Virology 535 (2019) 136–143

Contents lists available at ScienceDirect

Virology

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

HPV8 activates cellular gene expression mainly through Sp1/3 binding sites

Matthias Kirschberg[a,1], Sandra Heuser[a,1], Adnan S. Syed[a], Gertrud Steger[a], Slawomir Majewski[b], Martin Hufbauer[a], Baki Akgül[a,*]

[a] Institute of Virology, University of Cologne, Faculty of Medicine and University Hospital of Cologne, Germany
[b] Department of Dermatology and Venereology, Medical University of Warsaw, Warsaw, Poland

ARTICLE INFO

Keywords:
Human papillomavirus
SP1/3
Fibronectin
Transcription
Organotypic skin culture
Epidermodysplasia verruciformis

ABSTRACT

The human papillomavirus type 8 (HPV8) is associated with skin cancer development. The goal of this study was to investigate the effects of HPV8 oncoproteins on cellular gene expression and the identification of key regulators. We performed affymetrix microarray analyses to identify differentially expressed genes and common sequence motifs and identified Sp1/3 binding sites as being crucial. In transient transfection assays, we confirmed that HPV8-E7 stimulates the activity of Sp1/3 promoters. Interestingly, the HPV8-E7^{L23A} mutant, which cannot trigger keratinocyte invasion was unable to activate fibronectin gene expression. In skin models or HPV8 positive skin cancers we found a peculiar deposition of fibronectin in the dermal compartment, and a correlation of Sp3 and fibronectin in the nucleus of HPV8-positive keratinocytes.

Taken together, we identified that HPV8-E7 exerts control over cellular gene expression through Sp1/3 binding motifs, which may contribute to HPV8-mediated keratinocyte transformation and subsequent fibronectin-dependent invasion.

1. Introduction

Epidemiological and experimental data demonstrate an involvement of human papillomaviruses of genus betapapillomavirus (betaHPV) in the development of cutaneous squamous cell carcinoma (SCC) (Howley and Pfister, 2015; Tommasino, 2019). In particular, Epidermodysplasia verruciformis (EV) patients suffer from betaHPV mediated skin carcinogenesis. EV is a rare autosomal recessive hereditary skin disease characterized by increased susceptibility for betaHPV infection, such as HPV8 (Imahorn et al., 2017). It is also an accepted fact, that there is a direct link between betaHPV infection and cancer development in immunosuppressed organ-transplant recipients (OTR) (Bouwes Bavinck et al., 2018).

To further our understanding regarding the oncogenic potential of HPV8, we had previously generated a variety of HPV8 transgenic mouse models, the first of which is expressing the complete early genome region (CER) under the control of the keratin-14 promoter (K14). These mice displayed formation of skin papillomas with varying degrees of dysplasia as well as skin SCCs (Hufbauer et al., 2010; Schaper et al., 2005). K14-HPV8-E7 mice did not show papilloma formation, but exhibited carcinoma development (Heuser et al., 2016b).

Using monolayer cultures of primary human adult keratinocytes (PHK) the expression of HPV8-CER and HPV8-E7 induced an abnormal keratin expression pattern, that included simple epithelial (K8, K18, K19), hyperproliferation-specific (K6, K16), basal-specific (K14, K15) and differentiation-specific (K1, K10) keratins. The expression of hyperproliferation-associated keratins in HPV8-E7 cells was also paralleled by loss of G1/S control and cells were able to overcome the mitotic checkpoint (Akgül et al., 2007). Furthermore, we also demonstrated in the past that expression of HPV8-E7 is critical for the induction of keratinocyte invasion in organotypic skin cultures (OSC) (Akgül et al., 2005; Westphal et al., 2009). More recently, we could show that HPV8-E7 mediated keratinocyte invasion is triggered by the extracellular matrix protein fibronectin (FN), and an increase of integrin α3β1 surface presence. Intriguingly, we detected depositions of FN in adjacent tumoral stroma of HPV8-positive skin SCCs (Heuser et al., 2016b). Further evidence for the existence of the α3β1/FN axis arose from the observation that the HPV8-E7 mutant L23A (HPV8-E7^{L23A}) was neither capable of affecting α3β1 surface presence nor was it able to trigger keratinocyte invasion (Heuser et al., 2016b; Hufbauer and Akgül, 2017).

In this study, we aimed at a deeper understanding regarding the mechanisms involved in HPV8 mediated gene regulation.

* Corresponding author. Institute of Virology, University of Cologne, Fürst-Pückler-Str. 56, 50935, Cologne, Germany.
E-mail address: baki.akguel@uk-koeln.de (B. Akgül).
[1] These authors contributed equally.

https://doi.org/10.1016/j.virol.2019.06.019
Received 11 March 2019; Received in revised form 30 June 2019; Accepted 30 June 2019
Available online 01 July 2019
0042-6822/

2. Materials and methods

2.1. Cell culture

All cells were cultured at 37 °C and 6% CO_2. PHK were isolated from discarded skin acquired from surgeries. Isolated PHK were propagated on lethally irradiated NIH 3T3 feeder cells in passage 0 in keratinocyte culture media composed of three parts DMEM and one part Ham's F12 with 10% FCS and supplements as described elsewhere (Akgül et al., 2019). Before reaching confluence, cells were trypsinized, resuspended in FCS with 10% DMSO and stored in liquid nitrogen. The HPV-negative C33A cell line (Auersperg, 1969) was cultured in DMEM, supplemented with 10% FCS.

For retroviral transduction of PHKs at passage 1, cells were seeded out in defined keratinocyte serum-free medium (Thermofisher Scientific, Dreieich, Germany) and transduced with pLXSN-based recombinant retroviruses harboring either the empty pLXSN control vector, or the expression constructs for HPV8-E7 or HPV8-CER (Akgül et al., 2007). Following transduction, cells in total passage 2 were selected with G418, and positive clones expanded in passage 3. The use of these stable cell populations minimized possible variations due to the expected random viral genome integration into the host chromosomes.

