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Deregulation of TLR9 signalling pathway in human keratinocytes by E6 and E7 oncoproteins from beta human papillomavirus type 38

Laura Pacini

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Biologie Moléculaire Intégrative et Cellulaire

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par **Laura Pacini**

**Deregulation of TLR9 signalling pathway in human keratinocytes by E6
and E7 oncoproteins from beta human papillomavirus type 38**

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LIST OF ABBREVIATIONS

AP-1	Activator protein 1
CpG	Cytidine-phosphate-guanosine
DAMP	Danger-associated molecular pattern
DNA	Deoxyribonucleic acid
DNMT1	DNA methyltransferase 1
Doxo	Doxorubicin
Ds	Double-stranded
E	Early
EBV	Epstein Barr Virus
EV	Epidermodysplasia verruciformis
EZH2	Enhancer of zeste homolog 2
H ₂ O ₂	Hydrogen peroxide
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HFK/HPKs	Human primary foreskin keratinocyte
HHV-8	Human herpes virus type 8
HIV	Human immunodeficiency virus
HPV	Human papillomaviruses
HR	High-risk
HTERT	Human telomerase reverse transcriptase
HTLV-1	Human T lymphotropic virus 1
IFN	Interferon
IKK β	I κ B kinase beta
IL	Interleukin
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
KSHV	Kaposi sarcoma-associated herpes virus
L	Late
LCR	Long control region
LR	Low-risk
LRR	Leucine-rich repeats

MCPyV	Merkel cell polyomavirus
MyD88	Myeloid differentiation primary response gene 88
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIK	Naturally immortalized keratinocyte
NMSC	Non-melanoma skin cancer
ORF	Open Reading Frame
PAMP	Pathogen-associated molecular patterns
pDC	Plasmacytoid dendritic cells
pRb	Tumour suppressor protein retinoblastoma
PRR	Pattern recognition receptor
RE	Responsive element
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSV	Rous sarcoma virus
SSC	Skin squamous cell carcinoma
TA	Transit amplifying cells
TAK1	Transforming growth factor β -activated kinase 1
TIR	Toll/interleukin-1 receptor
TRAF6	Tumor necrosis factor receptor-associated factor 6
TRAM	TICAM2 Toll-like receptor adaptor molecule 2
TRIF	TIR-domain-containing adapter-inducing IFN- β
TLR	Toll-like receptor
UV	Ultraviolet light

ABSTRACT OF THE THESIS

RÉSUMÉ DE THÈSE

I. ABSTRACT OF THE THESIS

The human papillomaviruses (HPV) consist of a group of capsid-enclosed double-stranded deoxyribonucleic acid (dsDNA) viruses from the *Papillomaviridae* family that display a distinct tropism for mucosal or cutaneous squamous epithelia. Until now, more than 200 types of HPV have been isolated and grouped into a phylogenetic tree composed of 5 genera (alpha, beta, gamma, mu and nu papillomaviruses). Among them, the mucosal high-risk HPV types that belong to the genus alpha have been associated with cervical cancer as well as a subset of anogenital and head and neck carcinomas. They are responsible for approximately 5% of all virus-induced cancers.

Beta HPV types have a skin tropism and have been suggested to be involved, together with ultraviolet light (UV), in the development of non-melanoma skin cancer (NMSC). For instance, *in vitro* and *in vivo* experimental models highlight the transforming properties of beta HPV38 E6 and E7. Specifically, studies of transgenic mouse model, where HPV38 E6 and E7 are expressed in the undifferentiated basal layer of epithelia under the control of the Keratin 14 (K14) promoter, showed a very high susceptibility to UV-induced skin carcinogenesis in comparison to the wild-type animals.

Equally important as their ability to promote cellular transformation, oncogenic viruses have different strategies to overtake the host immune system thus guaranteeing persistent infection. Therefore, understanding whether potential oncogenic viruses have the ability to interfere with the immune response could provide additional evidence relating to their involvement in human carcinogenesis.

Here, we show that the E6 and E7 oncoproteins from HPV38 suppress the expression of the dsDNA innate immune sensor Toll-like receptor 9 (TLR9) by promoting the accumulation of $\Delta Np73\alpha$, an antagonist of p53 and p73. Chromatin immunoprecipitation experiments showed

that $\Delta Np73\alpha$ is part of a negative transcriptional regulatory complex that binds to a NF- κ B responsive element within the TLR9 promoter.

Interestingly, ectopic expression of TLR9 in HPV38 E6E7 cells resulted in an accumulation of the cell cycle inhibitors p21^{WAF/Cip1} and p27^{Kip1}, reduction of CDK2-associated kinase activity and inhibition of cellular proliferation. Together these data indicate that TLR9 is involved in additional events, besides the innate immune response. Accordingly, we observed that the treatment of human primary keratinocytes (HPKs) with different cellular stresses, e.g. UV irradiation, doxorubicin and H₂O₂ treatment, results in TLR9 up-regulation. This UV-induced event is mediated by the recruitment of several transcription factors to the TLR9 promoter, such as p53, NF- κ B p65 and c-Jun. The expression of HPV38 E6 and E7 strongly affect the recruitment of these transcription factors to the TLR9 promoter, with consequent impairment of TLR9 gene expression.

In summary, our data show that HPV38, similarly to other viruses with well-known oncogenic activity, can down-regulate TLR9. Most importantly, we highlight a novel function of TLR9 in controlling the cellular response to stresses and we show that HPV38 E6 and E7 are able to interfere with such mechanism. These findings further support the role of beta HPV types in skin carcinogenesis, providing additional insight into their precise contribution to the multi-step process of cancer development.

RÉSUMÉ DE THÈSE

Les oncoprotéines E6 et E7 du papillome humain de type 38 modifient la réponse cellulaire induite par les UV en inhibant l'expression du récepteur Toll-like 9

Les virus du papillome humain (HPV) sont des virus à ADN double-brin encapsidés appartenant à la famille des *Papillomaviridae* ayant un tropisme distinct pour les épithéliums squameux de type muqueux ou cutanés. Jusqu'à présent, plus de 200 types de HPV ont été isolés et regroupés dans un arbre phylogénétique composé de 5 genres nommés alpha, beta, gamma, mu et nu. Parmi eux, les types HPV muqueux à haut risque appartenant au genre alpha ont été associés au cancer du col de l'utérus ainsi qu'à des sous-groupes de carcinomes ano-génitaux et de la tête et du cou. Ces virus sont responsables d'environ 5% de tous les cancers viro-induits.

Les types bêta du HPV ont un tropisme pour la peau et pourraient être impliqués dans le développement du cancer de la peau non mélanique (NMSC), en association avec la lumière ultraviolette (UV). Ainsi, les modèles expérimentaux *in vitro* et *in vivo* ont démontré les propriétés de transformation des oncoprotéines E6 et E7 du type HPV bêta 38. De plus, des études sur le modèle de souris transgénique, où E6 et E7 du HPV38 sont exprimés au niveau de la couche basale non différenciée de l'épithélium sous le contrôle du promoteur du gène humain de la kératine (K14), ont montré une très forte susceptibilité de la peau à la carcinogenèse induite par les UV par rapport aux animaux de type sauvage.

Tout aussi important que leur capacité à promouvoir la transformation cellulaire, les virus oncogènes ont développé différentes stratégies pour prendre le dessus sur le système immunitaire de l'hôte, favorisant ainsi l'établissement d'une infection persistante. Par conséquent, savoir si des virus oncogènes potentiels ont la capacité d'interférer avec la

réponse immunitaire pourrait fournir des preuves supplémentaires de leur implication dans la cancérogenèse humaine.

Ici, nous montrons que les oncoprotéines E6 et E7 de HPV38 suppriment l'expression de Toll-like 9 (TLR9), récepteur des ADN double-brins, en favorisant l'accumulation de $\Delta Np73\alpha$, un antagoniste de p53 et p73. Des expériences d'immunoprécipitation de la chromatine ont montré que $\Delta Np73\alpha$ fait partie d'un complexe de régulation négative transcriptionnelle qui se lie à un élément de réponse NF- κ B dans le promoteur TLR9.

Fait intéressant, l'expression ectopique de TLR9 dans des cellules HPV38 E6E7 a entraîné une accumulation des inhibiteurs du cycle cellulaire p21^{WAF1/Cip1} et p27^{kip1}, une réduction de l'activité kinase associée à CDK2 et l'inhibition de la prolifération cellulaire. Ensemble, ces données indiquent que TLR9 est impliqué dans d'autres événements, en plus de la réponse immunitaire innée. Par conséquent, nous avons constaté que le traitement des kératinocytes humains primaires (HPK) avec différents stress cellulaires, par exemple l'irradiation aux UV, la doxorubicine et le traitement H₂O₂, conduisent à une induction de la transcription de TLR9. Cet événement induit par les UV est arbitré par le recrutement de plusieurs facteurs de transcription sur le promoteur TLR9, tels que p53, NF- κ B p65 et c-Jun. L'expression de E6 et E7 de HPV38 affecte fortement le recrutement de ces facteurs de transcription sur le promoteur TLR9, avec comme conséquence l'affaiblissement de l'expression du gène TLR9.

En résumé, nos données montrent que HPV38, de manière similaire à d'autres virus avec une activité oncogénique bien connue, peut inhiber l'expression de TLR9. Plus important encore, nous mettons en évidence une nouvelle fonction de TLR9 dans le contrôle de la réponse cellulaire aux stress et nous montrons que E6 et E7 de HPV38 sont capables d'interférer avec un tel mécanisme. Ces résultats confirment le rôle des types HPV bêta dans la carcinogénèse de la peau, en fournissant des informations supplémentaires sur leur contribution précise dans le processus multi-étapes de développement du cancer.

GENERAL INTRODUCTION

II. GENERAL INTRODUCTION

1. Virus and Cancer

a. Historical overview

The evidence for the recognition of cancer as a disease was found several thousand years ago. Different theories on the cause of cancer found favour over time, but nobody described infection aetiology until the 20th century. In 1907, the Italian physician Giuseppe Ciuffo described for the first time a viral aetiology for human warts when he showed that cell-free filtrates from such lesions could transmit the disease (1). Shortly thereafter in 1908, two Danish scientists, Vilhelm Ellerman and Olaf Bang, reported that inoculation of chicken leukemia cell filtrate into healthy chicken resulted in transmission of the disease (2). This experiment led to the discovery of the avian sarcoma leukosis. However, the finding was not well acknowledged before leukaemia was not generally accepted as a notable form of cancer. Peyton Rous discovered in 1911 that filtrate from a spontaneous spindle cell sarcoma of Plymouth Rock chicken could induce tumour in healthy chickens (3). The avian sarcoma induced by Rous sarcoma virus (RSV) was shown to represent a genuine cancer and led to the designation of RSV as the first known tumour virus (4). However, it took 40 years for the scientific community to fully understand the importance of these pioneering findings. Despite persistent dogma dismissing a possible role for viruses in cancer, researchers continued to identify new tumour viruses over the four decades following the discovery of RSV. Later identification of mammalian tumour viruses attracted the attention of scientific community on the identification of new viruses associated with human malignancies. In 1965, Tony Epstein and colleagues succeeded to isolate a virus from Burkitt's lymphoma patients

named Epstein Barr Virus (EBV) (5). This finding opened a new chapter in tumour virology and five other tumour viruses, namely Hepatitis B virus (HBV), Human T lymphotropic virus 1 (HTLV-1), Human papillomavirus (HPV), Hepatitis C virus (HCV), and Kaposi sarcoma-associated herpes virus (KSHV), were identified before the end of 20th century (6–10).

Such knowledge proved instrumental to the development of the first vaccines against cancers having an infectious aetiology. Moreover, tumour virologists recognised that viruses could serve as powerful discovery tools to reveal mechanisms that are involved in all human malignancies. This rich history promises that tumour virology will continue to contribute to our understanding of cancer and to the development of new therapeutic and preventive measures for this disease.

b. Human oncogenic viruses and associated diseases

Altogether chronic infections account for nearly 20% of cancer cases worldwide. This fraction is higher in less developed countries, reflecting the higher prevalence of the major oncogenic infections in these areas (11). Viruses are considered one of the most important risk factor for cancer development in human. In 2009 the International Agency for Research on Cancer (IARC) Monographs programme reviewed infectious agents that have been classified as carcinogenic to human. To date these viruses include HBV, HCV, HPV, EBV, HTLV-1 and KSHV (also known as human herpes virus type 8, HHV-8). The human immunodeficiency virus (HIV) causes cancer through immunosuppression, thereby enhancing the carcinogenic action of other viruses and it is considered a cofactor. Table 1 includes a short review of each viral infectious agent and its associated cancer sites.

Table 1. Major cancer sites associated with group infectious viral agents

Virus	Localization
HBV, HCV	Liver
HPV with or without HIV	Cervix uteri
HPV with or without HIV	Anogenital (penile, vulva, vagina, anus)
EBV	Nasopharynx
HHV-8 with or without tobacco or alcohol consumption	Oropharynx
HHV-8 with or without HIV	Kaposi's sarcoma
EBV with or without HIV, HCV, HTLV-1	Non-Hodgkin lymphoma
EBV with or without HIV	Hodgkin's lymphoma

Modified from (11).

Infectious agents can be classified as direct carcinogens, such as several types of the HPV family, HTLV-1, EBV and KSHV, or indirect carcinogens by causing chronic inflammation. The first group of viruses expressed oncogenes, of which their products play a direct role in the transformation of the infected cells, altering the regulation of key cellular pathways. Regarding the second group of viruses, chronic infection followed by chronic inflammation will lead to the production of chemokines and cytokines secreted by infected cells and/or inflammatory cells. This event will also lead to the production of reactive oxygen species (ROS), to the deregulation of immune system and to the promotion of angiogenesis, which is

essential for tumour survival. HBV and HCV have been shown to be carcinogens primarily through their induction of chronic inflammation.

A better understanding of the role of infectious agents in the aetiology of cancer and application of existing public health methods for infection prevention, such as vaccination, safer injection practice, or antimicrobial treatment, is an essential element in public health policy and could have an impact on future burden of cancer worldwide.

2. Human papillomaviruses

a. Classification and Phylogenetic

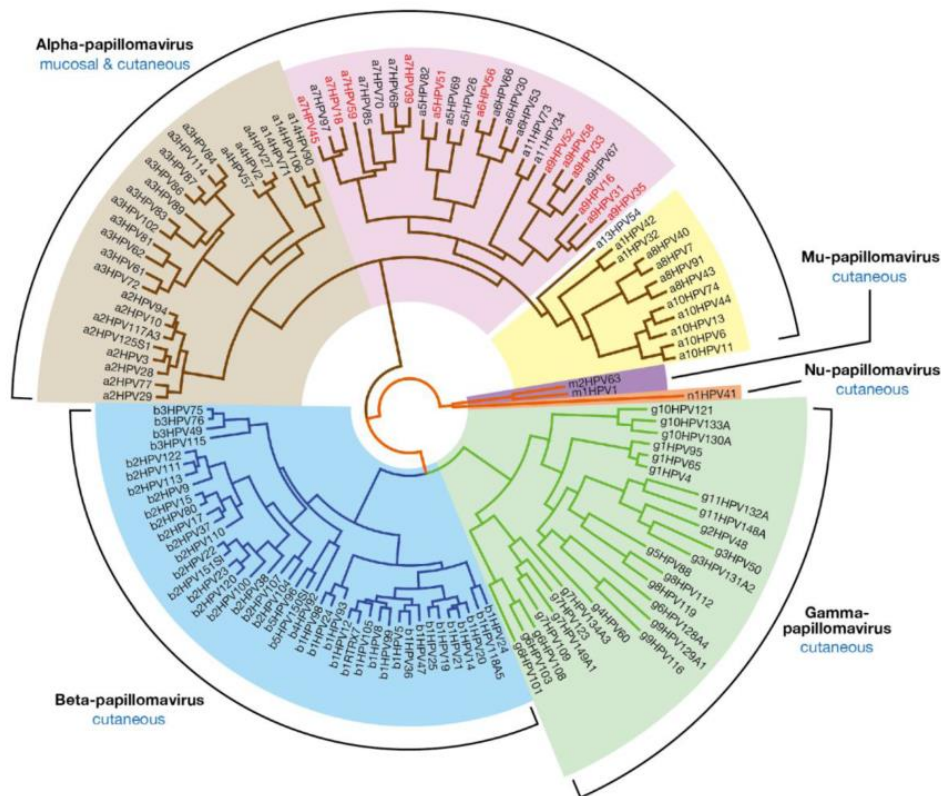
Papillomaviruses had originally been considered together with polyomaviruses as part of one single family, the *Papovaviridae*. As it was later recognized that the two virus groups show immense differences in their genetic organization and biological characteristics, they are now officially reconsidered as two separate families, *Papillomaviridae* and *Polyomaviridae*.

Papillomavirus (PV) have been discovered in a wide array of vertebrates. They are traditionally described as “types” based on their genome sequences and identified by a number. The PV types that have been studied the most extensively are cottontail rabbit papillomaviruses (CRPVs), bovine papillomaviruses (BPVs) and human papillomaviruses (HPVs). So far more than 300 PV types have been identified and completely sequenced, including over 200 HPVs. Many of these HPV types have been shown to be ubiquitous and globally distributed. The late (L) 1 Open Reading Frame (ORF) is the most conserved gene within the genome and has therefore been used for the identification of new PV types over the past 15 years. A new PV isolate is recognised as such if the DNA sequence of the L1 ORF

differs by more than 10% from the closest known PV type. Differences between 2% and 10% homology define a subtype and less than 2% a variant.

Based on sequence relatedness, the HPV phylogenetic tree is composed of five genera: alpha, beta, gamma, mu and nu (Fig. 1). They are, in turn, grouped into species and subdivided into types.

Fig. 1. HPV phylogenetic tree



The human papillomaviruses types are classified into five genera: alpha-, beta- (blue), gamma- (green), Nu- (orange) and Mu-papillomavirus (violet). The alpha-papillomaviruses are classified as benign cutaneous (light brown), low-risk mucosal (yellow) or high-risk (pink) according to their association with human diseases. The high-risk HPV types highlighted with red text are confirmed as “human carcinogens” on the basis of epidemiological data. From (12).

Currently, the most common HPV types are included in three genera: alpha, beta and gamma. HPVs exhibit a genotype-specific host-restriction and a strict tropism for epithelial cells of distinct anatomical sites, where they cause lesions with distinctive clinical pathologies. Based on their tissue tropism two main groups of HPVs are identified: mucosal and cutaneous. The mucosal HPV types known so far belong to the alpha genus. They are subdivided in high-risk (HR) and low-risk (LR) according to their ability to induce benign or malignant lesions. The LR HPV types are normally associated with benign genital warts, while the HR HPVs have been clearly linked to cervical cancer as well as a subset of other anogenital cancers and oropharyngeal cancers (13).

