken 12, 18, 24 and 30 days after UVA/B-irradiation.

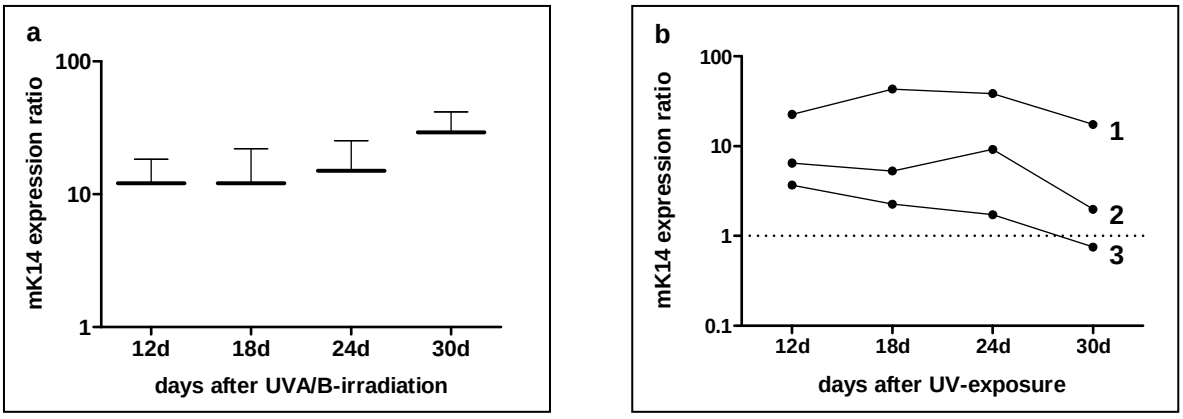


Figure 8: Papilloma growth is accompanied by enhanced mK14 expression in HPV8-E6 and E2 mice after UVA/B-irradiation.
The backs of shaved HPV8-E2 and E6 mice were irradiated with 10 J/cm^2 UVA and 1 J/cm^2 UVB on a 4 cm^2 sized skin area. Skin punch biopsies were taken at different points in time after UVA/B-irradiation out of the irradiated and non-irradiated skin. MK14 mRNA expression levels were measured in one microgram of reverse transcribed total cellular RNA by qRT-PCR. The mK14 level in irradiated skin was normalized to the mK14 level in untreated skin of the same mouse. **(a)** The mean mK14 expression ratio of three HPV8-E6 mice is plotted against time after irradiation. Error bars represent the standard deviation. **(b)** The mK14 expression ratio of three HPV8-E2 mice is plotted against time.

For HPV8-E6 mice enhanced transgene expression levels were measured and papilloma development was observed as seen for HPV8-CER mice (Figure 8a). However, only one HPV8-E2 mouse (1) developed papillomas and showed enhanced mK14 expression levels for all examined points in time (Figure 8b). The two other HPV8-E2 mice (2 and 3) did not develop papillomas and showed always lower mK14 expression levels compared to mouse 1.

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

It was previously shown that 0,36 J/cm^2 UVB-irradiation alone is sufficient to induce papillomatosis in HPV8-CER mice, while 10 J/cm^2 UVA-irradiation does not lead to papilloma development (Marcuzzi 2006). To check, if these two wavelengths also differ in their ability to stimulate transgene expression, mK14 levels were measured in three UVA and three UVB irradiated HPV8-CER and FVB/N wt mice.

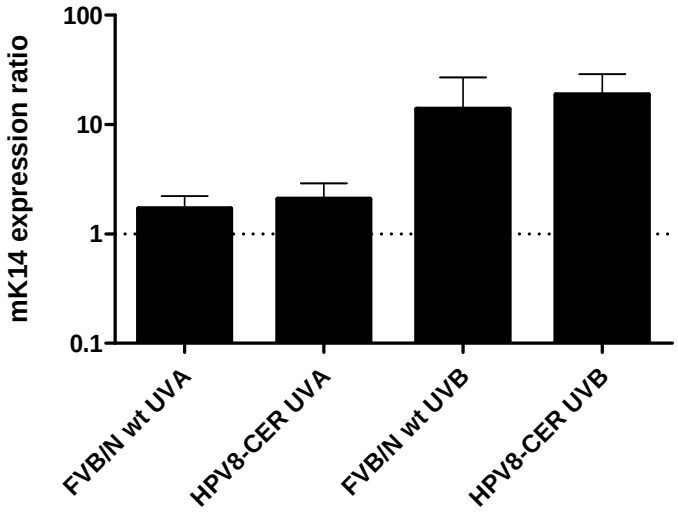


Figure 9: UVB-irradiation alone led to enhanced mK14 expression in HPV8-CER and FVB/N wt mice, while UVA-irradiation did not.
The backs (4 cm^2) of three shaved HPV8-CER and three shaved FVB/N wt mice were either UVA-irradiated (10 J/cm^2) or UVB-irradiated (0,36 J/cm^2). Skin punch biopsies were taken twelve days after UVA- or UVB-irradiation out of the irradiated and non-irradiated skin. MK14 mRNA expression levels were measured in one microgram of reverse transcribed total cellular RNA by qRT-PCR. The mK14 level in irradiated skin was normalized to the mK14 level in untreated skin of the same mouse. Bars represent the mean mK14 expression ratio of three FVB/N wt or three HPV8-CER mice. Error bars represent the standard deviation.

UVB-irradiation induced only small separated papillomas in HPV8-CER mice. For RNA analysis skin punch biopsies were taken out of papillomatous areas. Twelve days after UVB-irradiation both FVB/N wt and HPV8-CER mice showed an upregulated mK14 expression (Figure 9), which was similar to that observed for the combined UVA/B irradiation. In contrast, after UVA-irradiation mK14 expression was neither increased in FVB/N wt nor in HPV8-CER mice. This data demonstrates, that UVB radiation is responsible for the activation of the hK14-promoter and thus for the induction of the HPV8 oncogenes in transgenic mice.

4.1.7 Tape-stripping of HPV8-CER mouse skin induces papillomatosis and enhanced HPV8 oncogene expression

Wounding of HPV8-CER mice by taking skin punch biopsies also induced papilloma development (Marcuzzi 2006). In this study the influence of mechanical skin irritation on transgene expression was investigated by tape-stripping the mouse skin. In contrast to the area-wide irritation by UVA/B-irradiation, tape-stripping only leads to small, light wounds and therefore represents a softer experimental approach to irritate the skin.

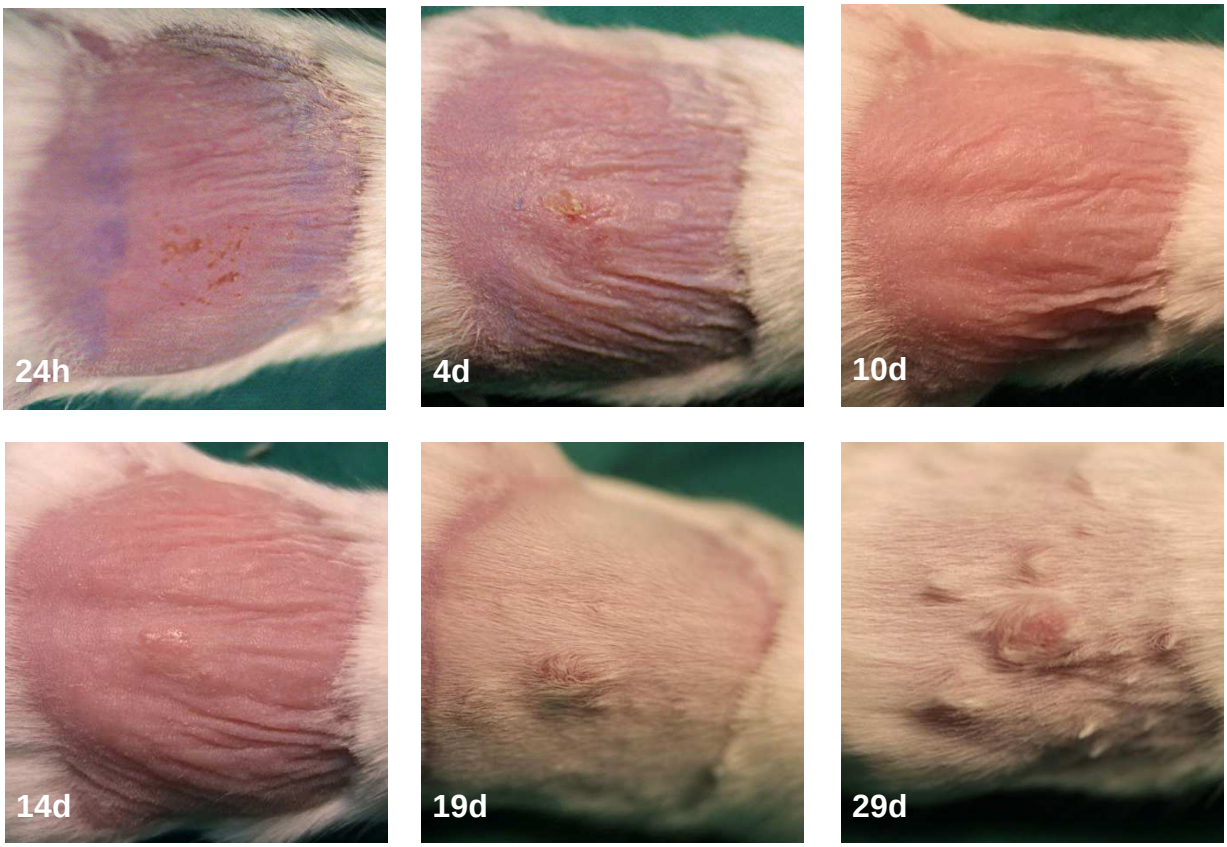


Figure 10: Induction of papilloma growth in HPV8-CER mice by mechanical skin irritation.
HPV8-CER mice were shaved and treated with depilatory cream on a 4 cm^2 sized skin area and then treated by attaching and detaching 15 times a new piece of tape. Pictures show treated skin areas of one mouse at different points in time.

Tape-stripping punctually led to wounds on small skin areas (Figure 10, 24h after treatment). These wounds macroscopically appeared to be healed up within ten days. However, starting from about day fourteen after treatment papilloma growth was visible particularly on skin areas, which had formerly been wounded more severely (Figure 10, 4d after treatment). These papillomas grew larger over the

following observation period. As from day 19 after treatment, additional, small, spike-shaped papillomas were detectable on the treated skin area.

The influence of tape-stripping on transgene expression was investigated in three HPV8-CER mice two days after treatment. Four punch biopsies were taken from each mouse to measure HPV8-E6 expression in areas with different grades of injury (Figure 11).

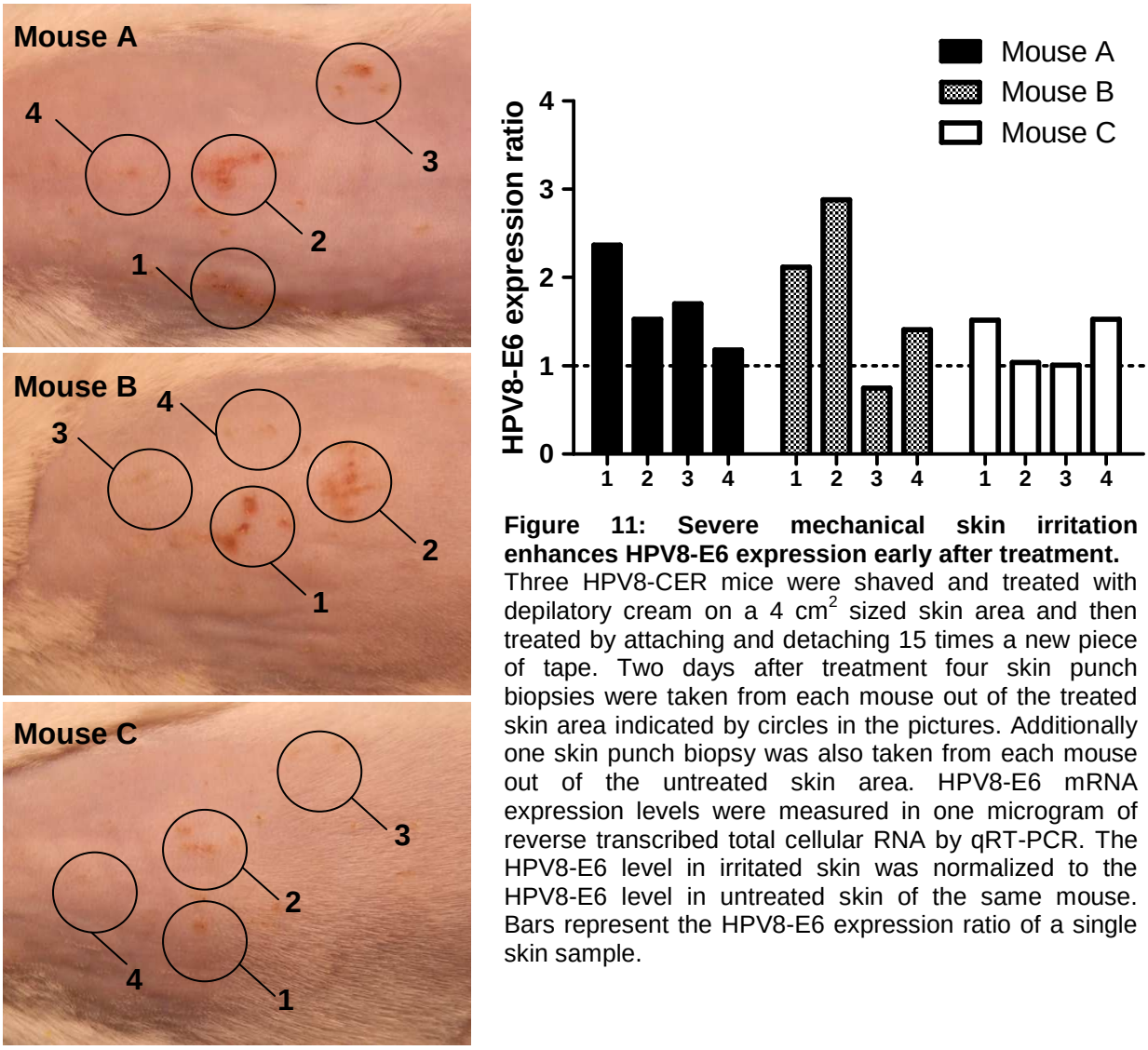


Figure 11: Severe mechanical skin irritation enhances HPV8-E6 expression early after treatment. Three HPV8-CER mice were shaved and treated with depilatory cream on a 4 cm^2 sized skin area and then treated by attaching and detaching 15 times a new piece of tape. Two days after treatment four skin punch biopsies were taken from each mouse out of the treated skin area indicated by circles in the pictures. Additionally one skin punch biopsy was also taken from each mouse out of the untreated skin area. HPV8-E6 mRNA expression levels were measured in one microgram of reverse transcribed total cellular RNA by qRT-PCR. The HPV8-E6 level in irritated skin was normalized to the HPV8-E6 level in untreated skin of the same mouse. Bars represent the HPV8-E6 expression ratio of a single skin sample.

Mouse A and B showed moderate to severe injuries and partially enhanced HPV8-E6 expression, while mouse C had only minor wounds and normal HPV8-E6 expression (Figure 11). Overall, samples with a macroscopically visible lesion (A1, A2, A3, B1, B2) showed enhanced (1.5 - 2.9 fold) HPV8-E6 expression, while samples with no visible or only tiny lesions (A4, B3, B4, C1, C2, C3, C4) showed normal or slightly enhanced (0.8 - 1.5 fold) HPV8-E6 expression.

4.2 HPV8-E6 knock-down by specific siRNAs in cell culture and skin of HPV8-CER mice

The similar phenotype of HPV8-E6 and HPV8-CER mice clearly demonstrates that E6 is the major oncogene in HPV8-CER mice (Marcuzzi 2006). In 4.1.2 and 4.1.7 it was shown that E6 expression is already enhanced one to two days after skin irritation in HPV8-CER mice, indicating that an early increase of E6 expression is of crucial importance for papilloma development. To substantiate this observation, experiments were designed to reduce or inhibit papilloma growth in HPV8-CER mice after irritation by topical application of HPV8-E6 specific siRNA. Prior to testing the designed siRNAs on the mouse skin, they had to be characterized in cell culture experiments regarding their ability to knock-down HPV8-E6. If successful this could also raise prospect for preventive treatment.

4.2.1 Characterization of HPV8-E6 specific siRNAs in monolayer cell culture

The silencing efficiency of a siRNA strongly depends on its target sequence. Therefore, four siRNAs with different target sites in HPV8-E6 were tested in cell culture to specify their silencing efficiency. All siRNAs (siRNA-HPV8E6-1, -2, -3, -4 and siRNA-control-fluorescein) were designed and manufactured by Qiagen (Hilden). HaCaT-pLXSN8-E6 cells were transfected with “Lipofectamine 2000” and 50 nM (final concentration) siRNA-HPV8E6-1, -2, -3, -4, siRNA-control-fluorescein, respectively, as described in 3.2.4. The transfection reagent alone and the non-silencing siRNA-control-fluorescein represent negative controls to rule out unspecific knock-down. RNA was isolated 24h after transfection and HPV8-E6 mRNA levels were measured by qRT-PCR. HPV8-E6 expression of control cells was comparable to that of untransfected HaCaT-pLXSN8-E6 cells, showing that there was no unspecific knockdown of HPV8-E6 expression (Figure 12). In contrast, HaCaT-pLXSN8-E6 cells transfected with siRNA-HPV8E6-1 showed the highest knock-down leading to a remaining HPV8-E6 expression of 15%, followed by siRNA-HPV8E6-2 with 17%, siRNA-HPV8E6-3 with 21% and siRNA-HPV8E6-4 with 25%. Therefore

siRNA-HPV8E6-1 and siRNA-HPV8E6-2 with the highest knock-down efficiencies were used in following cell culture experiments.

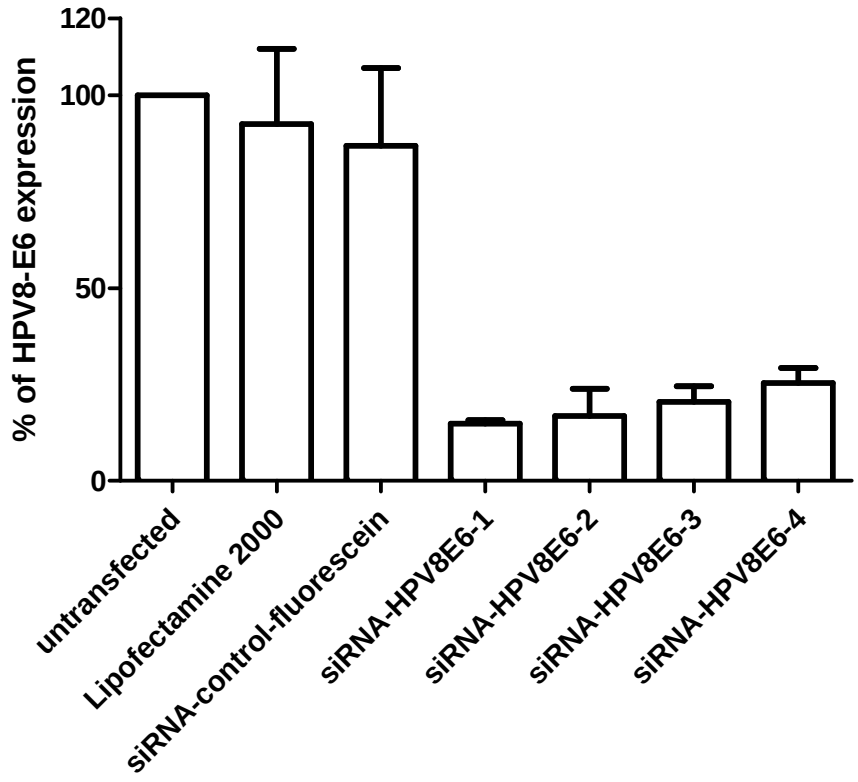


Figure 12: Silencing efficiency of four different siRNAs against HPV8-E6.
HaCaT-pLXSN8-E6 cells (3 x 10^4) were transfected with "Lipofectamine 2000" and siRNA-HPV8E6-1, 2, -3, -4 or siRNA-control-fluorescein, respectively, in a final concentration of 50 nM. RNA was isolated 24h after transfection and reverse transcribed. HPV8-E6 levels were measured by qRT-PCR and normalized to β-actin (human). HPV8-E6 expression of untransfected HaCaT-pLXSN8-E6 cells was set to 100 %. Experiments were done in duplicate.

Titration experiments were carried out with siRNA-HPV8E6-1, -2 and a combination of these two. Different concentrations of siRNAs were transfected in HaCaT-pLXSN8-E6 cells. There was no noticeable difference in knock-down efficiency between siRNA-HPV8E6-1 and -2 for all concentrations (Figure 13). Furthermore, a combination of these two siRNAs did not increase the HPV8-E6 knock-down. The knock-down increased with rising siRNA concentrations to about 10% HPV8-E6 expression with 50 nM siRNA, where E6 knock-down was at its maximum. A higher siRNA concentration (100 nM) did not further enhance the knock-down effect. From these results it is clearly seen that the knock-down efficiency depends on siRNA concentration.

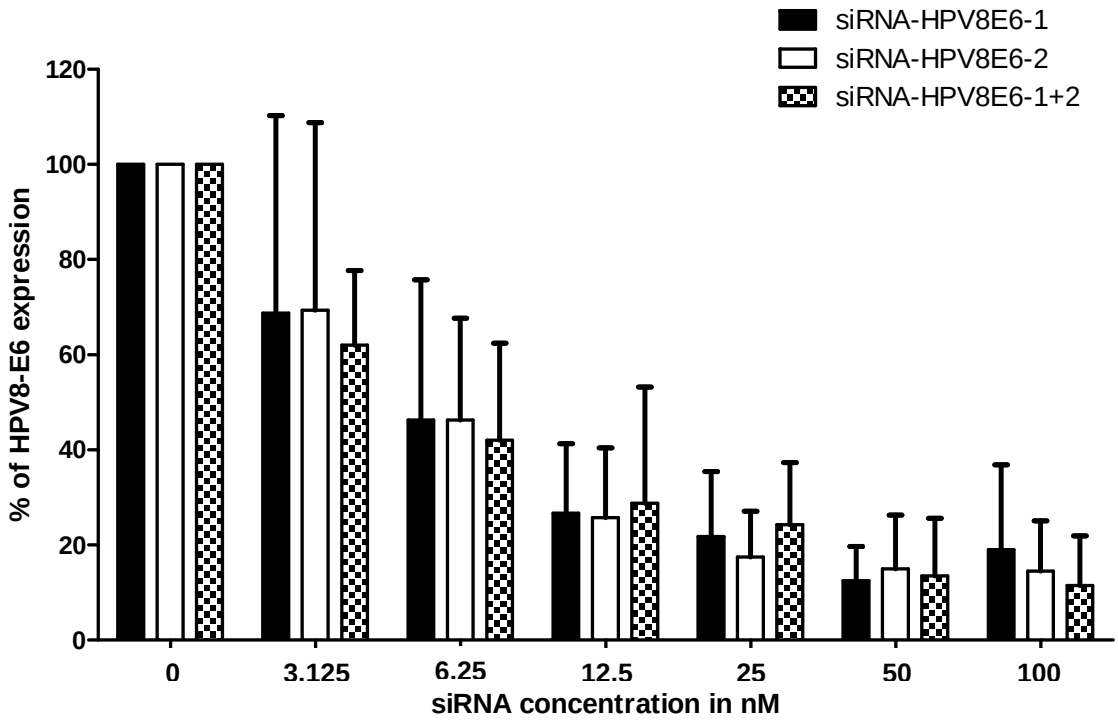


Figure 13: Titration of siRNA-HPV8E6-1 and siRNA-HPV8E6-2.
HaCaT-pLXSN8-E6 cells (3 x 10^4) were transfected with "Lipofectamine 2000" and siRNA-HPV8E6-1, -2 or a combination of these two siRNAs, respectively, in a final concentration of 3.125 nM; 6.25 nM; 12.5 nM; 25 nM; 50 nM or 100 nM. RNA was isolated 24h after transfection and reverse transcribed. HPV8-E6 levels were measured by qPCR and normalized to β-actin (human). HPV8-E6 expression of untransfected HaCaT-pLXSN8-E6 cells was set to 100 %. Experiments were done in quadruplicate.