2.2. Microarray analysis

For microarray analyses, total RNA was extracted from growing monolayer cultures of PHKs in total passage 3 (passage 2 after transduction) using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The RNA was analysed in biological triplicates from cells containing either the empty vector control (pLXSN), pLXSN-HPV8-E7 or pLXSN-HPV8-CER. Changes in gene expression were measured using the Human Genome U133A arrays according to Affymetrix protocols. Microarray data were analysed using Transcriptome Analysis Console software (Thermo Fisher, v4.0.1.36). For analysis of differential gene expression, we applied the following conditions to determine statistically relevant genes: a) ANOVA test: ebayes b) a fold change cut-off of < -2 or > 2, and c) determination of statistical significance with $p < 0.05$. Gene ontology analysis was carried out using the PANTHER (Protein ANalysis THrough Evolutionary Relationships) database version 14.1 (http://pantherdb.org/), which contains 15524 protein families, divided into 107627 functionally distinct protein subfamilies. The microarray data files have been uploaded to the Gene Expression Omnibus (GEO) database (GSE133813).

2.3. Plasmids

The human FN promoter fragment, which contains 1.2 kb of the 5' flanking region of the human FN gene originating from the human fibrosarcoma cell line HT1080 was cloned into the *Sma*I site of the pGL3 basic luciferase reporter vector. This led to the promoter construct pFN (1.2 kb)-LUC. This vector, as well as the truncation mutants pFN (0.5 kb)-LUC and pFN(0.2 kb)-LUC were kindly provided by Dr. J. Roman (Michaelson et al., 2002). To create the pFN(0.2 kb)-LUC-Sp1/3mut construct, the pFN(0.2 kb)-LUC plasmid was mutated at the Sp1/3 FN binding site by site-directed mutagenesis. The plasmid pALUC-Sp1/3 encompasses four synthetic HPV-E2 binding sites (CTAGACCGAAAA CGGTG) and two synthetic Sp1/3 binding sites (GATCTAAACCCCGCC CAGCCG) upstream of a minimal adenovirus major late promoter (Steger et al., 2002).

2.4. Transient reporter gene assays

For transient reporter gene assays, C33A or PHK were transiently transfected with FuGENE® 6 Transfection Reagent (Promega, Mannheim, Germany) in duplicates in 6 well dishes with 1 µg of luciferase reporter construct (pFN(1.2 kb)LUC, pFN(0.5 kb)LUC, pFN (0.2 kb)LUC, pFN(0.2 kb)LUC-SP1/3mut, pALUC-Sp1/3), pLXSN or pLXSN-8E7 (Akgül et al., 2005; Akgül et al., 2007) together with 0.25 µg reporter plasmid coding for renilla luciferase. The assays were performed using the Dual-Luciferase® Reporter Assay System (Promega) according to manufacturer's instructions. Luciferase assays were measured 48 h post-transfection and normalised to renilla values. Luciferase activity was measured using the Glomax® Luminometer (Promega).

2.5. Western blot

For Western blot analyses cells were trypsinized, pelleted by centrifugation and lysed on ice for 30 min in LSDB buffer (20% glycerol; 50 mM Tris-HCl, pH 7.9; 0.1% NP40; 100 mM KCl), supplemented with 1x protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Cell lysates were then sonicated and protein concentration was measured using the Bio-Rad DC™ Protein Assay (Bio-Rad, Puchheim, Germany). Cell extracts were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. After the membrane was blocked with 5% milk or 5% BSA in TBST (10 mM Tris/HCl, pH 8.0; 150 mM NaCl; 0.05% Tween 20) for 1 h, blots were probed with antibodies against Sp1 (GTX110593; Biozol, Eching, Germany) or Sp3 (Santa Cruz Clone G7, sc-365220). Tubulin (ab6160, Abcam, Cambridge, UK) or GAPDH (sc-365062 clone G9, Santa Cruz) were used as loading controls. Immunoreactive proteins were visualised using LICOR IRDye® labeled secondary antibodies. Fluorescence was detected using the LICOR Odyssey® FC device (LICOR, Bad Homburg, Germany).

2.6. RNA isolation, reverse transcription and real-time PCR

To quantify mRNA levels of cellular genes, quantitative reverse transcription-PCR (RT-qPCR) using the LightCycler system (Roche Diagnostics) was performed as previously described (Akgül et al., 2019). The primers used for RT-qPCR were: Sp3-fw: TTGCACCTGTCC CAACTGTA; Sp3-rev: TGTTGCTTCTTTTTCCCAAGA; HPRT1-fw: TGAC ACTGGCAAAACAATGCA; HPRT1-rev: GGTCCTTTTCACCAGCAAGCT.

2.7. Sections of EV skin lesions and organotypic skin cultures

Normal human skin as well as EV skin lesions were obtained during routine surgical excision, and embedded in paraffin (for detailed information regarding betaHPV typing and pathology results for EV tissue please see (Heuser et al., 2016a)). Ethical approval for the use of human samples was obtained from the Ethics Committee of the Medical University of Warsaw. The generation of OSCs of keratinocytes expressing HPV8-E7 was based on de-epidermalized human dermis serving as a dermal equivalent, which was then repopulated with keratinocytes. These 3D cultures were grown for 14 days at the air-liquid interphase, followed by fixing and embedding in paraffin (Westphal et al., 2009).