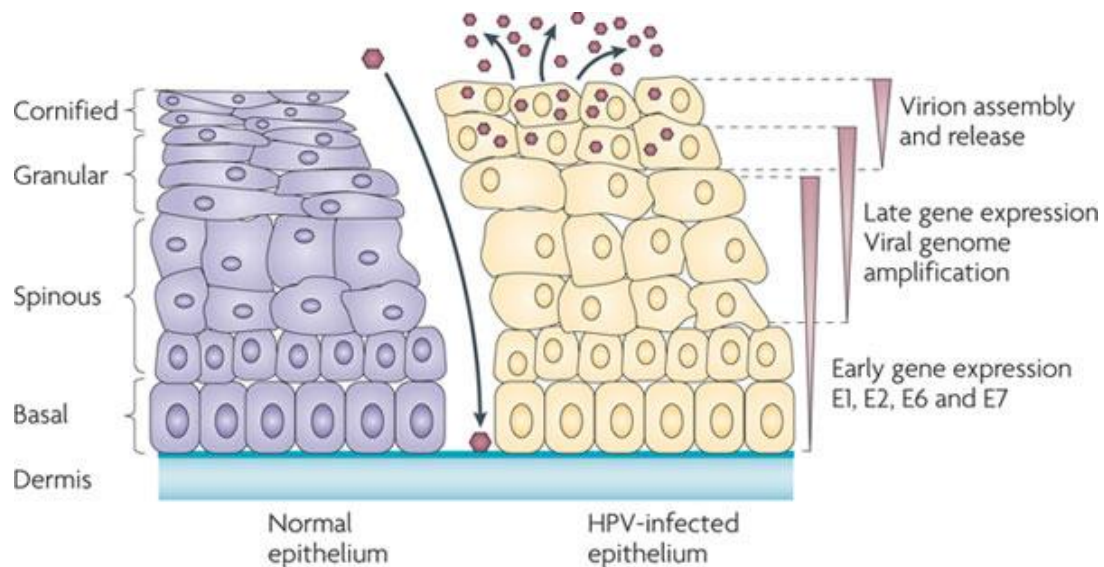
The genus beta includes a large number of types that preferentially infect the skin (cutaneous types). They were originally isolated in patients suffering from a rare autosomal recessive cancer-prone genetic disorder, named Epidermodysplasia verruciformis (EV) and are consistently detected in non-melanoma skin cancers from EV patients and immunocompromised and immunocompetent individuals (14). No information is available on the biological properties of the gamma HPV types that are highly prevalent on the skin of normal individuals.

b. HPV life cycle

HPV life cycle can be divided in two stages: non-productive and productive (Fig. 2). The non-productive stage occurs in proliferating basal layers of the epithelium where the virus is present as a low-copy-number nuclear plasmid and replicates its genome at low copy number. The productive stage occurs in the terminally differentiated layers of the epithelium (15,16). High-risk HPVs infect stratified squamous epithelia in the genital tract through micro-lesions (17). The viral capsid binds to the basement membrane and infection occurs as activated keratinocytes move into the wound (18). The viral particles interact with the cell surface *via*

interaction of the major capsid protein, L1, with heparin sulphate proteoglycans. Accumulating evidence suggests the involvement of a secondary receptor and a possible role for the minor capsid protein, L2, in cell surface interaction (19).

Fig. 2. HPV life cycle



HPVs infect keratinocytes in the basal layer of the epithelium exposed through microwounds. Uninfected epithelium (on the left) and HPV-infected epithelium (on the right) are shown. After infection, early viral genes are expressed. Differentiation of HPV-positive cells induces the productive phase of the viral life cycle, which requires cellular DNA synthesis machinery. The expression of E6 and E7 deregulates cell cycle control, pushing differentiating cells into S phase, allowing viral genome amplification in cells that normally would have exited the cell cycle. The L1 and L2 proteins encapsidate newly synthesized viral genomes and virions are shed from the uppermost layers of the epithelium (red hexagons). From (20).

Following entry and uncoating, HPV genomes are established as extrachromosomal elements in the nucleus and the copy number is increased and maintained at approximately 50-100 copies per cell (20). HPV replication requires the cellular machinery including DNA polymerase α /primase, DNA polymerase δ , replication protein A, Proliferating Cell Nuclear

Antigen (PCNA) and topoisomerases (21). Therefore, the replication occurs in the nucleus of infected cells and is dependent on S-phase entry.

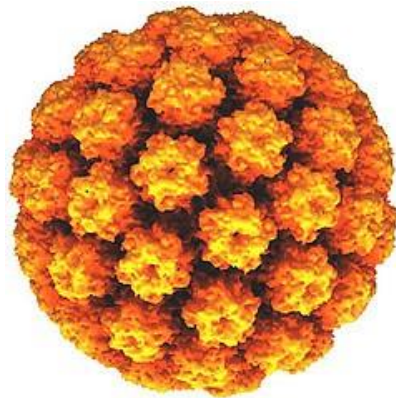
The HPV life cycle is intimately coordinated with the natural turnover of the epithelial cells and with skin architecture. Viral gene expression is differently regulated according to the degree of differentiation of the infected cells. In the skin, the only actively dividing cells are present in the basal and parabasal layers next to the basement membrane. The epithelia are composed of two main cell types: (i) the transit amplifying cells (TA), proliferating cells that can undergo terminal differentiation, and (ii) stem cells that divide rarely in order to refresh the TA pool. Upon cell division, TA cells produce daughter cells which migrate away from the basal layer and start to differentiate (17). As infected cells divide, viral DNA is equally distributed between both daughter cells. One of the daughter cell migrates and initiates a program of differentiation, while the other continues to divide in the basal layer and provides a reservoir of viral DNA for further cell division (22).

The early phase of the viral life cycle is based on the maintenance of the viral episomes in basal cells which is achieved by the expression of early (E) 1, E2, E6 and E7 proteins. When the HPV-infected cells divide and leave the basal layer undergoing differentiation, the activation of the late viral promoter occurs and the level of viral proteins increases dramatically. As a result, viral copy number amplifies from 50-200 copies to several thousands of copies per cell (23). In the late phase the virions are ensemble thanks to the production of the viral capsid proteins L1 and L2. The compartmentalisation of viral gene expression in the different skin layers is an important strategy the virus uses to have a long term infection.

c. Structure of HPV

Papillomaviruses are small, non-enveloped, icosahedral dsDNA viruses that have a diameter of 52–55 nm (Fig. 3). The protein capsid is composed of 72 pentameric capsomers and contained two structural proteins — L1 (55 kDa in size) and L2 (70 kDa) which are both virally encoded.

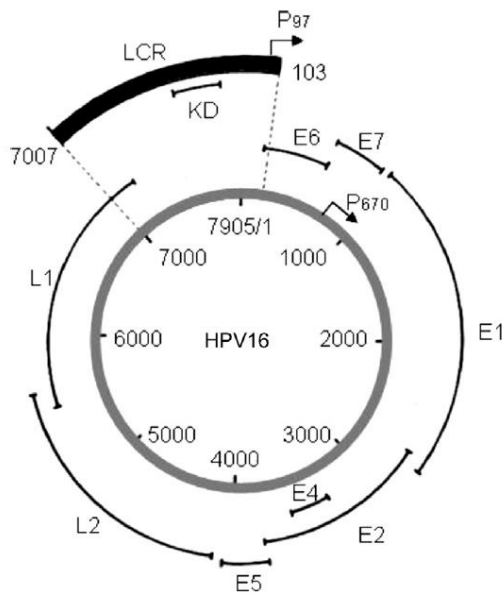
Fig. 3. HPV capsid



View of the molecular surface of the atomic model of HPV composed by 72 pentameric capsomer, each containing the 2 proteins L1 and L2. From (24).

The circular dsDNA genome is approximately 8 kb in size. All PVs share a common genetic structure that generally contains eight ORFs, all transcribed from a single DNA strand. The ORF can be divided into three functional parts: the early (E) region that encodes proteins (E1–E7) necessary for viral replication; the late (L) region that encodes the structural proteins (L1 and L2) that are required for virion assembly; and a long control region (LCR), localised between ORF L1 and E6, and containing most of the regulatory *cis* elements that are necessary for the replication and transcription of viral DNA (Fig. 4).

Fig. 4. The dsDNA HPV16 genome



Viral Protein	Functions and Features
E1	It forms a heterodimer complex with E2 and controls viral replication
E2	It regulates early gene promoter and together with E1 viral DNA replication
E4	It may mediate the viral particle release by destabilizing cytoskeleton network
E5	It stimulates mitogenic signals of growth factors
E6	It inactivates many cellular proteins and is one of the major viral oncoproteins
E7	It inactivates many cellular proteins and is one of the major viral oncoproteins
L1	It is the major capsid protein and is the component of the HPV prophylactic vaccine
L2	It is the minor capsid protein

The diagram indicates the ORFs of the early (E) and late (L) genes, the long control region (LCR) and the two major promoters that drive viral expression (P97 and P670). The main functions and features of the early and late gene products are listed in the table. From (25).

HPVs lack of a viral DNA polymerase activity and require the host DNA replication machinery for viral genome replication (26).

E1 and E2 are involved in viral DNA replication and the regulation of early transcription (27). Despite its name, E4 protein continues to be expressed in the terminally differentiated keratinocytes and is associated with virus assembly and release. E5, E6, and E7 are viral oncogenes that target a number of regulators of the cell cycle and therefore modulate the transformation process (26). Although HPV genera have basically the same genome organization, the majority of beta HPV types lack of the E5 protein. During the generation of progeny virions, the L1 and L2 proteins assemble in capsomers, which form icosahedral capsid around the viral genome (28).

d. Function of E6 and E7 oncoproteins

The HPV oncoproteins E6 and E7 are the primary viral factors responsible for initiation and progression of HPV-related cancer, and they act largely by promoting the alteration of host-cell pathways and by inducing genomic instability. E6 and E7 genes encode for small nuclear proteins of about 16-19 kDa and 10-14 kDa, respectively. Their functions are discussed in details below. Unless otherwise stated, the description of protein functions refers to HR HPV proteins and in particular to HPV16.

i. E6

The HPV E6 proteins are small polypeptides of approximately 151 amino acids and contain two zinc-finger motifs (Cys-X-X-Cys) conserved in all E6 HPV types (29). The E6 ORF of HPV16 is transcribed from the early promoter as a polycistronic messenger. From a variety of assays, it has been shown that the E6 proteins possess intrinsic transforming activity. The E6 protein of HR HPVs is classified as an oncoprotein that can transform human mammary cells (30) and that can cooperate with E7 in transforming primary human foreskin keratinocytes (31).

The best known property of the E6 proteins of HR HPVs is its ability to bind the tumour-suppressor protein p53 and to induce its degradation (32,33). HPV E6 is able to bind to a short leucine (L)-rich LxxLL consensus sequence within the cellular ubiquitin ligase E6-associated protein (E6-AP). Subsequently, E6/E6AP complex recruits p53 and promotes its ubiquitination (34,35). The LxxLL motif of E6AP renders the conformation of E6 competent for interaction with p53 by structuring a p53-binding cleft on E6 (36).

The loss of p53 significantly decreases the safeguard of the genome integrity, facilitating the accumulation of chromosomal abnormalities. The ability to degrade p53 appears to be specific

to the HR HPV E6 proteins, while no p53 degradation is observed in LR HPV-expressing keratinocytes and the majority of cutaneous types (37,38).

In addition, E6 from both HR and LR mucosal HPV types can binds to numerous other cellular proteins that can be divided into four broad classes: 1) transcriptional co-activators, such as p300 (39,40), myc (41) and IRF3 (Interferon regulatory factor 3) (42); 2) tumour suppressors and inducers of apoptosis, as p53 (34) and Bak (43); 3) proteins involved in cell polarity and motility, such as the membrane-associated guanylate kinase with inverted orientation (MAGI-1) (44,45) and the multiple PD2 protein 1 (MUPP1) (46); and 4) DNA replication and repair factors, like mcm7 (47,48), XRCC1 (49) and O⁶-methylguanine-DNA methyltransferase (50).

Another property of E6 that strongly contributes to prolong the lifespan of the infected cells is its ability to increase telomerase activity, by activating the transcription of the human telomerase reverse transcriptase (*hTERT*) gene (51). This activation of *hTERT* has been purported to be responsible for cell immortalization by E6, although the precise mechanism by which E6 achieves this effect is still unclear. Through the interactions described above, E6 can affect transcriptional pathways, disrupt cell adhesion and architecture, inhibit apoptosis, abrogate DNA damage responses, induce genome instability and immortalize cells.

ii. E7

E7 proteins are acid polypeptides of 98 amino acids with two zinc-binding motifs at the C-terminal. Three conserved region (CR) have been identified: CR1, CR2 and CR3 (52). All the three domains of the protein are important for its biological activity. The LXCXE domain located in the CR2 mediates the binding of E7 with the tumour suppressor protein retinoblastoma (pRb) (53). In the same domain is present a casein kinase II phosphorylation site (CKII) and a small motif of 3 amino acids required for the E7-induced pRb degradation

(54). The CR3 domain contains two CXXC motifs that bind zinc and appear important for the formation of a homodimer (52).

It has been reported that E7 from the mucosal HR HPV type 16, in cooperation with an activated cellular oncogene (e.g., *ras*), has the ability to induce full transformation of rodent and human primary cells, which are tumorigenic when injected into mice (55). The main characterised function of E7 is its interaction with the unphosphorylated form of pRb (53) and its related proteins p107 (56) and p130 (57). These proteins are all involved in cell cycle regulation. In fact, pRb directly associates and negatively regulates the activity of several transcription factors like E2F family members. In quiescent cells, pRb is hypophosphorylated and associates with E2F. When quiescent cells are exposed to mitogenic signals D-cyclins associate with and activate the cyclin-dependent kinases 4 and 6 (CDK4 and CDK6, respectively), which in turn phosphorylate pRb in mid-G1 phase, causing release of E2F (58). Ultimately, the free and active E2F promotes the transcription of a group of genes that encode proteins essential for cell cycle progression.

The interaction of HPV16 E7 with pRb, analogous to CDK-mediated phosphorylation, results in release of active E2Fs and stimulation of S-phase entry and unscheduled proliferation (59). The E7 of LR HPV types such as 6 or 11, which are rarely associated with malignant lesions, has reduced efficiency in binding pRb (60).

In addition to the pRb pathway, HPV16 E7 is able to associate with other cell cycle regulator proteins. For instance, E7 can abrogate the function of the cyclin-associated kinase inhibitors p21^{WAF/Cip1} and p27^{Kip1} via direct binding (61–64). Several other cellular partners of E7 contribute to its biological activity. Indeed, E7 can associate through its zinc finger with the histone deacetylase and modulate chromatin activation pathways (65). It can also interact with several transcription factors like AP-1(activator protein 1), TBP (TATA box binding protein) or c-Jun (66).

e. Oncogenic activities of high-risk HPV types in humans

The International Agency for Research on Cancer classified 12 different HR HPV types as carcinogenic to humans: types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 (67). HPV types 16 and 18 are the most frequently found in cervical cancers worldwide (in approximately 50% and 20% of squamous cell carcinoma, respectively, and in 35% of cervical adenocarcinoma) (68–70). High risk HPV types are also involved in a subset of other genital cancers, such as vulvar, vaginal, anal and penile cancers, as well as head and neck cancers. HPV16 is responsible for the majority (86-95%) of HPV-positive oropharyngeal carcinomas (71)

Although HPV16 E6 and E7 oncoproteins display strong transforming activities in *in vitro* and *in vivo* experimental models, only a minority of HPV16 infections in human results in the development of malignant lesions. For instance, more than 90% of HPV16 infections in the female genital tract remain asymptomatic and cleared by the immune system in approximately 18-24 months. Development of pre-malignant and malignant lesions is intimately linked to viral persistence. In high-grade cervical lesions HPV16 DNA is often found integrated in the host genome. This event results in the loss of the expression of several viral genes, with only preservation of the E6 and E7 oncogenes. Their constant expression in infected cells strongly facilitates the accumulation of chromosomal alterations, which in turn lead to cellular transformation.

An important achievement in HPV research was the development of a prophylaxis vaccine. To date, three prophylactic vaccines have been developed and are currently available: a bivalent vaccine (Cervarix) that protects against type 16 and 18 (72), a quadrivalent vaccine (Gardasil) that protects against HPV types 6, 11, 16 and 18 (73) and a nonavalent vaccine (Gardasil 9) that prevents infection with the same four HPV types plus five additional types (31, 33, 45, 52 and 58) (74). All these vaccines are based on the recombinant expression and self-assembly of the viral protein L1 into virus-like particles (VLPs). The expected outcome

of prophylactic vaccination is a reduction in the incidence of HPV-related genital diseases, including cervical, penile, vulvar, vagina, and anal cancer and precancerous lesions. Although their high efficacy, the available vaccines present some limitations. For instance, they do not protect against all HR HPV types and do not treat existing HPV infections. The long-term duration of protection to prevent cancer is unknown. However, the follow-up of young women did not detect evidence of waning immunity over 10 years and quadrivalent vaccine was shown to induce immune memory (75,76). Another important aspect to take into consideration is the cost of the vaccination that will probably limit the use of vaccine among low-income population. Interestingly, vaccination protocols with two or three doses resulted in a similar immune response, providing a strategy to reduce the vaccination cost. In addition, it will be important to evaluate the impact of the HPV vaccines on other genital and non-genital HPV-associated tumours and in others populations such as individuals at high risk for anal cancer (e.g. men who have sex with men).

f. Cutaneous HPV types

Approximately 75% of the human HPV types are cutaneotropic. The cutaneous HPV types are represented mainly by the beta and gamma genera, which are widely present in the skin of normal individuals. So far, more than 40 beta HPV types and 50 gamma HPV types have been isolated, and these numbers are continuously growing.

Beta HPV types were originally isolated in patients suffering from a rare autosomal recessive disorder, Epidermodysplasia verruciformis (EV) and are consistently detected in non-melanoma skin cancer (NMSC) of EV patients. The EV disease appears early in childhood and is characterised by disseminated polymorphic cutaneous lesions, that undergo malignant transformation at sun-exposed areas in more than 50% of patients starting around the third decade of life (77,78). Epidermodysplasia verruciformis causes a decrease in cell-mediated

immunity and makes the patient susceptible to HPV infections. Several studies have proved the role of HPV infection in the pathogenesis of skin cancer associated with EV (79).

g. Beta types and potential association with cancer

Recent studies have suggested the involvement of cutaneous HPV in skin carcinogenesis in non-EV patients. Infections with these viruses are frequently detected in immunocompromised individuals, for instance transplant recipients or HIV patients, and in NMSC in both immunocompetent and immunocompromised individuals (14,80). In contrast to HPV16 in cervical cancer, where the virus is important for the initiation and maintenance of cellular transformation, beta HPV appears to play a role only at an early stage of cancer development. Indeed, higher copy number of beta HPV genome is found in pre-malignant actinic keratosis lesions compared to skin squamous cell carcinoma (SSC) (14,81).