As the maximal knock-down was observed with 50 nM siRNA, this concentration was used to characterize the knock-down kinetics at different points in time after transfection. As shown in figure 14, no significant difference in HPV8-E6 expression was found between siRNA-HPV8E6-1 or -2 transfected cells. Already 6h post transfection a clear HPV8-E6 knock-down of 55% was detected, which increased to a maximum knock-down of 90% at 24h and 30h post transfection. 48h after transfection the HPV8-E6 expression began to rise indicating the end of the efficient knock-down effect.

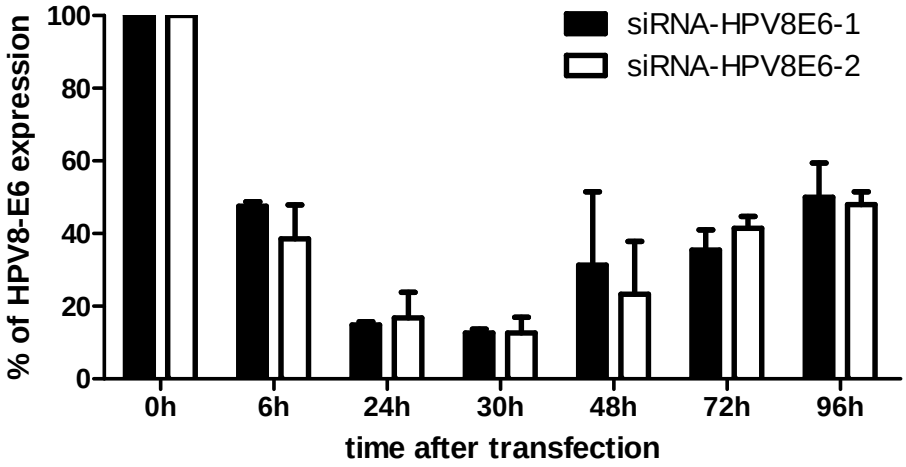


Figure 14: Duration of the siRNA induced HPV8-E6 knock-down in HaCaT-pLXSN8-E6 cells. HaCaT-pLXSN8-E6 cells (3 x 10^4) were transfected with "Lipofectamine 2000" and siRNA-HPV8E6-1 or -2, respectively, in a final concentration of 50 nM. RNA was isolated 6h, 24h, 30h, 48h, 72h and 96h after transfection and reverse transcribed. HPV8-E6 levels were measured by qPCR and normalized to β-actin (human). HPV8-E6 expression of untransfected HaCaT-pLXSN8-E6 cells was set to 100 %. Experiments were done in duplicate.

4.2.2 Topical application of a fluorescent siRNA on mouse skin

In order to deliver the siRNA to the K14-expressing target cells in the mouse epidermis, different transfection reagents, pretreatment and penetration methods were evaluated with a fluorescein-linked siRNA. Initial transfection experiments with siRNA-control-fluorescein (10 µM) and the transfection reagents "In vivo-jetPEI", "Lipofectamin 2000" or "MIKA-Nuclehicle-Kit", applied on shaved and depilated HPV8-CER mouse skin, did not yield fluorescence signals in the epidermis above H_2O treated mouse skin. Only trapped siRNA molecules in cornified cells and hair bulbs showed a distinct green fluorescence (Figure 15, left column). As an alternative, penetration of the skin by tattooing in cooperation with transfection reagents was tested to introduce siRNAs into the epidermal cells. The tattoo needles penetrate the dermis and epidermis, thereby delivering the siRNA to both layers and possibly allowing transfection of basal cells. SiRNA-control-fluorescein (10 µM) was used in combination with the different transfection reagents. These transfection-mixes were tattooed into shaved and depilated HPV8-CER mice. Skin samples were taken 24h after application.

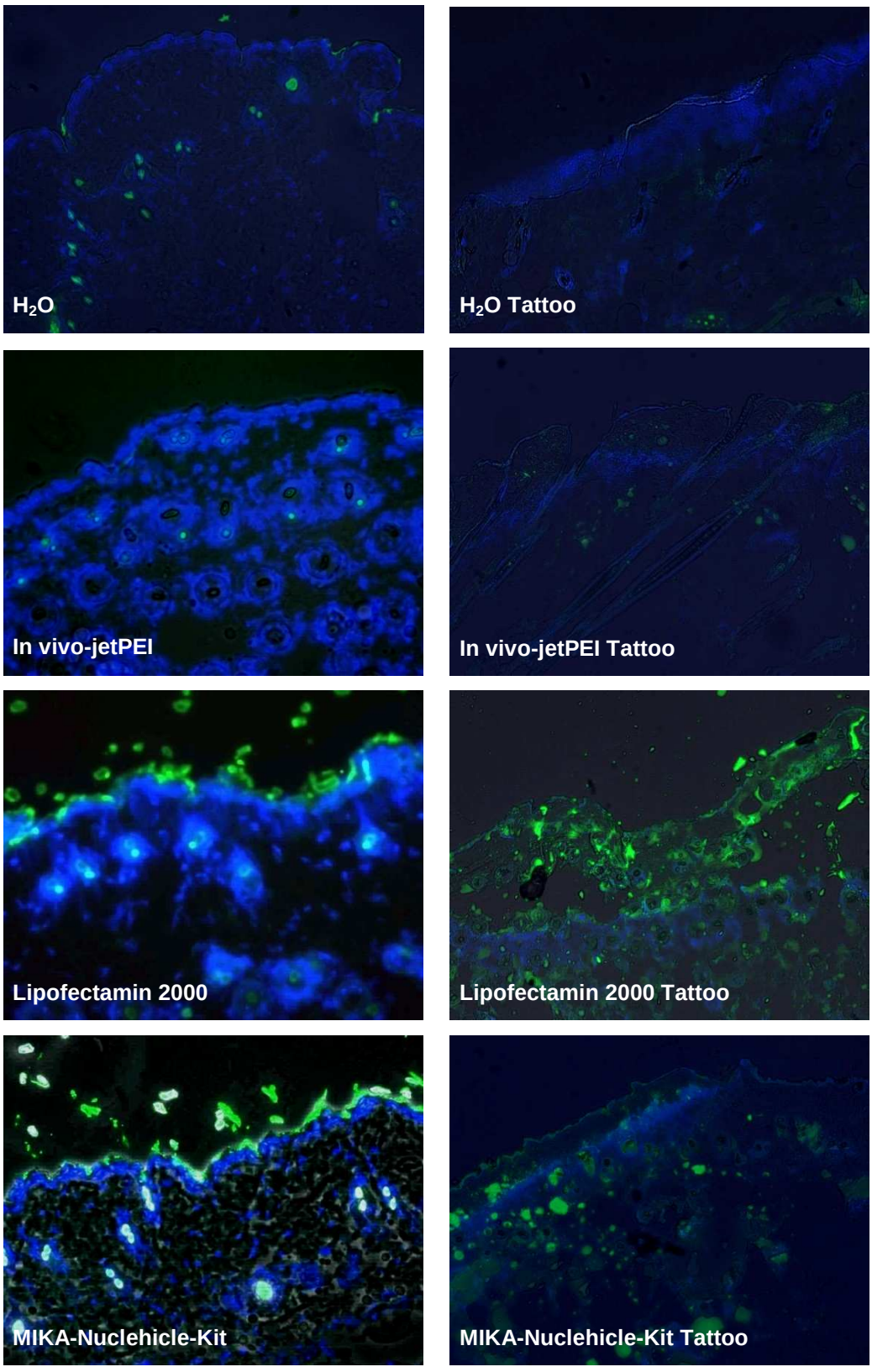
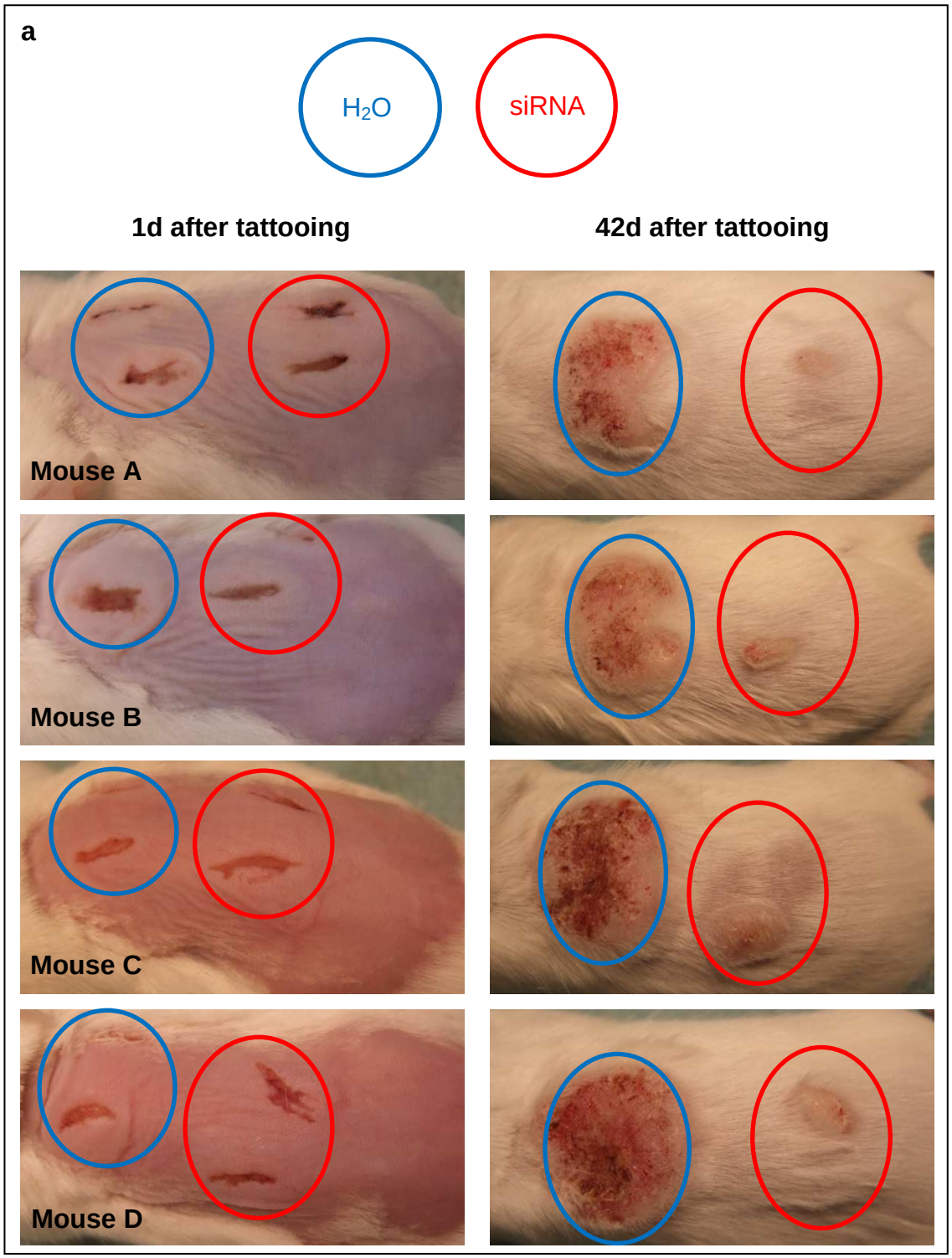


Figure 15: Tattoing is an effective method to introduce siRNAs into the epidermis.
HPV8-CER mice were shaved and depilated. Afterwards 20 µl of the transfection-mix (In vivo-jetPEI, Lipofectamin 2000, MIKA-Nuclehicle-Kit/siRNA-control-fluorescein (10 µM)) was either applied on the skin with a pipette or was tattooed into the mouse skin as described in 3.6.2. For each transfection reagent two mice were used. Skin samples were taken 24h after application, embedded into Tissue-Tek O.C.T. Compound and cut with a Leica CM3000 cryotome into 5 µm thick sections. Cell nuclei were stained with DAPI and appeared blue. Images show typical results and were taken with the fluorescence microscope Leica DMI 6000 B. Magnification: 100x

Tattooing the mouse skin with “In vivo-jetPEI”/siRNA-control-fluorescein mix did not lead to a noticeable fluorescence above H_2O-tattooed skin (Figure 15, right column). In contrast, siRNA-control-fluorescein tattooed with “Lipofectamin 2000” or “MIKA-Nuclehicle-Kit” was visible in the epidermis and dermis.

4.2.3 Knocking-down HPV8-E6 expression in HPV8-CER mice by tattooing gene specific siRNA

The most promising method to deliver siRNA into mouse skin was tattooing in cooperation with “Lipofectamin 2000”. In initial tests it was demonstrated that simply tattooing HPV8-CER mouse skin induced papilloma development within two to three weeks, similar to tape-stripping. It was now tested, if this tattooing-induced papillomatosis could be reduced or even inhibited by tattooing HPV8-E6 specific siRNAs into HPV8-CER mouse skin. Ten HPV8-CER mice were tattooed with H_2O and siRNA-HPV8E6-1(10 µM)/”Lipofectamin 2000”-mix on separate shaved and depilated skin areas at the same time as described in 3.6.2, thereby inducing 20 wounds respectively. Images of tattooed skin were taken at different points in time. The wounds caused by tattooing H_2O or siRNA/”Lipofectamin 2000” (Figure 16a) started to heal and the skin seemed to be restored around day ten after treatment. On day 18 after tattooing apparent differences between the two approaches were macroscopically visible. While there was only a small cicatricial spot in the siRNA-HPV8E6-1 tattooed skin, papilloma development already started in the H_2O treated area. Although on day 28 papilloma development was also visible on some wounds of the siRNA-HPV8E6-1 tattooed area it was remarkably slowed down and reduced. On day 42 a few siRNA-HPV8E6-1 tattooed areas still showed no papillomatosis and the emerged papillomas remained small and locally restricted to the wound areas in contrast to the pronounced papillomas in the H_2O treated areas, which overgrew the wound areas. The Kaplan-Meier curves in figure 16b show a delay of ten days in papilloma development between wounds induced by tattooing siRNA-HPV8E6-1 and H_2O into HPV8-CER mouse skin. The most prominent observation was that papillomatosis was detected in 15 out of 20 wounds (75%) induced by tattooing H_2O, while only 9 out of 20 wounds (45%) induced by tattooing siRNA-HPV8E6-1 developed papillomas.


a
H2O
siRNA
1d after tattooing
42d after tattooing
Mouse A
Mouse B
Mouse C
Mouse D

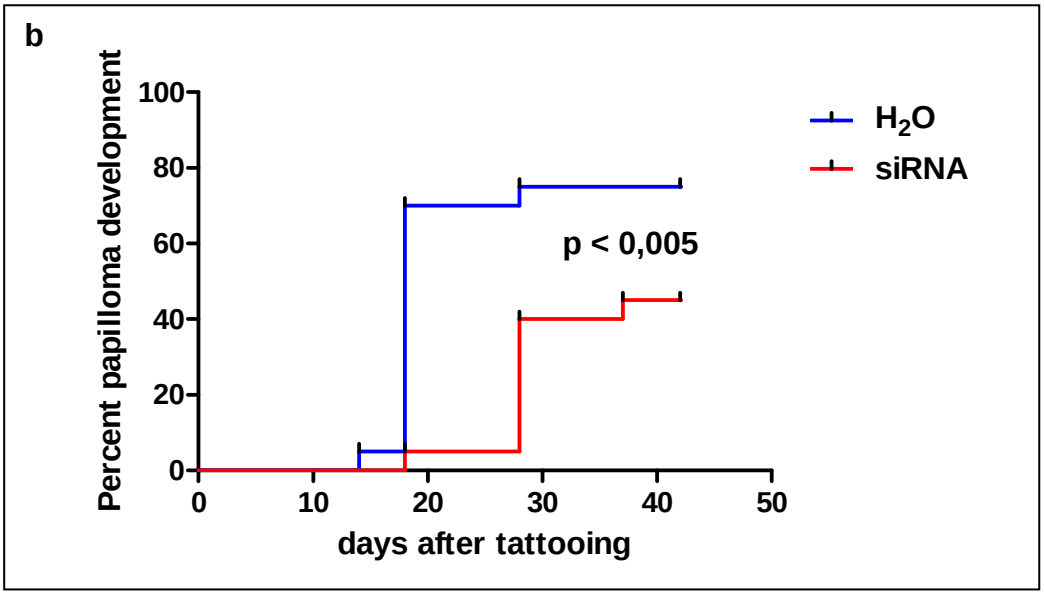


Figure 16: Tattoing HPV8-E6 specific siRNA slowed down papillomatosis in HPV8-CER mice. Ten HPV8-CER mice were shaved and depilated. Afterwards 20 µl H_2O and 20 µl of the transfection-mix siRNA-HPV8E6-1(10 µM)/Lipofectamin 2000 was tattooed into separate areas of the mouse skin as described in 3.6.2. **(a)** Images show tattooed skin areas of four mice one day and 42 days after tattooing. H_2O was tattooed cranial siRNA-HPV8E6-1 caudal. **(b)** Kaplan-Meier curves showing the percentage of papilloma development of 20 H_2O- and 20 siRNA-tattoed HPV8-CER skin areas plotted against time.

4.3 Cellular miRNA expression in HPV8 expressing cells

4.3.1 MiRNA expression levels in healthy skin of FVB/N wt and HPV8-CER mice

As described in 1.8.3 miRNAs are important regulators of cellular gene expression. Their deregulated expression has recently been associated with several types of cancer (Croce and Calin 2005), but also with viral infections for example HPV16 and hepatitis C virus (Wang *et al.* 2008; Braconi *et al.* 2010). To get a first insight into a possibly deregulated miRNA population mediated by HPV8, a comparative miRNA microarray (LC Sciences, Houston, USA) was performed with total RNA from non-lesional skin of HPV8-CER and FVB/N wt mice.

miRNA	FVB/N wt signal	HPV8-CER signal	ratio
mmu-miR-7	1	140,2	140,2
mmu-miR-17-5p	368,6	754,4	2,05
mmu-miR-20b	88,2	300,2	3,4
mmu-miR-21	7672,2	17313,4	2,26
mmu-miR-31	252,7	573,1	2,27
mmu-miR-106a	6,5	94,3	14,51
mmu-miR-133a*	131,7	17,4	0,13
mmu-miR-133a	1737,3	454,3	0,26
mmu-miR-133b	1599,6	444,9	0,28
mmu-miR-141	197,8	54	0,27
mmu-miR-145	3767,7	1351	0,36
mmu-miR-146b	480,1	1221,9	2,55
mmu-miR-150	473,7	194	0,41
mmu-miR-155	73,7	381	5,17
mmu-miR-181b	271	750,2	2,77
mmu-miR-200a	6898	1716,5	0,25
mmu-miR-206	25941,3	8176,4	0,32
mmu-miR-207	41,1	209	5,09
mmu-miR-218	19,3	165,5	8,58
mmu-miR-222	708,7	289,8	0,41
mmu-miR-224	36	121,3	3,37
mmu-miR-290	16,4	78,2	4,77
mmu-miR-346	154,7	611,9	3,96
mmu-miR-374-5p	11,1	116,3	10,48
mmu-miR-422b	726,2	294,3	0,41
mmu-miR-467a	100,1	365,6	3,65

mmu-miR-467b	165,4	841,8	5,09
mmu-miR-468	16,1	112,8	7,01
mmu-miR-652	205,1	72,3	0,35
mmu-miR-671	183,7	348,4	1,90
mmu-miR-674-5p	145	374	2,58
mmu-miR-680	45,3	276,1	6,09
mmu-miR-689	695,3	1974,5	2,84
mmu-miR-705	1503,4	3784,2	2,52
mmu-miR-721	1	70,9	70,90

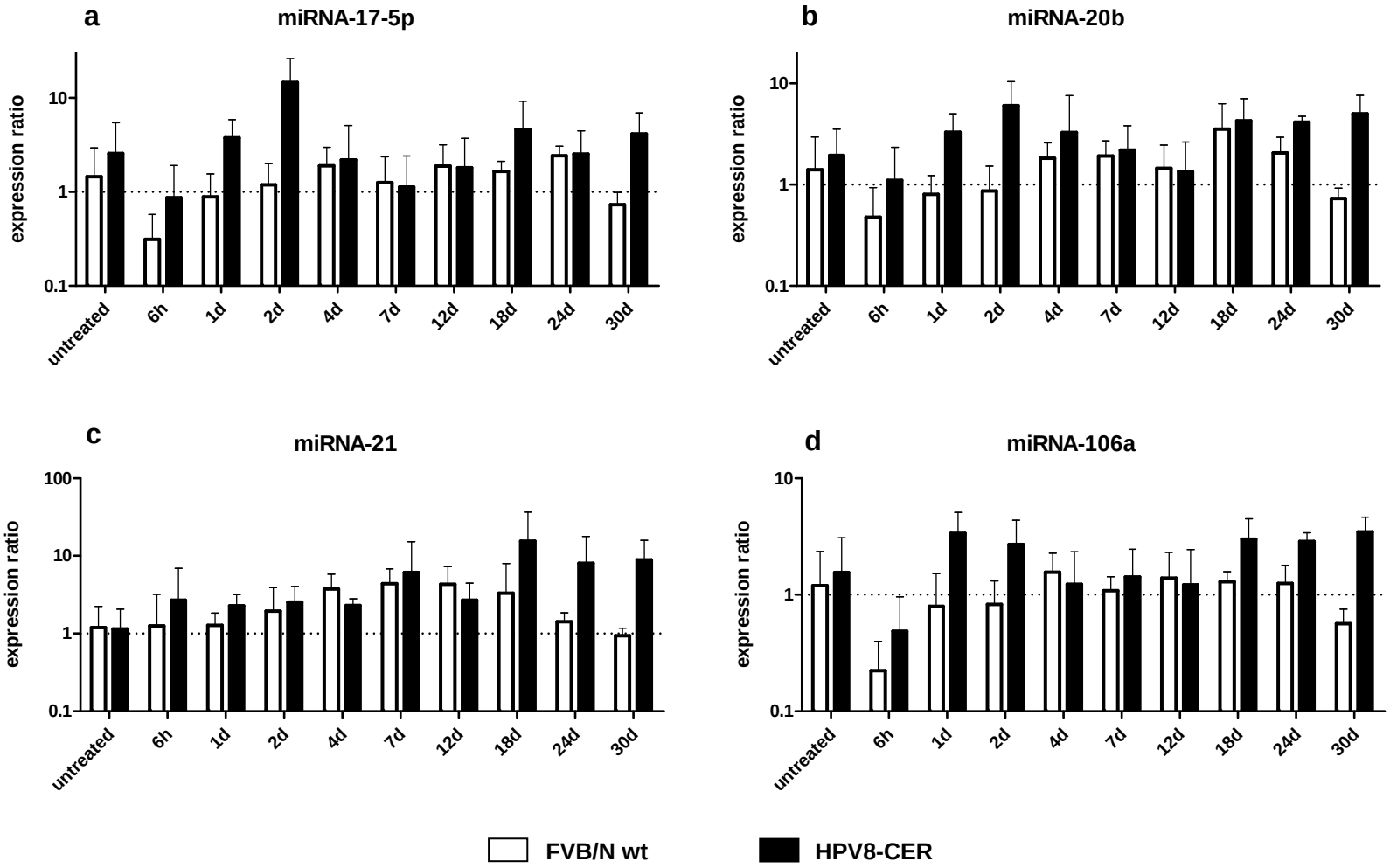
Table 2: 35 miRNAs were found to be more than two-fold deregulated in their expression in healthy skin of HPV8-CER mice compared to FVB/N wt mice.
Five microgram total RNA from healthy skin of one FVB/N wt and one HPV8-CER mouse were used to perform a comparative miRNA microarray (LC Sciences, Houston, USA). Signal intensity was measured in triplicate. Table shows mean intensity and HPV8-CER:FVB/N wt ratio.