3. Results

3.1. Global expression profiling of PHK expressing either HPV8-E7 or HPV8-CER

Microarray analyses were performed to explore the effects of HPV8 early gene expression on global cellular gene expression in PHKs. For this purpose, we performed Affymetrix microarray analyses with RNA extracted from PHK expressing either HPV8-E7 alone or the HPV8-CER. The data was analysed using the ANOVA test and genes were considered statistically relevant when the gene-level fold change was < -2 or > 2 and the $p < 0.05$ (Fig. 1, Supplementary Fig. 1). We observed 250 differentially expressed genes in the HPV8-E7 population compared to the control, whereas there were only 145 differentially expressed genes in the HPV8-CER population. Interestingly, the number of upregulated genes in the HPV8-E7 population was about two-fold

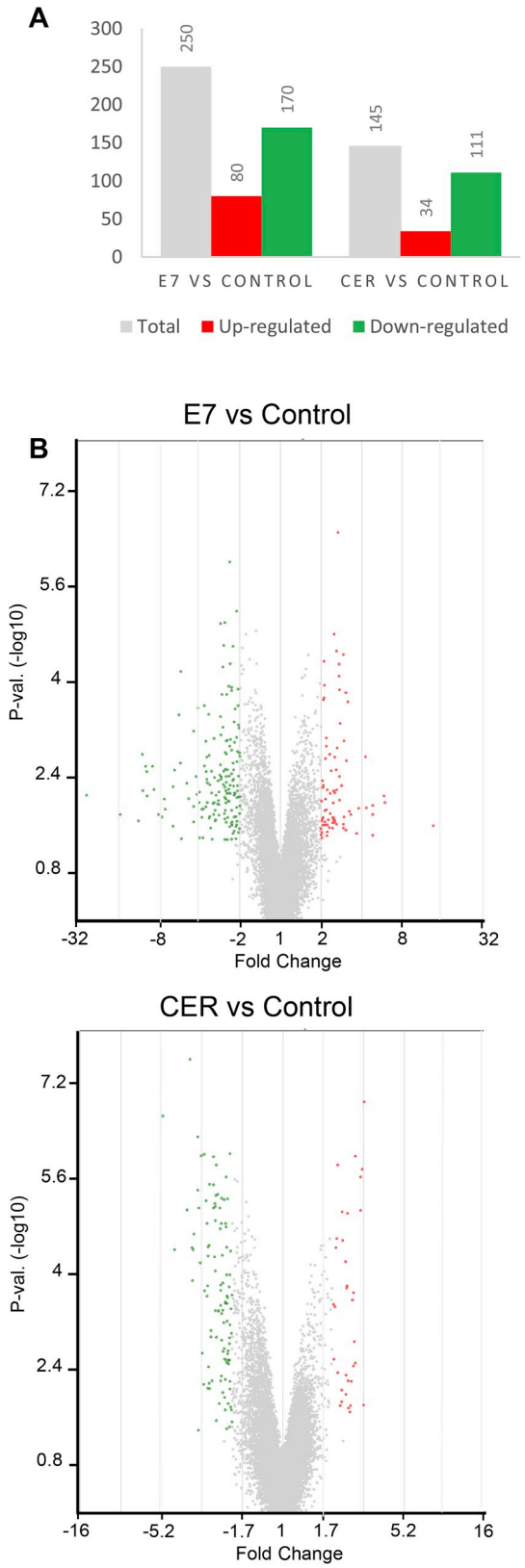


Fig. 1. Global gene expression profiling. A) Bar charts showing differentially expressed genes in HPV8-E7 vs. control or HPV8-CER vs. control (grey: total number of genes; red: up-regulated genes; green: down-regulated genes. B) Volcano plot depicting fold-change gene expression in HPV8-E7 vs control or HPV8-CER vs. control. (red: up-regulated genes; green: down-regulated genes).

higher than in CER (Fig. 1A). Furthermore, the overall fold changes in gene expression were also stronger in the HPV8-E7 versus control analysis as seen in the Vulcan plots (Fig. 1B). The gene ontology analysis sub-categorizing differentially genes in biological processes, cellular components and molecular functions are shown in Supplementary Fig. 2 and Fig. 3.

In order to understand how HPV8 might exert control over cellular gene expression, less stringent gene lists were translated to the Human9999 genome, which contained the first 9999bp of the upstream regions of all genes present in the ENSEMBL database. Regulatory sequences of five to eight base pairs between 10 and 500 bases upstream of dysregulated genes were matched with a $p < 0.05$ threshold. Afterwards, search criteria were further narrowed down to conserved regulatory sequences of 5–8 bp in length between 10 and 500 bp upstream of each of 295 picked genes, requesting exact matches with $p < 0.05$. This motif enrichment analysis led to the identification of four nucleotide sequences with 6 bp: 5′-CGCCCC-3′, 5′-CCGCCT-3′, 5′-CGCCTC-3′, 5′-TCCGCC-3’ (Fig. 2A). Interestingly, sequences 1 to 3 are complementary to the decanucleotide consensus sequence of the transcription factors Sp1/3: 5’-(G/T)GGGCGG(G/A) (G/A) (C/T)-3’ (Li and Davie, 2010; Nagaoka et al., 2001). The reverse complement orientation of sequence 4 also overlapped with the consensus Sp1/3 binding motif (Fig. 2B). The analysis of the microarray data led to the identification of only one Sp-family member, namely Sp3 as being 2.5-fold upregulated in HPV8-E7 expressing PHK. To confirm if HPV8-E7 has the ability to trans-activate promoters containing Sp1/3 binding motifs, we tested its activity on the synthetic pALuc-Sp1/3 promoter construct, which contains two Sp1/3 binding sites in front of a TATA box, as well as the luciferase gene (Fig. 2C). PHK were transiently co-transfected with pALuc-Sp1/3 and expression vectors coding for HPV8-E7. Luciferase activity of the promoter construct, measured 48 h post-transfection, was increased up to 8-fold in the presence of E7 (Fig. 2D). These results support our hypothesis that HPV8-E7 may exert control over cellular gene expression through Sp1/3 binding sites.

3.2. Expression of Sp3 and fibronectin correlates in HPV8 positive epithelia

Based on our own research we already know that FN expression is indeed modulated by HPV8-E7 (Heuser et al., 2016b) and is crucial for the invasive phenotype of E7 positive cells. To test whether Sp3 may be involved in the control of FN expression in betaHPV-positive differentiating epithelia, we first analysed the staining patterns in OSCs comprised either of PHK harboring the empty vector pLXSN, or PHK expressing the HPV8-E7. In epithelium repopulated with the control keratinocytes we only detected very weak Sp3 levels in the differentiating keratinocytes, and FN was only found, as expected, in the dermal compartment of the OSCs. Interestingly, compared to the control, Sp3 signals were more pronounced in the HPV8-E7 OSCs. Even more exciting though was the observation, that in the HPV8-E7 OSCs we observed strong stromal FN deposition as well as nuclear FN, which also partially correlated with Sp3 signals (Fig. 3A). In skin lesions from EV patients we observed an even more pronounced correlation in respect to the Sp3/FN staining patterns observed in the OSCs. In the EV lesions, a vast majority of keratinocytes in all epithelial layers were found to be expressing Sp3 as well as nuclear FN (Fig. 3B), which was in stark contrast to normal/healthy skin of non-EV patients, where we could not detect any FN deposition in the epidermal compartment at all. Interestingly, when staining skin sections from the same OSCs and EV tissues for Sp1 with several antibodies we did not observe any Sp1 signal anywhere throughout the skin (data not shown).