However, because of the plurality of HPV types found in normal and lesioned skin within the same biopsy and or skin abrasion, the role of cutaneous HPV types in skin cancer remains unclear and needs further investigations.

To date, only E6 and E7 proteins from two beta HPV types, 38 and 49, are able to immortalise human primary keratinocytes (82–84), as previously shown for HPV16 E6 and E7. HPV38 displays several transforming activities in *in vitro* and *in vivo* experimental model (80,85–87). In mouse immortalised fibroblast cell (NIH3T3) HPV38 E7 has similar biological properties to HPV16 E7. In fact, it has been demonstrated that HPV38 E7 associates with pRb, promotes its degradation and deregulates the G1/S phase transition (82). While in contrast to HPV16 E6, HPV38 E6 is not able to promote p53 degradation. HPV38 is still able to alter p53 functionality, but the mechanism used differs from the most studied HPV16. Indeed, HPV38 E6 and E7 induce the accumulation of $\Delta Np73\alpha$, a p53 transcriptional inhibitor which competes with p53 for the regulation of its target genes (85). It has been shown that HPV38

E7 promotes the formation of a transcriptional regulatory complex that inhibits the expression of several p53-regulated genes. Δ Np73 α plays a key role in the formation of this complex composed by the I κ B kinase beta (IKK β) and two epigenetic enzymes DNA methyltransferase 1 (DNMT1) and the enhancer of zeste homolog 2 (EZH2) (88). Δ Np73 α not only plays a role in HPV38-mediated immortalisation by altering p53 transcriptional functions but also by inducing the overexpression of *hTERT* in HPV38 E6 and E7 infected keratinocytes (83). Δ Np73 α is known to act as a dominant-negative inhibitor of p53 and its related proteins (*i.e.*, p63 and p73), which are strong repressors of *hTERT* expression (89). Moreover, Δ Np73 α also interferes with the E2F/pRb-mediated repression of *hTERT* transcription (90). Additionally, to the overexpression of Δ Np73 α , HPV38 has developed others mechanisms to prevent p53 transcriptional activation *via* binding and inactivation of histone acetyltransferase p300. Interestingly, this HPV38 property is shared with other beta HPV types (87).

h. Oncogenic viruses and immune response

Another important biological property of oncogenic viruses is the ability to evade the host immune system in order to guarantee persistent infection. They have developed several strategies, such as establishment of latency, sequestration of the viral genome, blockage of antigen presentation, evasion of antibody response, inhibition of apoptosis, and decrease in the production of cytokines and human type 1 interferon (IFN-1) (91). The latter are key molecules in the antiviral immune response involved in removal of viral components from infected cells and also conferring the resistance to uninfected cells against viral infection (92). In order to overcome the antiviral state, different oncogenic viruses have evolved numerous strategies to subvert the production of these innate immune effectors molecules, for instance, by directing their oncoproteins to target the molecules downstream of MyD88 (Myeloid differentiation primary response gene 88), such as IRF3 and IRF7, which are mainly

responsible for the production of IFN inducible genes (93,94). For example, HPV16 E6 oncoprotein is able to sequester IRF3 to reduce the production of IFN- β (42). Moreover, it has been reported that HPV16 E6 interacts with IRF9 to inhibit the IFN signalling (95). Another oncogenic virus, EBV, can also block type I IFN-mediated antiviral responses *via* LMP1-mediated inhibition of STAT1 (signal transducer and activator of transcription 1) and -2 phosphorylation (96).

3. Interaction of viruses with the immune system

a. Host response to viral infection

Vertebrates are constantly threatened by the invasion of microorganisms and have evolved systems of immune defence to eliminate infective pathogens in the body. The mammalian immune system is comprised of two branches: innate and acquired immunity. The innate immune system is the first line of host defence against pathogens. Viruses initially activate the innate immune system, which recognises viral components through pattern recognition receptors (PRRs) (97,98). Acquired immunity is involved in elimination of pathogens in the late phase of infection as well as the generation of immunological memory. In order to establish an infection, viruses need to modulate the innate immune system suppressing or escaping from the host defence.

The evasion of host immune defence is advantageous to viruses as it allows them to persist inside the host cell and maximise the production of the progeny. Virus and host genetic factors as well as conditions that lead to impairment of the immune system play key roles in the persistence of the infection.

b. Innate immunity

The innate immune system is an evolutionary conserved system acting as a first line of host defence against invading microbial pathogens. A range of PRRs recognize specific pathogen-associated molecular patterns (PAMPs) such as genomic DNA, single-stranded (ss) ribonucleic acid (RNA), dsRNA, RNA with 5'-triphosphate ends and viral proteins exclusively present on microbes. In addition, PRRs are involved in sensing endogenous danger signals by recognising danger-associated molecular pattern (DAMPs).

PRRs are classified into three families, namely Toll-like receptors (TLRs), Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and Nucleotide oligomerization domain (NOD)-like receptors (NLRs) (92,99). Among these receptor types, TLRs and RLRs are important for the production of IFN-1 and various cytokines, whereas NLRs are known to regulate interleukin-1 β (IL-1 β) maturation through activation of caspase-1 (99,100). Detection of PAMPs and DAMPs by the PRRs triggers an intracellular signalling cascades leading to the secretion of IFN-1, pro-inflammatory cytokines and chemokines. Type I IFNs activate intracellular signalling pathways *via* a type I IFN receptor and regulate the expression of a set of genes involved in eliminating viral components, inducing apoptosis of infected cells and conferring resistance to viral infection on uninfected cells.

PRRs display different expression pattern. They are primarily expressed by antigen presenting macrophage and plasmacytoid dendritic cells (pDCs) but can also be expressed by other cells (both immune and non-immune cells).

c. Toll-like receptors

The Toll-like receptors (TLRs) family is one of the best-characterised PRR families. The discovery of TLRs was an important event for immunology research and was recognised as such with the awarding of the 2011 Nobel Prize in Physiology or Medicine to Jules Hoffmann and Bruce Beutler for their ground breaking research within immunology.

TLRs are a group of highly conserved integral membrane glycoprotein found throughout evolution, from *Drosophila* to humans (101–104). They were first described in 1980 as receptor proteins on developing embryo of the *Drosophila* flies (105). After the characterisation of the first mammalian TLR, TLR4, several proteins that are structurally related to TLR4 were identified and named Toll-like receptors (106). The mammalian TLR family consists of 13 members (TLR1-TLR10 in humans, TLR1-TLR9 and TLR11-TLR13 in mice), each activated by a different evolutionary-conserved molecular structures unique to microorganisms, but triggering a common signalling cascade leading to the production of inflammatory cytokines. Based on the sequence homologies, vertebrate TLRs can be grouped into six subfamilies, TLR1/2/6/10, TLR3, TLR4, TLR5, TLR7/8/9, and TLR11/12/13 (107). TLRs are expressed on various immune cells including pDCs, macrophages, B and T cells and even non immune cells, such as epithelial cells and fibroblasts, and are localized on the cell surface or intracellularly. The TLRs that are expressed on the cell surface (TLR1, 2, 4, 5 and 6) largely recognise microbial membrane components, whereas the TLRs that are found almost exclusively in intracellular compartments (TLR3, 7, 8 and 9) recognise nucleic acids (Table 2).

Table 2. TLRs, ligands and cellular localization

TLRs	Localization	Ligands
TLR1 and TLR2	Cell surface	Peptidoglycan, lipoproteins, lipopolysaccharide
TLR2 and TLR6	Cell surface	Lipoproteins
TLR3	Endosome	Viral dsRNA
TLR4	Cell surface	Lipopolysaccharide
TLR5	Cell surface	Flagellin
TLR7 and TLR8	Endosome	Viral and bacterial ssRNA
TLR9	Endosome	Viral and bacterial CpG DNA
TLR10	Endosome	Unknown

Modified from (108).

d. Structure of TLRs

TLRs are type-I integral membrane receptors comprised of an extracellular N-terminal domain, a single trans-membrane helix, and a C-terminal cytoplasmic domain (Fig. 5). The extracellular N-terminal domain is constituted of 19-25 tandem copies of a leucine-rich repeats (LRRs) domain shared among members of TLRs family. The LRRs are arranged in horseshoe shape (109) and contain the sequences xLxxLxLxx, where x represents many amino acids and L is leucine. The TLRs require the LRRs for ligands recognition (110). The

crystal structure and 3D model of numerous TLRs (TLR1, 2, 3, 4, 8 and 9) extracellular domains with their agonist or antagonist PAMPs has been characterised (109,111–115).

Fig. 5. Structure of Toll-like receptors

All TLRs are integral membrane glycoproteins with a conserved cytoplasmic domain, a single transmembrane domain and an N-terminal ectodomain. The ectodomain of a TLR7, TLR8 and TLR9 family member is depicted with the LRR solenoid (in grey) and the N- and C- terminal flanking regions (in green and blue, respectively). An undefined region present in TLR7, TLR8 and TLR9, but not in the other TLRs, is shown as a light-blue string. Insertions within LRRs at position 10 (in red) might contribute to the formation of the pathogen-associated molecular pattern (PAMP) binding site. An insert at position 15 (in yellow) is expected to originate on the convex face of the TLR. The transmembrane domain is presumed to be a single α -helix. Modified from (116).

The TLRs cytoplasmic domain is designated the Toll/interleukin-1 receptor (TIR) domain due to shared homology with the signalling domains of IL-1R family members (117). This domain is composed of three highly conserved regions called box 1, 2 and 3 and is required for

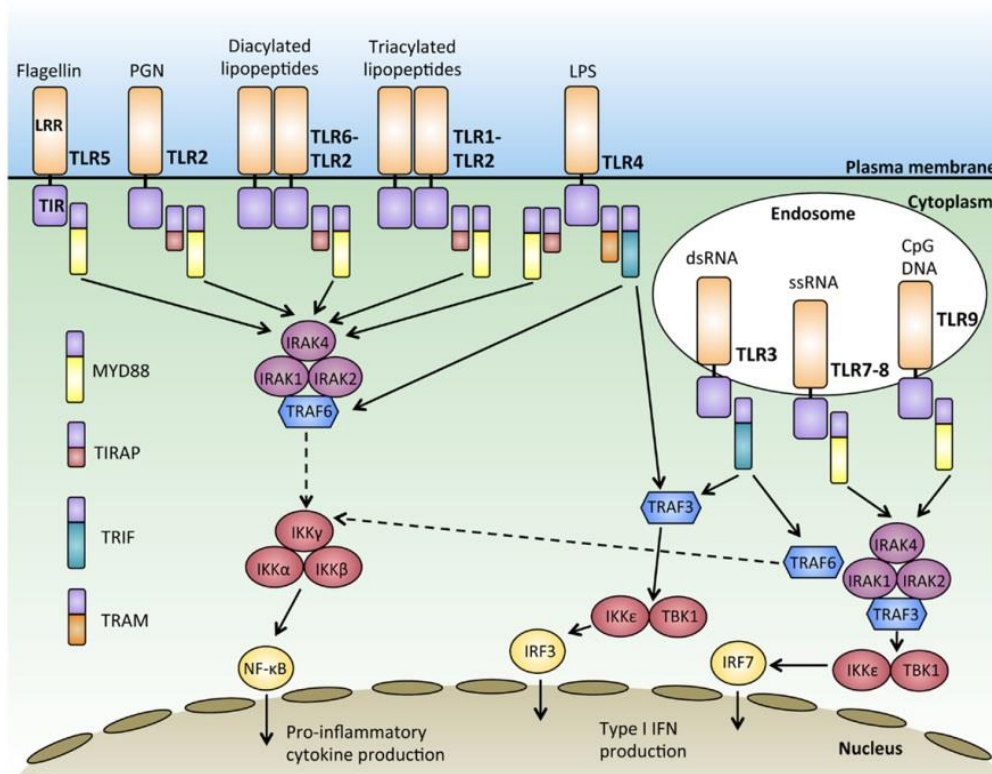
downstream signalling through interaction with other TIR domain containing proteins. In response to TLRs ligands, TIR domain is dimerised and recognized by TIR domains of adaptor proteins such as MyD88, MAL (Toll-like receptor 4 in MyD88 adapter-like), TRIF (TIR-domain-containing adapter-inducing IFN-beta), and TRAM (TICAM2 Toll-like receptor adaptor molecule 2) which then trigger downstream signalling pathways leading to the induction of inflammatory cytokines to initiate the adaptive immune response (118). TLRs are highly conserved and share structural and functional similarities.

e. Signalling pathway of TLRs

All TLRs activate a common signalling pathway leading to the production of proinflammatory cytokines *via* MyD88. Subsequent studies clearly demonstrated the existence of a MyD88-independent pathway which is peculiar to the TLR3 and TLR4 signalling pathway (119) (Fig. 6). MyD88 possesses a TIR domain in the C-terminal portion and a death domain in the N-terminal portion. In MyD88-dependent pathways, MyD88 associates with the TIR domain of TLRs. Upon ligand stimulation, MyD88 recruits interleukin-1 receptor-associated kinase (IRAK) through interaction of their death domains (120,121). Mammals have four IRAK family members, called IRAK1, IRAK2, IRAK3 and IRAK4. IRAK is activated by phosphorylation and associates with TRAF6 (tumor necrosis factor receptor-associated factor 6). This event induces TRAF6 polyubiquitination and TAK1 (transforming growth factor β -activated kinase 1) complex, TAK1/TAB (TAK1 binding protein), activation. This complex stimulates the I κ B kinase (IKK) complex, which consists of two catalytic components, IKK α and IKK β , and a regulatory component, NEMO (NF- κ B essential modifier, also known as IKK γ). Upon activation, the IKK complex triggers the rapid phosphorylation of specific serine residues of I κ B proteins, a family of inhibitor proteins which keep the NF- κ B transcription factors sequestered in the cytoplasm as an inactive form.

Phosphorylated I κ B proteins are subsequently polyubiquitinated and degraded by the 26S proteasome, allowing NF- κ B to move into the nucleus where it stimulates effector molecules for innate immunity (122).

Fig. 6. Signal transduction downstream of MyD88-dependent and independent pathways



Activation of Toll-like receptors (TLRs) leads to receptor dimerization and recruitment of adaptor proteins such as MyD88, TIRAP, TRIF, and TRAM. Most of the TLRs form homodimers while TLR2 can also form heterodimers with either TLR6 or TLR1. Receptor dimerization leads to the activation of IRAKs-TRAF6 and, consequently, of the IKK complex and culminates in the activation of NF- κ B and IRFs transcription factors, which regulate the production of pro-inflammatory cytokines and IFNs. From (123).

The NF- κ B family comprises dimeric transcription factors that contain Rel-homology domains (RHDs) presents in promoter and enhancer regions of various genes (124). In

mammalian cells, there are five members of the NF- κ B family: RelA (p65), RelB, C-Rel, p105 (NF- κ B1; a precursor of p50) and p100 (NF- κ B2; a precursor of p52). NF- κ B proteins form homo- or heterodimers and regulate expression of genes involved in innate and adaptive immunity, inflammation, anti-apoptosis, proliferation, stress responses and cancer progression (124,125). The most frequently activated form of NF- κ B in TLR signalling is a heterodimer composed of RelA and p50 (126). This pathway is called the ‘canonical pathway’ and is responsible for TLR-mediated induction of inflammatory cytokines such as tumour necrosis factor- α (TNF- α) and IL-6 (127).

In addition to the activation of the IKK complex, TAK1 can also recruit mitogen-activated protein kinases (MAPK), such as JNKs (c-Jun N-terminal kinases) and p38, for the activation of the transcription factor AP-1 (activator protein 1), which also triggers the production of pro-inflammatory response (128,129). Moreover, TLR7- and TLR9-mediated MyD88-dependent pathways also recruit another transcription factor, IRF7, to induce the type I IFN production (130).

On the other hand, in the MyD88-independent pathway lipopolysaccharides stimulation leads to the recruitment of TRAM and TRIF adaptor molecules, which in turn interact with TRAF3. TRAF3 induces the non-canonical IKKs, TANK-binding kinase 1 (TBK1) and IKK ϵ /IKK ι , which activate the transcription factor IRF3, and thereby induce IFN- β . IFN- β , in turn, activates STAT1, leading to the induction of several IFN-inducible genes (131,132).

f. Toll-like receptor 9 (TLR9)

TLR9 is the most studied member of TLRs family. It was firstly discovered in 1984 by Tokunaga and colleagues as a recogniser of bacterial DNA in human immune cells (133). Later on, Krieg et al. attributed the B cell activation to a cytidine-phosphate-guanosine (CpG) DNA motifs highly conserved in bacterial and viral genome (134). In 2000, Hemmi and colleagues showed for the first time that the cellular response to CpG DNA is arbitrated through TLR9 in mice (135). One year later Takeshita et al. cloned the human TLR9 (hTLR9) gene and revealed that TLR9 expression is a prerequisite for CpG DNA activation of human cells (136). The crystal structure of three forms of TLR9 (unliganded, bound to agonist CpG-DNA and bound to inhibitory DNA) has recently been determined (137). This structure serves as an important basis for improving our understanding of the ligand-binding and functional mechanism of TLR9.

i. TLR9 pathogen recognition and cellular tropism

The human TLR9 recognises un-methylated CpG islands found in bacterial and viral DNA (135). Along with other partners like TLR2 and TLR4, TLR9 is a key player of the innate and adaptive immune response against several bacterial species such as *Mycobacterium tuberculosis* (138), *Brucella* (139), *Streptococcus pneumoniae* (140), and *Helicobacter pylori* infection (141). Several DNA viruses containing CpG island in their genome such as adenovirus, herpes simplex virus (HSV) 149 and 2, murine gamma herpes virus (MHV) 68, mouse cytomegalovirus (MCMV) and HPV16 have been reported to be recognised by TLR9 (142–145).