Out of 358 tested miRNAs, 129 were found to be expressed in the FVB/N wt skin and 249 in the skin of HPV8-CER mice (data not shown). However, many of the detected miRNAs in HPV8-CER mouse skin were only slightly above the background noise and were therefore not considered. 35 miRNAs (Table 2) were found to be more than two-fold deregulated in their expression in the skin of HPV8-CER mice compared to FVB/N wt skin (24 upregulated and 11 downregulated). Many of these miRNAs showed low signal intensities indicating low level of these miRNAs in untreated skin, which makes it difficult to perform an exact measurement and subsequent evaluation. Based on this primary search the transcription kinetics of 30 miRNAs were determined in FVB/N wt and HPV8-CER mice after UV-irradiation, to check if the expression of certain miRNAs becomes altered during UVA/B-induced papilloma development and if this expression correlates with the rising HPV8 transgene expression. These 30 miRNAs were selected either because of their known involvement in carcinogenic processes or their prominent deregulation in HPV8-CER mice compared to FVB/N wt mice according to the microarray data.

In HPV8-CER mice miRNA-17-5p (Figure 17a) and miRNA-20b (Figure 17b) showed a moderately higher expression level than in FVB/N wt mice both in untreated skin and 6h, 1d and 2d after irradiation. In both mouse strains the total expression dropped six hours after UVA/B but rose again until day two after UVA/B-irradiation, in the case of HPV8-CER mice higher than in untreated skin. The remaining points in time were quite similar in FVB/N wt and HPV8-CER mice, except for day 30, where expression was again enhanced in HPV8-CER mice. In both mouse strains the expression of miRNA-21 (Figure 17c) rose until day 12 after UVA/B-irradiation.

However, starting on day 18 the expression returned to a basal level in FVB/N wt mice, whereas it persisted at an upregulated level in HPV8-CER mice. MiRNA-106a (Figure 17d) showed a similar expression profile like miRNA-20b, which are both members of the same miRNA cluster. But the difference between the enhanced expression in HPV8-CER mice compared to the unaltered or reduced expression in FVB/N wt mice on day 18, 24 and 30 after UVA/B-irradiation was more distinct for miRNA-106a. For miRNA-155 (Figure 17e) the expression was already lower in untreated HPV8-CER mouse skin compared to FVB/N wt mouse skin. This reduction of expression was maintained at all observed points in time after UVA/B-irradiation. The expression of miRNA-206 (Figure 17f) started to drop two days after UVA/B-irradiation in both mouse strains and was further decreased on day 18, 24 and 30. However, at all points in time after day seven miRNA-206 was lower in HPV8-CER mice than in FVB/N wt mice. A more drastic effect was observed for miRNA-374-5p (Figure 17g). Until day one after UVA/B-irradiation the expression was higher in HPV8-CER than in FVB/N wt mice. Starting from day two the expression decreased in HPV8-CER mice, significantly on day 30, while it stayed at basal level in FVB/N wt mice. Except for the untreated skin, the expression of miRNA-689 (Figure 17h) was at all points in time in HPV8-CER mice lower than in FVB/N wt mice, especially at late points in time.

These results demonstrate that the enhanced HPV8 oncogene expression after UVA/B-irradiation is paralleled by an expression deregulation of certain miRNAs.


a
miRNA-17-5p
b
miRNA-20b
c
miRNA-21
d
miRNA-106a
expression ratio
100
10
1
0.1
untreated
6h
1d
2d
4d
7d
12d
18d
24d
30d
FVB/N wt
HPV8-CER

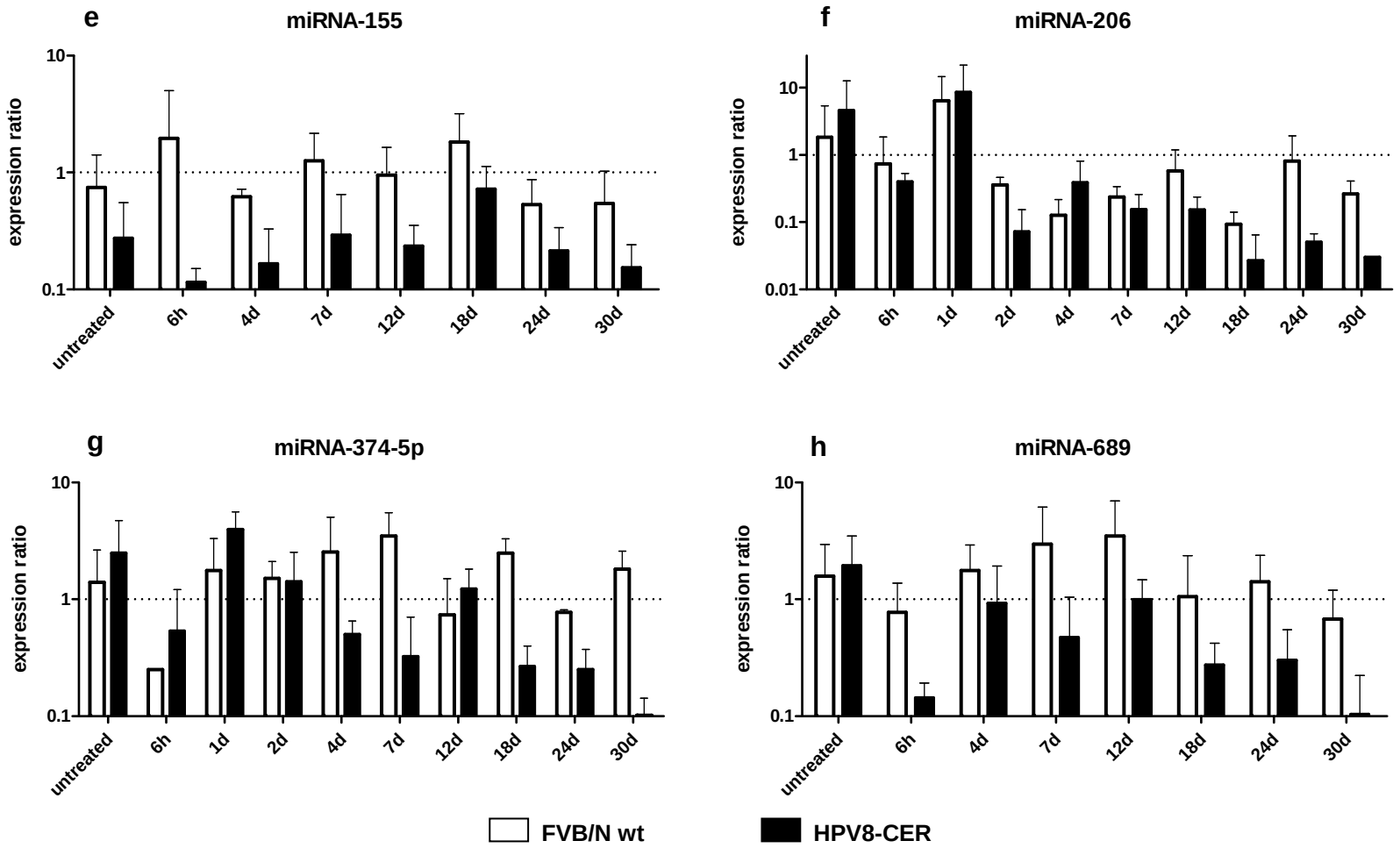
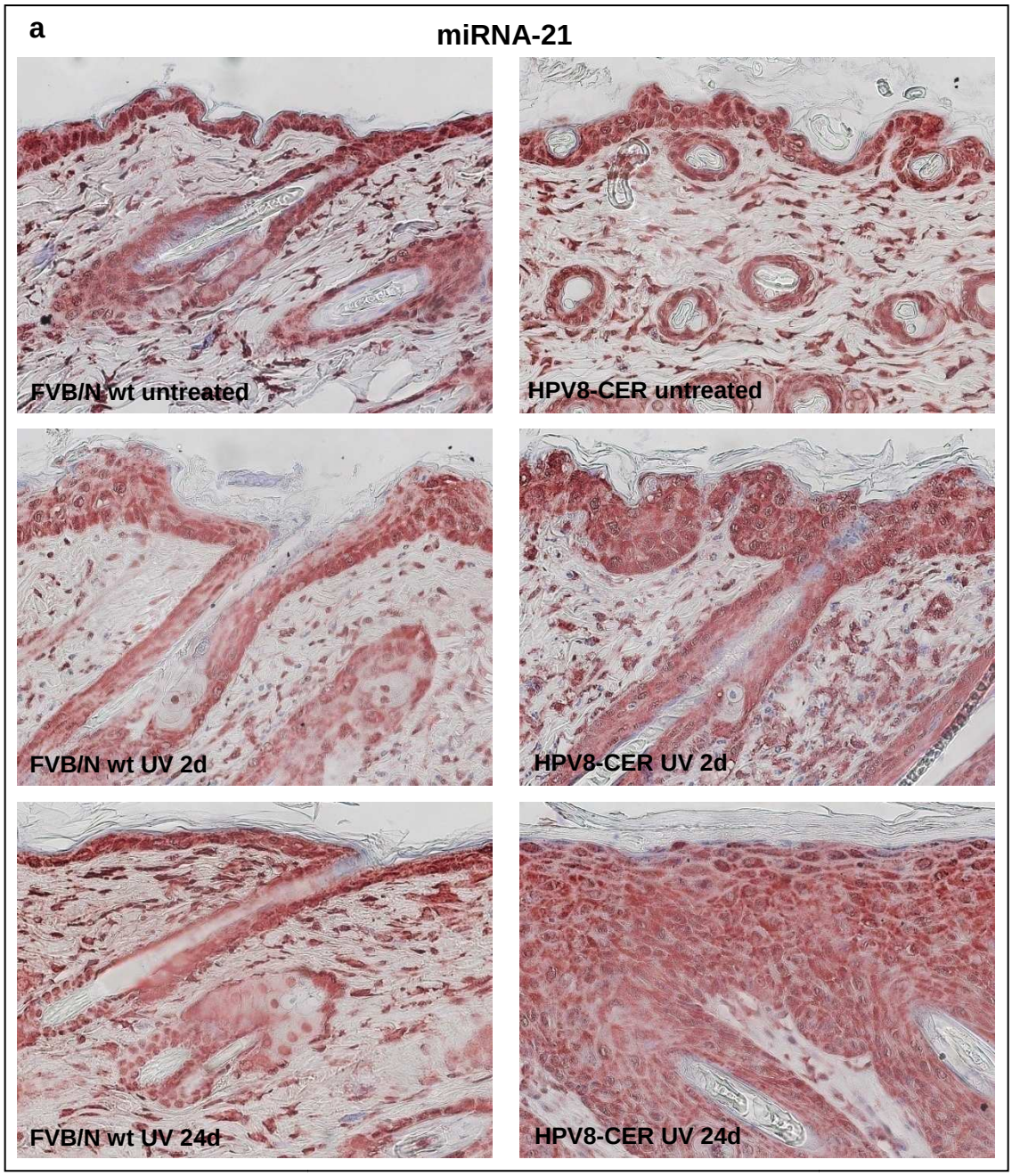
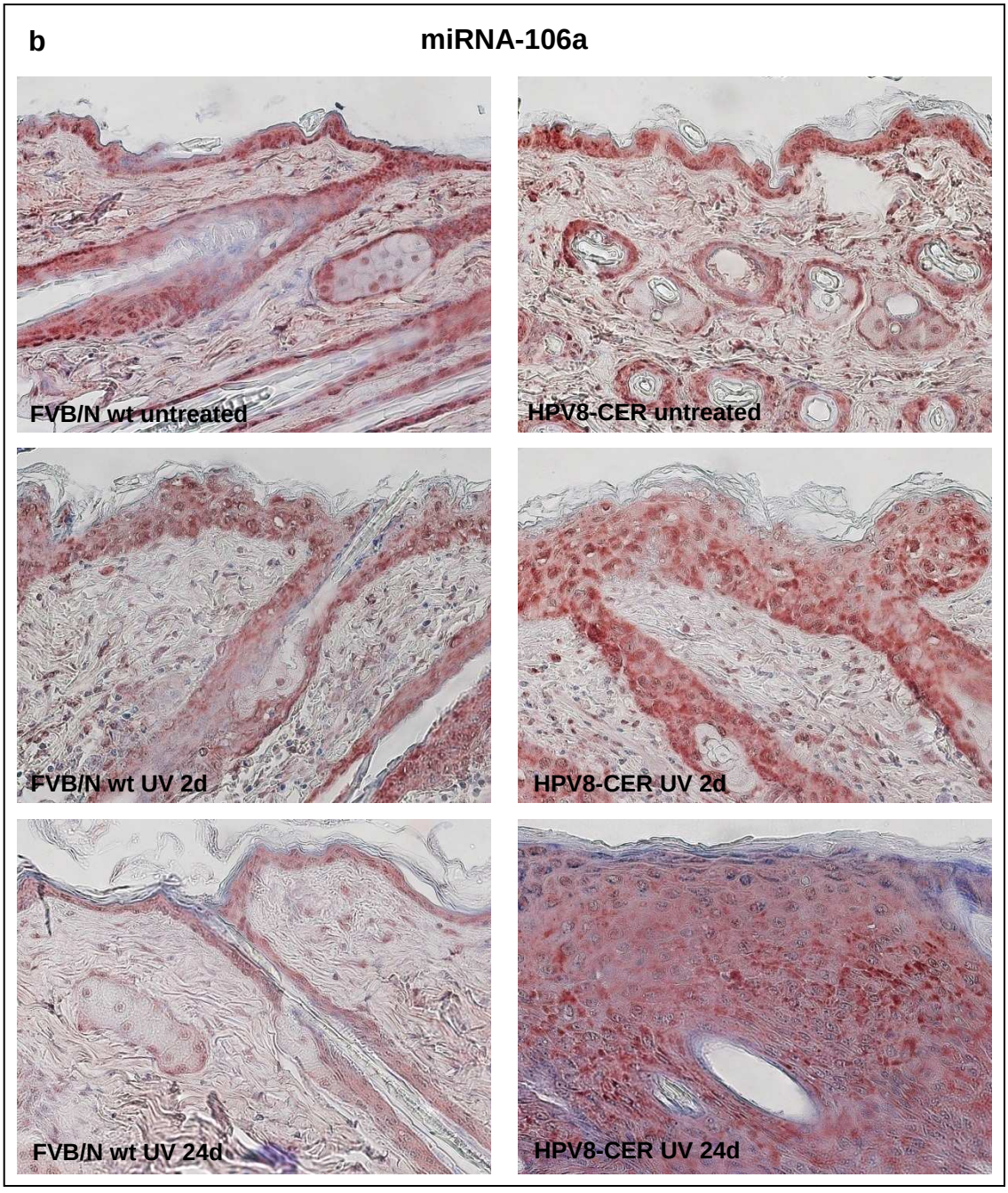


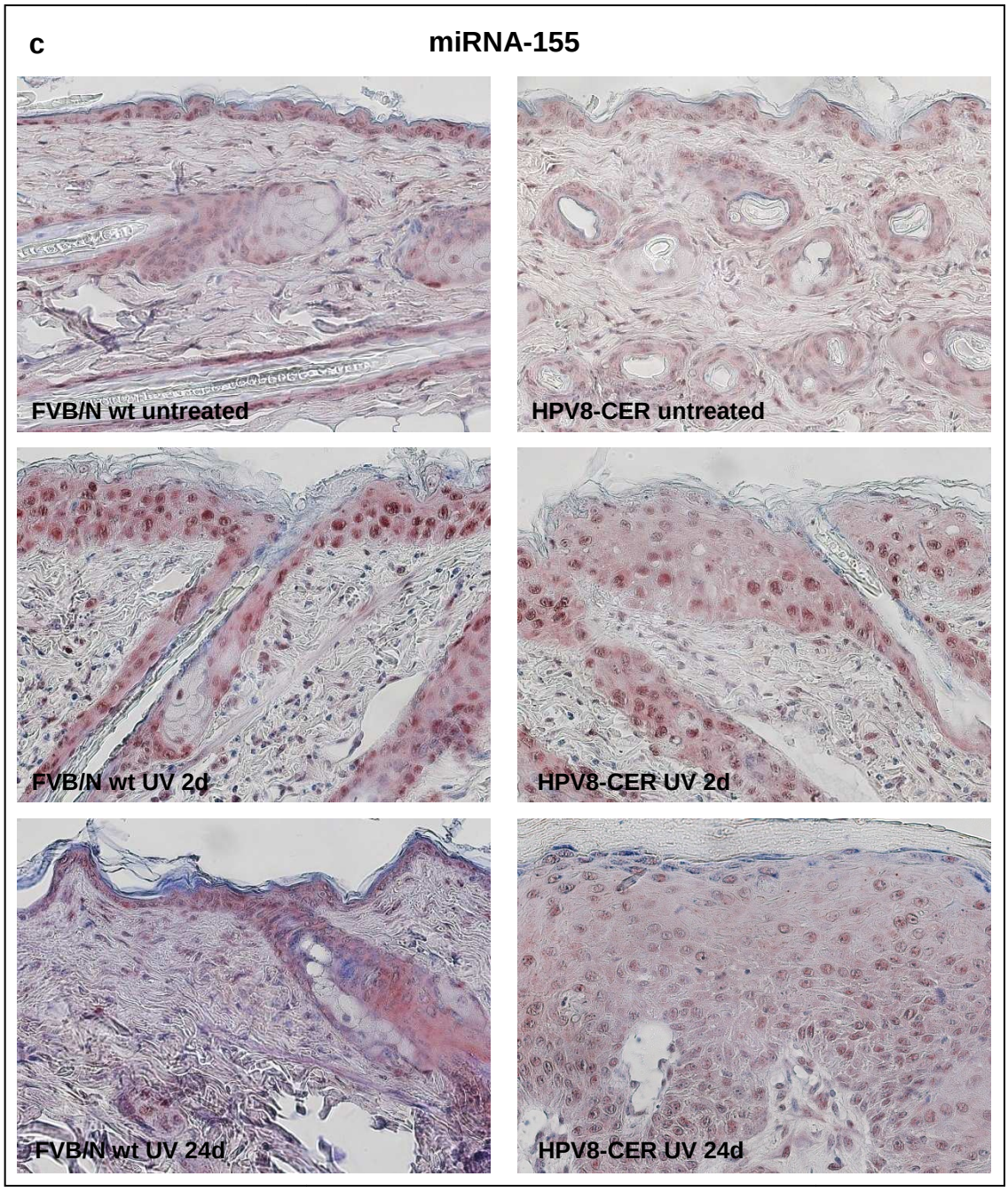
Figure 17a-h: Expression kinetics of deregulated miRNAs in UVA/B-irradiated HPV8-CER and FVB/N wt mice.
Back skin areas (4 cm^2) of shaved HPV8-CER and FVB/N wt mice were irradiated with 10 J/cm^2 UVA and 1 J/cm^2 UVB. Skin punch biopsies of three to six mice were taken at different points in time after UVA/B-irradiation out of the irradiated skin. Additionally skin biopsies from untreated skin of these mice were taken. MiRNA expression levels were measured in one microgram of reverse transcribed total cellular RNA by qRT-PCR and normalized to miRNA-127. The results were further normalized to the median expression of the respective miRNA in untreated FVB/N wt skin. The mean expression ratio of different miRNAs is plotted against time. Error bars represent standard deviation.

4.3.2 Localization of deregulated miRNA-21, -106a, -155, -206 in the skin of HPV8-CER and FVB/N wt mice

The qRT-PCR results (Figure 12c, d, e, f) point to a deregulation of the expression of miRNA-21, -106a, -155 and -206. To further characterize their localization in the epidermal cell layers, ISH experiments were performed on untreated and UVA/B-irradiated FVB/N wt and HPV8-CER mouse skin.




b
miRNA-106a
FVB/N wt untreated
HPV8-CER untreated
FVB/N wt UV 2d
HPV8-CER UV 2d
FVB/N wt UV 24d
HPV8-CER UV 24d


c
miRNA-155
FVB/N wt untreated
HPV8-CER untreated
FVB/N wt UV 2d
HPV8-CER UV 2d
FVB/N wt UV 24d
HPV8-CER UV 24d

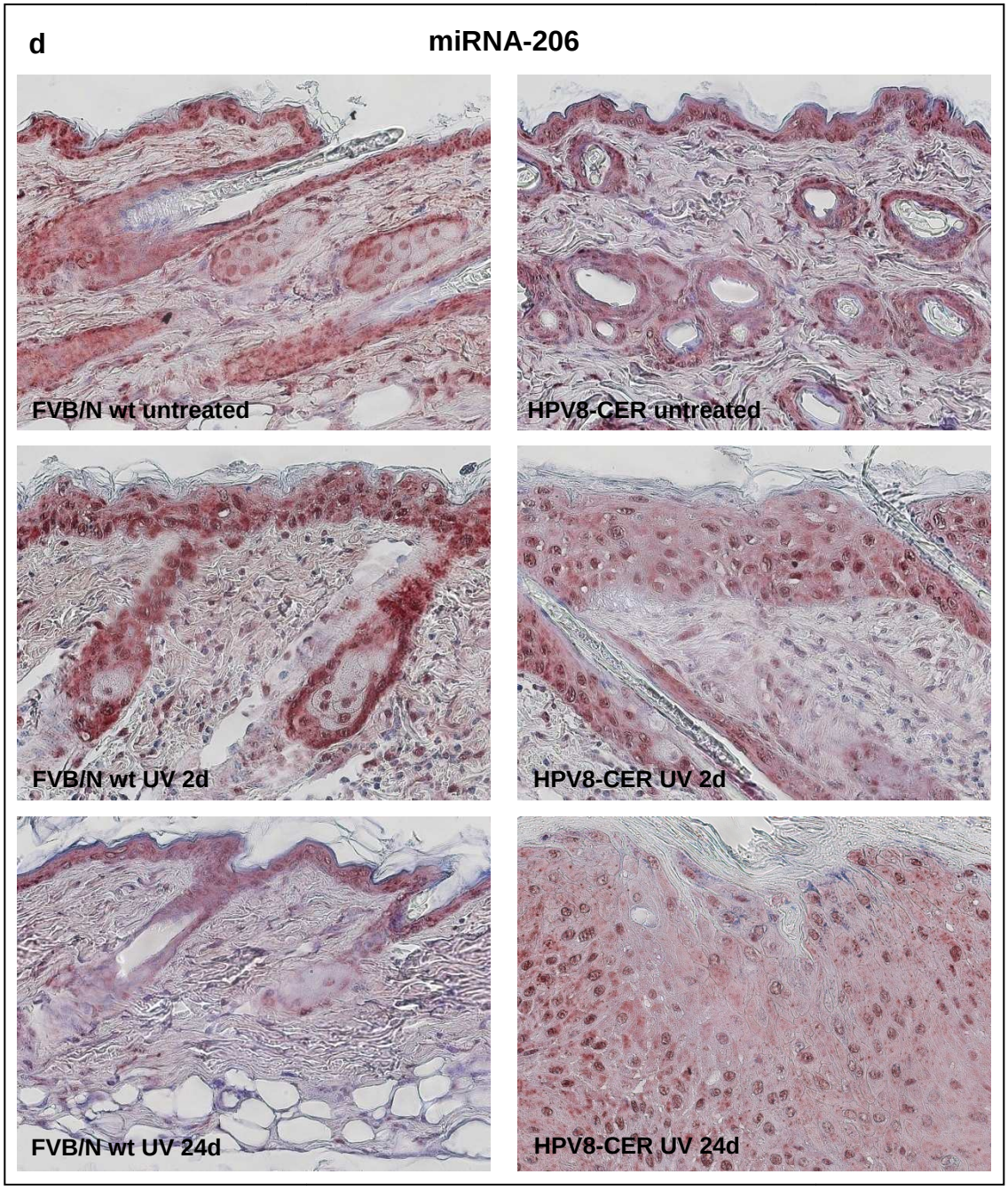


Figure 18: Localization of miRNA-21, -106a, -155 and -206 in FVB/N wt and HPV8-CER mice after UVA/B-irradiation.
Dorsal skin areas (4 cm^2) of shaved HPV8-CER and FVB/N wt mice were irradiated with 10 J/cm^2 UVA and 1 J/cm^2 UVB. Skin biopsies were taken from two HPV8-CER and two FVB/N wt mice out of the irradiated skin 2 and 24 days after UVA/B-irradiation. Additionally skin biopsies were also taken from two non-irradiated HPV8-CER and FVB/N wt mice, respectively. After formalin-fixation and paraffin embedding, samples were cut into sections. ISH against **(a)** miRNA-21, **(b)** miRNA-106a, **(c)** miRNA-155 and **(d)** miRNA-206 were performed. Sections were counterstained with hematoxylin. Magnification: 400x.