3.3. The fibronectin promoter is activated by HPV8-E7 through a Sp1/3 binding site

We next investigated the mechanism by which HPV8-E7 may exert control over FN expression. It is known that the human FN promoter

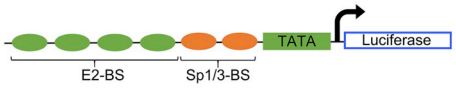
A

No.	Sequence	observed	p-value	random rate	observed rate	single p	tests
1	CGCCCC	135/295	0.00375	25.22%	31.54%	$2.3x10^{-7}$	16384
2	CCGCCT	104/295	0.0417	18.74%	23.34%	$2.6x10^{-6}$	16384
3	CGCCTC	106/295	0.00339	18.74%	22.75%	$2.07x10^{-7}$	16384
4	TCCGCC	98/295	0.000594	18.74%	19.74%	$3.62x10^{-8}$	16384

B

```
5'-(G/T)GGGCGG(G/A)(G/A)(C/T)-3'    Sp1/3 Consensus Binding Site
3'-(C/A)CCCGCC(C/T)(C/T)(G/A)-5'
          CGCC C    C              seq. 1
         CCGCC   T                 seq. 2
          CGCC   T  C              seq. 3
         CCGCC   T                 seq. 4
```

C



D

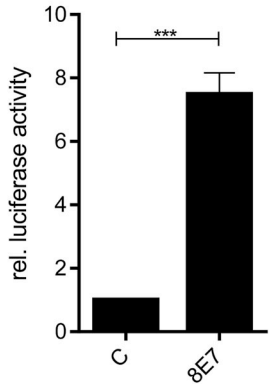


Fig. 2. Identification of regulatory sequences in HPV8-E7 regulated genes using upstream sequence analysis. (A) Sequence: Identified conserved sequence motif; Observed: number of genes in which the conserved motif was found; Random rate: % of genes (intrinsic probability) expected to contain the nucleotide combination if the nucleotide sequences were strictly random; Observed rate: the probability of the observed sequence occurring relative to the genes not regulated by HPV8; Single p: The probability that a particular sequence would be found, if only one test had been performed; Tests: number of oligomers tested having a length of the found sequence motif). (B) Sequence alignments of Seq. 1–4 (identified in A) with the Sp1/3 consensus binding site. (C) Schematic representation of a synthetic luciferase reporter construct composed of the minimal adenovirus major late promoter (MLP) in front of four E2 and two Sp1/3 binding sites. (D) The luciferase construct was transfected into PHK with HPV8-E7 expression vectors. Increased luciferase levels were measured in presence of HPV8-E7. Expression level of the corresponding co-transfected empty vector (control) was set as 1 (n = 3).

contains a Sp1/3 binding site at position (−136 to −127 (Michaelson et al., 2002) (Fig. 4A). To confirm that HPV8-E7 is capable of inducing the FN promoter, and in order to narrow down where the responsive element within the promoter region is, luciferase promoter assays were performed with FN promoter constructs pFN(1.2 kb)-LUC, or the truncated promoter constructs pFN(0.5 kb)-LUC, and pFN(0.2 kb)-LUC, respectively. PHKs were transiently transfected with the FN promoter constructs together with HPV8-E7 expression vector or empty vector control. HPV8-E7 activated all three tested FN promoter constructs about 2-fold (Fig. 4B). To confirm that the Sp1/3 binding site plays a pivotal role in FN promoter regulation in the presence of HPV8-E7, we generated a variant of the pFN(0.2 kb)-LUC with a mutated Sp1/3 binding motif (pFN(0.2 kb)-LUC-Sp1/3mut). This mutant failed to activate the FN promoter in the presence of HPV8-E7 as shown in Fig. 4B, indicating that the HPV8-E7 protein may exert control over Sp3 to enhance FN expression.

3.4. The invasion-deficient mutant HPV8-E7-L23A is incapable of transactivating the fibronectin promoter

To address the question whether the HPV8-$E7^{L23A}$ mutant may be defective in stimulating the FN promoter, the pFN(0.2 kb)-LUC and the pFN(0.2 kb)-LUC-Sp1/3mut constructs were co-transfected with expression vectors coding for HPV8-$E7^{wt}$ or -$E7^{L23A}$ into keratinocytes. As expected, in C33A and PHKs, HPV8-$E7^{wt}$ activated the pFN(0.2 kb)-LUC construct, whereas the HPV8-$E7^{L23A}$ mutant, which is known to be as stable as the wildtype E7 protein (Heuser et al., 2016b), failed to activate both pFN(0.2 kb)-LUC and pFN(0.2 kb)LUC-Sp1/3mut (Fig. 5A and B). Furthermore, in subsequent Western blots we could show that Sp3 protein levels were strongly increased in HPV8-$E7^{wt}$ expressing cells, whereas in HPV8-$E7^{L23A}$ positive cells there was a visible reduction Sp3 levels were less pronounced than in $E7^{WT}$ cells (Fig. 5C). The HPV8-$E7^{wt}$ dependent increase of Sp3 appears to be achieved through transcriptional activation, as Sp3 mRNA levels were significantly elevated in HPV8-$E7^{wt}$ cells compared to the control (Fig. 5C, ***p = 0.001). In addition, the invasion deficient HPV8-E7L23A mutant was less able to trigger Sp3 mRNA expression than HPV8-$E7^{WT}$ (Fig. 5D, *p = 0.0415). Unexpectedly, as neither the microarray data nor immunofluorescence tissue staining had shown Sp1 as a significant target, Sp1 protein levels were also found to be elevated in both HPV8-E7wt and HPV8-E7L23A PHK compared to the control (Fig. 5D). However, we observed that Sp1 fluctuates between different keratinocyte cell lines expressing HPV8-E7 (data not shown).