Like other TLRs, TLR9 is expressed by numerous cells of the immune system such as B cells, lymphocytes, monocytes, natural killer (NK) cells and pDCs. TLR9 expression has also been

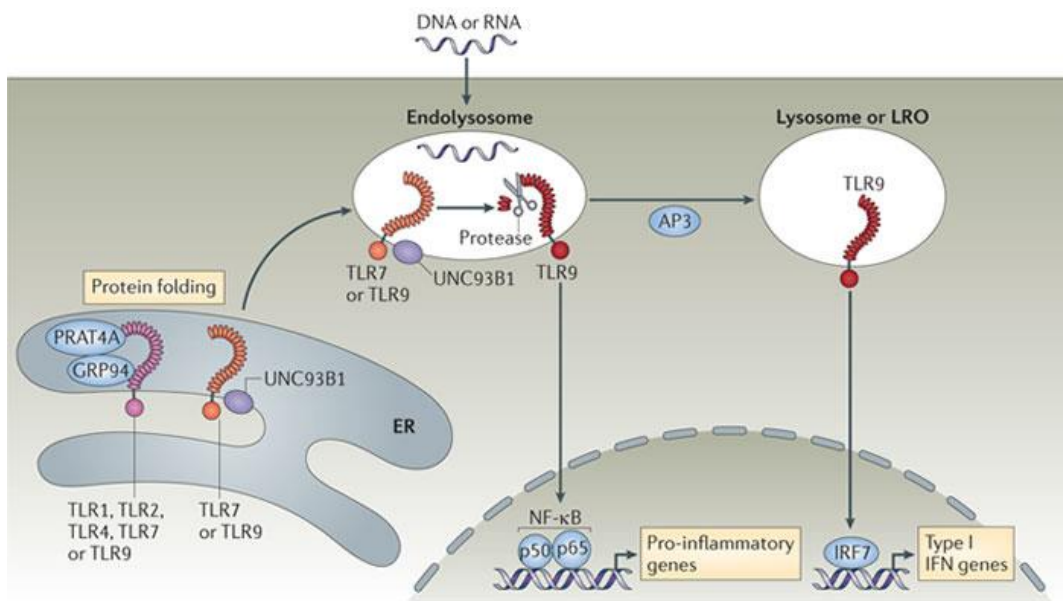
reported in nonimmune cells including human keratinocytes, cardiomyocytes and neurons (146–148).

ii. TLR9 activation

Recent studies have shown that binding of DNA containing CpG, but not of DNA lacking CpG, to TLR9 dimers resulted in conformational changes in the TLR9 cytoplasmic signalling domains (121,149) that most likely promote the recruitment of signalling adaptor molecules. CpG specificity facilitates recognition of a broad array of microbes but introduces the potential for recognition of self-nucleic acids which contributes to autoimmune diseases such as systemic lupus erythematosus (SLE) (150,151). Discrimination between self and microbial nucleic acids cannot be achieved solely through recognition of distinct features, but instead relies on differential delivery of these potential ligands to TLRs (152). All of the TLRs capable of nucleic acid recognition localise within endosomal compartments which sequesters these receptors away from self-nucleic acids in the extracellular space (152).

In quiescent cells, TLR9 are exclusively located in the endoplasmic reticulum (ER). Recognition of CpG DNA activates TLR9 that translocates from the ER to endolysosomes *via* the Golgi apparatus (153,154) (Fig. 7). This translocation is mainly mediated by endoplasmic reticulum membrane protein UNC93B1 (155,156). Once routed to the endolysosomes, TLR9 is cleaved by resident proteases and the truncated form of the receptor is able to bind MyD88 and initiate the downstream signalling (157).

Fig. 7. Trafficking and processing factors for TLRs



Toll-like receptor 1 (TLR1), TLR2, TLR4, TLR7 and TLR9 folding and function are regulated by the endoplasmic reticulum (ER) luminal chaperones glucose-regulated protein of 94 kDa (GRP94) and protein associated with TLR4 A (PRAT4A). The ER membrane protein uncoordinated 93 homolog B1 (UNC93B1) is required for the translocation of TLR7 and TLR9 to endolysosomes, where these TLRs are cleaved by endopeptidases. The cleaved TLRs can bind to their ligands, which trigger the activation of downstream components leading to production of pro-inflammatory cytokines. From (158).

iii. TLR9 downstream signalling

Upon translocation to the endosome, TLR9 receptor recognises ligands and recruits its adapter protein MyD88, which leads to the activation of its downstream pathways including NF-κB and MAPK pathway (159). After stimulation, MyD88 recruits and activates IRAK4 through the death domains. The activation of IRAK4 results in its dissociation from MyD88 and in its association with TRAF6, which activates (i) IRF5 and the NF-κB RelA-p50 heterodimer for inflammatory cytokine induction and (ii) IRF7 for IFN-1 induction (160,161).

The transcription factors IRFs play an important role in the type I IFN production mediated by TLR9 (162,163). Following the initial identification of two structurally related members,

IRF1 and IRF2, seven additional members have now been reported. IRF7 is one of the most important IRF family members in type I IFN. Indeed, it has been shown that IRF7 is constitutively expressed in pDCs, and, consistently, IRF7-deficient mice fail to produce IFN- α from pCDs in response to stimuli (130). Notably, type I INF induction through TLR7 and TLR9 absolutely depends on MyD88 (164).

Cells expressing TLR9 mainly produce Th1-like pro-inflammatory cytokines, interferons, and chemokines. However cytokine production depends on many factors such as cell type and type of CpG stimuli (165). The varying amount of cytokine secretion by different cells is due to different expression level of TLR9. In B cells, ODN CpG induces IL-6 and IL-10 production within a few hours (166). pDCs are considered the most efficient producer of cytokines in TLR signalling (167). In response to CpG DNA, pDCs secrete cytokines (IL-6, TNF-a), interferon (IFN- a), and chemokine (IL-8) (165).

iv. Transcriptional regulation of TLR9

The transcriptional regulation of TLR9 has not been extensively studied. In 2004, Takeshita et al. cloned the 5'-flanking region of hTLR9 gene and characterised the activity of its promoter by comparing hTLR9 deletion mutants to the full length hTLR9 promoter (-3227 to -1 relative to transcription site) in reporter gene assay performed in human myeloma cell line RPMI-8226. Interestingly, deletion (-3227 to -800 bp) on the 5'-end did not change the promoter activity showing that critical elements for transcriptional regulation of hTLR9 are located within 800 bp upstream region (168). In the same study they showed that the transcription regulation of hTLR9 was controlled by four *cis*-acting elements namely CRE, 5'-PU box, 3'-PU box and a C/EBP site. The transcription factors CREB1 (cyclin AMP-responsive element-binding protein 1), Ets2, Elf1 (E74-like factor 1), Elk1, and C/EBP α (CCAAT-enhancer-binding protein α) *trans*-activate the promoter by interacting with these *cis*-acting elements,

while it appeared that suppression was mediated *via* Spi1 (PU.1), c-Jun and NF- κ B p65 (168). Later on, several factors were reported to activate the transcription of hTLR9 in various cell types. For instance, it was found that the transforming growth factor (TGF) α , a growth and differentiation factor that is present during wound healing and in psoriasis, increased the expression and activity of TLR9 and TLR5 in keratinocytes (146).

Interestingly, different viruses have shown the abilities to deregulate TLR9 transcription, as a potential mechanism to escape the immune evasion (144,169–171). The E6 and E7 oncoproteins of HPV16 abolish the functionality of TLR9 in human keratinocytes by down-regulating the TLR9 promoter activity (144,169). This event is variable among different HPV types, as HR HPV16 strongly down-regulates TLR9, while low-risk type, such as HPV6, do not share this ability. Later on, LMP1 oncoprotein of EBV was also reported to down-regulate the TLR9 expression in B cells, through LMP1-mediated activation of the NF- κ B pathway (171). And another study showed that a recently discovered oncogenic virus, Merkel cell polyomavirus (MCPyV), is also able to deregulate TLR9 expression in epithelial and Merkel cell carcinoma-derived cells through the large T (LT) antigen (170). All these results provide evidence that TLR9 down-regulation is likely a crucial step in virus-driven carcinogenesis.

Recent data provide new insight on the role of TLR9 in non-immuno-related pathways. It has been shown that TLR9 expression is strongly activated *via* p53 in primary human blood lymphocytes, alveolar macrophages and cancer cells upon exposure to different types of DNA-damaging (172,173). These findings demonstrate that the human innate immune system can be modulated by DNA metabolic stress, having many implications for health and disease.

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AIM OF THE STUDY

III. AIM OF THE STUDY

The role of cutaneous beta HPV types in carcinogenesis is still under debate. However, several lines of evidence support their role in NMSC.

Some studies have shown that different well-established oncogenic viruses down-regulate the innate immune sensor TLR9. In this study we aimed (i) to determine whether beta HPV38 E6 and E7, which are highly transforming in *in vitro* and *in vivo* experimental models, share the same ability to inhibit the expression of TLR9 and (ii) to elucidate the possible mechanism.

Furthermore, independent studies provide evidence for the involvement of TLR9 in the response to cellular stress. Based on this, we aimed (i) to determine whether similar events occurred in primary human keratinocytes, which are the natural host of beta HPV types, (ii) to characterise the underlying mechanism and (iii) to evaluate whether HPV38 is able to interfere with such mechanism.

RESULTS

IV. RESULTS

Paper I. Down-regulation of Toll-like receptor 9 expression by beta human papillomavirus type 38 and implications for cell cycle control

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Downregulation of Toll-Like Receptor 9 Expression by Beta Human Papillomavirus 38 and Implications for Cell Cycle Control

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ABSTRACT

Innate immunity is the first line of host defense against infections. Many oncogenic viruses can deregulate several immune-related pathways to guarantee the persistence of the infection. Here, we show that the cutaneous human papillomavirus 38 (HPV38) E6 and E7 oncoproteins suppress the expression of the double-stranded DNA sensor Toll-like receptor 9 (TLR9) in human foreskin keratinocytes (HFK), a key mediator of the antiviral innate immune host response. In particular, HPV38 E7 induces TLR9 mRNA downregulation by promoting accumulation of Δ Np73 α , an antagonist of p53 and p73. Inhibition of Δ Np73 α expression by antisense oligonucleotide in HPV38 E6/E7 HFK strongly rescues mRNA levels of TLR9, highlighting a key role of Δ Np73 α in this event. Chromatin immunoprecipitation experiments showed that Δ Np73 α is part of a negative transcriptional regulatory complex with I κ B kinase beta (IKK β) that binds to a NF- κ B responsive element within the TLR9 promoter. In addition, the Polycomb protein enhancer of zeste homolog 2 (EZH2), responsible for gene expression silencing, is also recruited into the complex, leading to histone 3 trimethylation at lysine 27 (H3K27me3) in the same region of the TLR9 promoter. Ectopic expression of TLR9 in HPV38 E6/E7 cells resulted in an accumulation of the cell cycle inhibitors p21^{WAF1} and p27^{Kip1}, decreased CDK2-associated kinase activity, and inhibition of cellular proliferation. In summary, our data show that HPV38, similarly to other viruses with well-known oncogenic activity, can downregulate TLR9 expression. In addition, they highlight a new role for TLR9 in cell cycle regulation.

IMPORTANCE

The mucosal high-risk HPV types have been clearly associated with human carcinogenesis. Emerging lines of evidence suggest the involvement of certain cutaneous HPV types in development of skin squamous cell carcinoma, although this association is still under debate. Oncogenic viruses have evolved different strategies to hijack the host immune system in order to guarantee the persistence of the infection. Their capability to evade the immune system is as important as their ability to promote cellular transformation. Therefore, understanding the viral mechanisms involved in viral persistence is a valid tool to evaluate their potential role in human carcinogenesis. Here, we show that E6 and E7 oncoproteins from the cutaneous HPV38 downregulate the expression of the double-stranded DNA sensor TLR9 of innate immunity. We also present evidence that the HPV38-mediated downregulation of TLR9 expression, in addition to its potential impact on the innate immune response, is linked to cell cycle de-regulation.

In addition to the well-characterized mucosal high-risk human papillomaviruses (HPV), a subgroup of cutaneous HPV types belonging to the genus beta of the HPV phylogenetic tree appears to be associated with human carcinogenesis (1–3). These HPV types are suspected to be involved together with UV radiation in the development of nonmelanoma skin cancer (4, 5). Beta HPV types were originally isolated in patients suffering from a rare autosomal recessive cancer-prone genetic disorder, epidermodysplasia verruciformis (EV), and are consistently detected in nonmelanoma skin cancer from EV patients and immunocompromised and healthy individuals (1). More than 40 different beta HPV types have been identified so far, but only a few have been studied for the characterization of their biological properties (6). In particular, several studies have demonstrated that E6 and E7 oncoproteins from beta HPV 38 (HPV38) display transforming activities in *in vitro* and *in vivo* experimental models (7–12). The transforming activity of HPV38 is explained partly by the ability of E7 to induce the accumulation of Δ Np73 α , which antagonizes p53 functions in activating the transcription of genes encoding cell cycle inhibitors or proapoptotic regulators (9, 10).

HPV38 E7 induces the accumulation of I κ B kinase beta (IKK β) in the nucleus, where it, in turn, binds and phosphorylates the Δ Np73 α protein at serine 422 (S422), resulting in a large increase in the half-life of Δ Np73 α (10). The IKK β / Δ Np73 α complex binds p53 responsive elements together with two epigenetic enzymes, DNA methyltransferase 1 (DNMT1) and enhancer of zeste homolog 2 (EZH2), and inhibits the expression of some p53-regulated genes, such as the PIG3 gene (13).

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TABLE 1 Sequences of different siRNAs used for gene silencing

Target	siRNA sequence or description (source)
Scrambled (negative control)	5'-GGUGGAAGAGGUGGUGAGC-3'
IKK β	5'-CGUACGCGGAAUACUUCGA-3'
EZH2	5'-AGUGGUGCUGAAGCCUCAUGUUUA-3'
p65	siGenome SMART pool M-003533-02-0005, human RELA, NM_021975 (Thermo Scientific)
p38 (MAPK14)	Silencer Select predesigned siRNA, catalog no. 4392420, ID ^a s3587 (Life Technologies)
S Δ Np73 α	5'-accgACGTACAGcatg-3' ^b
AS Δ Np73 α	5'-ccatGCTGTACGtcggT-3' ^b

^a ID, identification number.

^b For the Δ Np73 α sense (S) and antisense (AS) oligonucleotides, the capital letters indicate phosphorothioate nucleotides.

Studies with transgenic mice expressing HPV38 E6 and E7 in the basal layer of the epidermis further highlighted its transforming properties. In fact, these transgenic animals, upon chronic UV irradiation, developed actinic keratosis-like lesions, which are considered precursors of squamous cell carcinomas (SCC) in humans, and subsequently SCC. In contrast, wild-type animals subjected to identical treatments did not develop any type of skin lesions (12).

However, despite the well-characterized oncogenic properties of HPV38 in *in vivo* and *in vitro* experimental models, its role in human carcinogenesis remains to be proven.

In addition to their ability to promote cellular transformation, human cancer-associated viruses deregulate pathways linked to the host immune response, thus favoring the persistence of the infection, which is an essential condition for cancer development (14–16). Mucosal high-risk HPV16, Epstein-Barr virus (EBV), Merkel cell polyomavirus, and hepatitis B virus alter the expression of Toll-like receptors (TLRs), which are fundamental players in the innate immune response, acting as pattern recognition receptors (PRRs) (17, 18). In particular, all four of these oncogenic viruses, using distinct mechanisms, downregulate the transcription of TLR9, which resides in the endosomal compartments of the cell and senses viral double-stranded DNA (16, 19–24). To gain further insights into the possible role of HPV38 in human carcinogenesis, in this study, we investigated whether HPV38 E6 and E7 have the ability to deregulate TLR9 expression. Our data show that HPV38, similarly to the well-established oncogenic viruses, efficiently downregulated the expression of TLR9.

MATERIALS AND METHODS

Plasmid constructs. The genes of interest were expressed in the following expression vectors: pcDNA3 (Invitrogen), pLXSN (Clontech, Le Pont-de-Claix, France), and pBabe (25). The pLXSN-HPV38 E6/E7, pLXSN-HPV16 E6/E7, pcDNA3 HA- Δ Np73 α , and pBabe-puro- Δ N- $\text{IkB}\alpha$ (lacking the first 36 N-terminal amino acids) constructs and the TLR9 promoter luciferase construct, full-length (–3227/–1) or with deletions (–1017/–1 and –290/–1), have been previously described (7, 10, 16, 24). The pBabe-puro-TLR9 construct was generated during this study using standard molecular biology techniques.

Cell culture, retroviral infection, and treatment. Cell culture, antibiotic selection, and generation of high-titer retroviral supernatants were carried out as previously described (7). For treatment, cells were incubated in medium containing SB203580 (Sigma) with a final concentration of 5 μ M for 3 h. For fluorescence-activated cell sorter (FACS) staining, cells were collected, washed twice in PBS, and then stained with pro-

TABLE 2 Sequences of primers used for RT-PCR analyses, ChIP, and oligonucleotide pulldown

Promoter or gene	Primer sequence ^a
TLR9 promoter	F: 5'-TGGGTCTGTACCTGTGTGTGCA-3'
NF- κ B site A	R: 5'-TCATTCCCTCCATCCACCTC-3'
TLR9 promoter	F: 5'-AGGAGCTCAGGAGTGCCAG-3'
NF- κ B site B	R: 5'-TGGGATGTGCTGTTCCCTC-3'
TLR9 promoter	F: 5'-GAGAGCACTCAGGGGAACAG-3'
NF- κ B site C	R: 5'-GGTCACATTCAGCCCCTAGA-3'
TLR9 promoter	F: 5'-AGGCCCTGCAGAACTTGGAG-3'
NF- κ B site D	R: 5'-TCAGGCAGAGAGCAGGGAGA-3'
GAPDH	F: 5'-AAGGTGGTGAAGCAGGCGT-3'
	R: 5'-GAGGAGTGGGTGCTGCTGTT-3'
TLR9	F: 5'-CGTCTGAAGCCTGGTGTGA-3'
	R: 5'-CTGGAAGGCCTGGTTTGTGA-3'
IKK β	F: 5'-GCTGCAACTGATGCTGATGT-3'
	R: 5'-TGTCACAGGGTAGGTGTGGA-3'
p65	F: 5'-GTCACCGGATTGAGGAGAAA-3'
	R: 5'-GCTCAGGGATGACGTAAAGG-3'
EZH2	F: 5'-ACGTCAGATGGTGCCAGCAATA-3'
	R: 5'-CCCTGACCTCTGTCTTACTTGTGGA-3'
Probes for oligonucleotide pulldown assay	F: 5'-Bln-GAGAGCACTCAGGGGAACAG-3'
	R: 5'-GGTCACATTCAGCCCCTAGA-3'

^a F, forward; R, reverse.

pidium iodide (PI) at a final concentration of 5 μ g/ml. Subsequently, cells were analyzed by FACS CANTO (Becton Dickinson).