There was no difference in miRNA-21 expression pattern in untreated skin of HPV8-CER and FVB/N wt mice. Both mouse lines showed a panepithelial staining and signals in the dermal compartment (Figure 18a). On day 2 and 24 after UVA/B-irradiation the expression was enhanced in HPV8-CER mice compared to FVB/N wt animals. MiRNA-106a showed overall a more cytoplasmatic expression (Figure 18b). Similar to miRNA-21, miRNA-106a showed no difference between the expression pattern of non-lesional HPV8-CER and FVB/N wt mouse skin. While the expression was weaker in FVB/N wt mice 2 and 24 days after UVA/B-irradiation it was generally enhanced in HPV8-CER mice but gradually decreased in the uppermost differentiated cell layers. MiRNA-155 showed an overall low expression. In untreated skin of HPV8-CER mice miRNA-155 expression was slightly lower than in untreated skin of FVB/N wt mice (Figure 18c). Two days after UVA/B-irradiation it was enhanced in FVB/N wt mice, while it was reduced in HPV8-CER mice, particularly in the suprabasal cells of the epidermis. Twenty-four days after irradiation miRNA-155 signals were reduced in epidermal cells and almost exclusively nuclear in HPV8-CER mice compared to FVB/N wt mice, where also cytoplasmatic signals were visible. A slight reduction was also observed in dermal cells of HPV8-CER mice compared to FVB/N wt mice. The expression of miRNA-206 was equivalent in untreated skin of both mouse strains (Figure 18d). There was an increase of miRNA-206 expression detectable two days after UVA/B-irradiation in FVB/N wt mice, which decreased 24 days after UVA/B-irradiation, but was still higher than in HPV8-CER mice, with predominantly cytoplasmatic localization. In contrast, in HPV8-CER mice a decreased and primarily nuclear miRNA-206 expression was found on day two and 24 after UVA/B-irradiation.

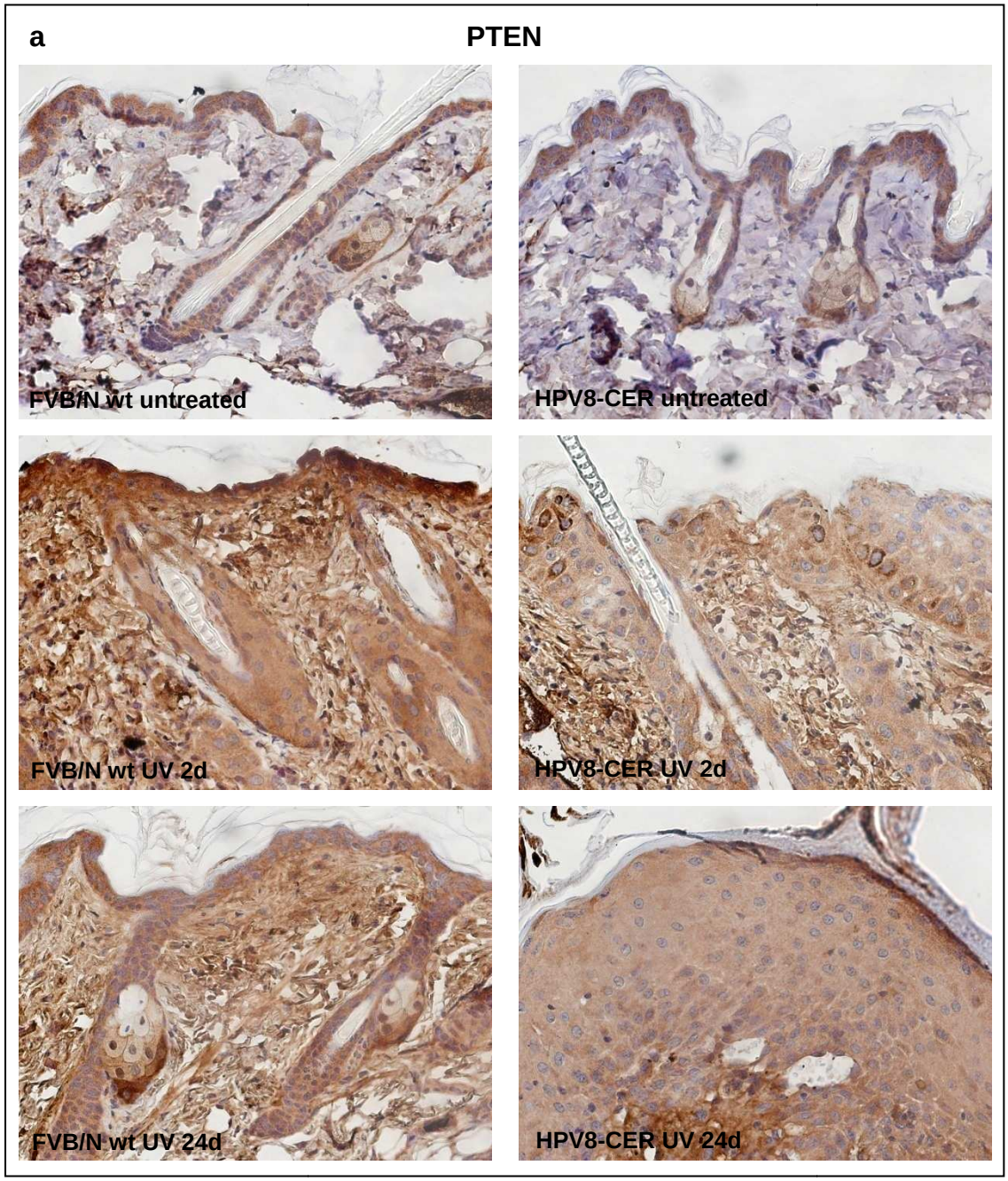
Overall, the ISH data confirm the qRT-PCR results and therefore further confirms the notion of deregulated miRNAs in HPV8 oncogene expressing cells.

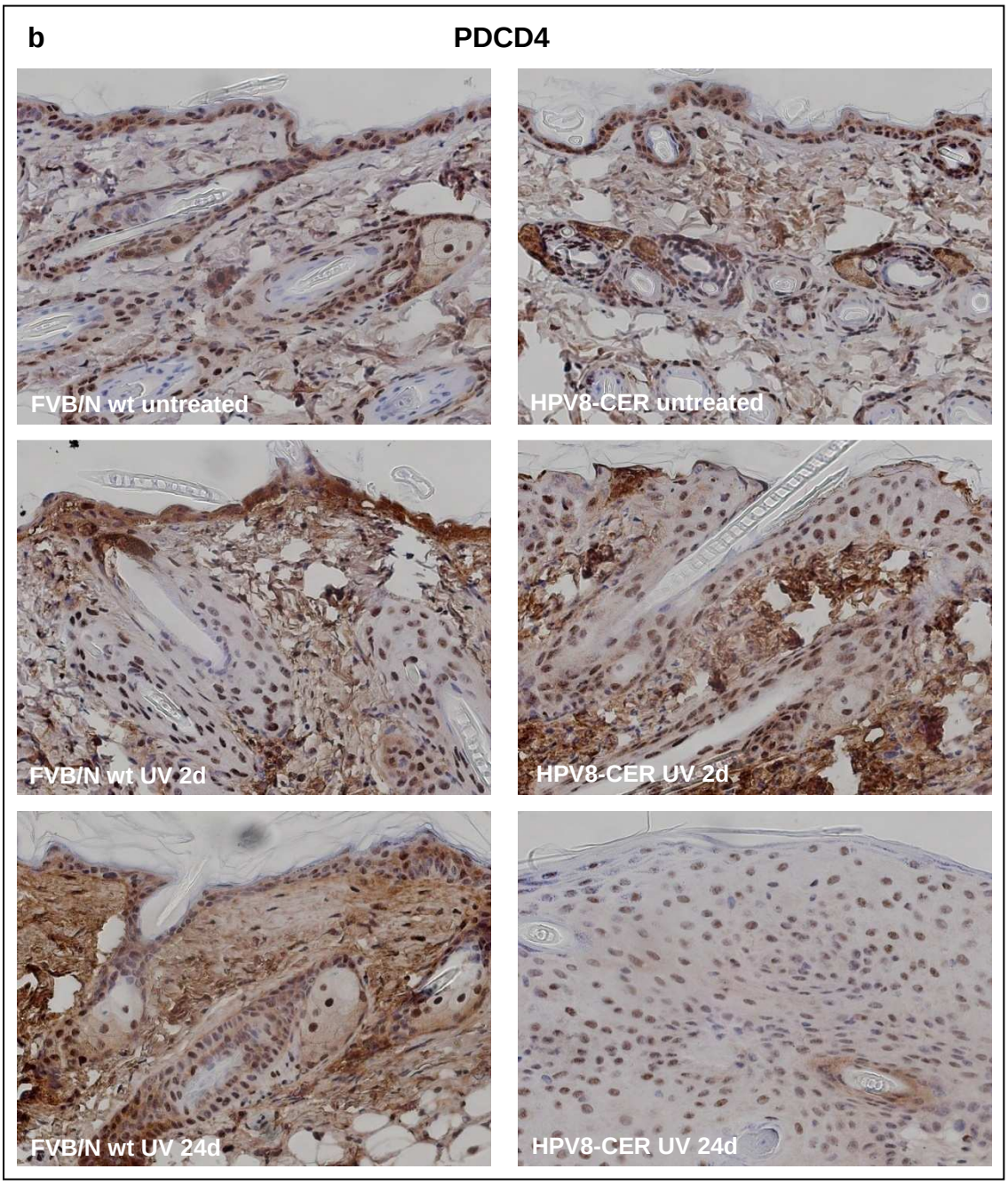
4.3.3 Expression alterations of cellular targets of deregulated miRNAs in HPV8-CER mice

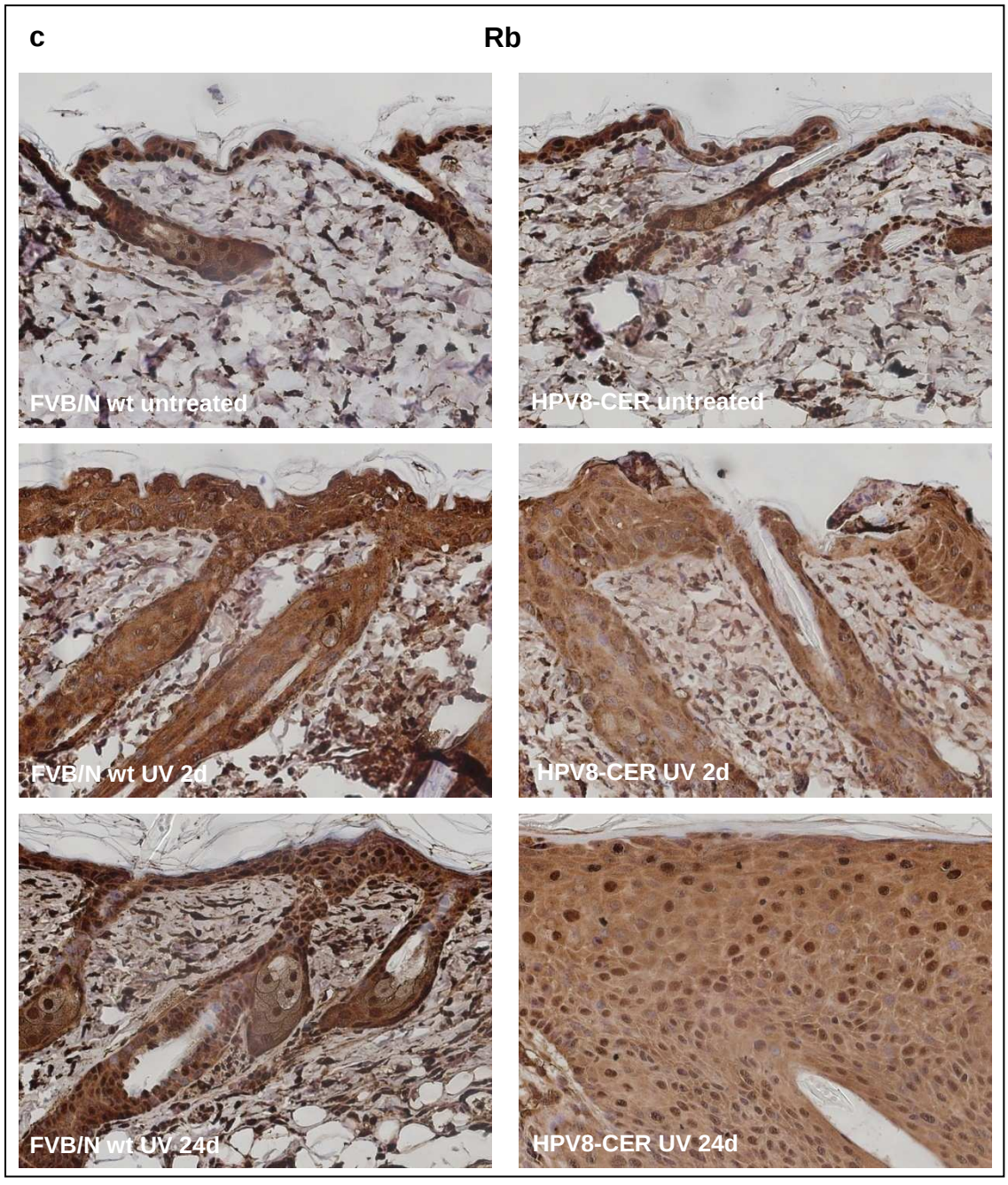
To test, whether deregulated miRNAs observed in HPV8-CER mice by qRT-PCR and ISH actually affected the expression of their respective cellular targets IHC stainings were performed. MiRNA-17-5p and miRNA-21 are both able to downregulate the

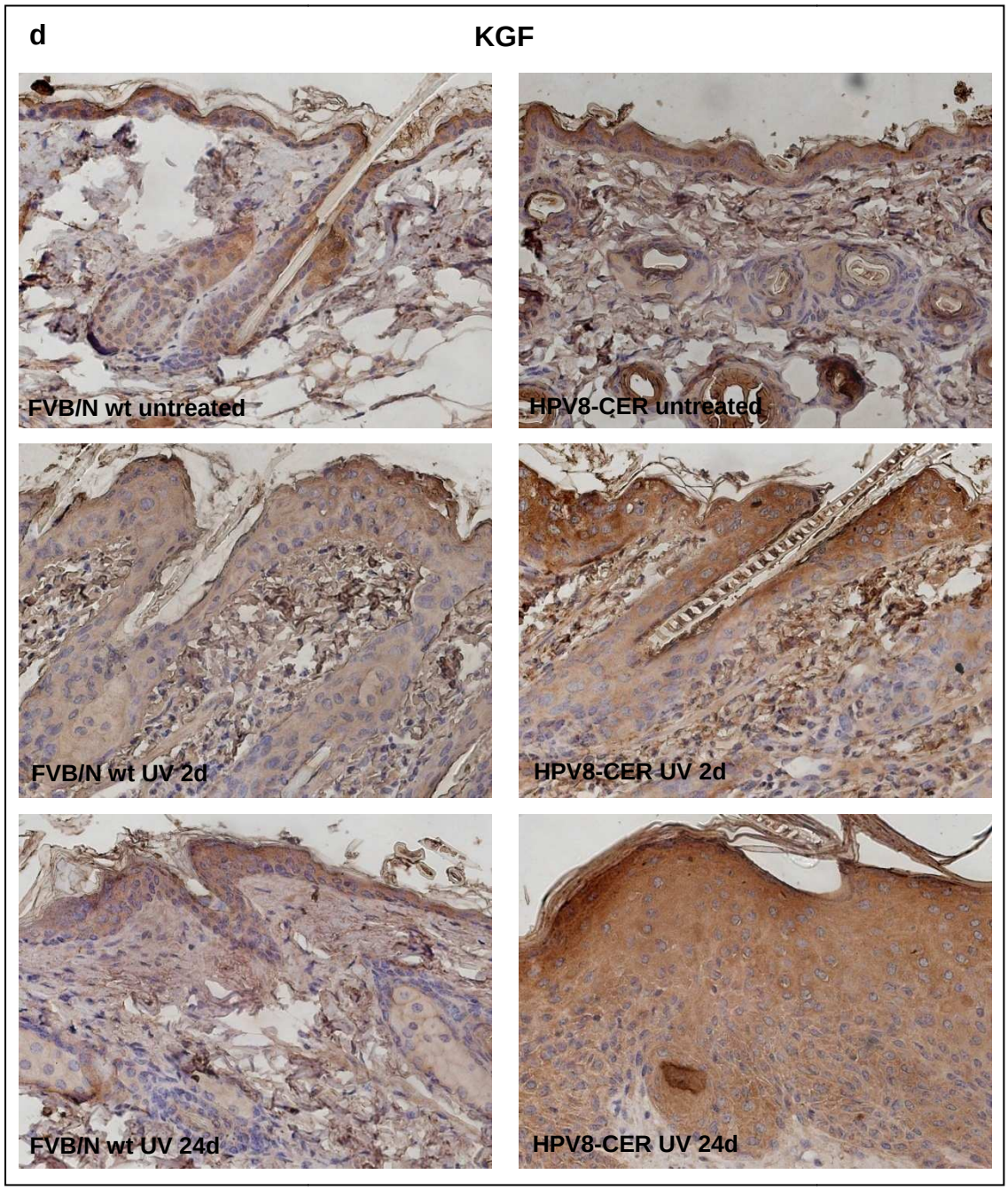
phosphatase and tensin homolog (PTEN) (Lewis *et al.* 2003; Meng *et al.* 2007). Since these two miRNAs were upregulated in HPV8-CER mice after UVA/B-irradiation in contrast to FVB/N wt animals (Figure 17a, c) the protein level of PTEN was expected to be downregulated in HPV8-CER mice. There was no difference in the PTEN protein level between untreated FVB/N wt and HPV8-CER mouse skin. Both showed distinct signals in the epidermis and hair follicles (Figure 19a). Two days after UVA/B-irradiation the PTEN level was enhanced in FVB/N wt mice whereas it was reduced in HPV8-CER mouse skin. This expression pattern was preserved 24 days after UVA/B-irradiation in both mouse lines. PTEN was detected in the cytoplasm at all points in time in both mouse strains. Another target of miRNA-21 is the tumor suppressor programmed cell death 4 (PDCD4) (Frankel *et al.* 2008), which was found in the nucleus and in the cytoplasm in epidermal cells of untreated skin of FVB/N wt and HPV8-CER mice (Figure 19b). Two days after UVA/B-irradiation the staining was more nuclear in HPV8-CER mice compared to FVB/N wt mice. Remarkably, 24 days after irradiation, PDCD4 was exclusively nuclear and reduced in HPV8-CER mice, while it returned to its basal level with nuclear and cytoplasmatic localization in FVB/N wt mice. For the tumor suppressor Rb, a known cellular target for miRNA-106a (Volinia *et al*. 2006), a pronounced expression was detected in the cytoplasm and the nucleus of untreated skin of FVB/N wt and HPV8-CER mice (Figure 19c). Two and twenty-four days after UVA/B-irradiation, where miRNA106a expression is upregulated in HPV8-CER mice (Figure 17d), Rb expression is reduced, especially in the lower epidermal cell layers of these mice. At the same points in time staining was intensive in FVB/N wt mice. It was shown that miRNA-155 downregulates the expression of the keratinocyte growth factor (KGF), a member of the fibroblast growth factor family (Pottier *et al*. 2009). Therefore the KGF expression was expected to be upregulated in HPV8-CER mice since miRNA-155 levels were reduced (Figure 17e). KGF expression in HPV8-CER rose after UVA/B-irradiation in a differentiation-dependant manner showing a higher expression in the uppermost differentiated cells. In FVB/N wt mice the KGF expression was not changed after UVA/B-irradiation (Figure 19d). In both mouse strains KGF was detected exclusively in the cytoplasm for all points in time. Recently, it could be shown that miRNA-206 downregulates the proto-oncogene mesenchymal-epithelial transition factor (MET) also known as hepatocyte growth factor receptor (Yan *et al*. 2009). The decrease of miRNA-206 in HPV8-CER mice starting two days after

UVA/B-irradiation (Figure 17f) should therefore lead to an enhanced expression of MET. In untreated skin of FVB/N wt and HPV8-CER mice no difference in MET expression was detected, both mice showed cytoplasmatic staining in the epidermis and hair follicles (Figure 19e). In FVB/N wt mice this staining was not remarkably changed during UVA/B-irradiation. However, along with a downregulation of miRNA-206 expression in HPV8-CER mice an upregulation of cytoplasmatic MET became evident in the epidermis and hair follicles after UVA/B-irradiation, which exceeded the MET level detected in irradiated FVB/N wt mice.




b
PDCD4
FVB/N wt untreated
HPV8-CER untreated
FVB/N wt UV 2d
HPV8-CER UV 2d
FVB/N wt UV 24d
HPV8-CER UV 24d


c
Rb
FVB/N wt untreated
HPV8-CER untreated
FVB/N wt UV 2d
HPV8-CER UV 2d
FVB/N wt UV 24d
HPV8-CER UV 24d


d
KGF
FVB/N wt untreated
HPV8-CER untreated
FVB/N wt UV 2d
HPV8-CER UV 2d
FVB/N wt UV 24d
HPV8-CER UV 24d

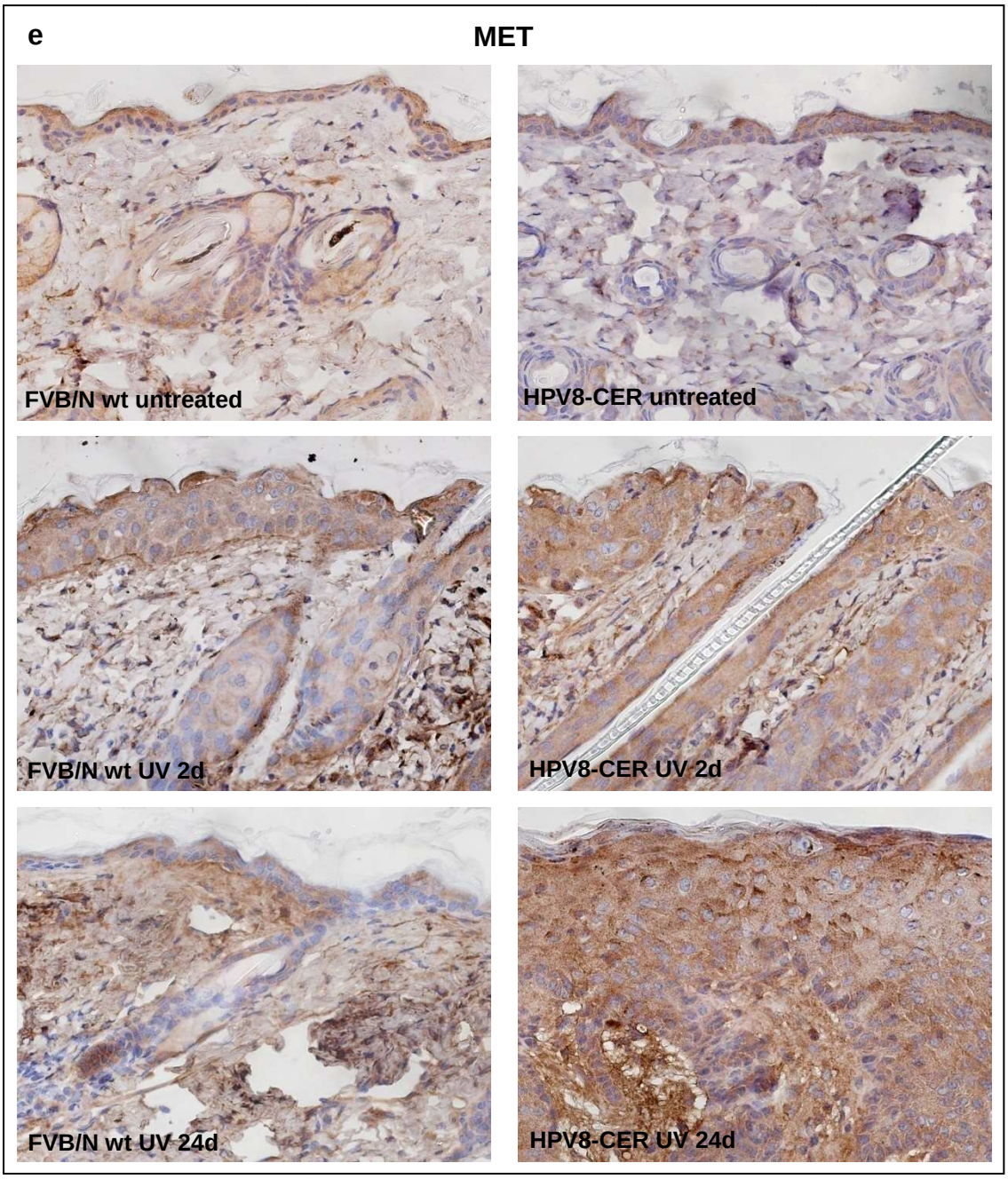


Figure 19: Confirmation of expression alteration of cellular miRNA targets in HPV8-CER and FVB/N wt mice after UVA/B-irradiation.
Dorsal skin areas (4 cm^2) of shaved HPV8-CER and FVB/N wt mice were irradiated with 10 J/cm^2 UVA and 1 J/cm^2 UVB. Skin biopsies were taken from two HPV8-CER and two FVB/N wt mice out of the irradiated skin 2 and 24 days after UVA/B-irradiation. Additionally skin biopsies were also taken from two non-irradiated HPV8-CER and FVB/N wt mice, respectively. After formalin-fixation and paraffin embedding, samples were cut into sections. Sections were marked against **(a)** PTEN, **(b)** PDCD4, **(c)** Rb, **(d)** KGF, **(e)** MET and counterstained with hematoxylin. Magnification: 400x.