4. Discussion

In this study, we explored the effects of HPV8 early proteins on cellular gene expression. Differentially expressed genes were identified by microarray analyses of RNA from PHK expressing either HPV8-E7 alone or all early proteins simultaneously. By comparing these two populations we saw an about two-fold enrichment of upregulated genes in E7 cells, indicating that the co-expression of E7 with E1^E4, E2, E6 and E8^E2 may affect the transcriptional outcome in oncogene positive cells.

The main objective of this study, however, was the identification of upstream regulators hijacked by HVP8 to manipulate cellular gene expression. For this purpose, we used the generated microarray data, and identified the transcription Sp3 as a key regulator of cellular genes in HPV8 positive PHK. Sp3 belongs to a transcription factor family with four known members, namely: Sp1, Sp2, Sp3 and Sp4. Despite being one transcription factor family, there is only very little functional overlap amongst the Sp proteins, with the exception of Sp1 and Sp3, which play a dual role in the regulation of gene expression through Sp1/Sp3 binding sites (Huang et al., 2015). Both Sp1 and Sp3 together control more than 12.000 genes, and are therefore often collectively referred to as Sp1/3 (Li and Davie, 2010). Depending on the binding

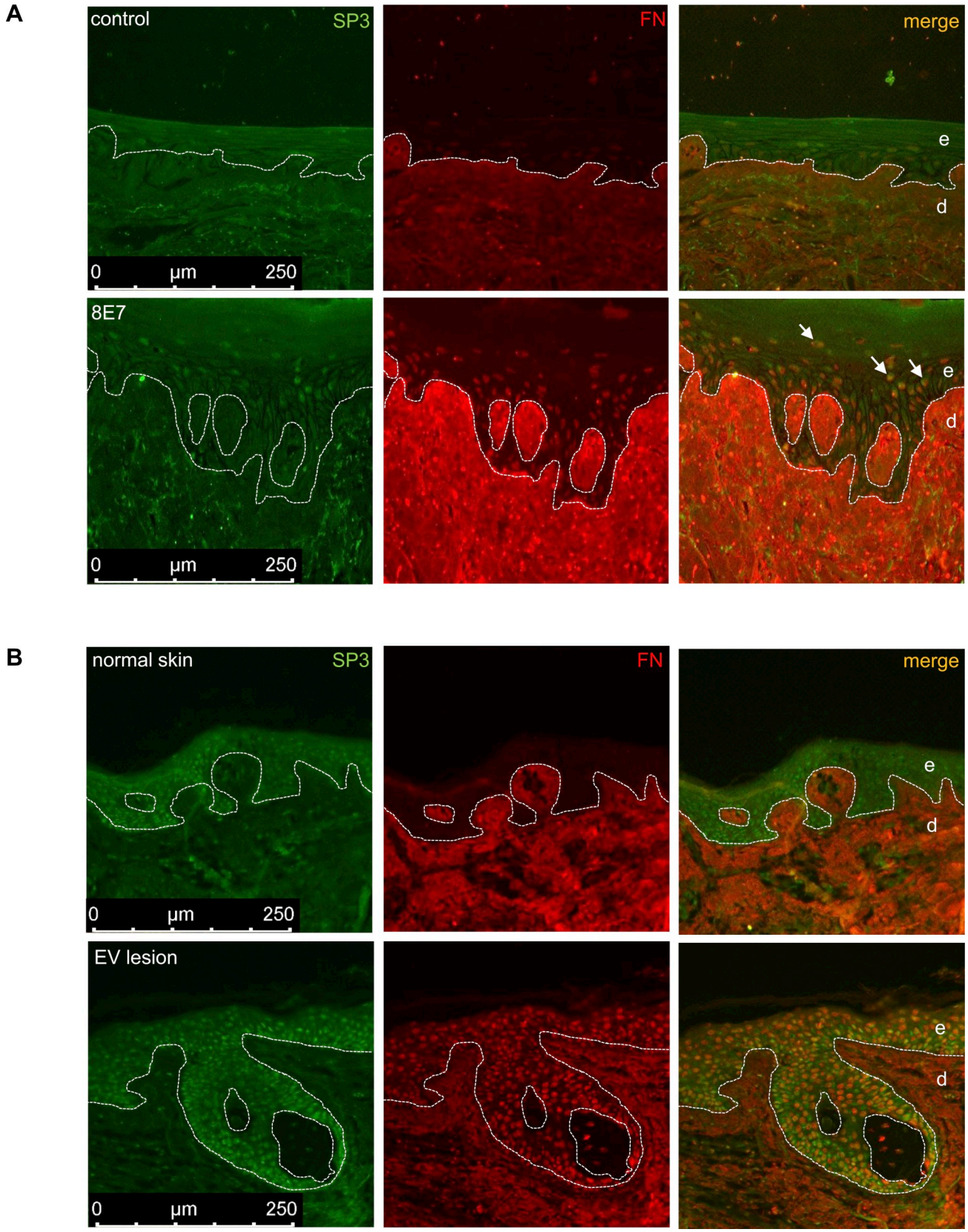


Fig. 3. Sp3 and FN expression patterns in HPV8 positive skin compared to control. (A) Representative immunofluorescence staining of Sp3 and FN in OSCs, which were repopulated with PHK harboring the empty retroviral vector pLXSN (control) or pLXSN-8E7, respectively, grown for 14 days at the air-liquid interphase (n = 3). (B) Representative immunofluorescence staining of Sp3 and FN in healthy skin and skin SCCs from EV patients positive for HPV5, 8, 20, 23, 36, 50 (green: Sp3, red: FN; dashed line: basement-membrane zone; d: dermis; e: epidermis; white arrows: cells double-positive for Sp3 and FN).