Gene silencing. Downregulation of Δ Np73 α was achieved by transfecting the antisense (AS) oligonucleotide as previously described (9). Silencing of HPV38 E6/E7 gene expression was obtained using the pRetro-Super construct (pRS), expressing small hairpin RNAs (shRNAs) for the polycistronic HPV38 E6 and E7 mRNA (pRS 38E6/E7) as previously described (9, 13). Gene silencing of p65 and IKK β was achieved using synthetic small interfering RNA (siRNA) (Table 1). siRNA or scrambled RNA at a concentration of 50 nM was transfected using Lipofectamine 2000 according to the standard protocol (Invitrogen). EZH2 and p38 silencing was performed using specific stealth siRNA as previously described (13).

ELISA. To measure secreted cytokines, HPV16 E6/E7 or HPV38 E6/E7 human foreskin keratinocytes (HFK) were seeded at 4×10^4 cells/96 wells in 200 μ l of growth medium. The next day, cells were washed with PBS, and Gpc or CpG 2006 was added. Twenty-four hours later, supernatants were collected for analysis of interleukin 8 (IL-8) and MIP3 α secretion using Quantikine enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) as previously described (22).

Luciferase assay. Transient transfections were conducted using XtremeGene 9 (Roche) according to the manufacturer's protocol. RPMI 8226 cells were cotransfected with firefly luciferase TLR9 promoter vector (0.5 μ g) and the vector of interest. pRL-TK *Renilla* reporter vector (15 ng) was used as an internal control. After 48 h, cells were lysed and luciferase activity was measured using a dual-luciferase reporter assay system (Promega). The expression of firefly luciferase relative to that of *Renilla* luciferase was expressed in relative luminescence units (RLU).

Reverse transcription-PCR (RT-PCR) and qPCR. Total RNA was extracted using the Absolutely RNA Miniprep kit (Stratagene). The ob-

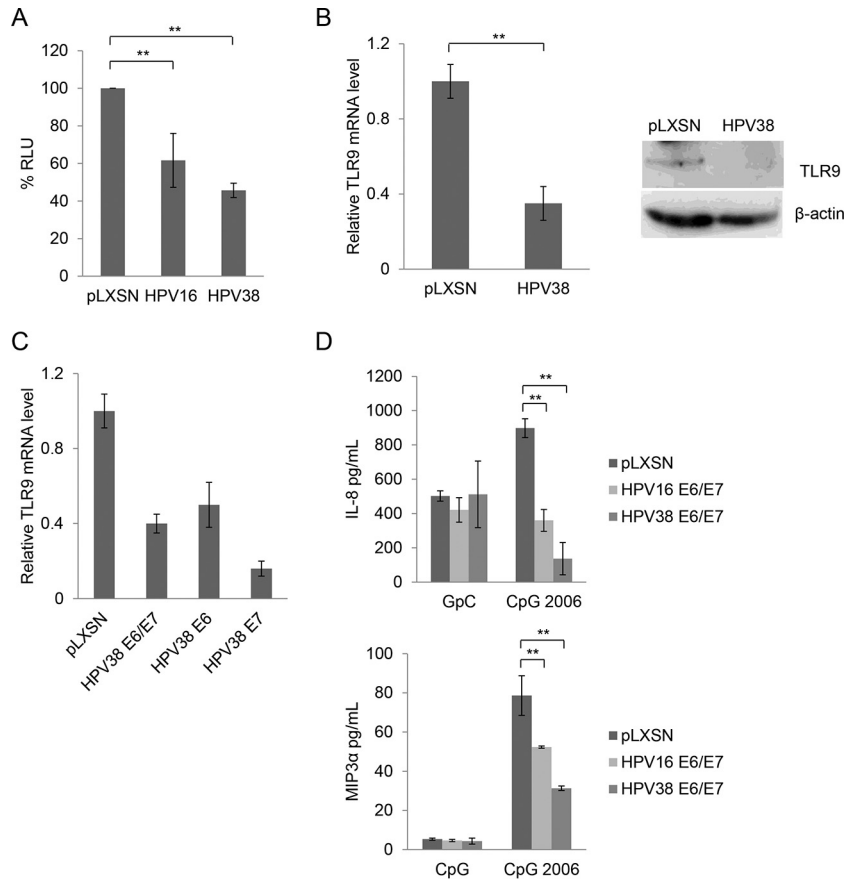


FIG 1 HPV38 E6 and E7 downregulate TLR9 expression. (A) RPMI 8226 cells were cotransfected with the TLR9 promoter construct ($-3227/-1$) fused with the luciferase reporter gene and with pLXSN empty vector (pLXSN), pLXSN HPV16 E6/E7 (HPV16), or HPV38 E6/E7 (HPV38). After 48 h, cells were harvested and luciferase activity was measured. Data are the means from three independent experiments performed in triplicate. **, $P < 0.01$. (B) Total RNA and total proteins from HFK and HPV38 E6/E7 HFK were extracted, and TLR9 expression levels were measured by RT-qPCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels (left); in addition, TLR9 protein levels were determined by immunoblotting (right). Error bars on the left side represent standard deviations from three biological replicates. **, $P < 0.01$. (C) Total RNA from control HFK or the indicated transduced HFK was extracted. TLR9 expression levels were measured by RT-qPCR. Error bars represent standard deviations from three biological replicates. (D) HFK transduced with pLXSN, HPV38 E6/E7, or HPV16 E6/E7 were treated with GpC or CpG 2006. After 24 h, the supernatants were collected to measure IL-8 or MIP3 α secretion. Data are the means from three independent experiments performed in triplicate. **, $P < 0.01$.

tained RNA was reverse transcribed to cDNA with the RevertAid H Minus Moloney murine leukemia virus (M-MuLV) reverse transcriptase kit (Fermentas) according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed using the MesaGreen qPCR MasterMix Plus for SYBR Assay (Eurogentec) with the primers listed in Table 2.

IB. Total protein extraction, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and immunoblotting (IB) were conducted as described previously (9). Antibodies to the following antibodies were used: β -actin (C4; MP Biomedicals), TLR9 (2254; Cell Signaling), p73 (OP108; Calbiochem), hemagglutinin (HA; 3F10; Roche), IKK β (2684; Cell Signaling), NF- κ B p65 (3034; Cell Signaling), p21^{WAF/Cip1} (2946; Cell Signaling), p27^{Kip1} (2552; Cell Signaling), p38 mitogen-activated protein kinase (MAPK; 9212; Cell Signaling), and phospho-p38 MAPK Thr180/Tyr182 (9211; Cell Signaling). Images were taken using the ChemiDoc XRS imaging system (Bio-Rad).

ChIP. Chromatin immunoprecipitation (ChIP) was performed with Shearing ChIP and OneDay ChIP kits (Diagenode) according to the manufacturer's protocols. Briefly, cells were sonicated to obtain DNA fragments of 200 to 500 bp. Sheared chromatin was immunoprecipitated with isotype control IgG or antibodies to the following: EZH2 (AC22; Cell Signaling), p73 (OP108; Calbiochem), IKK β (2684; Cell Signaling), DNMT1 (60B1220; Abnova), H3Lys27me3 (4039; Epigentek), and

NF- κ B p65 (3034; Cell Signaling). The eluted DNA was used as a template for qPCR. Primers for qPCR are listed in Table 2.

Oligonucleotide pulldown. Cells were lysed and sonicated in HKMG buffer (10 mM HEPES [pH 7.9], 100 mM KCl, 5 mM MgCl₂, 10% glycerol, 1 mM dithiothreitol [DTT], and 0.5% Nonidet P-40) containing protease and phosphatase inhibitors. After centrifugation at 12,000 \times g for 10 min, protein extracts were precleared with streptavidin-agarose beads. The TLR9 promoter was used as a template to amplify the NF- κ B RE region containing wild-type or mutated NF- κ B RE. PCR amplification was performed using a biotinylated forward primer and a nonbiotinylated reverse primer (listed in Table 2). Amplicons were extracted from an agarose gel by using the MinElute gel extraction kit (Qiagen) and quantified. Then, 2 mg of prepared protein extract was incubated with 1 μ g of biotin-TLR9 promoter probes and 10 μ g of poly(dI-dC)-poly(dI-dC) for 16 h at 4°C. DNA-bound proteins were collected with streptavidin-agarose beads for 1 h and washed five times with HKMG buffer. DNA-bound proteins were then analyzed by IB.

Colony formation assay. For colony formation assays, the different types of cells were seeded at different dilutions (10^4 , 10^3 , and 10^2) and cultured for 7 days. Cells were then stained with crystal violet, and the average numbers of cells per colony were determined.

Time course. As cells were split for selection, 10^5 cells were seeded in 6-well plates and allowed to grow for 4 days. The growth was monitored daily by cell trypsinization and counting with trypan blue. Double determinations were performed in each independent experiment.

Kinase assay. CDK2 kinase assays were performed using 1 mg of keratinocyte lysate. CDK2 complexes were immunoprecipitated using an anti-CDK2 antibody (sc-748; Santa Cruz). Immunopellets were washed three times in lysis buffer and twice in kinase buffer (50 mM Tris [pH 7.5], 10 mM $MgCl_2$, 1 mM DTT, and 100 μ M ATP). Samples were then resuspended in 10 μ l of kinase buffer containing 10 μ Ci of [γ - 32 P]ATP (PerkinElmer) and incubated for 15 min at room temperature in the presence of 2 μ g of histone H1 (Millipore). Samples were subsequently analyzed by SDS-PAGE (12%), transferred to nitrocellulose, and visualized on X-ray films.

Statistical analysis. Statistical significance was determined by the Student *t* test. Statistically significant *P* values ($P < 0.05$ [*] and $P < 0.01$ [**]) for each experiment are indicated in the corresponding figure legends. Error bars in the graphs represent the standard deviations.

RESULTS

Beta HPV38 E6 and E7 downregulate TLR9 expression. We aimed to test our hypothesis that, as previously shown for many other viral oncoproteins, beta HPV38 E6 and E7 have the ability to inhibit TLR9 promoter activity in RPMI 8226 human myeloma cells. These cells express high levels of TLR9 and have previously been used to demonstrate the ability of E6 and E7 from mucosal HPV16 to inhibit TLR9 promoter activity (22). We found that HPV38 E6 and E7 inhibited TLR9 promoter activity in transiently transfected RPMI 8226 cells with an efficiency similar to that of human papillomavirus 16 (HPV16) E6 and E7 (Fig. 1A). Next, we analyzed TLR9 mRNA and protein levels in human keratinocytes, the natural host of the virus. Primary human foreskin keratinocytes (HFK) were transduced with a recombinant retrovirus expressing HPV38 E6 and E7 oncoproteins or empty retrovirus (pLXSN), and TLR9 mRNA or protein levels were determined by quantitative RT-PCR or immunoblotting, respectively. TLR9 expression was decreased in HPV38 E6/E7 HFK compared with pLXSN HFK (Fig. 1B). When expressed alone, both oncoproteins were able to inhibit TLR9 expression, although E7 appeared to be more efficient than E6 (Fig. 1C). Finally, we checked whether the viral proteins could inhibit TLR9 functionality. pLXSN and HPV38 E6/E7 HFK were exposed to a TLR9 synthetic ligand (CpG oligodeoxynucleotide 2006) for 24 h, and the concentrations of secreted cytokines, IL-8 and MIP3 α , were determined by ELISA. The secretion of both cytokines was significantly inhibited in HPV38 E6/E7 HFK compared with control cells (Fig. 1D). These data show that, similar to the case with several oncogenic viruses, HPV38 E6 and E7 block TLR9 function by inhibiting its transcription.

Δ Np73 α is directly involved in TLR9 downregulation. HPV38 E7 oncoprotein promotes the accumulation of the p53 antagonist Δ Np73 α , repressing the transcription of p53-regulated genes (9, 10). Recent studies have demonstrated that TLR9 expression can be positively regulated by the p53 transcription factor (26, 27). Therefore, we next determined whether Δ Np73 α can modulate TLR9 transcription. Δ Np73 α expression inhibited TLR9 promoter activity in transient-transfection experiments performed with RPMI 8226 cells (Fig. 2A). In addition, downregulation of Δ Np73 α expression in HPV38 E6/E7 HFK using an antisense (AS) oligonucleotide led to an increase of both mRNA and protein levels of TLR9 (Fig. 2B and C). Finally, downregulation of viral

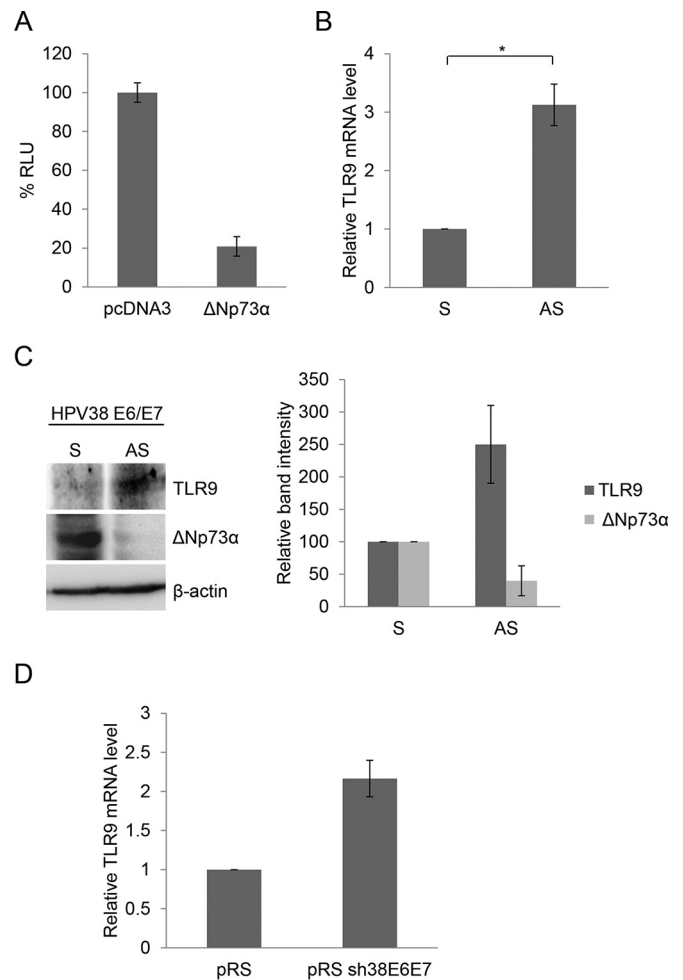


FIG 2 Δ Np73 α negatively regulates TLR9 expression. (A) RPMI 8226 cells were cotransfected with the TLR9 promoter construct (–3227/–1) cloned in front of the luciferase reporter gene with pcDNA3 empty vector or expressing Δ Np73 α . After 48 h, cells were harvested and luciferase activity was measured. Data are the means from three independent experiments performed in triplicate. (B and C) HPV38 E6/E7 HFK were transfected with Δ Np73 α sense (S) and antisense (AS) oligonucleotides. (B) Expression of TLR9 and GAPDH was determined by RT-qPCR. Results are representative of those from three independent experiments. *, $P < 0.05$. (C) Protein extracts (40 μ g) were analyzed by immunoblotting (IB) with indicated antibodies (left side). Band intensities were quantified and normalized to β -actin levels (right side). Data are the means from three independent experiments. (D) HFK and HPV38 E6/E7 HFK transduced with pRetroSuper (pRS) or shRNA-HPV38E6E7 (pRS sh38E6E7) were processed for RNA extraction. The mRNA levels of TLR9 were determined by RT-qPCR.

oncoproteins by shRNA resulted in an increase of TLR9 mRNA levels (Fig. 2D).

Previous studies have shown that Δ Np73 α also has the ability to modulate the activity of NF- κ B (28), a key transcription factor involved in the regulation of TLR9 expression (16, 22, 29). We therefore determined by chromatin immunoprecipitation (ChIP) experiments whether Δ Np73 α is recruited to or near to the four characterized NF- κ B responsive elements (RE), termed A, B, C, and D (16, 22, 29). We found that Δ Np73 α binds mainly the promoter region near NF- κ B RE C (Fig. 3A). To corroborate these findings, we performed oligonucleotide pulldown experiments using biotinylated DNA fragments of the TLR9 pro-