4.3.4 HPV8-E6 mice show similar miRNA expression deregulations after UVA/B-irradiation as HPV8-CER mice

To compare the UVA/B-induced miRNA expression alterations of HPV8-CER mice with the miRNA expression of HPV8-E6 mice, known to have a similar tumor phenotype, punch biopsies were taken out of the irradiated and non-irradiated skin of HPV8-E6 animals 7 and 24 days after treatment.

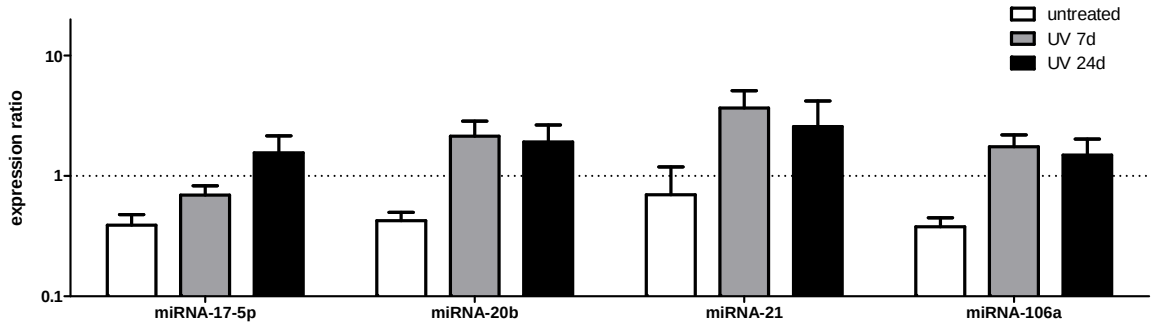


Figure 20: HPV8-E6 mice showed similar miRNA expression deregulations as HPV8-CER mice. Back skin areas (4 cm^2) of shaved HPV8-E6 mice were irradiated with 10 J/cm^2 UVA and 1 J/cm^2 UVB. Skin punch biopsies were taken 7 and 24 days after UVA/B-irradiation from four mice each, out of the irradiated and non-irradiated skin. MiRNA expression levels were measured in one microgram of reverse transcribed total cellular RNA by qRT-PCR and normalized to miRNA-127. The results were further normalized to the median expression of the respective miRNA in untreated FVB/N wt skin. The mean expression ratio of different miRNAs is plotted against time. Error bars represent standard deviation.

In untreated skin of HPV8-E6 mice the expression of miRNA-17-5p (Figure 20) was lower than in FVB/N wt or HPV8-CER mice. On day seven after UVA/B-irradiation expression started to increase and was further enhanced on day 24 after UVA/B-irradiation similar to HPV8-CER mice. Expressions of miRNA-20b and -106a were reduced in untreated skin of HPV8-E6 mice compared to HPV8-CER or FVB/N wt mice, but they were enhanced on day 7 and 24 after UVA/B-irradiation just like in HPV8-CER mice. A similar miRNA-21 expression profile was measured in untreated and UVA/B-irradiated skin of HPV8-E6 and -CER mice.

4.3.5 The tendency of miRNA alterations in HPV8 transgenic mice is mirrored in HPV8-CER expressing human keratinocytes

To verify the miRNA expression alterations observed in HPV8 transgenic mice in a human system, monolayer cell culture experiments were performed with the human keratinocyte line PM1. These cells were retrovirally infected with viruses coding for the empty retroviral vector pLXSN or for the complete early region of HPV8 pLXSN-8-CER. Total RNA was isolated from drug selected stable cells and miRNA expression levels were measured by qRT-PCR.

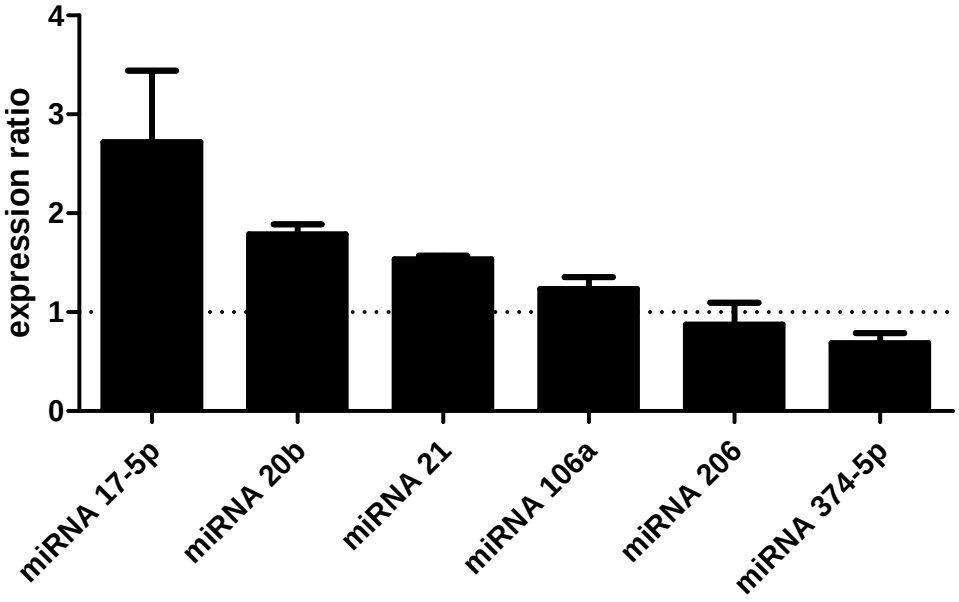


Figure 21: The miRNA expression alterations measured in UV-irradiated HPV8-CER mice were also observed in the human keratinocyte cell line PM1-pLXSN8-CER.
Total RNA was isolated from PM1-pLXSN cells and PM1-pLXSN8-CER cells. MiRNA expression levels were measured in one microgram of reverse transcribed total cellular RNA by qRT-PCR and normalized to miRNA-127. The results were further normalized to the expression of the respective miRNA in control cells. Bars represent mean miRNA expression ratios between PM1-pLXSN8-CER cells and PM1-pLXSN cells. Error bars represent standard deviation.

MiRNA-17-5p, -20b, -21 and -106a were upregulated in PM1-pLXSN8-CER cells compared to the control cells, in contrast to miRNA-206 and -374-5p which were unaffected or downregulated (Figure 21). These results are partially consistent with the miRNA data obtained from untreated skin of HPV8-CER mice and highly conformable with the miRNA data obtained from UVA/B-irradiated HPV8-CER mice. Although the alterations of miRNA expression were minor in cell culture, they showed the same tendency, supporting an HPV8-dependent deregulation of miRNA expression in humans.

5 Discussion

The oncogenic potential of HPV8 in the skin of transgenic mouse lines was recently shown (Schaper *et al*. 2005; Marcuzzi 2006; Pfefferle *et al*. 2008). UV-irradiation and mechanical wounding leads to a synchronized papilloma development in these mice within three weeks. However, little is known about the triggers of tumorous skin growth. To clarify the role of skin wounding and its influence on HPV expression in the early stages of tumor development in the HPV8 transgenics, the kinetics and levels of transgene expression were analyzed in response to exogenous skin irritations induced by UVA/B-irradiation or mechanical wounding.

In prevenient experiments the expression levels of the oncogenes HPV8-E2, -E6 and -E7 were found to be equally expressed from the hK14-promoter. Therefore it can be presumed that a deregulation of one of these genes implicates the same deregulation of the other genes. These results are in contrast to a previous study, where it was reported that the E2 transcript level was at least ten times higher than the E6 and E7 levels (Schaper *et al.* 2005). This discrepancy, however, can be explained by the use of different primers for reverse transcription (Stahlberg *et al.* 2004). Whereas Schaper *et al*. (2005) used oligo-dT_{23}-primers, a mixture of oligo-dT_{23}-primers and random nonamers was used in the present study. The long HPV8 transcript, in particular the 5' proximal genes E6 and E7, was obviously more efficiently reverse transcribed by this primer combination. Hence the combination of oligo-dT_{23}-primers and random nonamers was used to measure kinetics of transgene expression in the skin of HPV8 transgenic mice irritated by tape-stripping or UV.

It is known that UV-irradiation, the main risk factor for SCC development (Leiter and Garbe 2008), can induce the cellular K14-promoter (Kinouchi *et al.* 2002) as well as the viral oncogene promoter of HPV5 and 8 (Akgül *et al.* 2005). Therefore, mK14 and HPV8-E6 levels were determined in transgenic and FVB/N wt skin during UV-induced papillomatosis. Irradiation induced enhanced mK14 expression levels in both strains, reaching maximum levels two to four days after induction. However, whereas the level of mK14 mRNA gradually returned to normal starting around day twelve in the FVB/N wt mice, the high level of mK14 mRNA persisted in the transgenics. Analogously to the mK14, the HPV8-E6 expression and thus the HPV8-E2 and -E7 expression were also constantly enhanced starting from day one after skin irritation.

In parallel, hyperplasia induced by UVA/B-irradiation resolved within three weeks in FVB/N wt mice, while the enhanced transgene expression led to papilloma development in HPV8-CER mice. Perpetuation of epidermal growth and enhanced transgene expression were also seen for UVA/B-irradiated mice of the HPV8-E6 strain and underline the necessity of elevated transgene expression for papilloma development. IHC staining for HPV8-E2 protein in sections from UVA/B-irradiated HPV8-CER skin confirmed increased transgene expression already one day after induction. These data suggest that increased HPV8 oncogene expression is crucial in the early steps of papilloma development. The observation that almost all keratinocytes which emerged during papillomatosis were positive for HPV8-E2 protein (E2 is only expressed in cells with an active K14-promoter) together with the finding of enhanced mK14 levels (K14, a marker for basal cells) in irradiated HPV8-CER mice may indicate a gain of a basal cell phenotype. It is tempting to speculate that HPV8 is able either to maintain proliferative signals or to circumvent cell differentiation to preserve the basal phenotype. It is also noteworthy that HPV8-E2 was predominantly localized in the cytoplasm until day ten after UVA/B-irradiation, when it was additionally detectable in the nucleus. Since HPV8-E2 is a transcription factor, it is reasonable to find it in the nucleus. E2 can regulate HPV8 as well as cellular gene expression. For example, E2 downregulates the cellular gene β4-integrin, which leads to the egress of keratinocytes from the basal to suprabasal layers (Oldak *et al.* 2004).

In the HPV8-CER mice, UVB-irradiation alone induced only small separated papillomas, whereas UVA plus UVB induced confluent papilloma development in the irradiated skin area. However, the HPV8 transgene levels in papillomatous areas elicited by either UVB or UVA/UVB irradiation were similar. UVA-irradiation alone enhanced neither mK14 expression nor papilloma development. Thus, UVB is the decisive cause of UVA/B-induced enhanced transgene expression and tumor growth, but synergistic effects of UVA apparently further promote UVB-induced papilloma development (Marcuzzi 2006), possibly by causing oxidative stress (Burke and Wei 2009).

Papillomas also developed spontaneously in non-irradiated HPV8-CER mice, but at slower rates. Without UVA/B-irradiation, spontaneous tumors arose mostly dorsal caudal and more frequently in male HPV8-CER mice, despite a constitutive, low level transgene expression in the entire mouse epidermis (Schaper *et al*. 2005; Marcuzzi

2006; Pfefferle *et al*. 2008). Since male mice fight more than female mice, it was speculated that fight wounds might enhance tumor development. Several reports showed enhanced K14 expression in hyperplastic skin (Coussens *et al.* 1996; Rossiter *et al.* 2001; Sethi and Palefsky 2004). Hence, the question came up, if skin regeneration processes in general might be sufficient to induce papillomas in HPV8-CER mice. To test this, mice were wounded by tape-stripping and papilloma development was indeed observed. In contrast to the area wide papillomatous growth elicited by UVA/B-irradiation, tape-stripping elicited only small discrete papillomas. The HPV8-E6 expression levels observed in tape-stripped skin areas of HPV8-CER mice were overall less pronounced than those observed in UVA/B-irradiated areas. This may point to only few 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. In line with this, the severity of skin wounding by tape-stripping appeared to correlate with enhanced transgene expression two days after treatment and with the probability of papilloma induction. A similar situation could be supposed for UVB-irradiated mice, where enhanced mK14 levels were found in papillomatous skin areas and presumably low levels in non-lesional but also UVB-irradiated skin areas. However, this has not yet been confirmed experimentally. Since wounds from tape-stripping are comparable to wounds from scratching or fighting, it is conceivable that many of the spontaneous papillomas actually arose from wounds. This concept is further supported by previous reports, in which enhanced amounts of viral DNA and subsequent papilloma development was observed after activation of latent mastomys natalensis papillomavirus and cottontail rabbit papillomavirus (CRPV) infections by mild mechanical skin irritation (Siegsmund *et al.* 1991; Amella *et al.* 1994). In a later report, it was shown that CRPV could also be activated by UVA/B-irradiation (Zhang *et al.* 1999). Regarding enhanced expression levels, the conversion of a latent beta-HPV infection into an activated infection can be mirrored by the hK14-HPV8 transgenic animals. With this model it was demonstrated that enhanced expression levels of HPV8 lead to a perpetuation of tumorous processes in the course of skin regeneration processes and may be a key step in early skin carcinogenesis.

To substantiate this assumption HPV8-E6-specific siRNAs were topically applied on HPV8-CER mouse skin to knock down E6 expression at early points in time. The delivery of the siRNA to its target cells, the basal cells, was checked with a

fluorescent control siRNA. To overcome the densely packed corneocytes an invasive delivery with a high siRNA concentration is recommended (Geusens *et al.* 2009). Accordingly, the attempt to introduce the fluorescent siRNA into the skin of HPV8-CER mice using only transfection reagents did not work, whereas in combination with a tattoo machine definite fluorescence was observed in the epidermis. Therefore the tattoo approach was used for the knock-down experiment in HPV8-CER mice. In the H_2O-tattooed skin area papillomas developed in 75% of the cases and were macroscopically visible approximately on day 18 after tattooing. A significant difference was achieved by tattooing E6 specific siRNA, where papilloma development was reduced or even inhibited. In these areas papillomas developed only in 45%, which were not macroscopically visible until approximately day 28 after tattooing. In these animals papilloma development may be due to an inconsistent delivery of the siRNA. This would support the assumption that only few HPV8-E6 positive cells are necessary to induce papillomatosis. As shown in cell culture experiments the efficient HPV8-E6 knock-down lasts about four days after transfection, with a maximal level around 30h after siRNA transfection. Similar observations were reported for siRNAs injected into mice, where the knock-down decreased after reaching its maximal level on day one or two after injection (Layzer *et al.* 2004; Nishina *et al.* 2008). When assuming that the knock-down also lasts only few days in the skin of HPV8-CER mice, the knock-down of induced HPV8-E6 expression seems to have a great impact on papilloma development in HPV8-CER mice. This would be a strong indication that HPV8 gene expression is crucial in the early steps of papillomatosis in mice and most probably also in immunosuppressed and epidermodysplasia verruciformis patients.

The reduced penetrance of tumor development after the less efficient induction of transgene expression following abrasive skin irritations compared to UVA/B-irradiation furthermore points to a dose-response relationship between oncogene expression and tumor development. This notion is supported by the finding that the level of HPV8-E2 mRNA in different HPV8-E2 transgenic mouse lines has an influence on the prevalence and rate of tumor development in these mice (Pfefferle *et al.* 2008). These data together with the observation that only HPV8-E2 mice with a high E2 expression level after UVA/B-irradiation developed tumors indicate the necessity of a certain threshold of oncogene expression for transformation of keratinocytes. Interestingly, also for EV patients, who are at a strongly increased risk

for SCC development, high beta-HPV DNA levels could be shown (Dell'Oste *et al.* 2009). Thus, within the first steps of HPV associated skin carcinogenesis enhanced beta-HPV oncogene levels, caused either by promoter induction in the mouse model or in humans by immunosuppression or genetic defects, seems to elevate the risk for tumor development.

Kinetics of selected cellular miRNAs and their respective targets were determined in HPV8-CER and FVB/N wt mice after UVA/B-irradiation to detect a possible deregulation, as it has been reported for several types of cancer (Croce and Calin 2005), but also for viral infections for example HPV16 and hepatitis C virus (Wang *et al.* 2008; Braconi *et al.* 2010). The miRNA kinetics obtained from irradiated HPV8-CER mice were checked for a correlation with the enhanced transgene expression. It was noted that there were differences between microarray and qRT-PCR results for some miRNAs in untreated skin of FVB/N wt and HPV8-CER mice. Differences between microarray and qRT-PCR data have often been described (Canales *et al.* 2006; Chen *et al.* 2009; Zhang *et al.* 2009; Buchtova *et al.* 2010). Microarrays have a lower sensitivity than qRT-PCRs, which makes an exact measurement of low miRNA quantities by microarray impossible. Therefore, it is necessary to confirm or confute the microarray data by qRT-PCR, which is accepted to be the most appropriate method for this purpose because of its high sensitivity and accuracy (Provenzano and Mocellin 2007; VanGuilder *et al.* 2008; Li and Ruan 2009). The array detected just marginal levels for miRNA-106a and -374-5p in untreated skin of FVB/N wt mice compared to HPV8-CER mice, whereas ISH and qRT-PCR results clearly showed that there is no difference between untreated skin of FVB/N wt and HPV8-CER mice. The miRNA-155 results obtained from the array are also precarious, because both, qRT-PCR and ISH showed a downregulation of miRNA-155 expression in untreated skin of HPV8-CER mice, which is in contrast to the array. In the microarray, a lower level of miRNA-206 was measured in untreated skin of HPV8-CER mice compared to FVB/N wt mice, but this reduction was not measured until day two after irradiation in qRT-PCR and ISH. This discrepancy may be due to the fact that the HPV8-CER skin used in the microarray already exhibited inconspicuous skin alterations, leading to a reduced miRNA-206 expression. Moreover, the array was performed with only one FVB/N wt and one HPV8-CER mouse, while the qRT-PCR experiments with untreated skin were done with at least six mice, respectively. These findings endorse the accuracy of the qRT-PCR results.

MiRNA-15a, -16 and -34a are commonly deregulated in cancer tissue (Cimmino *et al.* 2005; Iorio *et al.* 2005), but they were not altered in irradiated HPV8-CER mice (data not shown).

MiRNA-17-5p is a member of the miRNA-17-92 cluster on chromosome 13q31 along with miRNA-18a, -19a, -19b-1, -20a and -92a-1. These miRNAs promote proliferation and inhibit apoptosis and are commonly overexpressed in lymphoma, colon, lung, breast and pancreas cancer (Hayashita *et al.* 2005; He *et al.* 2005; Volinia *et al.* 2006; Mendell 2008). Important for the anti-apoptotic effects of the miRNA-17-92 cluster is the down-modulation of the tumor suppressor PTEN (reviewed in Garzon *et al.* 2009). PTEN negatively regulates phosphatidylinositol-3,4,5-trisphosphate (PIP_3) levels and the AKT signaling pathway (Jiang and Liu 2009). Therefore, the upregulation of miRNA-17-5p in HPV8-CER mice at early points in time after UV-irradiation may favor papilloma development by downregulation of PTEN (Figure 22), which was verified by IHC two and twenty-four days after UVA/B-irradiation. Confirmed by microarray other miRNAs from the miRNA-17-92 cluster were also upregulated in untreated skin of HPV8-CER mice compared to FVB/N mice although not as strong as miRNA-17-5p (data not shown).

The oncogenic miRNA-21 was found to be upregulated in a wide variety of hematological and solid cancers (Chan *et al*. 2005; Volinia *et al*. 2006) and overexpression of this miRNA in the breast cancer cell line MCF-7 promoted soft agar colony formation (Lu *et al.* 2008). MiRNA-21 inhibits the translation of the tumor suppressor genes PTEN (Meng *et al*. 2007) and PDCD4 (Frankel *et al*. 2008). The enhanced miRNA-21 expression in irradiated HPV8-CER mice, proven by qRT-PCR and ISH, should therefore lead to reduced PTEN and PDCD4 levels. The moderate downregulation of PTEN was already described above and is possibly a result of a combined effect of overexpressed miRNA-17-5p and miRNA-21 (Figure 22). Moreover cytoplasmatic PDCD4 protein level was strongly reduced in HPV8-CER mice 24 days after UV-irradiation as confirmed by IHC. PDCD4 inhibits tumor-promoter induced neoplastic transformation, cell cycle progression and proliferation by regulating multiple proteins at the transcriptional and translational level. For example PDCD4 induces p21, inhibits AKT and interferes with the mitogen-activated protein kinase (MAPK) pathway resulting in activator protein 1 (AP-1) inhibition (Lankat-Buttgereit and Goke 2009). Targeting cytoplasmic PDCD4 therefore may support tumorigenic processes (Figure 22).

Volinia *et al*. (2006) found a strong functional interaction between the Rb 3′UTR and miRNA-106a. This finding suggests a posttranscriptional mechanism for regulation of Rb. Rb was found to be downregulated in gastric, prostate and lung tumor samples and miRNA-106a was overexpressed. Thus, the immunohistochemically confirmed Rb reduction in HPV8-CER mouse skin two and twenty-four days after UV-irradiation could be caused by the upregulated miRNA-106a expression at these points in time. This is supported by the finding that miRNA-106a and Rb showed an inverse complementary expression pattern in ISH and IHC stainings. The minor expression of Rb in the lower epidermal cell layers correlated with the higher expression of miRNA-106a in these cells. Rb prevents the cell from progression through the cell cycle when it is bound to a transcription factor of the E2F-family. The loss of Rb in HPV8-CER mice and with it a controlled cell cycle, drives the cell into excessive growth (Figure 22). The fact that Rb is also downregulated in primary human adult keratinocytes expressing all HPV8 early genes is supporting the HPV8-mediated Rb downregulation (Akgül *et al.* 2007). The upregulation of miRNA-20b in irradiated HPV8-CER mice is substantiating the overexpression measured for miRNA-106a, because these miRNAs are both members of the same miRNA cluster.