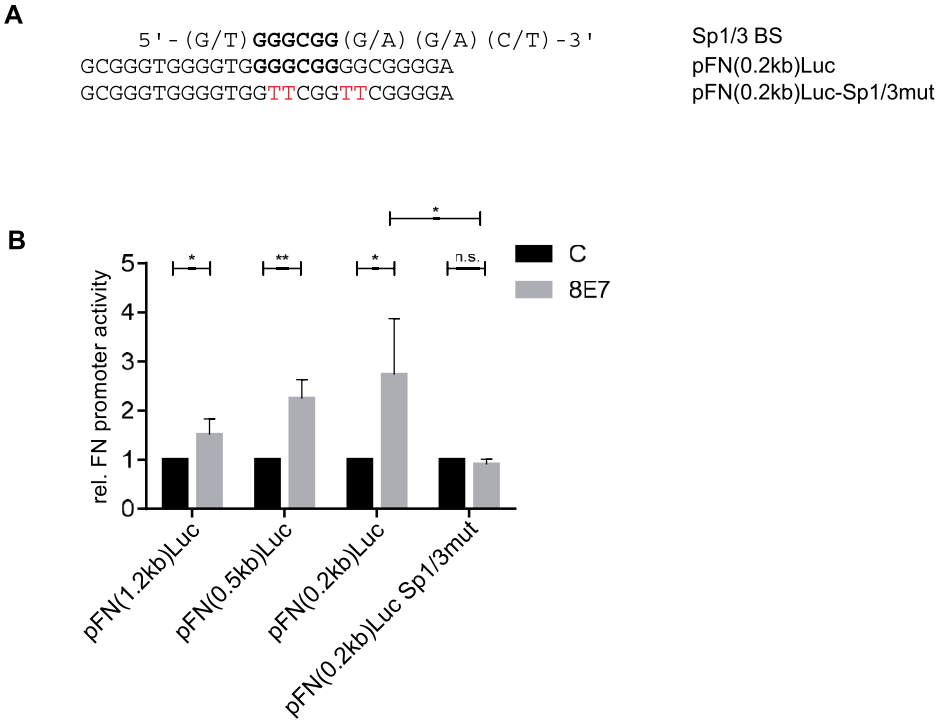


Fig. 4. The fibronectin promoter activity is increased by HPV8-E7. (A) Schematic presentation of putative transcription factor binding sequence within the 200 bp fragment of the human FN promoter. The sequence alignment shows the consensus Sp1/3 binding site and the mutation strategy for the Sp1/3 binding site (highlighted in red). (B) FN promoter constructs were transfected into C33A-keratinocytes with pLXSN-8E7 expression vectors. The expression level of the corresponding co-transfected empty pLXSN vector (control) was set as 1. FN: FN (n = 3). (C) FN promoter activation by HPV8-E7 is dependent on a functional Sp1/3 binding site.

region within a given promoter or Sp1/3 relative ratios, Sp3 may act as either an activator or repressor of gene expression. There is also growing evidence that Sp proteins play a critical role in tumorigenesis and metastatic potential in many tumour types by regulating expression of cell cycle genes, expression of the vascular endothelial growth factor, tumorigenesis, and apoptotic processes (Mertens-Talcott et al., 2007). Taken together, their transcriptional activity depend on a) the cell type b) the DNA binding site and finally c) Sp1/3 relative ratios. Considering