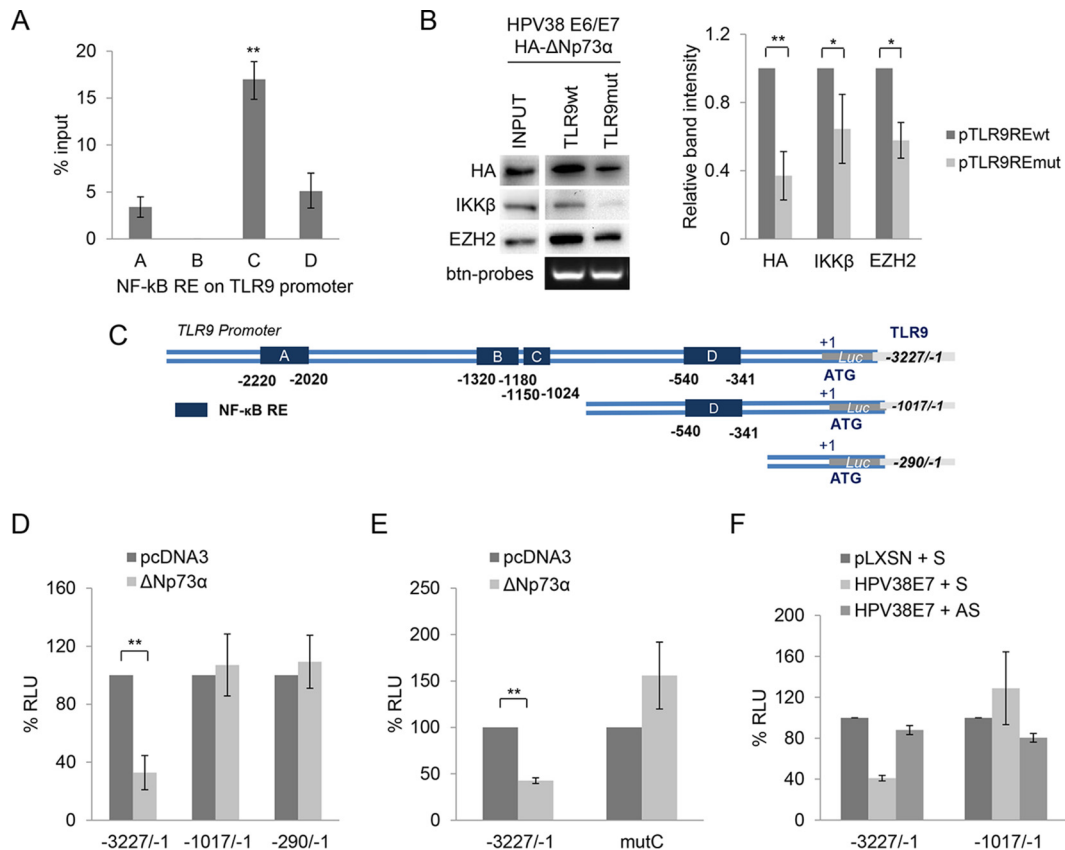


FIG 3 Δ Np73 α binds to TLR9 promoter on NF- κ B site C. (A) ChIP was performed in HPV38 E6/E7 HFK using anti- Δ Np73 α antibody. Simultaneously, part of the total chromatin fraction (1/10) was used as input. qPCR was performed using specific primers flanking the NF- κ B RE within the TLR9 promoter. The histogram shows the relative amount of the promoter bound by Δ Np73 α after subtraction of the background of nonspecific IgG control expressed as a percentage of the input. Data are the means from three independent experiments. **, $P < 0.01$. (B) HPV38 E6/E7 HFK transiently expressing HA- Δ Np73 α was processed for the oligonucleotide pulldown assay. Cell lysate was incubated with biotinylated (btn) probes containing the NF- κ B RE of the TLR9 promoter, either wild type (pTLR9wt) or mutated (pTLR9mut). DNA-associated proteins were recovered by precipitation with streptavidin beads and analyzed by IB (left side). The intensity of the protein bands in three independent experiments was quantified (right side). *, $P < 0.05$; **, $P < 0.01$. (C) Scheme of the TLR9 promoter luciferase constructs: the full-length (-3227/-1) construct containing the 4 NF- κ B RE (sites A, B, C, and D) and the deletion (-1017/-1 and -290/-1) constructs. (D) RPMI 8226 cells were cotransfected with TLR9 promoter constructs (-3227/-1, -1017/-1, and -290/-1) cloned in front of the luciferase reporter gene with pcDNA3 empty vector or expressing Δ Np73 α . After 48 h, cells were harvested and luciferase activity was measured. Data are the means from three independent experiments performed in triplicate. **, $P < 0.01$. (E) RPMI 8226 cells were cotransfected with TLR9 promoter constructs wild-type (-3227/-1) or NF- κ B point mutated in site C (mutC) cloned in front of the luciferase reporter gene with pcDNA3 empty vector or expressing Δ Np73 α . After 48 h, cells were harvested and luciferase activity was measured. Data are the means from three independent experiments performed in triplicate. **, $P < 0.01$. (F) RPMI 8226 cells were cotransfected with TLR9 promoter constructs (-3227/-1 or -1017/-1) cloned in front of the luciferase reporter gene with pLXSN empty vector or pLXSN HPV38E7 construct and Δ Np73 α sense (S) or AS oligonucleotides. After 48 h, cells were harvested and luciferase activity was measured. Data are the means from three experiments. **, $P < 0.01$.

moter (-1150/-1024) that encompass wild-type or mutated NF- κ B RE C. The two biotinylated DNA probes were incubated with cellular extracts of HPV38 E6/E7 HFK overexpressing hemagglutinin (HA)-tagged Δ Np73 α . The TLR9 promoter fragment containing the wild-type NF- κ B RE C coprecipitated with Δ Np73 α and IKK β as well as EZH2, which was previously shown to be part of the Δ Np73 α repressive transcriptional complex (Fig. 3B) (13). In contrast, mutation of NF- κ B RE C significantly decreased the binding of the Δ Np73 α /IKK β /EZH2 complex to the DNA fragment, indicating that the DNA binding region of Δ Np73 α coincides with or partially overlaps with NF- κ B RE C. Transient-transfection experiments using two mutants of the TLR9 promoter cloned in front of the luciferase reporter gene showed that deletion of the region that includes NF- κ B RE C abolished the inhibitory function of

Δ Np73 α (Fig. 3C and D). Point mutations of RE C had the same effect (Fig. 3E). Finally, downregulation of Δ Np73 α by antisense oligonucleotide and/or deletion of promoter region containing RE C resulted in the loss of HPV38 E7-mediated inhibition of TLR9 promoter activity (Fig. 3F). Together, these findings highlight the crucial role of Δ Np73 α in the HPV38-induced downregulation of TLR9 transcription.

Δ Np73 α and NF- κ B p65 inversely regulate TLR9 expression. We recently showed that Δ Np73 α forms a complex with the Polycomb group (PcG) 2 member EZH2 and DNA methyltransferase DNMT1 to repress the transcription of p53-regulated genes (13). Therefore, we performed ChIP experiments to determine which cellular proteins were recruited to the TLR9 promoter region near NF- κ B RE C. We observed that in addition to Δ Np73 α and IKK β , the subunit of NF- κ B transcription complex p65 as well as

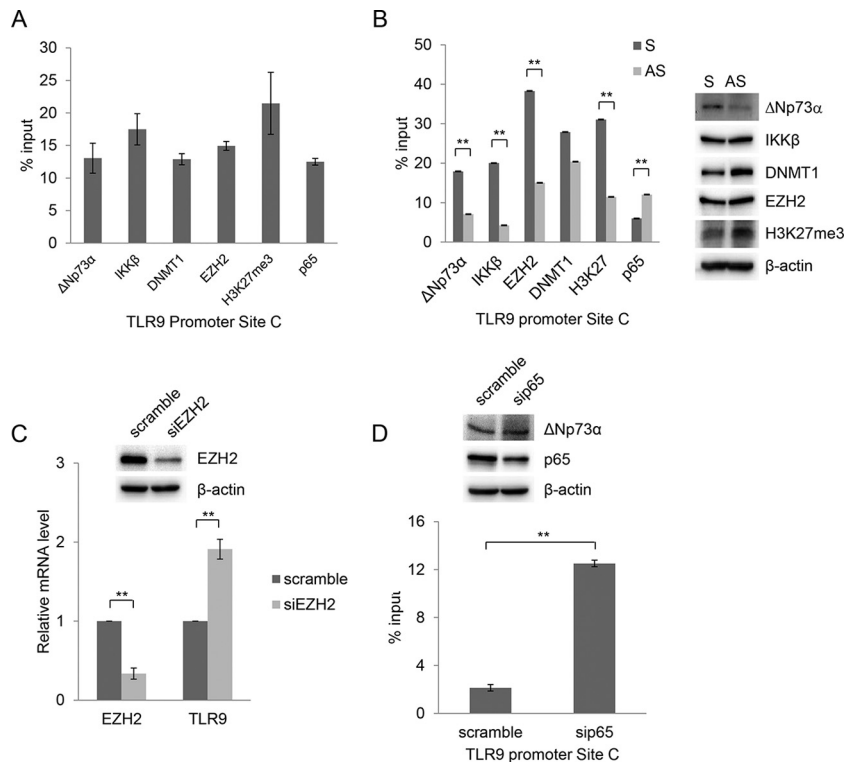


FIG 4 Δ Np73 α and p65 compete for binding to the TLR9 promoter. (A) HPV38 E6/E7 HFK were processed for ChIP using Δ Np73 α , IKK β , DNMT1, EZH2, H3K27me3, and p65 antibodies. qPCR was performed using specific primers flanking NF- κ B RE site C within the TLR9 promoter. Data are the means from three independent experiments. All the bindings shown are statistically significant compared to the negative control, with a P value of <0.01 . (B) HPV38 E6/E7 HFK were transfected with Δ Np73 α S and AS oligonucleotides, and the levels of indicated cellular proteins were determined by IB (right side). Total cellular extracts were processed for ChIP with the indicated antibodies. Data are from one representative experiment of two independent experiments. **, $P < 0.01$. (C) HPV38 E6/E7 HFK were transfected with a control siRNA (scramble) or siRNA specific for EZH2 (siEZH2). After 72 h, total RNA was extracted and EZH2 or TLR9 mRNA levels were analyzed by RT-qPCR. Error bars represent standard deviations from three biological replicates. **, $P < 0.01$. The inset shows the EZH2 protein levels determined by IB. (D) HPV38 E6/E7 HFK were transfected with a control siRNA (scramble) or siRNA specific for p65 (sip65), and p65 as well as Δ Np73 α levels were determined by IB (inset). Total cellular extracts were processed for ChIP using anti- Δ Np73 α antibody. The results were analyzed by qPCR. Data are the means from three independent experiments. **, $P < 0.01$.

DNMT1 and EZH2 was associated with the TLR9 promoter in the NF- κ B RE C region (Fig. 4A). Consistent with the recruitment of EZH2, histone 3 was found to be trimethylated at lysine 27 (H3K27me3) in the same region of the TLR9 promoter (Fig. 4A). Downregulation of Δ Np73 α by AS oligonucleotide experiments resulted in a significant decrease in the amount of IKK β and EZH2 recruited to the TLR9 promoter as well as of H3K27 (Fig. 4B). The recruitment of DNMT1 appeared to be less affected by the decrease in Δ Np73 α levels, suggesting that it may also be associated with another, as-yet-unidentified complex. Similarly to the downregulation of Δ Np73 α , silencing the expression of EZH2 increased TLR9 mRNA levels (Fig. 4C). All these findings show that Δ Np73 α plays a key role in the inhibition of TLR9 expression by promoting the formation of a repressive transcriptional complex.

We also observed that higher levels of p65 were associated with the NF- κ B RE C region when Δ Np73 α was downregulated, indicating that Δ Np73 α and p65 compete in binding the same or a partially overlapping DNA region within the TLR9 promoter (Fig. 4B). Consistent with these conclusions, downregulation of p65 by siRNA increased the recruitment of Δ Np73 α in the same promoter region (Fig. 4D). Experiments with HPV38 E6/E7 HFK in which the Δ Np73 α levels were downregulated provided evidence for the positive role of p65 in TLR9 transcriptional regulation. In

fact, although the decrease in Δ Np73 α protein levels is associated with reactivation of TLR9 expression, the simultaneous silencing of p65 expression repressed TLR9 transcription (Fig. 5A). Consistent with these findings, inhibition of NF- κ B signaling in HPV38 E6/E7 HFK by overexpressing a nondegradable deletion mutant of I κ B α (Δ N-I κ B α) that lacks the first 36 amino acids at the N terminus containing the IKK-phosphorylated amino acid further decreased TLR9 mRNA levels (Fig. 5B). Similar results were obtained with cells expressing p65 or IKK β siRNA (Fig. 5C).

In summary, these findings showed that p65 and Δ Np73 α compete for binding to the TLR9 promoter, leading to an inverse regulation of its activity.

TLR9 ectopic expression affects cellular proliferation in HPV38 E6/E7 HFK. To gain more insights on the biological significance of HPV38-mediated downregulation of TLR9 transcription, we reexpressed TLR9 in HPV38 E6/E7 HFK using a retrovirus expression system. As expected, TLR9 mRNA and protein were detected only in cells transduced with TLR9 recombinant retrovirus (Fig. 6A). No changes in viral gene expression were observed in mock-treated cells or TLR9 HPV38 E6/E7 HFK (Fig. 6A). We first determined whether TLR9 expression affected the proliferation of HPV38 E6/E7 HFK by performing a colony for-

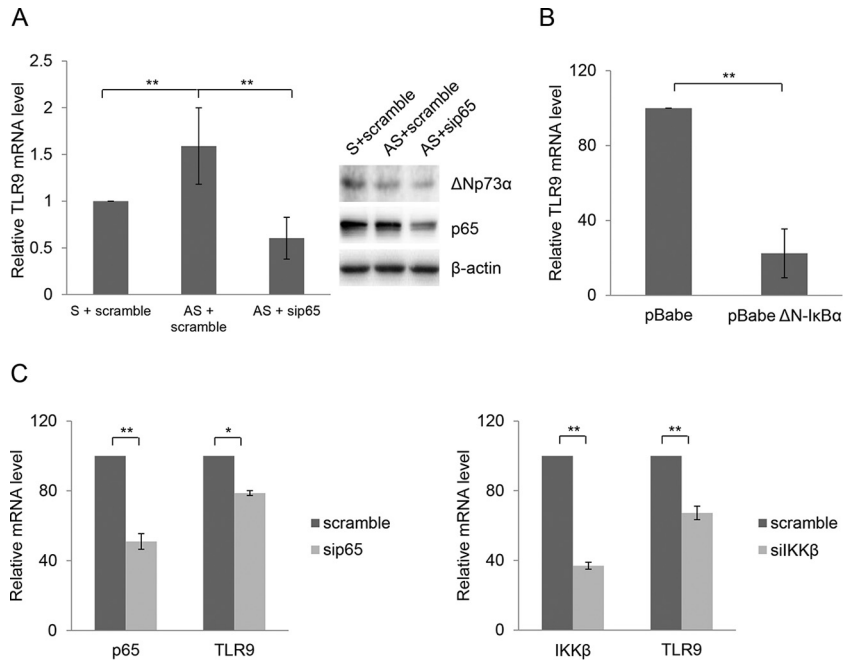


FIG 5 Δ Np73 α and p65 inversely regulate TLR9 expression. (A) HPV38 E6/E7 HFK were cotransfected with 1 μ g of Δ Np73 α S or AS oligonucleotides and 50 pmol of a control siRNA (scramble) or siRNA specific for p65 (sip65). After 36 h, total RNA was extracted and analyzed by RT-qPCR. Data are the means from three independent experiments. **, $P < 0.01$. (B) HPV38 E6/E7 HFK were transduced with pBabe or pBabe- Δ N-I κ B α superrepressor (Δ N-I κ B α). Total RNA was extracted and TLR9 levels were analyzed by RT-qPCR. Data are the means from three independent experiments. **, $P < 0.01$. (C) HPV38 E6/E7 HFK were transfected with scramble or p65 siRNA (left) or with scramble or IKK β siRNA (right). After 40 h, total RNA was extracted and TLR9 mRNA levels were analyzed by RT-qPCR. Data are the means from three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

mation assay. Although we detected approximately the same numbers of colonies in TLR9-expressing and control cells, the colony size was significantly decreased in the presence of TLR9 expression (Fig. 6B). The negative impact of TLR9 overexpression on the proliferation of HPV38 E6/E7 HFK was also observed in a short-term growth curve (Fig. 6C). Analysis of cell cycle profile by flow cytometry of HPV38 E6/E7 HFK overexpressing TLR9 revealed a significant decrease of cells in S and G₂/M phase compared to the value for the mock-treated cells (Fig. 6D). Immunoblotting for many cell cycle regulators showed that the cell cycle inhibitors p21^{WAF1} and p27^{Kip1} are strongly accumulated in TLR9-expressing HPV38 E6/E7 HFK (Fig. 6E).

Consistent with p21^{WAF1} levels, lower CDK2-associated kinase activity was detected in these cells than in mock-treated cells (Fig. 7A). However, TLR9 expression did not lead to significant changes in p21^{WAF1} or p27^{Kip1} mRNA levels (Fig. 7B). Based on these data, we investigated whether posttranslational mechanisms are implicated in the TLR9-induced accumulation of p21^{WAF1} and p27^{Kip1}. Interestingly, it is known that p38 MAPK is activated by TLR9 signaling and positively regulate the p21^{WAF1} protein levels by posttranslational mechanisms (30). Accordingly, we observed that the phosphorylated form (Thr180/Tyr182) of p38 MAPK was strongly accumulated in TLR9-expressing HPV38 E6/E7 HFK (Fig. 7C). Moreover, both p21^{WAF1} and p27^{Kip1} levels were reduced in these cells treated with a chemical inhibitor of p38 MAPK, SB203580 (Fig. 7D), or with a specific siRNA against p38 (Fig. 7E). Taken together, these findings highlight a novel function of TLR9 in inhibiting cellular proliferation via accumulation of the cell cycle inhibitors p21^{WAF1} and p27^{Kip1}.

DISCUSSION

Previous studies demonstrated that several oncogenic viruses use different mechanisms to inhibit the expression of TLR9 (16, 19–24). Here, we describe a novel mechanism of beta HPV38 in repressing TLR9 expression that is mediated by Δ Np73 α , a well-characterized antagonist of p53. Thus, beta HPV38, in addition to its ability to promote cellular transformation, also shares with oncogenic viruses the property of repressing the TLR9 signaling pathway. The high conservation of this property among human oncogenic viruses underlines the importance of downregulation of TLR9 expression in virus-mediated carcinogenesis. It is likely that loss of the functionality of TLR9 signaling has a positive impact on completion of the viral life cycle and/or evasion of the host immune response. However, the fact that the inhibition of TLR9 gene transcription occurs after the translocation of viral DNA into the nucleus and is dependent on the expression of the viral oncoproteins does not fully support an exclusive role of the event in the evasion of the innate immune response to viral infection. In fact, at an early stage of infection and before viral gene expression, TLR9 is functional and is activated by nonmethylated CpG islands present in the viral genome (16, 22). A possible explanation is that by inhibiting TLR9 expression, oncogenic viruses prevent its further activation by the microflora present at specific anatomical sites, which may have a negative impact on viral infection. In agreement with this hypothesis, it has been reported that commensal bacterial subspecies could be protective against HPV infection (31). In addition, it is plausible that inactivation of TLR9 by oncogenic viruses may play an additional role in infected cells. Previous findings from our group showed that EBV infection of