MiRNA-155 knockout mice are immunodeficient, have defective B- and T-lymphocyte and dendritic cell functions, and show abnormal cytokine and chemokine expression (Rodriguez *et al*. 2007). Another group could show that Th cell differentiation in BIC/miRNA-155$^{-/-}$ mice is shifted towards Th2 cells (Thai *et al*. 2007). Therefore, the reduced miRNA-155 expression level in HPV8-CER mice at all observed points in time compared to FVB/N wt mice may indicate that HPV8 has a possible negative influence on the mouse immune system. When transferring these findings to humans it is tempting to speculate that beside the known immunosuppressive effects of UV-light on the skin additional HPV8-mediated immunosuppressive effects through downregulation of miRNA-155 expression may synergistically contribute to a local immunosuppression, which can promote papillomatosis. Furthermore, miRNA-155 also inhibits the translation of KGF and consequently a downregulation of miRNA-155 leads to an increase of KGF protein (Pottier *et al.* 2009). Several studies reported enhanced KGF expression in different cancer specimen, which is accompanied by proliferative, invasive and cell survival effects. KGF is produced in cells of mesenchymal origin through stimulation of interleukin 1-α (IL1-α) secreted from keratinocytes and acts exclusively through the KGF receptor, which is

expressed primarily by epithelial cells (Figure 22). KGF has diverse roles, for instance regulating cell proliferation, migration, differentiation during vertebrate development, as well as tissue repair (Finch and Rubin 2006). Interestingly transgenic mice expressing KGF under the hK14-promoter showed hyperthickening and alterations of the differentiation pattern of epidermal tissue (Guo *et al.* 1993). Furthermore, engineered skin equivalents with KGF-expressing human keratinocytes showed increased proliferation of basal cells and keratinocytes residing in the normally quiescent suprabasal layers of the epidermis (Andreadis *et al.* 2001). Similar hyperproliferative activities were observed in UV-irradiated HPV8-CER mice where enhanced KGF protein levels were observed by IHC, may be caused by the reduced miRNA-155 level in mesenchmal cells. This downregulation of miRNA-155 is possibly accomplished by an enhanced transforming growth factor-β (TGF-β) secretion, which is known to downregulate miRNA-155 (Pottier *et al.* 2009). Indeed, upregulation of TGF-β expression has been reported for UV-irradiated keratinocytes immortalized with HPV38 (Dell'oste *et al.* 2008).

In a recent study, it was shown that miRNA-206 downregulates the proto-oncogene MET (Yan *et al*. 2009). In contrast, this factor is upregulated in a variety of tumors including oral squamous cell cancer, but also after skin injuries (Di Renzo *et al.* 1994; Ferracini *et al.* 1995; Natali *et al.* 1996; Morello *et al.* 2001; Yoshida *et al.* 2003). While MET is primarily expressed in epithelial and lymphatic cells, its ligand hepatocyte growth factor (HGF) is only expressed and secreted from cells of mesenchymal origin through stimulation of IL-1α secreted from keratinocytes (Zarnegar 1995). The MET/HGF signaling pathway plays an important role in embryonic development and tumor growth by inducing mitogenesis, motogenesis, morphogenesis and angiogenesis (Bladt *et al*. 1995; Birchmeier and Gherardi 1998). Furthermore it is involved in wound healing, invasive growth and metastasis (Danilkovitch-Miagkova and Zbar 2002; Chmielowiec *et al*. 2007). The AKT signaling pathway plays a central role in the anti-apoptotic response induced by MET, for example in the HGF-mediated inhibition of UVB-induced apoptosis of human keratinocytes (Mildner *et al.* 2002; Tulasne and Foveau 2008). It was also demonstrated that supernatants of UVB-irradiated keratinocytes strongly induce HGF production in fibroblasts, and that this effect was primarily mediated by IL-1α (Mildner *et al.* 2007). The oncogenic potential of this pathway becomes especially apparent in transgenic mice expressing HGF in a variety of tissues. These mice develop

metastatic tumors (Takayama *et al*. 1997). Cells co-expressing MET and HGF become tumorigenic, when implanted into nude mice (Rong *et al*. 1994). The decrease of miRNA-206 in HPV8-CER mice confirmed by qRT-PCR and ISH starting two days after UV-irradiation should therefore lead to an enhanced expression of MET. This upregulation of MET was verified by IHC. Because MET is involved in many processes, which can lead to tumorigenesis, if dysregulated, the upregulation of this factor in HPV8-CER mice after UV-irradiation is a further effect of HPV8 which promotes papilloma development (Figure 22).

The targets of the downregulated miRNA-374-5p and miRNA-689 are currently not known. Table 3 shows a list of predicted targets generated with a software tool from http://www.microrna.org. It is noteworthy that both miRNAs are possibly targeting the TGF-β pathway (miRNA-374 - LTBP1; miRNA-689 - TGFBR2). The reduced expression of these miRNAs would result in an activation of this pathway, thereby possibly promoting papillomatosis.

The expression alterations measured for miRNA-17-5p, -20b, -21 and -106a in irradiated HPV8-E6 mice are supporting the results obtained from irradiated HPV8-CER mice, which showed similar deregulations of miRNA expression. Furthermore, this shows that the similar phenotype of HPV8-CER and -E6 mice is reflected by comparable miRNA deregulations.

The observation that human keratinocytes transduced with HPV8 showed the same tendency in expression alterations of miRNA17-5p, -20b, -21, -106a, -206 and -374-5p as UV-irradiated HPV8-CER mice, is approving the assumption that HPV8 also deregulates these miRNAs in humans.

It is remarkable that the deregulated miRNA targets KGF, MET, PDCD4, PTEN and Rb are all involved in the AKT signaling pathway which influences a broad spectrum of essential cell functions known to cause carcinogenesis, if dysregulated (Figure 22). This indicates that the AKT pathway is important for the HPV8-mediated papillomatosis. However, it is most likely that the MAPK pathway is also influenced by most of these factors. In summary it was shown that the increase of HPV8 oncogene expression was paralleled by a deregulation of tumorigenic and oncogenic miRNAs and their respective targets in UV-irradiated HPV8-CER mice. If cellular processes interacting with the induction of tumor growth are initiated by the enhanced expression of the HPV8 oncogene or by UVA/B-irradiation/wounding remains to be elucidated.

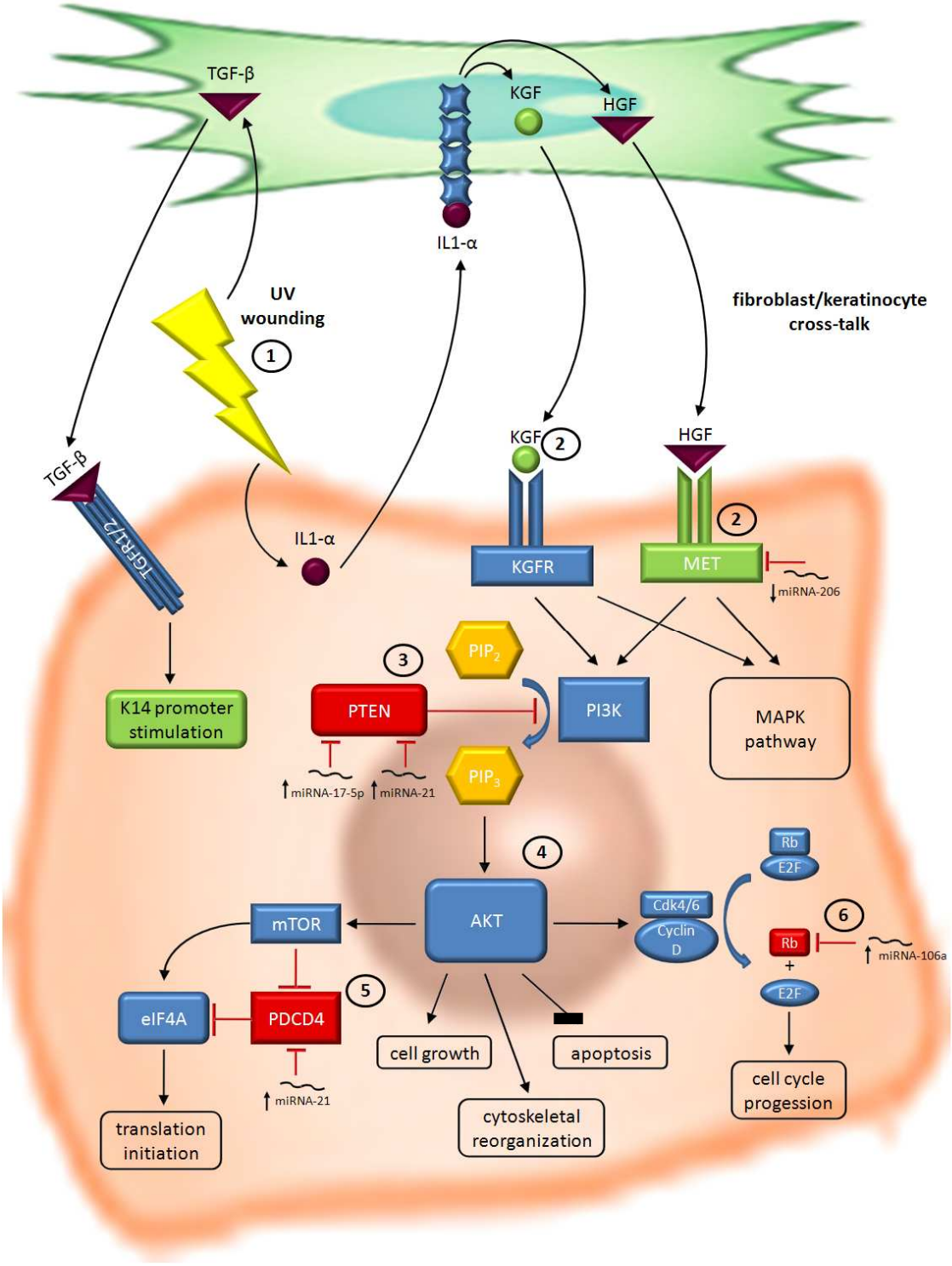


Figure 22: Model how HPV8 can interfere with cellular gene expression via miRNA deregulation
Green objects represent confirmed upregulation, red objects represents confirmed downregulation. (1) After UV-irradiation keratinocytes release IL-1α which in turn leads to secretion of KGF, HGF and TGF-β from the fibroblasts. TGF-β activates the K14-promoter in keratinocytes. (2) The enhanced expression of KGF and MET due to miRNA-206 downregulation lead to the increased phosphorylation of PIP_2 to PIP_3 by PI3K, but also to the activation of the MAPK pathway. (3) The phosphorylation of PIP_2 can be inhibited by PTEN, however PTEN is downregulated by miRNA-17-5p and miRNA-21. (4) PIP_3 recruits AKT to the cell membrane where it is activated by phosphorylation. (5) The PDCD4-mediated block of this translation initiation via inhibition of the helicase activity of EIF4A, is prevented by the reduced expression of PDCD4 mediated through miRNA-21. (6) The progression of the cell cycle is induced by phosphorylation of Rb which releases the transcription factor E2F. The release of E2F from Rb is furthermore facilitated by the downregulation of Rb by overexpressed miRNA-106a.

mRNAs Targeted by mmu-miR-374

- **Onecut2,** one cut domain, family member 2, **Mus musculus** [view alignment details]
- **Taok1,** TAO kinase 1, **Mus musculus** [view alignment details]
- **Mstn,** myostatin, **Mus musculus** [view alignment details]
- **Rsf1,** remodeling and spacing factor 1, **Mus musculus** [view alignment details]
- **AI593442,** expressed sequence AI593442, **Mus musculus** [view alignment details]
- **Stk38l,** serine/threonine kinase 38 like, **Mus musculus** [view alignment details]
- **Stk38l,** serine/threonine kinase 38 like, **Mus musculus** [view alignment details]
- **Med12l,** mediator of RNA polymerase II transcription, subunit 12 homolog (yeast)-like, **Mus musculus** [view alignment details]
- **Atp6ap2,** ATPase, H+ transporting, lysosomal accessory protein 2, **Mus musculus** [view alignment details]
- **Ltbp1,** latent transforming growth factor beta binding protein 1, **Mus musculus** [view alignment details]
- **Ltbp1,** latent transforming growth factor beta binding protein 1, **Mus musculus** [view alignment details]
- **Slc6a5,** solute carrier family 6 (neurotransmitter transporter, glycine), member 5, **Mus musculus** [view alignment details]
- **Homer1,** homer homolog 1 (Drosophila), **Mus musculus** [view alignment details]
- **Homer1,** homer homolog 1 (Drosophila), **Mus musculus** [view alignment details]
- **Homer1,** homer homolog 1 (Drosophila), **Mus musculus** [view alignment details]
- **Trp53inp1,** transformation related protein 53 inducible nuclear protein 1, **Mus musculus** [view alignment details]
- **Trp53inp1,** transformation related protein 53 inducible nuclear protein 1, **Mus musculus** [view alignment details]
- **Stk17b,** serine/threonine kinase 17b (apoptosis-inducing), **Mus musculus** [view alignment details]
- **Qk,** quaking, **Mus musculus** [view alignment details]
- **Qk,** quaking, **Mus musculus** [view alignment details]

mRNAs Targeted by mmu-miR-689

- **4933413J09Rik,** RIKEN cDNA 4933413J09 gene, **Mus musculus** [view alignment details]
- **A930004D18Rik,** RIKEN cDNA A930004D18 gene, **Mus musculus** [view alignment details]
- **LOC628101,** hypothetical LOC628101, **Mus musculus** [view alignment details]
- **Plekha7,** pleckstrin homology domain containing, family A member 7, **Mus musculus** [view alignment details]
- **Plekha7,** pleckstrin homology domain containing, family A member 7, **Mus musculus** [view alignment details]
- **Arhgef12,** Rho guanine nucleotide exchange factor (GEF) 12, **Mus musculus** [view alignment details]
- **Arhgef12,** Rho guanine nucleotide exchange factor (GEF) 12, **Mus musculus** [view alignment details]
- **2810011L19Rik,** RIKEN cDNA 2810011L19 gene, **Mus musculus** [view alignment details]
- **Csmd2,** CUB and Sushi multiple domains 2, **Mus musculus** [view alignment details]
- **4930577N17Rik,** RIKEN cDNA 4930577N17 gene, **Mus musculus** [view alignment details]
- **Armet,** arginine-rich, mutated in early stage tumors, **Mus musculus** [view alignment details]
- **Armet,** arginine-rich, mutated in early stage tumors, **Mus musculus** [view alignment details]
- **Ccdc32,** coiled-coil domain containing 32, **Mus musculus** [view alignment details]
- **E130112L23Rik,** RIKEN cDNA E130112L23 gene, **Mus musculus** [view alignment details]
- **AW121686,** expressed sequence AW121686, **Mus musculus** [view alignment details]
- **AI851790,** expressed sequence AI851790, **Mus musculus** [view alignment details]
- **Phf1,** PHD finger protein 1, **Mus musculus** [view alignment details]
- **Tgfbr2,** transforming growth factor, beta receptor II, **Mus musculus** [view alignment details]
- **Tgfbr2,** transforming growth factor, beta receptor II, **Mus musculus** [view alignment details]
- **Slco2a1,** solute carrier organic anion transporter family, member 2a1, **Mus musculus** [view alignment details]

Table 3: Predicted targets for miRNA-374-5p and miRNA-689.
The list is prepared with a prediction tool from http://www.microrna.org

6 References

Akgül, B., J. C. Cooke and A. Storey (2006). "HPV-associated skin disease." J Pathol **208**(2): 165-75.

Akgül, B., L. Ghali, D. Davies, H. Pfister, I. M. Leigh and A. Storey (2007). "HPV8 early genes modulate differentiation and cell cycle of primary human adult keratinocytes." Exp Dermatol **16**(7): 590-9.

Akgül, B., P. Karle, M. Adam, P. G. Fuchs and H. J. Pfister (2003). "Dual role of tumor suppressor p53 in regulation of DNA replication and oncogene E6-promoter activity of epidermodysplasia verruciformis-associated human papillomavirus type 8." Virology **308**(2): 279-90.

Akgül, B., W. Lemme, R. Garcia-Escudero, A. Storey and H. J. Pfister (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): 145-51.

Alam, M. and D. Ratner (2001). "Cutaneous squamous-cell carcinoma." N Engl J Med **344**(13): 975-83.

Ambros, V. (2003). "MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing." Cell **113**(6): 673-6.

Ambros, V. (2004). "The functions of animal microRNAs." Nature **431**(7006): 350-5.

Amella, C. A., L. A. Lofgren, A. M. Ronn, M. Nouri, M. J. Shikowitz and B. M. Steinberg (1994). "Latent infection induced with cottontail rabbit papillomavirus. A model for human papillomavirus latency." Am J Pathol **144**(6): 1167-71.

Andreadis, S. T., K. E. Hamoen, M. L. Yarmush and J. R. Morgan (2001). "Keratinocyte growth factor induces hyperproliferation and delays differentiation in a skin equivalent model system." FASEB J **15**(6): 898-906.

Antonsson, A., S. Karanfilovska, P. G. Lindqvist and B. G. Hansson (2003). "General acquisition of human papillomavirus infections of skin occurs in early infancy." J Clin Microbiol **41**(6): 2509-14.

Arias-Pulido, H., C. L. Peyton, N. E. Joste, H. Vargas and C. M. Wheeler (2006). "Human papillomavirus type 16 integration in cervical carcinoma in situ and in invasive cervical cancer." J Clin Microbiol **44**(5): 1755-62.

Baker CC (1993). The genomes of papillomaviruses. In: *S.J. O'Brien* (Herausg.), Genetic maps: locus maps of complex genomes. *Cold Spring Harbor Laboratory Press, Cold Spring Harbor*, N.Y., 1.134-1.146.

Banerjee, N. S., N. J. Genovese, F. Noya, W. M. Chien, T. R. Broker and L. T. Chow (2006). "Conditionally activated E7 proteins of high-risk and low-risk human papillomaviruses induce S phase in postmitotic, differentiated human keratinocytes." J Virol **80**(13): 6517-24.

Bartel, D. P. (2004). "MicroRNAs: genomics, biogenesis, mechanism, and function." Cell **116**(2): 281-97.

Bayne, E. H. and R. C. Allshire (2005). "RNA-directed transcriptional gene silencing in mammals." Trends Genet **21**(7): 370-3.

Behm-Ansmant, I., J. Rehwinkel, T. Doerks, A. Stark, P. Bork and E. Izaurralde (2006). "mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes." Genes Dev **20**(14): 1885-98.

Berezikov, E., E. Cuppen and R. H. Plasterk (2006). "Approaches to microRNA discovery." Nat Genet **38 Suppl**: S2-7.

Berezutskaya, E. and S. Bagchi (1997). "The human papillomavirus E7 oncoprotein functionally interacts with the S4 subunit of the 26 S proteasome." J Biol Chem **272**(48): 30135-40.

Berkhout, R. J., J. N. Bouwes Bavinck and J. ter Schegget (2000). "Persistence of human papillomavirus DNA in benign and (pre)malignant skin lesions from renal transplant recipients." J Clin Microbiol **38**(6): 2087-96.

Bernard, H. U. (2005). "The clinical importance of the nomenclature, evolution and taxonomy of human papillomaviruses." J Clin Virol **32 Suppl 1**: S1-6.

Bernstein, E., A. A. Caudy, S. M. Hammond and G. J. Hannon (2001). "Role for a bidentate ribonuclease in the initiation step of RNA interference." Nature **409**(6818): 363-6.

Birchmeier, C. and E. Gherardi (1998). "Developmental roles of HGF/SF and its receptor, the c-Met tyrosine kinase." Trends Cell Biol **8**(10): 404-10.

Birnboim, H. C. and J. Doly (1979). "A rapid alkaline extraction procedure for screening recombinant plasmid DNA." Nucleic Acids Res **7**(6): 1513-23.

Bladt, F., D. Riethmacher, S. Isenmann, A. Aguzzi and C. Birchmeier (1995). "Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud." Nature **376**(6543): 768-71.

Bohnsack, M. T., K. Czaplinski and D. Gorlich (2004). "Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs." RNA **10**(2): 185-91.

Borchert, G. M., W. Lanier and B. L. Davidson (2006). "RNA polymerase III transcribes human microRNAs." Nat Struct Mol Biol **13**(12): 1097-101.

Boukamp, P., R. T. Petrussevska, D. Breitkreutz, J. Hornung, A. Markham and N. E. Fusenig (1988). "Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line." J Cell Biol **106**(3): 761-71.

Bouvard, V., A. Storey, D. Pim and L. Banks (1994). "Characterization of the human papillomavirus E2 protein: evidence of trans-activation and trans-repression in cervical keratinocytes." EMBO J **13**(22): 5451-9.

Bracht, J., S. Hunter, R. Eachus, P. Weeks and A. E. Pasquinelli (2004). "Trans-splicing and polyadenylation of let-7 microRNA primary transcripts." RNA **10**(10): 1586-94.

Braconi, C., N. Valeri, P. Gasparini, N. Huang, C. Taccioli, G. Nuovo, T. Suzuki, C. M. Croce and T. Patel (2010). "Hepatitis C Virus Proteins Modulate MicroRNA Expression and Chemosensitivity in Malignant Hepatocytes." Clin Cancer Res.

Bryan, J. T. and D. R. Brown (2001). "Transmission of human papillomavirus type 11 infection by desquamated cornified cells." Virology **281**(1): 35-42.

Buchtova, M., W. P. Kuo, S. Nimmagadda, S. L. Benson, P. Geetha-Loganathan, C. Logan, T. Au-Yeung, E. Chiang, K. Fu and J. M. Richman (2010). "Whole genome microarray analysis of chicken embryo facial prominences." Dev Dyn **239**(2): 574-91.

Burke, K. and H. Wei (2009). "Synergistic damage by UVA radiation and pollutants." Toxicol Ind Health **25**(4-5): 219-24.

Cai, X., C. H. Hagedorn and B. R. Cullen (2004). "Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs." RNA **10**(12): 1957-66.

Calin, G. A., C. D. Dumitru, M. Shimizu, R. Bichi, S. Zupo, E. Noch, H. Aldler, S. Rattan, M. Keating, K. Rai, L. Rassenti, T. Kipps, M. Negrini, F. Bullrich

and C. M. Croce (2002). "Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia." Proc Natl Acad Sci U S A **99**(24): 15524-9.

Calin, G. A., C. Sevignani, C. D. Dumitru, T. Hyslop, E. Noch, S. Yendamuri, M. Shimizu, S. Rattan, F. Bullrich, M. Negrini and C. M. Croce (2004). "Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers." Proc Natl Acad Sci U S A **101**(9): 2999-3004.

Canales, R. D., Y. Luo, J. C. Willey, B. Austermiller, C. C. Barbacioru, C. Boysen, K. Hunkapiller, R. V. Jensen, C. R. Knight, K. Y. Lee, Y. Ma, B. Maqsodi, A. Papallo, E. H. Peters, K. Poulter, P. L. Ruppel, R. R. Samaha, L. Shi, W. Yang, L. Zhang and F. M. Goodsaid (2006). "Evaluation of DNA microarray results with quantitative gene expression platforms." Nat Biotechnol **24**(9): 1115-22.

Carleton, M., M. A. Cleary and P. S. Linsley (2007). "MicroRNAs and cell cycle regulation." Cell Cycle **6**(17): 2127-32.

Caudy, A. A., R. F. Ketting, S. M. Hammond, A. M. Denli, A. M. Bathoorn, B. B. Tops, J. M. Silva, M. M. Myers, G. J. Hannon and R. H. Plasterk (2003). "A micrococcal nuclease homologue in RNAi effector complexes." Nature **425**(6956): 411-4.

Chan, J. A., A. M. Krichevsky and K. S. Kosik (2005). "MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells." Cancer Res **65**(14): 6029-33.

Chen, Y., J. A. Gelfond, L. M. McManus and P. K. Shireman (2009). "Reproducibility of quantitative RT-PCR array in miRNA expression profiling and comparison with microarray analysis." BMC Genomics **10**: 407.

Chmielowiec, J., M. Borowiak, M. Morkel, T. Stradal, B. Munz, S. Werner, J. Wehland, C. Birchmeier and W. Birchmeier (2007). "c-Met is essential for wound healing in the skin." J Cell Biol **177**(1): 151-62.

Chung, C. T. and R. H. Miller (1988). "A rapid and convenient method for the preparation and storage of competent bacterial cells." Nucleic Acids Res **16**(8): 3580.

Cimmino, A., G. A. Calin, M. Fabbri, M. V. Iorio, M. Ferracin, M. Shimizu, S. E. Wojcik, R. I. Aqeilan, S. Zupo, M. Dono, L. Rassenti, H. Alder, S. Volinia, C. G. Liu, T. J. Kipps, M. Negrini and C. M. Croce (2005). "miR-15 and miR-16 induce apoptosis by targeting BCL2." Proc Natl Acad Sci U S A **102**(39): 13944-9.

Clifford, G. M., J. S. Smith, M. Plummer, N. Munoz and S. Franceschi (2003). "Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis." Br J Cancer **88**(1): 63-73.