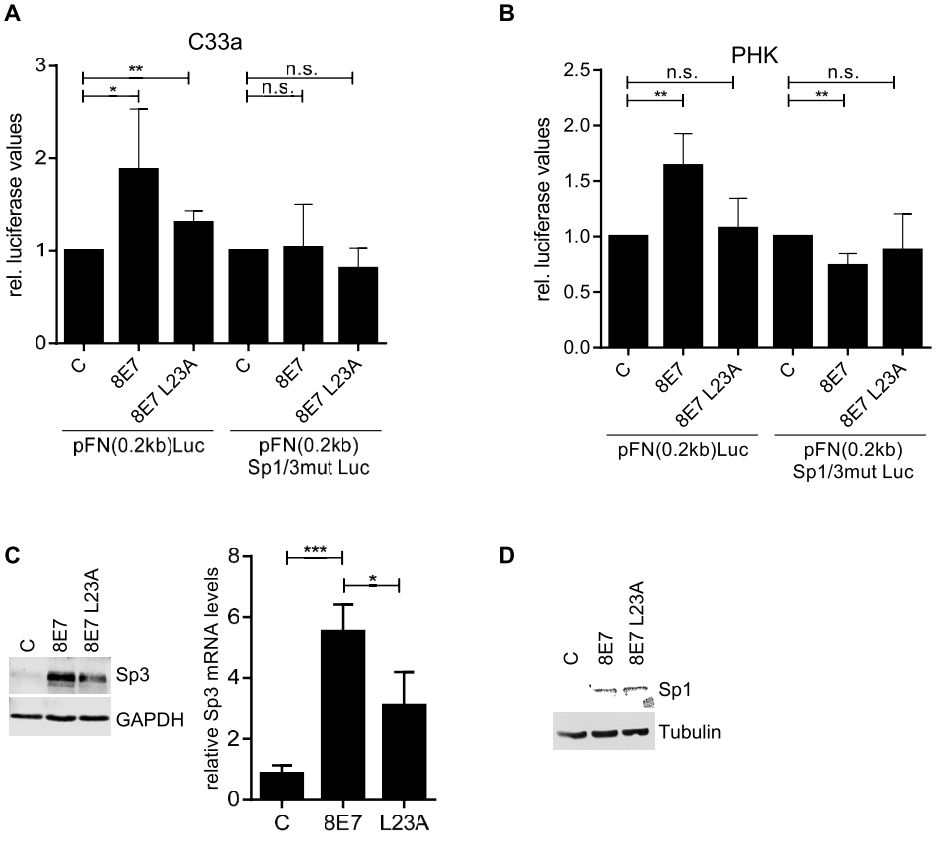


Fig. 5. HPV8-E7^{L23A} lacks the ability to activate the FN promoter. (A, B) pFN(0.2 kb)LUC and pFN (0.2 kb)LUC-Sp1/3mut constructs were transfected into C33A and PHK together with pLXSN-8E7^{wt}, pLXSN-8E7^{L23A} or control vector and luciferase was measured (n = 3). (C) Representative immunoblot of RIPA extracts from PHK expressing HPV8-E7^{wt} or HPV8-E7^{L23A} tested for Sp3 protein expression (n = 3). Equal loading was confirmed by immunoblotting for GAPDH. Relative mRNA levels of Sp3 in PHK (n = 3) expressing either E7^{WT} or E7^{L23A}. Sp3 mRNA levels were normalized to the HPRT1 mRNA levels. The relative ratio of the control was set to 1. (D) Representative immunoblot of RIPA extracts from PHK expressing HPV8-E7^{wt} or HPV8-E7^{L23A} tested for Sp1 protein expression (n = 3). Equal loading was confirmed by immunoblotting for tubulin.

that we were only able to detect Sp3, but not Sp1 in the epidermal layers of HPV8 positive skin of EV patients and HPV8-E7 positive OSCs suggests that HPV8 may alter Sp1/Sp3 relative ratios.

When grown on FN, HPV8-E7 expressing keratinocytes undergo epithelial-mesenchymal transition (EMT), a process that is accompanied by a downregulation of E-cadherin and upregulation of N-cadherin, which enhances both cell motility and thus also invasion (Heuser et al., 2016b). FN production did not only result from E7 expression in keratinocytes, but also from stimulated fibroblasts in monolayer cultures (Heuser et al., 2016b). These previous observations highlighted the importance of cell-matrix interactions, especially the involvement of FN in betaHPV induced keratinocyte transformation. Utilizing FN promoter truncation mutants, we could also show that HPV8-E7 not only activates the FN promoter, but also that the HPV8-E7 responsive element is most likely located within the 0.2 kb upstream promoter region. This particular fragment still showed luciferase activity comparable to pFN(1.2 kb)-LUC in the presence of HPV8-E7 (Fig. 4B). By mutating the Sp1/3 binding site within this region and testing for HPV8-E7-mediated activation, we could prove that HPV8-E7 mediates FN promoter activation through the Sp1/3 binding motif (Fig. 4B). Since we could demonstrate that HPV8-E7^{wt} upregulates Sp3 and Sp1 protein levels in monolayer cultures of PHKs we hypothesize that E7 mediated changes of Sp1/3 ratios may be the crucial underlying regulatory mechanism for the control of cellular gene expression. Since the HPV8-E7^{L23A} was less proficient at inducing Sp3 and FN expression (Fig. 5), we speculate that Sp3 may be involved in controlling FN dependent processes regulating keratinocyte invasion.

In addition, we could show in OSC, that FN is deposited in the dermal compartment beneath HPV8-E7 positive epithelial layers. In addition, nuclear FN was found in these HPV8-E7 positive keratinocytes and more strikingly, staining patterns of both Sp3 and nuclear FN were found to be correlating (Fig. 3). This effect was even more pronounced in EV skin SCCs, which was in stark contrast compared to the FN/Sp3 distribution patterns found in healthy skin, where we observed no FN deposition in the epidermal compartment. Our results provide compelling data that hint at a previously unknown regulation of the transcription factor Sp1/3 by HPV8-E7, which may be the driving element behind the observed FN over-expression in HPV8-E7 positive keratinocytes.

It was reported decades ago that cellular FN apparently can be part of the nuclear matrix of cancer cells. In hepatocellular carcinoma cells FN was even found in the nuclear matrix of cells cultivated in FN deprived medium and that it was even preferentially associated with the nuclear matrix (Jagirdar et al., 1985). Interestingly, such nuclear deposition has also been described in HeLa cells. In cervical carcinomas FN staining patterns were described as diffuse (Goldberg et al., 1998), which does not exclude the possibility that nuclear FN may indeed also be found in cervical cancers (Zerlauth et al., 1988). In that light, it is a quite intriguing finding that we now show enhanced levels of nuclear FN and a correlation with over-expression of the transcription factor Sp3 in betaHPV positive skin SCC (Fig. 3). Regarding the modulation of FN expression by other HPV it has previously been described that both the HPV16-E6 and -E7 proteins are able to promote FN expression in monolayer cultures of primary keratinocytes (Hellner et al., 2009). Interestingly, in our study FN was also found to be deposited in the tumor stroma but also in the nucleus of betaHPV positive skin cancers from non-EV patients (Heuser et al., 2016b) as well as skin lesions from EV patients as shown in Fig. 3B. These observations are of particular relevance in respect to immunosuppressed individuals such as organ transplant recipients, who frequently suffer from high betaHPV loads in their skin as well as a greater susceptibility to develop skin tumours (Bouwes Bavinck et al., 2018; Dell'Oste et al., 2009).

While it has been known that extracellular FN regulates keratinocyte homeostasis, it is completely unknown, whether the presence of nuclear FN may be a result of changes to the FN molecule itself – altering its' binding affinities – or if it is caused by alterations of the tumour nuclear matrix. It is also entirely unknown whether nuclear FN expression might influence skin cell motile behavior. Taken together, our results warrant further studies to confirm the clinical importance of FN deregulation for wound healing and betaHPV mediated skin carcinogenesis.

Acknowledgements

The authors thank Dr Jesse Roman (University of Louisville, Louisville, USA) for providing the fibronectin promoter constructs. This study was supported by the Wilhelm Sander Stiftung für Krebsforschung (grant number: 2012.105.3). MH was supported by the German Cancer Aid (grant number: 111087), ASS was supported by the German Research Foundation (grant number: AK 42/7-1).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.06.019.

References

Akgül, B., Garcia-Escudero, R., Ghali, L., Pfister, H.J., Fuchs, P.G., Navsaria, H., Storey, A., 2005. The E7 protein of cutaneous human papillomavirus type 8 causes invasion of human keratinocytes into the dermis in organotypic cultures of skin. Cancer Res. 65, 2216–2223.