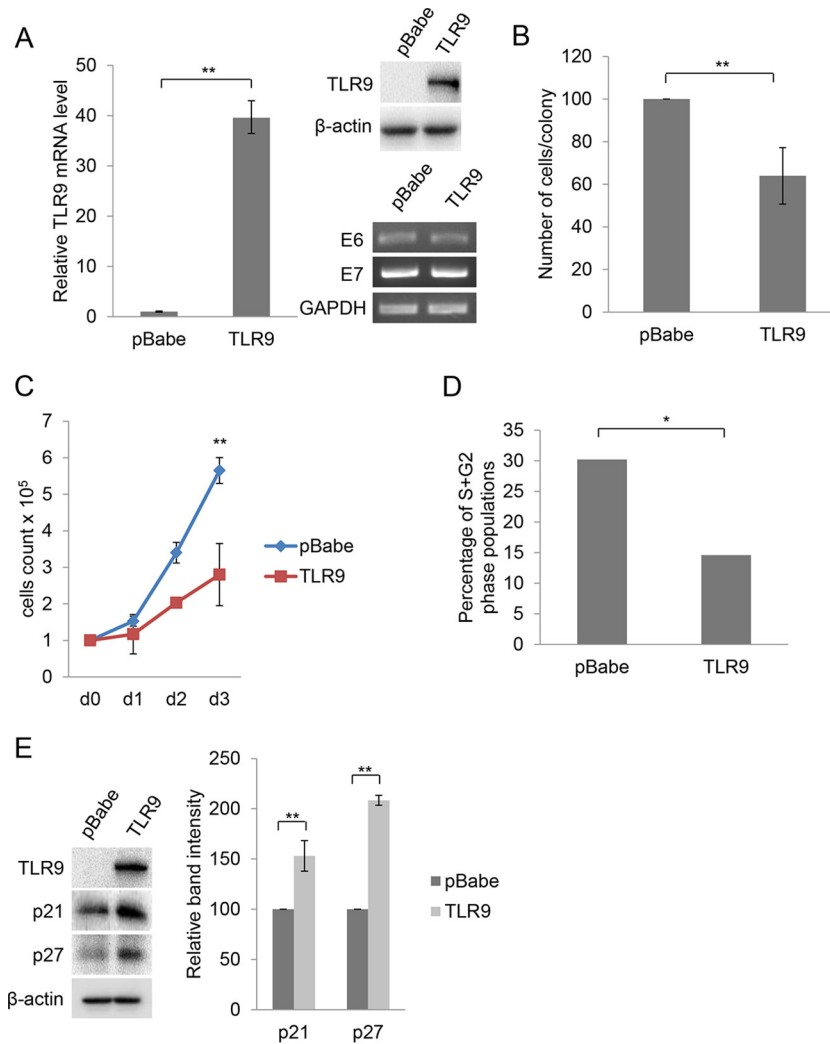


FIG 6 TLR9 expression in HPV38 E6/E7 HFK inhibits cellular proliferation by the accumulation of p21^{WAF1} and p27^{Kip1}. (A) HPV38 E6/E7 HFK were transduced with pBabe empty vector or expressing TLR9. The efficiency of transduction was verified by RT-PCR (left) and IB (right). Data on the left are the means from three independent experiments. **, $P < 0.01$. The bottom right shows the levels of HPV38 E6 and E7 mRNA determined by qPCR. (B) Colony formation assay. After 6 to 8 days of culture in puromycin-containing medium, colonies were fixed in 20% methanol and stained with crystal violet. The number of cells per colony was determined by cell counting. Results are the mean counts from 10 colonies randomly selected from three independent experiments. Double-blind counting was performed. **, $P < 0.01$. (C) Growth time course. The growth of all cell populations was monitored for 3 days as described in Materials and Methods. The growth was assessed by counting live cells with trypan blue. Data represent the means from two independent experiments, each performed in duplicate. **, $P < 0.01$. (D) Flow cytometry analysis of cells described for panel C (day 3) stained by propidium iodide. Data are from one representative experiment of three independent experiments. *, $P < 0.05$. (E) Total proteins (20 μ g) from HPV38 E6/E7 HFK transduced with pBabe or pBabe-TLR9 were analyzed by IB (left) and band intensities quantified and normalized to β -actin levels (right). Data are the means from three independent experiments. **, $P < 0.01$.

primary B cells resulted in a rapid decrease of TLR9 mRNA levels, which became more pronounced upon immortalization, suggesting that the decrease in TLR9 expression may be linked to cellular transformation (19).

In this study, we provided evidence for a novel function of TLR9 in controlling cellular proliferation. Reexpression of TLR9 in HPV38 E6/E7 HFK resulted in a strong accumulation of the cell cycle inhibitors p21^{WAF1} and p27^{Kip1} and a clear decrease in cellular proliferation. It is likely that the inhibitory role of TLR9 on cellular proliferation is a response to the cellular stress induced by the expression of the viral oncogenes. The precise mechanism of this novel TLR9 function is still unknown. Ongoing studies by our group are focused on deter-

mining whether the TLR9-induced p21^{WAF1} accumulation requires TLR9 engagement with endogenous ligands, for example, damage-associated molecular patterns (DAMP) generated during virus-induced stress. In support of the hypothesis that TLR9 promotes antiproliferative events in response to cellular stresses, it has recently been shown that TLR9 expression is strongly activated via p53 in primary human blood lymphocytes and alveolar macrophages upon exposure to different types of DNA-damaging insults (27). Based on these findings, it is likely that UV irradiation, the main risk factor for the development of skin squamous cell carcinoma, can induce TLR9 upregulation via p53 activation in keratinocytes. The fact that HPV38 E7 induces Δ Np73 α accumulation may prevent activa-

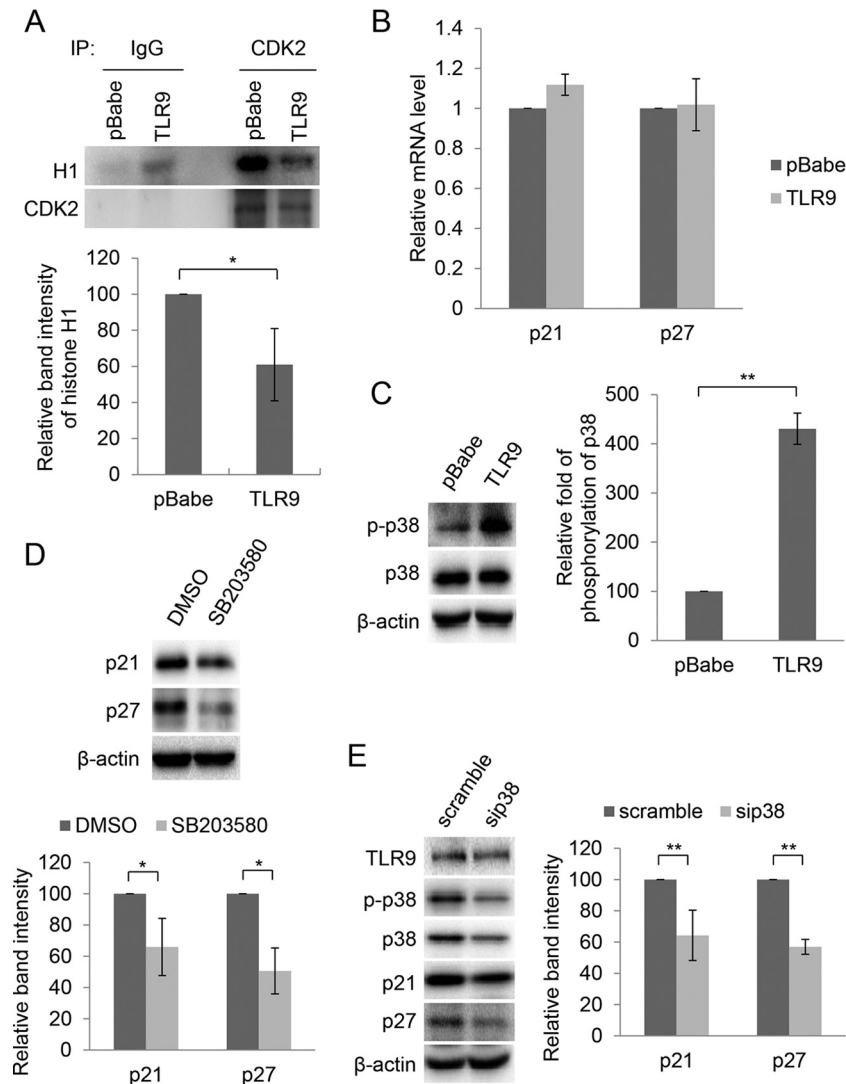


FIG 7 TLR9-induced accumulation of p21^{WAF1} and p27^{Kip1} is mediated by activation of p38 signaling. (A) Kinase assay. CDK2 was immunoprecipitated from 1 mg of total proteins in whole-cell lysate. As a control, an identical reaction was carried out with a negative IgG antibody. Immunocomplexes were then incubated with purified histone H1 in the presence of [γ -³²P]ATP. After SDS-PAGE followed by autoradiography (top), band intensity was quantified (bottom). Data are the means from three independent experiments. *, $P < 0.05$. (B) Total RNA from indicated cell lines was extracted and p21^{WAF1} and p27^{Kip1} expression levels were measured by RT-qPCR. Error bars represent standard deviations from four biological replicates. (C) Total proteins (20 μ g) from HPV38 E6/E7 HFK transduced with pBabe or pBabe-TLR9 were analyzed by IB (left). The intensity of the protein bands in three independent experiments was quantified (right). **, $P < 0.01$. (D) TLR9-expressing HPV38 E6/E7 HFK were treated with the p38 inhibitor SB203580 (5 μ M) or dimethyl sulfoxide (DMSO). After 3 h, total proteins were extracted and analyzed by IB using the indicated antibodies (top). The intensity of the protein bands in three independent experiments was quantified (bottom). *, $P < 0.05$. (E) TLR9-expressing HPV38 E6/E7 HFK were transfected with siRNA scramble or siRNA specific for p38 (sip38). After 48 h, total proteins were extracted and analyzed by IB (left). Band intensity was quantified in three independent experiments and is shown as a histogram (right). **, $P < 0.01$.

tion of TLR9 in cells exposed to UV irradiation. This model is in line with the concept of a synergy between UV irradiation and beta HPV38 in skin carcinogenesis (12).

In conclusion, we have demonstrated that beta HPV38, as observed for several well-established oncogenic viruses, has the ability to inhibit the expression of TLR9. Interestingly, the HPV38 mechanism elucidated here differs substantially from those previously characterized for other oncogenic viruses. Most importantly, our study highlights a novel function of TLR9 in negatively regulated cellular proliferation via accumulation of the cell cycle inhibitors p21^{WAF1} and p27^{Kip1}.

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Paper II. E6 and E7 of human papillomavirus 38 alter the UV-induced cellular response by inhibiting the expression of Toll-like receptor 9

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**E6 and E7 of human papillomavirus type 38 inhibit Toll-like receptor 9 expression
induced by UV irradiation**

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Running title: HPV38 E6 and E7 inhibit UV-induced TLR9 expression

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ABSTRACT

Several lines of evidence indicate that cutaneous human papillomavirus (HPV) types belonging to genus beta of the HPV phylogenetic tree synergise with ultra-violet (UV) irradiation in the development of skin cancer. Accordingly, the E6 and E7 oncoproteins from some beta HPV types are able to deregulate pathways related to immune response and cellular transformation. Toll-like receptor 9 (TLR9), in addition to its role in the innate immunity, has been shown to be involved in the cellular stress response. Here, using human primary keratinocytes as experimental models, we showed that UV irradiation, or other cellular stresses, activates TLR9 expression. This event is closely linked to p53 activation. Silencing the expression of p53, or deletion of its gene, affected the activation of TLR9 expression after UV irradiation. Using different strategies, we also showed that the transcription factors p53 and c-Jun are recruited onto a specific region of the TLR9 promoter after UV irradiation. Importantly, E6 and E7 oncoproteins from beta HPV38, suggested to be a skin co-carcinogen of UV irradiation, prevent the UV-mediated recruitment of these transcription factors onto the TLR9 promoter, with subsequent impairment of TLR9 gene expression.

This study provides new insight into the mechanism that mediates TLR9 up-regulation in response to cellular stresses. In addition, we showed that HPV38 E6 and E7 are able to interfere with such mechanisms, providing another explanation for a possible cooperation of beta HPV types with UV irradiation in skin carcinogenesis.

INTRODUCTION

Toll-like receptor 9 (TLR9) is a transmembrane receptors which plays a fundamental role in innate immunity. It is expressed in endolysosome by various human immune cells including dendritic B and on nonimmune cells, such as epithelial cells. TLR9 senses highly conserved unmethylated CpG motif on viral and bacterial DNA and its activation results in the production of proinflammatory cytokines and IFN-1.

Previous studies showed that the basal levels of TLR9 expression are down-regulated by different viruses, such as the alpha human papillomavirus (HPV) type 16, the beta HPV38, Epstein-Barr virus (EBV) and Merkel cell polyomavirus (MCPyV) ¹⁻⁵. The blocking of TLR9 function by several oncogenic viruses signifies a crucial event related to host immune evasion and guarantees the persistence of the infection. However, the down-regulation of TLR9 expression requires viral gene expression and occurs after virus entry and activation of the innate immune response. Thus, it is plausible that inhibition of TLR9 signalling may also be required to deregulate additional events and to generate ideal conditions for completion of the viral life cycle. In a previous study, we showed that the over-expression of TLR9 in HPV38-immortalized keratinocytes results in an accumulation of the cell cycle inhibitor p21^{WAF1/Cip1} and a decrease in cellular proliferation ². The inhibitory functions of TLR9 on cell cycle progression was also observed in head and neck cancer cell lines, in which its expression resulted in a reduction of cellular proliferation and inhibition of their ability to grow in anchorage-independent manner ⁶. It has been also shown that TLR9 signalling can be activated by the damage-associated molecular pattern (DAMPs) released by cells after exposure to several types of stresses ⁷. Since the DNA replication of HPV and other oncogenic viruses is entirely dependent on the proliferative status of the infected cells, inhibition of TLR9 expression may be needed to counteract its negative role on cellular proliferation and to allow an efficient viral replication. Beta HPV types are suspected,

together with ultra-violet (UV) radiation, to promote the development of non-melanoma skin cancer (NMSC) ⁸. Interestingly, it has been shown that exposure of different cancer-derived cells to genotoxic stresses, including UV irradiation, resulted in an up-regulation of TLR9 expression that was partially mediated by activation of the transcription factor p53 ⁹. However, the possible interplay between beta HPVs, UV irradiation and TLR9 signalling in human keratinocytes has not been yet elucidated. Here, we show that, UV irradiation induces activation of TLR9 expression in human primary keratinocytes (HPKs), the natural host cell of HPV. This event is mediated by the recruitment of p53 and c-Jun to the TLR9 promoter. Importantly, beta HPV38 E6 and E7 oncoproteins prevent the UV-mediated activation of TLR9.

RESULTS

TLR9 transcription is induced by several cellular stresses in primary human keratinocytes

We have previously shown that beta HPV38 E6 and E7 repress the basal TLR9 transcriptional levels in HPKs, which are naturally infected by the virus ². As it has also been shown that UV irradiation and other stresses activate TLR9 expression in several cell lines⁸, we were interested in determining whether the viral proteins could also interfere with this UV-mediated transcriptional activation. As first step, we evaluated the effect of UV irradiation on TLR9 expression in HPKs. Cells were irradiated with UVB (25 mj/cm²), and after 8 hours they were processed for the preparation of total RNA and protein extract. Quantitative RT-PCR showed that UV irradiation significantly increases the expression of TLR9 (Figure 1a, left panel). Similarly, TLR9 protein levels were increased in UV-irradiated cells in comparison to mock cells (Figure 1a, right panel). As expected, p21^{WAF1/Cip1}, a marker for

activation of the cellular response to stresses, was accumulated after UV irradiation (Figure 1a, right panel).

We also assessed whether other cellular stresses led to TLR9 transcriptional activation in HPKs. Similarly to UV irradiation, doxorubicin (0.1 $\mu\text{g/ml}$) and H_2O_2 (500 μM) induced accumulation of TLR9 mRNA and protein levels (Figure 1b and c).

p53 plays a key role in UV-mediated TLR9 transcriptional activation

Next we determined whether the activation of TLR9 by UV irradiation and other cellular stresses in HPKs is mediated by p53, as previously observed in human cancer cells⁸. As a first strategy, we generated p53 knockout HPKs using CRISPR/Cas9 technology. To increase the yield of p53 knockout cells, we used HPKs expressing the human telomerase reverse transcriptase (*hTERT*) gene, which extends the life span of primary cells. Expression of p53 was efficiently silenced when the gene was deleted, compared to the mock cells that were transfected with a CRISPR/Cas9 vector containing a scramble sequence (Figure 2a). Upon UV irradiation, no significant activation of TLR9 expression was observed in p53^{-/-} HPKs (crip53) in comparison to the mock cells (scramble) in three independent experiments (Figure 2a). In line with lower RNA expression levels, TLR9 protein was not accumulated in UV-irradiated p53^{-/-} HPKs (Figure 2b, top and bottom panels). To eliminate the possibility that the *hTERT* could interfere with TLR9 expression in the different conditions, we performed additional experiments in HPKs in which p53 expression was silenced by small interfering (si) RNA. Here, we also observed the key role of p53 in UV-mediated TLR9 transcriptional activation (Figure 2c). A similar scenario was observed in *hTERT* p53^{-/-} HPKs exposed to doxorubicin treatment (Figure 2d). However, H_2O_2 -mediated TLR9 mRNA up-regulation appeared to have a different mechanism as it was independent of the presence of p53 (Figure 2e).

The UV-induced TLR9 transcriptional activation is mediated by p53 and c-Jun recruitment at a specific region of TLR9 promoter

Subsequently, we performed transient transfection experiments in the spontaneous immortalized keratinocytes, NIKS, using a construct containing different regions of TLR9 promoter cloned in front of the luciferase reporter gene (Figure 3a). Consistent with our observations in HPKs, UV irradiation activated the TLR9 promoter (Figure 3b). The UV-mediated activation is dependent on a promoter region of approximately 2200 nucleotides, as its deletion prevented the increase of luciferase activity upon UV irradiation (Figure 3b). This region contains three NF- κ B responsive elements (RE) named A, B and C^{1,4}. Our previous data showed that RE C is involved in the basal transcriptional repression mediated by beta HPV38 E6 and E7 *via* the accumulation of Δ Np73 α ². Chromatin immune precipitation (ChIP) experiments showed that, upon UV irradiation, p53 is mainly recruited near the NF- κ B RE C (Figure 4a). Interestingly, we observed that two additional transcription factors, p65 and c-Jun, were recruited in the same region of the TLR9 promoter after UV irradiation (Figure 4a). We targeted these transcription factors as c-Jun is well known to be activated by UV irradiation¹⁰, while p65 has been shown to play a positive and negative role in TLR9 expression^{3,11,12}. The ChIP data was confirmed by oligonucleotide pull-down experiments using biotinylated DNA fragments of the TLR9 promoter (-1150/-1024) which encompass the wild-type or mutated NF- κ B RE C confirmed the ChIP data (Figure 4b). Both wild-type and mutated biotinylated DNA probes were incubated with cellular extracts of *hTERT* HPKs irradiated or not irradiated with UV. Only the wild-type DNA probe co-precipitated p53 and c-Jun (Figure 4b). In the same assay, we also observed that p53 and c-Jun binding to the TLR9 promoter region increased upon UV irradiation.

Silencing the expression of p65 by a specific siRNA revealed that it was not essential to UV-induced transcriptional activation (Figure 4c). In contrast, inhibition of c-Jun activation by a chemical inhibitor eliminated the UV-induced TLR9 expression at mRNA and protein level (Figure 4d, top and bottom panels, respectively).

Down-regulation of p53 by a specific siRNA resulted in a significant decrease in the amount of c-Jun recruited to the TLR9 promoter in HPKs irradiated with UV (Figure 4e), indicating that c-Jun and p53 may directly interact at the TLR9 promoter. The same result was observed in *hTERT* p53^{-/-} HPKs (crip53) (Figure 4f).