Corona, R., E. Dogliotti, M. D'Errico, F. Sera, I. Iavarone, G. Baliva, L. M. Chinni, T. Gobello, C. Mazzanti, P. Puddu and P. Pasquini (2001). "Risk factors for basal cell carcinoma in a Mediterranean population: role of recreational sun exposure early in life." Arch Dermatol **137**(9): 1162-8.

Coussens, L. M., D. Hanahan and J. M. Arbeit (1996). "Genetic predisposition and parameters of malignant progression in K14-HPV16 transgenic mice." Am J Pathol **149**(6): 1899-917.

Croce, C. M. and G. A. Calin (2005). "miRNAs, cancer, and stem cell division." Cell **122**(1): 6-7.

Danilkovitch-Miagkova, A. and B. Zbar (2002). "Dysregulation of Met receptor tyrosine kinase activity in invasive tumors." J Clin Invest **109**(7): 863-7.

de Fougerolles, A., H. P. Vornlocher, J. Maraganore and J. Lieberman (2007). "Interfering with disease: a progress report on siRNA-based therapeutics." Nat Rev Drug Discov **6**(6): 443-53.

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

Dell'Oste, V., B. Azzimonti, M. De Andrea, M. Mondini, E. Zavattaro, G. Leigheb, S. J. Weissenborn, H. Pfister, K. M. Michael, T. Waterboer, M. Pawlita, A. Amantea, S. Landolfo and M. Gariglio (2009). "High beta-HPV DNA loads and strong seroreactivity are present in epidermodysplasia verruciformis." J Invest Dermatol **129**(4): 1026-34.

Dell'oste, V., B. Azzimonti, M. Mondini, M. De Andrea, C. Borgogna, R. Mesturini, R. Accardi, M. Tommasino, S. Landolfo, U. Dianzani and M. Gariglio (2008). "Altered expression of UVB-induced cytokines in human papillomavirus-immortalized epithelial cells." J Gen Virol **89**(Pt 10): 2461-6.

Demeret, C., M. Yaniv and F. Thierry (1994). "The E2 transcriptional repressor can compensate for Sp1 activation of the human papillomavirus type 18 early promoter." J Virol **68**(11): 7075-82.

DePinho, R. A. (2000). "The age of cancer." Nature **408**(6809): 248-54.

Di Renzo, M. F., M. Olivero, D. Katsaros, T. Crepaldi, P. Gaglia, P. Zola, P. Sismondi and P. M. Comoglio (1994). "Overexpression of the Met/HGF receptor in ovarian cancer." Int J Cancer **58**(5): 658-62.

Diederichs, S. and D. A. Haber (2007). "Dual role for argonautes in microRNA processing and posttranscriptional regulation of microRNA expression." Cell **131**(6): 1097-108.

Dong, W., U. Kloz, R. Accardi, S. Caldeira, W. M. Tong, Z. Q. Wang, L. Jansen, M. Durst, B. S. Sylla, L. Gissmann and M. Tommasino (2005). "Skin hyperproliferation and susceptibility to chemical carcinogenesis in transgenic mice expressing E6 and E7 of human papillomavirus type 38." J Virol **79**(23): 14899-908.

Dong, X. P., F. Stubenrauch, E. Beyer-Finkler and H. Pfister (1994). "Prevalence of deletions of YY1-binding sites in episomal HPV 16 DNA from cervical cancers." Int J Cancer **58**(6): 803-8.

Doorbar, J., S. Ely, J. Sterling, C. McLean and L. Crawford (1991). "Specific interaction between HPV-16 E1-E4 and cytokeratins results in collapse of the epithelial cell intermediate filament network." Nature **352**(6338): 824-7.

Doorbar, J. (2006). "Molecular biology of human papillomavirus infection and cervical cancer." Clin Sci (Lond) **110**(5): 525-41.

Dyson, N., P. M. Howley, K. Munger and E. Harlow (1989). "The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product." Science **243**(4893): 934-7.

Eckert, R. L., J. F. Crish and N. A. Robinson (1997). "The epidermal keratinocyte as a model for the study of gene regulation and cell differentiation." Physiol Rev **77**(2): 397-424.

Einstein, M. H. and G. L. Goldberg (2002). "Human papillomavirus and cervical neoplasia." Cancer Invest **20**(7-8): 1080-5.

Elbel, M., S. Carl, S. Spaderna and T. Iftner (1997). "A comparative analysis of the interactions of the E6 proteins from cutaneous and genital papillomaviruses with p53 and E6AP in correlation to their transforming potential." Virology **239**(1): 132-49.

Enzenauer, C., G. Mengus, A. Lavigne, I. Davidson, H. Pfister and M. May (1998). "Interaction of human papillomavirus 8 regulatory proteins E2, E6 and E7 with components of the TFIID complex." Intervirology **41**(2-3): 80-90.

Evander, M., I. H. Frazer, E. Payne, Y. M. Qi, K. Hengst and N. A. McMillan (1997). "Identification of the alpha6 integrin as a candidate receptor for papillomaviruses." J Virol **71**(3): 2449-56.

Favre, M., F. Breitburd, O. Croissant and G. Orth (1977). "Chromatin-like structures obtained after alkaline disruption of bovine and human papillomaviruses." J Virol **21**(3): 1205-9.

Fehrmann, F. and L. A. Laimins (2003). "Human papillomaviruses: targeting differentiating epithelial cells for malignant transformation." Oncogene **22**(33): 5201-7.

Feltkamp, M. C., R. Broer, F. M. di Summa, L. Struijk, E. van der Meijden, B. P. Verlaan, R. G. Westendorp, J. ter Schegget, W. J. Spaan and J. N. Bouwes Bavinck (2003). "Seroreactivity to epidermodysplasia verruciformis-related human papillomavirus types is associated with nonmelanoma skin cancer." Cancer Res **63**(10): 2695-700.

Ferracini, R., M. F. Di Renzo, K. Scotlandi, N. Baldini, M. Olivero, P. Lollini, O. Cremona, M. Campanacci and P. M. Comoglio (1995). "The Met/HGF receptor is over-expressed in human osteosarcomas and is activated by either a paracrine or an autocrine circuit." Oncogene **10**(4): 739-49.

Finch, P. W. and J. S. Rubin (2006). "Keratinocyte growth factor expression and activity in cancer: implications for use in patients with solid tumors." J Natl Cancer Inst **98**(12): 812-24.

Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver and C. C. Mello (1998). "Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans." Nature **391**(6669): 806-11.

Forstemann, K., Y. Tomari, T. Du, V. V. Vagin, A. M. Denli, D. P. Bratu, C. Klattenhoff, W. E. Theurkauf and P. D. Zamore (2005). "Normal microRNA maturation and germ-line stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein." PLoS Biol **3**(7): e236.

Fournier, N., K. Raj, P. Saudan, S. Utzig, R. Sahli, V. Simanis and P. Beard (1999). "Expression of human papillomavirus 16 E2 protein in Schizosaccharomyces pombe delays the initiation of mitosis." Oncogene **18**(27): 4015-21.

Frankel, L. B., N. R. Christoffersen, A. Jacobsen, M. Lindow, A. Krogh and A. H. Lund (2008). "Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells." J Biol Chem **283**(2): 1026-33.

Frattini, M. G., S. D. Hurst, H. B. Lim, S. Swaminathan and L. A. Laimins (1997). "Abrogation of a mitotic checkpoint by E2 proteins from oncogenic human papillomaviruses correlates with increased turnover of the p53 tumor suppressor protein." EMBO J **16**(2): 318-31.

Gammoh, N., H. S. Grm, P. Massimi and L. Banks (2006). "Regulation of human papillomavirus type 16 E7 activity through direct protein interaction with the E2 transcriptional activator." J Virol **80**(4): 1787-97.

Gardiol, D., C. Kuhne, B. Glaunsinger, S. S. Lee, R. Javier and L. Banks (1999). "Oncogenic human papillomavirus E6 proteins target the discs large tumour suppressor for proteasome-mediated degradation." Oncogene **18**(40): 5487-96.

Garland, S. M. (2002). "Human papillomavirus update with a particular focus on cervical disease." Pathology **34**(3): 213-24.

Garzon, R., G. A. Calin and C. M. Croce (2009). "MicroRNAs in Cancer." Annu Rev Med **60**: 167-79.

Geusens, B., N. Sanders, T. Prow, M. Van Gele and J. Lambert (2009). "Cutaneous short-interfering RNA therapy." Expert Opin Drug Deliv **6**(12): 1333-49.

Gewin, L. and D. A. Galloway (2001). "E box-dependent activation of telomerase by human papillomavirus type 16 E6 does not require induction of c-myc." J Virol **75**(15): 7198-201.

Giampieri, S. and A. Storey (2004). "Repair of UV-induced thymine dimers is compromised in cells expressing the E6 protein from human papillomaviruses types 5 and 18." Br J Cancer **90**(11): 2203-9.

Giroglou, T., L. Florin, F. Schafer, R. E. Streeck and M. Sapp (2001). "Human papillomavirus infection requires cell surface heparan sulfate." J Virol **75**(3): 1565-70.

Gregory, R. I., T. P. Chendrimada, N. Cooch and R. Shiekhattar (2005). "Human RISC couples microRNA biogenesis and posttranscriptional gene silencing." Cell **123**(4): 631-40.

Grossman, S. R., R. Mora and L. A. Laimins (1989). "Intracellular localization and DNA-binding properties of human papillomavirus type 18 E6 protein expressed with a baculovirus vector." J Virol **63**(1): 366-74.

Guo, L., Q. C. Yu and E. Fuchs (1993). "Targeting expression of keratinocyte growth factor to keratinocytes elicits striking changes in epithelial differentiation in transgenic mice." EMBO J **12**(3): 973-86.

Halbert, C. L., G. W. Demers and D. A. Galloway (1991). "The E7 gene of human papillomavirus type 16 is sufficient for immortalization of human epithelial cells." J Virol **65**(1): 473-8.

Han, J., Y. Lee, K. H. Yeom, Y. K. Kim, H. Jin and V. N. Kim (2004). "The Drosha-DGCR8 complex in primary microRNA processing." Genes Dev **18**(24): 3016-27.

Harfe, B. D. (2005). "MicroRNAs in vertebrate development." Curr Opin Genet Dev **15**(4): 410-5.

Harwood, C. A., C. M. Proby, J. M. McGregor, M. T. Sheaff, I. M. Leigh and R. Cerio (2006). "Clinicopathologic features of skin cancer in organ transplant recipients: a retrospective case-control series." J Am Acad Dermatol **54**(2): 290-300.

Harwood, C. A., T. Surentheran, J. M. McGregor, P. J. Spink, I. M. Leigh, J. Breuer and C. M. Proby (2000). "Human papillomavirus infection and non-melanoma skin cancer in immunosuppressed and immunocompetent individuals." J Med Virol **61**(3): 289-97.

Hayashita, Y., H. Osada, Y. Tatematsu, H. Yamada, K. Yanagisawa, S. Tomida, Y. Yatabe, K. Kawahara, Y. Sekido and T. Takahashi (2005). "A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation." Cancer Res **65**(21): 9628-32.

He, L., J. M. Thomson, M. T. Hemann, E. Hernando-Monge, D. Mu, S. Goodson, S. Powers, C. Cordon-Cardo, S. W. Lowe, G. J. Hannon and S. M. Hammond (2005). "A microRNA polycistron as a potential human oncogene." Nature **435**(7043): 828-33.

Iftner, T., S. Bierfelder, Z. Csapo and H. Pfister (1988). "Involvement of human papillomavirus type 8 genes E6 and E7 in transformation and replication." J Virol **62**(10): 3655-61.

Iftner, T., M. Elbel, B. Schopp, T. Hiller, J. I. Loizou, K. W. Caldecott and F. Stubenrauch (2002). "Interference of papillomavirus E6 protein with single-strand break repair by interaction with XRCC1." EMBO J **21**(17): 4741-8.

Iorio, M. V., M. Ferracin, C. G. Liu, A. Veronese, R. Spizzo, S. Sabbioni, E. Magri, M. Pedriali, M. Fabbri, M. Campiglio, S. Menard, J. P. Palazzo, A. Rosenberg, P. Musiani, S. Volinia, I. Nenci, G. A. Calin, P. Querzoli, M. Negrini and C. M. Croce (2005). "MicroRNA gene expression deregulation in human breast cancer." Cancer Res **65**(16): 7065-70.

Jablonska, S. and S. Majewski (1994). "Epidermodysplasia verruciformis: immunological and clinical aspects." Curr Top Microbiol Immunol **186**: 157-75.

Jackson, S. and A. Storey (2000). "E6 proteins from diverse cutaneous HPV types inhibit apoptosis in response to UV damage." Oncogene **19**(4): 592-8.

Jiang, B. H. and L. Z. Liu (2009). "PI3K/PTEN signaling in angiogenesis and tumorigenesis." Adv Cancer Res **102**: 19-65.

Johnson, S. M., H. Grosshans, J. Shingara, M. Byrom, R. Jarvis, A. Cheng, E. Labourier, K. L. Reinert, D. Brown and F. J. Slack (2005). "RAS is regulated by the let-7 microRNA family." Cell **120**(5): 635-47.

Joyce, J. G., J. S. Tung, C. T. Przysiecki, J. C. Cook, E. D. Lehman, J. A. Sands, K. U. Jansen and P. M. Keller (1999). "The L1 major capsid protein of human papillomavirus type 11 recombinant virus-like particles interacts with heparin and cell-surface glycosaminoglycans on human keratinocytes." J Biol Chem **274**(9): 5810-22.

Kim, D. H., P. Saetrom, O. Snove, Jr. and J. J. Rossi (2008). "MicroRNA-directed transcriptional gene silencing in mammalian cells." Proc Natl Acad Sci U S A **105**(42): 16230-5.

Kim, Y. K. and V. N. Kim (2007). "Processing of intronic microRNAs." EMBO J **26**(3): 775-83.

Kinouchi, M., H. Takahashi, Y. Itoh, A. Ishida-Yamamoto and H. Iizuka (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): 634-41.

Kisielow, M., S. Kleiner, M. Nagasawa, A. Faisal and Y. Nagamine (2002). "Isoform-specific knockdown and expression of adaptor protein ShcA using small interfering RNA." Biochem J **363**(Pt 1): 1-5.

Kiyono, T., K. Nagashima and M. Ishibashi (1989). "The primary structure of major viral RNA in a rat cell line transfected with type 47 human papillomavirus DNA and the transforming activity of its cDNA and E6 gene." Virology **173**(2): 551-65.

Kuo, S. R., J. S. Liu, T. R. Broker and L. T. Chow (1994). "Cell-free replication of the human papillomavirus DNA with homologous viral E1 and E2 proteins and human cell extracts." J Biol Chem **269**(39): 24058-65.

Lagos-Quintana, M., R. Rauhut, W. Lendeckel and T. Tuschl (2001). "Identification of novel genes coding for small expressed RNAs." Science **294**(5543): 853-8.

Lankat-Buttgereit, B. and R. Goke (2009). "The tumour suppressor Pdcd4: recent advances in the elucidation of function and regulation." Biol Cell **101**(6): 309-17.

Lau, N. C., L. P. Lim, E. G. Weinstein and D. P. Bartel (2001). "An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans." Science **294**(5543): 858-62.

Layzer, J. M., A. P. McCaffrey, A. K. Tanner, Z. Huang, M. A. Kay and B. A. Sullenger (2004). "In vivo activity of nuclease-resistant siRNAs." RNA **10**(5): 766-71.

Lazarczyk, M. and M. Favre (2008). "Role of Zn2+ ions in host-virus interactions." J Virol **82**(23): 11486-94.

Lazarczyk, M., C. Pons, J. A. Mendoza, P. Cassonnet, Y. Jacob and M. Favre (2008). "Regulation of cellular zinc balance as a potential mechanism of EVER-mediated protection against pathogenesis by cutaneous oncogenic human papillomaviruses." J Exp Med **205**(1): 35-42.

Lee, R. C. and V. Ambros (2001). "An extensive class of small RNAs in Caenorhabditis elegans." Science **294**(5543): 862-4.

Lee, S. S., B. Glaunsinger, F. Mantovani, L. Banks and R. T. Javier (2000). "Multi-PDZ domain protein MUPP1 is a cellular target for both adenovirus E4-ORF1 and high-risk papillomavirus type 18 E6 oncoproteins." J Virol **74**(20): 9680-93.

Lee, Y., C. Ahn, J. Han, H. Choi, J. Kim, J. Yim, J. Lee, P. Provost, O. Radmark, S. Kim and V. N. Kim (2003). "The nuclear RNase III Drosha initiates microRNA processing." Nature **425**(6956): 415-9.

Lee, Y., M. Kim, J. Han, K. H. Yeom, S. Lee, S. H. Baek and V. N. Kim (2004). "MicroRNA genes are transcribed by RNA polymerase II." EMBO J **23**(20): 4051-60.

Leiter, U. and C. Garbe (2008). "Epidemiology of melanoma and nonmelanoma skin cancer--the role of sunlight." Adv Exp Med Biol **624**: 89-103.

Lewandowsky, F. and W. Lutz (1922). Ein Fall einer bischer nicht beschriebenen Hauterkrankung *(Epidermodysplasia verruciformis)*. Arch Dermatol Syphilol **141**: 193-203.

Lewis, B. P., C. B. Burge and D. P. Bartel (2005). "Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets." Cell **120**(1): 15-20.

Lewis, B. P., I. H. Shih, M. W. Jones-Rhoades, D. P. Bartel and C. B. Burge (2003). "Prediction of mammalian microRNA targets." Cell **115**(7): 787-98.

Li, W. and K. Ruan (2009). "MicroRNA detection by microarray." Anal Bioanal Chem **394**(4): 1117-24.

Liu, J., F. V. Rivas, J. Wohlschlegel, J. R. Yates, 3rd, R. Parker and G. J. Hannon (2005). "A role for the P-body component GW182 in microRNA function." Nat Cell Biol **7**(12): 1261-6.

Longworth, M. S. and L. A. Laimins (2004). "Pathogenesis of human papillomaviruses in differentiating epithelia." Microbiol Mol Biol Rev **68**(2): 362-72.

Lu, Z., M. Liu, V. Stribinskis, C. M. Klinge, K. S. Ramos, N. H. Colburn and Y. Li (2008). "MicroRNA-21 promotes cell transformation by targeting the programmed cell death 4 gene." Oncogene **27**(31): 4373-9.

Lund, E., S. Guttinger, A. Calado, J. E. Dahlberg and U. Kutay (2004). "Nuclear export of microRNA precursors." Science **303**(5654): 95-8.

Majewski, S., S. Jablonska and G. Orth (1997). "Epidermodysplasia verruciformis. Immunological and nonimmunological surveillance mechanisms: role in tumor progression." Clin Dermatol **15**(3): 321-34.

Maniataki, E. and Z. Mourelatos (2005). "A human, ATP-independent, RISC assembly machine fueled by pre-miRNA." Genes Dev **19**(24): 2979-90.

Marcuzzi, P. (2006). “Analyse des onkogenen Potentials der frühen Gene E6 und E7 des humanen Papillomvirus Typ 8 durch die Etablierung transgener Mausmodelle.“ *Doktorarbeit*, Institut für Virologie, Universität zu Köln.

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

Maroney, P. A., Y. Yu, J. Fisher and T. W. Nilsen (2006). "Evidence that microRNAs are associated with translating messenger RNAs in human cells." Nat Struct Mol Biol **13**(12): 1102-7.

Martinez, I., A. S. Gardiner, K. F. Board, F. A. Monzon, R. P. Edwards and S. A. Khan (2008). "Human papillomavirus type 16 reduces the expression of microRNA-218 in cervical carcinoma cells." Oncogene **27**(18): 2575-82.

Masini, C., P. G. Fuchs, F. Gabrielli, S. Stark, F. Sera, M. Ploner, C. F. Melchi, G. Primavera, G. Pirchio, O. Picconi, P. Petasecca, M. S. Cattaruzza, H. J. Pfister and D. Abeni (2003). "Evidence for the association of human papillomavirus infection and cutaneous squamous cell carcinoma in immunocompetent individuals." Arch Dermatol **139**(7): 890-4.

Mendell, J. T. (2008). "miRiad roles for the miR-17-92 cluster in development and disease." Cell **133**(2): 217-22.

Meng, F., R. Henson, H. Wehbe-Janek, K. Ghoshal, S. T. Jacob and T. Patel (2007). "MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer." Gastroenterology **133**(2): 647-58.

Michel, A., A. Kopp-Schneider, H. Zentgraf, A. D. Gruber and E. M. de Villiers (2006). "E6/E7 expression of human papillomavirus type 20 (HPV-20) and HPV-27 influences proliferation and differentiation of the skin in UV-irradiated SKH-hr1 transgenic mice." J Virol **80**(22): 11153-64.

Mildner, M., L. Eckhart, B. Lengauer and E. Tschachler (2002). "Hepatocyte growth factor/scatter factor inhibits UVB-induced apoptosis of human keratinocytes but not of keratinocyte-derived cell lines via the phosphatidylinositol 3-kinase/AKT pathway." J Biol Chem **277**(16): 14146-52.

Mildner, M., V. Mlitz, F. Gruber, J. Wojta and E. Tschachler (2007). "Hepatocyte growth factor establishes autocrine and paracrine feedback loops for the protection of skin cells after UV irradiation." J Invest Dermatol **127**(11): 2637-44.

Morello, S., M. Olivero, M. Aimetti, M. Bernardi, S. Berrone, M. F. Di Renzo and S. Giordano (2001). "MET receptor is overexpressed but not mutated in oral squamous cell carcinomas." J Cell Physiol **189**(3): 285-90.

Morgan, I. M., G. J. Grindlay and M. S. Campo (1998). "Epithelial specific transcriptional regulation of the bovine papillomavirus 4 promoter by E2." J Gen Virol **79 (Pt 3)**: 501-8.

Mullis, K. B. and F. A. Faloona (1987). "Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction." Methods Enzymol **155**: 335-50.

Münger, K., J. R. Basile, S. Duensing, A. Eichten, S. L. Gonzalez, M. Grace and V. L. Zacny (2001). "Biological activities and molecular targets of the human papillomavirus E7 oncoprotein." Oncogene **20**(54): 7888-98.

Munoz, N. (2000). "Human papillomavirus and cancer: the epidemiological evidence." J Clin Virol **19**(1-2): 1-5.

Nakagawa, S. and J. M. Huibregtse (2000). "Human scribble (Vartul) is targeted for ubiquitin-mediated degradation by the high-risk papillomavirus E6 proteins and the E6AP ubiquitin-protein ligase." Mol Cell Biol **20**(21): 8244-53.

Natali, P. G., M. Prat, M. R. Nicotra, A. Bigotti, M. Olivero, P. M. Comoglio and M. F. Di Renzo (1996). "Overexpression of the met/HGF receptor in renal cell carcinomas." Int J Cancer **69**(3): 212-7.

Nishina, K., T. Unno, Y. Uno, T. Kubodera, T. Kanouchi, H. Mizusawa and T. Yokota (2008). "Efficient in vivo delivery of siRNA to the liver by conjugation of alpha-tocopherol." Mol Ther **16**(4): 734-40.

Oldak, M., H. Smola, M. Aumailley, F. Rivero, H. Pfister and S. Smola-Hess (2004). "The human papillomavirus type 8 E2 protein suppresses beta4-integrin expression in primary human keratinocytes." J Virol **78**(19): 10738-46.

Oliveira, J. G., L. A. Colf and A. A. McBride (2006). "Variations in the association of papillomavirus E2 proteins with mitotic chromosomes." Proc Natl Acad Sci U S A **103**(4): 1047-52.

Orth, G. (2005). "Human papillomaviruses associated with epidermodysplasia verruciformis in non-melanoma skin cancers: guilty or innocent?" J Invest Dermatol **125**(1): xii-xiii.

Orth, G. (2006). "Genetics of epidermodysplasia verruciformis: Insights into host defense against papillomaviruses." Semin Immunol **18**(6): 362-74.

Parker, J. S., S. M. Roe and D. Barford (2006). "Molecular mechanism of target RNA transcript recognition by Argonaute-guide complexes." Cold Spring Harb Symp Quant Biol **71**: 45-50.