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

Akgül, B., Kirschberg, M., Storey, A., Hufbauer, M., 2019. Human papillomavirus type 8 oncoproteins E6 and E7 cooperate in downregulation of the cellular checkpoint kinase-1. Int. J. Cancer 145 (3), 797–806.

Auersperg, N., 1969. Histogenetic behavior of tumors. I. Morphologic variation in vitro and in vivo of two related human carcinoma cell lines. J. Natl. Cancer Inst. 43, 151–173.

Bouwes Bavinck, J.N., Feltkamp, M.C.W., Green, A.C., Fiocco, M., Euvrard, S., Harwood, C.A., Nasir, S., Thomson, J., Proby, C.M., Naldi, L., Diphoorn, J.C.D., Venturuzzo, A., Tessari, G., Nindl, I., Sampogna, F., Abeni, D., Neale, R.E., Goeman, J.J., Quint, K.D., Halk, A.B., Sneek, C., Genders, R.E., de Koning, M.N.C., Quint, W.G.V., Wieland, U., Weissenborn, S., Waterboer, T., Pawlita, M., Pfister, H., group, E.-H.-U.-C., 2018. Human papillomavirus and posttransplantation cutaneous squamous cell carcinoma: a multicenter, prospective cohort study. Am. J. Transplant. 18, 1220–1230.

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

Goldberg, I., Davidson, B., Lerner-Geva, L., Gotlieb, W.H., Ben-Baruch, G., Novikov, I., Kopolovic, J., 1998. Expression of extracellular matrix proteins in cervical squamous cell carcinoma–a clinicopathological study. J. Clin. Pathol. 51, 781–785.

Hellner, K., Mar, J., Fang, F., Quackenbush, J., Munger, K., 2009. HPV16 E7 oncogene expression in normal human epithelial cells causes molecular changes indicative of an epithelial to mesenchymal transition. Virology 391, 57–63.

Heuser, S., Hufbauer, M., Marx, B., Tok, A., Majewski, S., Pfister, H., Akgül, B., 2016a. The levels of epithelial anchor proteins beta-catenin and zona occludens-1 are altered by E7 of human papillomaviruses 5 and 8. J. Gen. Virol. 97, 463–472.

Heuser, S., Hufbauer, M., Steiger, J., Marshall, J., Sterner-Kock, A., Mauch, C., Zigrino, P., Akgül, B., 2016b. The fibronectin/alpha3beta1 integrin axis serves as molecular basis for keratinocyte invasion induced by betaHPV. Oncogene 35, 4529–4539.

Howley, P.M., Pfister, H.J., 2015. Beta genus papillomaviruses and skin cancer. Virology 479–480, 290–296.

Huang, Y., Shen, P., Chen, X., Chen, Z., Zhao, T., Chen, N., Gong, J., Nie, L., Xu, M., Li, X., Zeng, H., Zhou, Q., 2015. Transcriptional regulation of BNIP3 by Sp3 in prostate cancer. Prostate 75, 1556–1567.

Hufbauer, M., Akgül, B., 2017. Molecular mechanisms of human papillomavirus induced skin carcinogenesis. Viruses 9, 187.

Hufbauer, M., Lazic, D., Akgül, B., Brandsma, J.L., Pfister, H., Weissenborn, S.J., 2010. Enhanced human papillomavirus type 8 oncogene expression levels are crucial for skin tumorigenesis in transgenic mice. Virology 403, 128–136.

Imahorn, E., Yuksel, Z., Spoerri, I., Gurel, G., Imhof, C., Saracoglu, Z.N., Koku Aksu, A.E., Rady, P.L., Tyring, S.K., Kempf, W., Itin, P.H., Burger, B., 2017. Novel TMC8 splice site mutation in epidermodysplasia verruciformis and review of HPV infections in patients with the disease. J. Eur. Acad. Dermatol. Venereol. 31, 1722–1726.

Jagirdar, J., Ishak, K.G., Colombo, M., Brambilla, C., Paronetto, F., 1985. Fibronectin patterns in hepatocellular carcinoma and its clinical significance. Cancer 56, 1643–1648.

Li, L., Davie, J.R., 2010. The role of Sp1 and Sp3 in normal and cancer cell biology. Ann. Anat. 192, 275–283.

Mertens-Talcott, S.U., Chintharlapalli, S., Li, X., Safe, S., 2007. The oncogenic microRNA-

27a targets genes that regulate specificity protein transcription factors and the G2-M checkpoint in MDA-MB-231 breast cancer cells. Cancer Res. 67, 11001–11011.
Michaelson, J.E., Ritzenthaler, J.D., Roman, J., 2002. Regulation of serum-induced fibronectin expression by protein kinases, cytoskeletal integrity, and CREB. Am. J. Physiol. Lung Cell Mol. Physiol. 282, L291–L301.
Nagaoka, M., Shiraishi, Y., Sugiura, Y., 2001. Selected base sequence outside the target binding site of zinc finger protein Sp1. Nucleic Acids Res. 29, 4920–4929.
Schaper, I.D., Marcuzzi, G.P., Weissenborn, S.J., Kasper, H.U., Dries, V., Smyth, N., Fuchs, P., Pfister, H., 2005. Development of skin tumors in mice transgenic for early genes of human papillomavirus type 8. Cancer Res. 65, 1394–1400.
Steger, G., Schnabel, C., Schmidt, H.M., 2002. The hinge region of the human papillomavirus type 8 E2 protein activates the human p21(WAF1/CIP1) promoter via interaction with Sp1. J. Gen. Virol. 83, 503–510.
Tommasino, M., 2019. HPV and skin carcinogenesis. Papillomavirus Res 7, 129–131.
Westphal, K., Akgül, B., Storey, A., Nindl, I., 2009. Cutaneous human papillomavirus E7 type-specific effects on differentiation and proliferation of organotypic skin cultures. Cell. Oncol. 31, 213–226.
Zerlauth, G., Wesierska-Gadek, J., Sauermann, G., 1988. Fibronectin observed in the nuclear matrix of HeLa tumour cells. J. Cell Sci. 89 (Pt 3), 415–421.