Pasquinelli, A. E., B. J. Reinhart, F. Slack, M. Q. Martindale, M. I. Kuroda, B. Maller, D. C. Hayward, E. E. Ball, B. Degnan, P. Muller, J. Spring, A. Srinivasan, M. Fishman, J. Finnerty, J. Corbo, M. Levine, P. Leahy, E. Davidson and G. Ruvkun (2000). "Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA." Nature **408**(6808): 86-9.

Petersen, C. P., M. E. Bordeleau, J. Pelletier and P. A. Sharp (2006). "Short RNAs repress translation after initiation in mammalian cells." Mol Cell **21**(4): 533-42.

Pfefferle, R., G. P. Marcuzzi, B. Akgul, H. U. Kasper, F. Schulze, I. Haase, C. Wickenhauser and H. Pfister (2008). "The human papillomavirus type 8 E2 protein induces skin tumors in transgenic mice." J Invest Dermatol **128**(9): 2310-5.

Pfister, H. (2003). "Chapter 8: Human papillomavirus and skin cancer." J Natl Cancer Inst Monogr(31): 52-6.

Pfister H, and Fuchs P (1987). Papillomaviruses: particles , genome organisation and proteins. In: *Papillomaviruses and human disease*. Herausg: K. Syrijänen, L. Gissmann, L.G. Koss, *et al.*

Pfister, H. and P. G. Fuchs (1994). "Anatomy, taxonomy and evolution of papillomaviruses." Intervirology **37**(3-4): 143-9.

Pfister, H. and J. Ter Schegget (1997). "Role of HPV in cutaneous premalignant and malignant tumors." Clin Dermatol **15**(3): 335-47.

Pillai, R. S., S. N. Bhattacharyya, C. G. Artus, T. Zoller, N. Cougot, E. Basyuk, E. Bertrand and W. Filipowicz (2005). "Inhibition of translational initiation by Let-7 MicroRNA in human cells." Science **309**(5740): 1573-6.

Pottier, N., T. Maurin, B. Chevalier, M. P. Puissegur, K. Lebrigand, K. Robbe-Sermesant, T. Bertero, C. L. Lino Cardenas, E. Courcot, G. Rios, S. Fourre, J. M. Lo-Guidice, B. Marcet, B. Cardinaud, P. Barbry and B. Mari (2009). "Identification of keratinocyte growth factor as a target of microRNA-

155 in lung fibroblasts: implication in epithelial-mesenchymal interactions." PLoS One **4**(8): e6718.

Provenzano, M. and S. Mocellin (2007). "Complementary techniques: validation of gene expression data by quantitative real time PCR." Adv Exp Med Biol **593**: 66-73.

Ramoz, N., L. A. Rueda, B. Bouadjar, L. S. Montoya, G. Orth and M. Favre (2002). "Mutations in two adjacent novel genes are associated with epidermodysplasia verruciformis." Nat Genet **32**(4): 579-81.

Ramoz, N., A. Taieb, L. A. Rueda, L. S. Montoya, B. Bouadjar, M. Favre and G. Orth (2000). "Evidence for a nonallelic heterogeneity of epidermodysplasia verruciformis with two susceptibility loci mapped to chromosome regions 2p21-p24 and 17q25." J Invest Dermatol **114**(6): 1148-53.

Reinartz, M. (2007). "Quantitative Analyse kleiner regulierender Ribonukleinsäuren in Normalgewebe und Hauttumoren." *Diplomarbeit*, Institut für Virologie, Universität zu Köln.

Rodriguez, A., S. Griffiths-Jones, J. L. Ashurst and A. Bradley (2004). "Identification of mammalian microRNA host genes and transcription units." Genome Res **14**(10A): 1902-10.

Rodriguez, A., E. Vigorito, S. Clare, M. V. Warren, P. Couttet, D. R. Soond, S. van Dongen, R. J. Grocock, P. P. Das, E. A. Miska, D. Vetrie, K. Okkenhaug, A. J. Enright, G. Dougan, M. Turner and A. Bradley (2007). "Requirement of bic/microRNA-155 for normal immune function." Science **316**(5824): 608-11.

Rong, S., S. Segal, M. Anver, J. H. Resau and G. F. Vande Woude (1994). "Invasiveness and metastasis of NIH 3T3 cells induced by Met-hepatocyte growth factor/scatter factor autocrine stimulation." Proc Natl Acad Sci U S A **91**(11): 4731-5.

Rossiter, H., S. Beissert, C. Mayer, M. P. Schon, B. G. Wienrich, E. Tschachler and T. S. Kupper (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): 3619-26.

Roth D (2000) Untersuchungen des zelltransformiereneden Potentials hautspezifischer Papillomviren. *Diplomarbeit.* Institut für Virologie, Universität zu Köln.

Ruby, J. G., C. Jan, C. Player, M. J. Axtell, W. Lee, C. Nusbaum, H. Ge and D. P. Bartel (2006). "Large-scale sequencing reveals 21U-RNAs and additional microRNAs and endogenous siRNAs in C. elegans." Cell **127**(6): 1193-207.

Saito, K., A. Ishizuka, H. Siomi and M. C. Siomi (2005). "Processing of pre-microRNAs by the Dicer-1-Loquacious complex in Drosophila cells." PLoS Biol **3**(7): e235.

Sambrook, J., Fritsch, E. F., Maniatis. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y, 1989.

Sampson, V. B., N. H. Rong, J. Han, Q. Yang, V. Aris, P. Soteropoulos, N. J. Petrelli, S. P. Dunn and L. J. Krueger (2007). "MicroRNA let-7a down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells." Cancer Res **67**(20): 9762-70.

Sanger, F., S. Nicklen and A. R. Coulson (1977). "DNA sequencing with chain-terminating inhibitors." Proc Natl Acad Sci U S A **74**(12): 5463-7.

Schaper, I. D., G. P. Marcuzzi, S. J. Weissenborn, H. U. Kasper, V. Dries, N. Smyth, P. Fuchs and H. Pfister (2005). "Development of skin tumors in mice

transgenic for early genes of human papillomavirus type 8." Cancer Res **65**(4): 1394-400.

Scheffner, M., J. M. Huibregtse, R. D. Vierstra and P. M. Howley (1993). "The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53." Cell **75**(3): 495-505.

Schmitt, A., J. B. Harry, B. Rapp, F. O. Wettstein and T. Iftner (1994). "Comparison of the properties of the E6 and E7 genes of low- and high-risk cutaneous papillomaviruses reveals strongly transforming and high Rb-binding activity for the E7 protein of the low-risk human papillomavirus type 1." J Virol **68**(11): 7051-9.

Schwarz, D. S., G. Hutvagner, T. Du, Z. Xu, N. Aronin and P. D. Zamore (2003). "Asymmetry in the assembly of the RNAi enzyme complex." Cell **115**(2): 199-208.

Selinka, H. C., T. Giroglou and M. Sapp (2002). "Analysis of the infectious entry pathway of human papillomavirus type 33 pseudovirions." Virology **299**(2): 279-287.

Sethi, N. and J. Palefsky (2004). "Transcriptional profiling of dysplastic lesions in K14-HPV16 transgenic mice using laser microdissection." FASEB J **18**(11): 1243-5.

Shah, K. and p. M. Howley (1996). Papillomaviruses. Fields, Virology. Third edition.

Shi, R. and V. L. Chiang (2005). "Facile means for quantifying microRNA expression by real-time PCR." Biotechniques **39**(4): 519-25.

Siegsmund, M., K. Wayss and E. Amtmann (1991). "Activation of latent papillomavirus genomes by chronic mechanical irritation." J Gen Virol **72 (Pt 11)**: 2787-9.

Smola-Hess S, and Pfister H (2002). Interaction of papillomaviral oncoproteins with cellular factors. In: *Structure-function relationships of human pathogeneic viruses*. New York, London, Dodrecht, Boston, Moscow. Kluver Academic/PlenumPublishers.

Somasundaram, K. (2000). "Tumor suppressor p53: regulation and function." Front Biosci **5**: D424-37.

Stahlberg, A., J. Hakansson, X. Xian, H. Semb and M. Kubista (2004). "Properties of the reverse transcription reaction in mRNA quantification." Clin Chem **50**(3): 509-15.

Steger, G., J. Ham and M. Yaniv (1996). "E2 proteins: modulators of papillomavirus transcription and replication." Methods Enzymol **274**: 173-85.

Steger, G. and H. Pfister (1992). "In vitro expressed HPV 8 E6 protein does not bind p53." Arch Virol **125**(1-4): 355-60.

Stern, R. S. (1999). "The mysteries of geographic variability in nonmelanoma skin cancer incidence." Arch Dermatol **135**(7): 843-4.

Stubenrauch, F. and L. A. Laimins (1999). "Human papillomavirus life cycle: active and latent phases." Semin Cancer Biol **9**(6): 379-86.

Stubenrauch, F., J. Malejczyk, P. G. Fuchs and H. Pfister (1992). "Late promoter of human papillomavirus type 8 and its regulation." J Virol **66**(6): 3485-93.

Takayama, H., W. J. LaRochelle, R. Sharp, T. Otsuka, P. Kriebel, M. Anver, S. A. Aaronson and G. Merlino (1997). "Diverse tumorigenesis associated with aberrant development in mice overexpressing hepatocyte growth factor/scatter factor." Proc Natl Acad Sci U S A **94**(2): 701-6.

Tan, S. H., B. Gloss and H. U. Bernard (1992). "During negative regulation of the human papillomavirus-16 E6 promoter, the viral E2 protein can displace Sp1 from a proximal promoter element." Nucleic Acids Res **20**(2): 251-6.

Tan, Y., B. Zhang, T. Wu, G. Skogerbo, X. Zhu, X. Guo, S. He and R. Chen (2009). "Transcriptional inhibiton of Hoxd4 expression by miRNA-10a in human breast cancer cells." BMC Mol Biol **10**: 12.

Thai, T. H., D. P. Calado, S. Casola, K. M. Ansel, C. Xiao, Y. Xue, A. Murphy, D. Frendewey, D. Valenzuela, J. L. Kutok, M. Schmidt-Supprian, N. Rajewsky, G. Yancopoulos, A. Rao and K. Rajewsky (2007). "Regulation of the germinal center response by microRNA-155." Science **316**(5824): 604-8.

Thomas, J. T. and L. A. Laimins (1998). "Human papillomavirus oncoproteins E6 and E7 independently abrogate the mitotic spindle checkpoint." J Virol **72**(2): 1131-7.

Thomas, M., R. Laura, K. Hepner, E. Guccione, C. Sawyers, L. Lasky and L. Banks (2002). "Oncogenic human papillomavirus E6 proteins target the MAGI-2 and MAGI-3 proteins for degradation." Oncogene **21**(33): 5088-96.

Tijsterman, M., R. F. Ketting and R. H. Plasterk (2002). "The genetics of RNA silencing." Annu Rev Genet **36**: 489-519.

Tinsley, J. M., C. Fisher and P. F. Searle (1992). "Abnormalities of epidermal differentiation associated with expression of the human papillomavirus type 1 early region in transgenic mice." J Gen Virol **73 (Pt 5)**: 1251-60.

Tulasne, D. and B. Foveau (2008). "The shadow of death on the MET tyrosine kinase receptor." Cell Death Differ **15**(3): 427-34.

Turek, L. P. (1994). "The structure, function, and regulation of papillomaviral genes in infection and cervical cancer." Adv Virus Res **44**: 305-56.

VanGuilder, H. D., K. E. Vrana and W. M. Freeman (2008). "Twenty-five years of quantitative PCR for gene expression analysis." Biotechniques **44**(5): 619-26.

Volinia, S., G. A. Calin, C. G. Liu, S. Ambs, A. Cimmino, F. Petrocca, R. Visone, M. Iorio, C. Roldo, M. Ferracin, R. L. Prueitt, N. Yanaihara, G. Lanza, A. Scarpa, A. Vecchione, M. Negrini, C. C. Harris and C. M. Croce (2006). "A microRNA expression signature of human solid tumors defines cancer gene targets." Proc Natl Acad Sci U S A **103**(7): 2257-61.

Voorhoeve, P. M., C. le Sage, M. Schrier, A. J. Gillis, H. Stoop, R. Nagel, Y. P. Liu, J. van Duijse, J. Drost, A. Griekspoor, E. Zlotorynski, N. Yabuta, G. De Vita, H. Nojima, L. H. Looijenga and R. Agami (2007). "A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors." Adv Exp Med Biol **604**: 17-46.

Walboomers, J. M., M. V. Jacobs, M. M. Manos, F. X. Bosch, J. A. Kummer, K. V. Shah, P. J. Snijders, J. Peto, C. J. Meijer and N. Munoz (1999). "Human papillomavirus is a necessary cause of invasive cervical cancer worldwide." J Pathol **189**(1): 12-9.

Wang, X., S. Tang, S. Y. Le, R. Lu, J. S. Rader, C. Meyers and Z. M. Zheng (2008). "Aberrant expression of oncogenic and tumor-suppressive microRNAs in cervical cancer is required for cancer cell growth." PLoS One **3**(7): e2557.

Weissenborn, S. J., M. N. De Koning, U. Wieland, W. G. Quint and H. J. Pfister (2009). "Intrafamilial transmission and family-specific spectra of cutaneous betapapillomaviruses." J Virol **83**(2): 811-6.

Weissenborn, S. J., R. Neale, M. N. de Koning, T. Waterboer, D. Abeni, J. N. Bouwes Bavinck, U. Wieland and H. J. Pfister (2009). "Prevalence and multiplicity of cutaneous beta papilloma viruses in plucked hairs depend on cellular DNA input." J Virol Methods **161**(2): 280-3.

Wells, S. I., D. A. Francis, A. Y. Karpova, J. J. Dowhanick, J. D. Benson and P. M. Howley (2000). "Papillomavirus E2 induces senescence in HPV-positive cells via pRB- and p21(CIP)-dependent pathways." EMBO J **19**(21): 5762-71.

Werness, B. A., A. J. Levine and P. M. Howley (1990). "Association of human papillomavirus types 16 and 18 E6 proteins with p53." Science **248**(4951): 76-9.

Yan, D., E. Dong Xda, X. Chen, L. Wang, C. Lu, J. Wang, J. Qu and L. Tu (2009). "MicroRNA-1/206 targets c-Met and inhibits rhabdomyosarcoma development." J Biol Chem **284**(43): 29596-604.

Yekta, S., I. H. Shih and D. P. Bartel (2004). "MicroRNA-directed cleavage of HOXB8 mRNA." Science **304**(5670): 594-6.

Yi, R., M. N. Poy, M. Stoffel and E. Fuchs (2008). "A skin microRNA promotes differentiation by repressing 'stemness'." Nature **452**(7184): 225-9.

Yoshida, S., Y. Yamaguchi, S. Itami, K. Yoshikawa, Y. Tabata, K. Matsumoto and T. Nakamura (2003). "Neutralization of hepatocyte growth factor leads to retarded cutaneous wound healing associated with decreased neovascularization and granulation tissue formation." J Invest Dermatol **120**(2): 335-43.

Zarnegar, R. (1995). "Regulation of HGF and HGFR gene expression." EXS **74**: 33-49.

Zhang, P., M. Nouri, J. L. Brandsma, T. Iftner and B. M. Steinberg (1999). "Induction of E6/E7 expression in cottontail rabbit papillomavirus latency following UV activation." Virology **263**(2): 388-94.

Zhang, Z. H., Z. M. Wang, M. E. Crosby, K. F. Kang, J. Luan, W. Huang, L. H. Xiang and Z. Z. Zheng (2009). "Reassessment of microarray expression data of porokeratosis by quantitative real-time polymerase chain reaction." J Cutan Pathol.

Zhao, Y. and D. Srivastava (2007). "A developmental view of microRNA function." Trends Biochem Sci **32**(4): 189-97.

Zhu, S., M. L. Si, H. Wu and Y. Y. Mo (2007). "MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1)." J Biol Chem **282**(19): 14328-36.

zur Hausen, H. (2000). "Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis." J Natl Cancer Inst **92**(9): 690-8.

zur Hausen, H. (2002). "Papillomaviruses and cancer: from basic studies to clinical application." Nat Rev Cancer **2**(5): 342-50.

7 List of abbreviations

AA	amino acid
AEC	3-amino-9-ethylcarbazole
AK	actinic keratosis
AP-1	activator protein 1
ATP	adenosine triphosphate
B2M	beta-2-microglobulin
bp	base pair
BSA	bovine serum albumin
CDK	cyclin-dependent kinase
CRPV	cottontail rabbit papillomavirus
DAB	3,3'-Diaminobenzidine
DAPI	4′,6-diamidino-2-phenylindol
Dig	digoxigenin
DMBA	dimethylbenzanthracene
DMEM	Dulbecco’s modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dsRBD	double-stranded RNA-binding domain
dsRNAs	double stranded RNAs
E6-AP	E6-associated protein
E. coli	Escherichia coli
EDTA	ethylenediaminetetraacetate
EIF4A	eukaryotic translation initiation factor 4A
EV	epidermodysplasia verruciformis
FCS	fetal calf serum
FVB/N	Friend leukaemia virus B strain
g	gravitational acceleration
CER	complete early region (of the HPV8 genome)
h	human
hDlg	human discs large (drosophila) homolog

HE-staining	hematoxylin and eosin staining
HGF	hepatocyte growth factor
HPLC	high pressure liquid chromatography
HPRT1	hypoxanthine phosphoribosyltransferase 1
HPV	human papillomvirus
HPV8-CER	mouse strain transgenic for the complete early region of HPV8
hScrib	human scribble (drosophila) homolog
Hz	hertz
IHC	immunohistochemistry
IL1-α	interleukin 1-α
ISH	in situ hybridization
K14	keratin 14
kb	kilobase
kDa	kilodalton
KGF	keratinocyte growth factor
LB-medium	Luria Bertani medium
LTBP1	latent transforming growth factor beta binding protein 1
LTR	long terminal repeat
µM	micromolar
m	murine
MAGUK	membrane associated guanylate kinase
MAPK	mitogen-activated protein kinase
MET	mesenchymal-epithelial transition factor
min	minute
miRNA	microRNA
mM	millimolar
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MUPP1	multiple-PDZ domain protein
MW	molecular weight
NCR	non-coding region
nM	nanomolar
NMSC	non-melanoma skin cancer
nt	nucleotide

OD	optical density
ORF	open reading frame
ori	origin of replication
P-bodies	processing bodies
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDCD4	programmed cell death 4
PDZ	PSD-95, Dlg, Zo-1
PEG	polyethylene glycol
PI3K	phosphoinositide-3-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol-3,4,5-trisphosphate
POD	peroxidase
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PTEN	phosphatase and tensin homolog
PV	papillomvirus
qPCR	quantitative polymerase chain reaction
qRT-PCR	quantitative real-time polymerase chain reaction
Rb	retinoblastoma protein 1
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
rpm	rounds per minute
RT	room temperature
SCC	squamous cell carcinoma
SDS	sodium dodecyl sulfate
siRNA	small interfering RNA
Sp1	Specific protein 1
TBP	TATA binding protein
TGF-β	transforming growth factor-β
TGFBR2	transforming growth factor β receptor 2
TPA	12-O-tetradecanoylphorbol-13-acetate
TRBP	trans-activation-responsive RNA-binding protein

Tris	tris(hydroxymethyl)aminomethane
TSB-medium	transformation and storage medium
u	atomic mass unit
UV	ultraviolet
VLP	virus like particles
WHO	World Health Organization
wt	wild type

8 Abstract

An active role of cutaneous human papillomaviruses (HPV) in the pathogenesis of non-melanoma skin cancer (NMSC) in the general population is currently discussed. At least in immunosuppressed and epidermodysplasia verruciformis patients, an association between cutaneous HPV and NMSC is accepted. An oncogenic potential of the cutaneous HPV type 8 could be demonstrated in a transgenic mouse model, expressing all early genes of HPV8 (HPV8-CER), which shows spontaneous papilloma development, predominantly on scratched skin areas. A single UVA/B-dose induces papillomatosis within three weeks in these mice, while the skin of FVB/N wild type mice heals completely within this time. In order to clarify the role of skin wounding and its influence on HPV gene expression in the early stages of tumor development in this mouse model, the kinetics and levels of transgene expression were analyzed in response to exogenous skin irritations induced by UVA/B-irradiation or mechanical wounding. Already on day one after irradiation enhanced HPV8 mRNA and protein levels were determined in the skin of HPV8-CER mice, which were preserved during the whole observation period of 30 days. Analogously in HPV8-E2 and -E6 mice papilloma development was also paralleled by enhanced transgene expression. Mechanically irritated skin also showed an enhanced HPV8 oncogene expression in HPV8-CER mice two days after irritation, which could be knocked-down by tattooing HPV8-E6-specific small interfering RNA leading to a delay and a lower incidence of papilloma development. This substantiates the hypothesis that the early HPV8 gene expression after wounding is crucial for papillomatosis in mice. The expression kinetics of selected cellular microRNAs (miRNAs) and their targets were determined after induction of papillomatosis to check for a correlation with HPV8 oncogene expression. Several miRNAs (miRNA-17-5p, -21, -106a, -155, -206) and their targets (PTEN, PDCD4, Rb, KGF, MET) showed expression alterations often found to be associated with carcinogenesis. The alterations occurred in parallel to the increase of HPV8 oncogene expression, which raises the assumption that HPV8 can directly or indirectly induce these alterations.

9 Zusammenfassung

Eine aktive Rolle von kutanen humanen Papillomviren (HPV) bei der Pathogenese von nicht-melanozytärem Hautkrebs (NMHK) wird zurzeit diskutiert. Eine Verbindung zwischen kutanen HPV und NMHK ist zumindest für immunsupprimierte und Epidermodysplasia verruciformis Patienten akzeptiert. Für den kutanen HPV Typ 8 konnte ein onkogenes Potential in einem transgenen Mausmodell gezeigt werden, welches alle frühen Gene von HPV8 exprimiert (HPV8-GFR). Diese Tiere entwickeln spontan Papillome, überwiegend auf zerkratzten Hautarealen. Eine einzige Bestrahlung mit UVA/B induziert in diesen Mäusen eine Papillomatose innerhalb von drei Wochen, während die Haut von FVB/N Wildtyp Mäusen in dieser Zeit vollständig verheilt. Um die Rolle der Hautverletzung und ihren Einfluss auf die HPV Genexpression in den frühen Stadien der Tumorentwicklung in diesem Mausmodell zu klären, wurden die Kinetik und Höhe der Transgenexpression analysiert als Antwort auf exogene Hautreizungen, verursacht durch UVA/B-Bestrahlung oder mechanische Verwundung. Bereits einen Tag nach Bestrahlung wurden erhöhte HPV8 mRNA und Protein Spiegel in der Haut von HPV8-GFR Mäusen ermittelt, die während der gesamten Beobachtungsdauer von 30 Tagen erhalten blieben. Analog dazu war die Papillomentwicklung in HPV8-E2 und HPV8-E6 Mäusen ebenfalls begleitet von einer erhöhten Transgenexpression. Mechanisch gereizte Haut zeigte ebenfalls eine erhöhte HPV8 Onkogenexpression in HPV8-GFR Mäusen zwei Tage nach der Reizung. Diese Expression konnte durch tätowieren einer HPV8-E6-spezifischen small interfering RNA unterdrückt werden, was die Papillomentwicklung verzögerte und reduzierte. Dies untermauert die Hypothese, dass die frühe HPV8 Genexpression nach der Verwundung entscheidend für die Papillomatose in Mäusen ist. Die Expressionskinetiken von ausgewählten microRNAs (miRNAs) und deren Zielgenen wurden nach Induktion der Papillomatose ermittelt, um zu prüfen, ob sie mit der HPV8 Onkogenexpression korrelieren. Mehrere miRNAs (miRNA-17-5p, -21, -106a, -155, -206) und deren Zielgene (PTEN, PDCD4, Rb, KGF, MET) zeigten Expressionsveränderungen die bekanntermaßen mit Karzinogenese assoziiert sind. Die Veränderungen wurden von einem Anstieg der HPV8 Onkogenexpression begleitet, was die Vermutung aufkommen lässt, dass HPV8 diese Veränderungen direkt oder indirekt induzieren kann.

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11 Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen - noch nicht veröffentlich worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Herrn Prof. Dr. Dr. h.c. Herbert Pfister betreut worden.

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12 Lebenslauf

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