HPV38 E6 and E7 prevent the UV-mediated activation of TLR9 expression

Our previous data showed that beta HPV38 E6 and E7 oncoproteins inhibit the basal expression of TLR9 mRNA. Therefore, we evaluated whether the viral proteins could also interfere with the UV-induced activation of TLR9 transcription. The *hTERT* HPKs were retro-transduced with a recombinant retrovirus expressing HPV38 E6 and E7 oncoproteins or empty retrovirus (pLXSN). After total RNA preparation, TLR9 mRNA levels were determined by quantitative RT-PCR. The data show that TLR9 expression decreased in HPV38 E6 and E7 HPKs irradiated with UV compared with pLXSN HPKs (Figure 5a). Similar results were observed in HPKs retro-transduced with beta HPV38 E6 and E7 or empty pLXSN (Figure 5b), indicating that the results are independent of the *hTERT* expression. Similarly, TLR9 protein levels decreased in *hTERT* HPKs expressing the two viral oncogenes in comparison to mock cells (pLXSN) (Figure 5c). ChIP experiments revealed that recruitment of p53 and c-Jun induced by UV irradiation was severely affected by the viral oncoproteins (Figure 5d). Moreover, recruitment of Δ Np73 α on the same RE of the TLR9 promoter was significantly increased in HPV38 E6 and E7 HPKs after UV irradiation

compare to the non- irradiated cells (Figure 5e). This suggested that p53 and $\Delta Np73\alpha$ compete for binding to the TLR9 promoter leading to an inverse regulation of its activity.

DISCUSSION

Beta HPV types are suspected to play a role, together with UV irradiation, in the development of NMSC, the most common cancer among Caucasian^{9,13,14}. *In vitro* and *in vivo* mouse models revealed that E6 and E7 from certain beta HPV type have the ability, as the high-risk alpha HPV types, to deregulate molecular pathways related to cell survival, proliferation and apoptosis^{9,15}. Transgenic mice expressing beta HPV oncoproteins under the control of the keratin 14 (K14) promoter showed higher susceptibility to UV-induced carcinogenesis than the wild-type animals¹⁶. Although K14 HPV38 E6/E7 transgenic mice did not develop any skin lesions during their lifespan, their exposure to low doses of UV irradiation for many weeks resulted in the development of skin pre-malignant lesions and subsequently squamous cell carcinoma¹⁶. These findings indicate that beta HPV types have developed mechanisms to alter the response of normal cells to UV irradiation, favouring skin carcinogenesis. In this study, we provide evidence implicating TLR9 in the HPK responses to UV irradiation and other stresses. Exposure of HPKs to UV irradiation increases TLR9 expression, which is mediated by p53 and c-Jun. The product of *c-jun* protooncogene was initially discovered as component of the AP-1 transcription complex that is activated by several extracellular stimuli, such as mitogenic factors and different forms of cellular stresses¹⁷, including UV irradiation¹⁰. Many findings underline the complexity of the c-Jun network, which is involved in the control of cell cycle progression and apoptosis^{18,19}. It has been shown that c-Jun, via the activation of the expression of cyclin D1 gene, promotes the G1/S transition of cell cycle¹⁸. In addition, c-Jun is also able to prevent apoptosis induced by UV irradiation¹⁸. The use of c-

jun^{+/+} and *c-jun*^{-/-} fibroblasts allowed a more detailed characterization of c-Jun's role in the UV-induced cellular response¹⁹. The study showed that c-Jun negatively regulates the p53 recruitment to p21^{WAF1} promoter. Accordingly, the loss of c-Jun induces a prolonged cell-cycle arrest in UV-irradiated fibroblasts and p21^{WAF1} accumulation, with no signs of apoptotic events. Constitutive expression of c-Jun in *c-jun*^{-/-} fibroblasts counteracts cell cycle arrest and promotes apoptosis by an unknown mechanism¹⁹. Based on these findings, it has been proposed that c-Jun may facilitate the recovery of the cell after the stress induced by UV irradiation. This function appears to be specific to UV-irradiated cells, as c-Jun expression has little effect on the response to ionizing radiation¹⁹. It would be interesting to evaluate the role of TLR9 in the context of the c-Jun network and stress induced by UV irradiation. It is likely that TLR9 accumulation induced by UV irradiation is required to co-ordinate the cell cycle arrest or apoptosis, according the extent of damage. Independent of the precise role, HPV38 E6 and E7 prevent the activation of UV-induced TLR9 expression which indicates that TLR9 exerts anti-proliferative functions in presence of cellular stresses.

Our data also demonstrate that, the activation of TLR9 expression induced by UV irradiation or the DNA damaging agent doxorubicin is mediated by p53. However, oxidative stress induced by H₂O₂ leads to TLR9 accumulation in a p53 independent manner. It is plausible to hypothesize that different types of stress can lead to the accumulation of endogenous TLR9 ligands, activating specific signalling pathways. The physiological ligands which may induce TLR9 activation remain poorly characterized. We speculate that the inhibitory role of TLR9 on cellular proliferation is may be induced by DAMP molecules, normally released by damaged or stressed tissues to alert the immune system of tissue injury. A recent study in a model of hepatocellular carcinoma showed that hypoxia induced HMGB1 with released of mtDNA lead to the activation of TLR9-mediated tumour growth²⁰. Nucleic acids have been shown to be a potent trigger of innate inflammatory responses²¹. Similar to CpG ODN,

mtDNA is rich in CpG motifs, and thus it is highly possible that TLR9 senses mtDNA in a similar way as CpG ODN and other nucleic acids.

In a previous study, we provided evidence that HPV38 oncoproteins also inhibit the basal transcription of TLR9, as the mucosal high-risk HPV E6 and E7. In the context of HPV infection, we speculate that TLR9 expression is activated in response to the oncogenic stress. Thus, the virus has to inhibit this event in order to guarantee the survival and proliferation of the infected cell. In line with this hypothesis, TLR9 re-expression in HPV38 E6 and E7-immortalize keratinocytes showed a significant decrease in cellular proliferation and an increase of protein levels of the cell cycle inhibitor p21^{WAF1/Cip1}². Consistently, other studies have shown that the loss of TLR9 expression may also disable its ability to control the cell cycle and events that may control transformation⁶. These results highlight the association of TLR9 in cell cycle control in cancer cells. Consistently, clinical studies showed that TLR9 expression is reduced in head and neck cancers⁶.

Interestingly, another pathway involved in the antiviral response, the cyclic guanosine monophosphate-adenosine monophosphate synthase/STING pathway, is hampered by oncogenic viruses²². cGAS detects intracellular DNA and subsequently signals *via* the adapter protein STING. E7 oncoprotein protein from the mucosal high-risk HPV16 is able to directly interact with STING inhibiting its normal functions. Thus the DNA sensors, TLR9 and STING, appear to be key targets for oncogenic viruses. However, there is no evidence as to whether STING is also influenced by other HPV types, such as beta HPV38.

In conclusion, our data, in agreement with other studies, support a role of TLR9 in the response induced by different stresses. In particular, our study provides possible explanations for the cooperation of UV irradiation and beta HPV types in cellular transformation.

MATERIALS AND METHODS

Cell culture and treatment

Experiments were carried out in human primary foreskin keratinocytes (HPKs) and in the spontaneously immortalized human keratinocyte cell line, NIKs. Cell culture, antibiotic selection and generation of high-titer retroviral supernatants were carried out using a previously described method ²³. Transient transfection experiments were performed using Lipofectamine 2000 (Invitrogen) transfection reagent according to manufacturer protocols.

Cells were exposed to 25 mj/cm² UVB dose with a thin layer of phosphate-buffered saline (PBS) using the BIO SUN UV Light Irradiation apparatus (Vilber Lourmat). Cells were then incubated at 37°C in a humidified chamber and harvested after 8 hours.

Cells were incubated in media containing H₂O₂ at different concentration (Sigma) for 1 hour and doxorubicin (0.1 µg/ml) (Sigma) for 8 hour.

For c-Jun inhibition, cells were incubated in media containing JNK inhibitor II (CAS 129-56-6 Merckmillipore) with a final concentration of 30 µM for 1 hour, then irradiated at 25 mj/cm² UVB doses and incubated in media containing 5 µM JNK inhibitor II for 8 hours.

Gene silencing

Gene silencing of p53 and p65 was achieved using synthetic small interfering RNA (siRNA) (Table 1). siRNA or scrambled RNA at a concentration of 40 nM was transfected using Lipofectamine 2000 according to the standard protocol (Invitrogen).

The plasmids for CRISPR/Cas9 were obtained from Addgene. All sgRNAs were designed by Thermo Fisher Scientific. The target sequence information is shown in Table 1. All the CRISPR/Cas9 vectors were generated according to manufacturer protocols and then transiently transfected in keratinocytes. The purification step of the cells carrying the

CRISPR/Cas9 vectors was performed 48 hours after transfection following the manufacturer's protocol (GeneArt® CRISPR Nuclease Vector Kit, Life Technologies).

Luciferase assay

Transient transfections were conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Naturally immortalized keratinocytes (NIKs) were transfected with firefly luciferase TLR9 promoter vectors (0.5 µg). A pRL-TK Renilla reporter vector (15 ng) was used as an internal control. After 40 hours, cells were irradiated with UVB as previously described, then lysed and luciferase activity was measured using a dual-luciferase reporter assay system (Promega). The expression of firefly luciferase relative to Renilla luciferase was expressed in relative luminescence units (RLU).

RT-PCR and QPCR

Total RNA was extracted using the Absolutely RNA Miniprep kit (Stratagene). The RNA obtained was reverse-transcribed to cDNA with the RevertAid H Minus M-MuLV Reverse Transcriptase kit (Fermentas) according to the manufacturer's protocols. Real-time qPCR was performed using the MesaGreen qPCR MasterMix Plus for SYBR Assay (Eurogentec) with the primers listed in Table 2.

Immunoblotting

Total protein extracts, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and immunoblotting (IB) were prepared as described previously²⁴. The following antibodies were used: β-actin (C4, MP Biomedicals), TLR9 (2254, Cell Signaling), p21^{WAF1/Cip1} (2946, Cell Signaling), p53 DO-1 (sc-126), phospho-p53 (Ser15) (9284, Cell Signaling), NF-κB p65 (3034, Cell Signaling), c-Jun (H-79, Santa Cruz), phosphor-c-Jun

(Ser73) (9164, Cell Signaling). Images were taken using the ChemiDoc XRS imaging system (Bio-Rad).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed with Shearing ChIP and OneDay ChIP kits (Diagenode) according to the manufacturer's instruction. Briefly, cells were sonicated to obtain DNA fragments of 200–500 bp. Sheared chromatin was immunoprecipitated with isotype control IgG or the indicated antibodies. The eluted DNA was used as a template for qPCR. Primers for qPCR are listed in Table 2.

Oligonucleotide pulldown

Cells were lysed and sonicated as previously described ². The TLR9 promoter, wild-type or mutated, was used as a template to amplify the NF- κ B RE region near the Site C. PCR amplification was performed using a biotinylated forward primer and a non-biotinylated reverse primer (listed in Table 2). Amplicons were extracted from agarose gel by using the MinElute gel extraction kit (Qiagen) and quantified. Then, 1 mg of prepared protein extract was incubated with 2 μ g of biotin-TLR9 promoter probes and 10 μ g of poly(dI-dC)-poly(dI-dC) for 16 hours at 4°C. DNA-bound proteins were collected with streptavidin-agarose beads for 1 hour and washed five times with HKMG buffer. DNA-bound proteins were then analysed by IB.

Statistical analysis

Statistical significance was determined by Student t-tests. Statistically significant p values of each experiment are indicated in the corresponding Figure legends, $P < 0.05$ (*); $P < 0.01$ (**); ns = non-significant. Error bars in the graphs represent the standard deviation.

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FIGURE LEGENDS

Figure 1

TLR9 transcription is induced by several cellular stresses in primary human keratinocytes

(a) Total RNA and total proteins from HPKs irradiated with UVB (UV) were extracted. TLR9 expression levels were measured by RT-qPCR and normalized to GAPDH levels (left panel) and protein extracts were analysed by immunoblotting for the indicated antibodies (right panel). (b, c) Total RNA and total proteins from HPKs treated with (b) 0.1 $\mu\text{g/ml}$ doxorubicin (doxo) for 8 hours or (c) H_2O_2 at the indicated concentration for 1 hour were extracted. TLR9 expression levels were measured by RT-qPCR (left panel) and total proteins extract analysed by IB for the indicated antibodies (right panel). RT-qPCR data shown are the mean of three independent experiments. IB figures shown are from one representative experiment of three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

Figure 2

p53 plays a key role in UV-mediated TLR9 transcriptional activation

(a, b) The *hTERT* HPKs expressing wt p53 (scramble) or CRISPR/Cas9 deleted p53 (crip53) were irradiated with UVB (UV). (a) Total RNA was extracted and TLR9 and p53 levels measured by RT-qPCR. Data shown are the mean of three independent experiments. **, $P < 0.01$; ns, non-significant. (b) Total protein extracts were analysed by IB with indicated antibodies (upper panel). Band intensities were quantified and normalized to β -actin levels (lower panel). Data shown are the mean of three independent experiments. **, $P < 0.01$; ns, non-significant. (c) HPKs were transfected with a control siRNA (scramble) or siRNA specific for p53 (sip53). After 40 hours, cells were irradiated with UVB as indicated in Material and Method. Total RNA was then extracted and p53 or TLR9 mRNA levels analysed

by RT-qPCR. Error bars represent standard deviation of three biological replicates. **, $P < 0.01$; ns, non-significant. (d, e) RT-qPCR analysis of the TLR9 mRNA level (upper panel) or IB analysis with the indicated antibodies (lower panel) of *hTERT* HPKs treated with (c) 0.1 $\mu\text{g/ml}$ doxorubicin for 8 hours or (d) 500 μM H_2O_2 for 1 hour. The data shown are representative of three independent experiments. **, $P < 0.01$; ns, non-significant.

Figure 3

The UV-induced TLR9 transcriptional activation is mediated by p53 and c-Jun recruitment at a specific region of TLR9 promoter

(a) Scheme of the TLR9 promoter luciferase constructs: the full length (-3227/-1) construct containing the 4 NF- κ B RE (site A, B, C and D) and the deleted (-1017/-1; -290/-1) constructs. (b) NIKS were transfected with the TLR9 promoter constructs (-3227/-1; -1017/-1; -290/-1) cloned in front of the luciferase reporter gene. After 40 hours, cells were irradiated with UVB 25 mJ/cm^2 and incubated at 37°C in a humidified chamber. Cells were harvested after 8 hours from UVB irradiation and luciferase activity was measured. Data shown are the mean of three independent experiments performed in triplicate. **, $P < 0.01$; ns, non-significant.

Figure 4

The UV-induced TLR9 transcriptional activation is mediated by p53 and c-Jun recruitment at a specific region of TLR9 promoter

(a) ChIP was performed in not irradiated (-) and UVB-irradiated HPKs (+) using p53, p65 and c-Jun antibody. Simultaneously, part of the total chromatin fraction (1/10) was used as input. qPCR was performed using specific primers flanking the NF- κ B RE site C within the TLR9 promoter. The histogram shows the relative amount of the promoter bound by the different

antibodies after subtracting the background of nonspecific IgG control expressed as a percentage of the input. The data shown are representative of three independent experiments. **, $P < 0.01$. (b) The *hTERT* HPKs UVB-irradiated or not irradiated were processed for the oligonucleotide pulldown assay. Cell lysate was incubated with biotinylated probes containing the NF- κ B RE C of the TLR9 promoter wild-type (NF- κ Bwt) or mutated (NF- κ Bmut). DNA-associated proteins were recovered by precipitation with streptavidin beads and analysed by IB. The data shown are representative of two independent experiments. (c) HPKs were transfected with a control siRNA (scramble) or siRNA specific for p65 (sip65). After 40 hours, cells were irradiated with UVB as described in Material and Method. Total RNA was then extracted and p65 or TLR9 mRNA levels analysed by RT-qPCR. Error bars represent standard deviation of two biological replicates. **, $P < 0.01$. (d) HPKs were treated with the JNK inhibitor II or DMSO and irradiated with UVB as described in Material and Method. After 8 hours, total RNA was extracted and TLR9 level analysed by RT-qPCR (top panel). Simultaneously, total proteins were extracted and analysed by IB using the indicated antibodies (bottom panel). Error bars represent standard deviation of two biological replicates. *, $P < 0.05$; ns, non-significant. (e) HPKs were transfected with a control siRNA (scramble) or siRNA specific for p53 (sip53). After 40 hours, cells were irradiated with UVB as indicated in Material and Method. Total cellular extracts were processed for ChIP with the indicated antibodies. (f) The *hTERT* HPKs expressing wt p53 (scramble) or CRISPR/Cas9 deleted p53 (crip53) were irradiated with UVB and processed for ChIP with the indicated antibodies.

Figure 5

HPV38 E6 and E7 prevent the UV-mediated activation of TLR9 expression

(a) The *hTERT* HPKs were transduced with pLXSN empty vector or expressing HPV38 E6 and E7 and irradiated with UVB as indicated in Material and Method. The efficiency of

transduction and TLR9 mRNA levels were verified by RT-qPCR **, $P < 0.01$; ns, non-significant. (b) HPKs were transduced with pLXSN empty vector or expressing HPV38 E6 and E7 and irradiated with UVB as described in Material and Method. After 8 hours, total RNA was extracted and TLR9 and viral protein levels analysed by RT-qPCR **, $P < 0.01$; ns, non-significant. (c) Total protein extracts from *hTERT* HPKs transduced with pLXSN empty vector or expressing HPV38 E6 and E7 and irradiated with UVB were analysed by IB. Data shown are representative of three independent experiments in *hTERT* HPKs and HPKs. **, $P < 0.01$. (d) HPKs and HPV38 E6 and E7 HPKs were irradiated or not irradiated with UVB. Total cellular extracts were processed for ChIP with the indicated antibodies. Error bars represent standard deviation of three biological replicates. **, $P < 0.01$; ns, non-significant. (e) HPV38 E6 and E7 HPKs were irradiated or not irradiated with UVB. Total cellular extracts were processed for ChIP with anti-p73 antibody (OP108, Calbiochem). Error bars represent standard deviation of two biological replicates. *, $P < 0.05$.

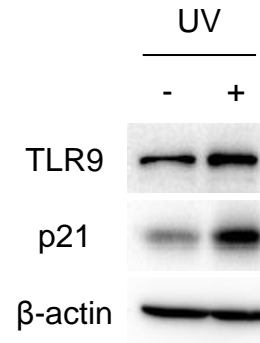
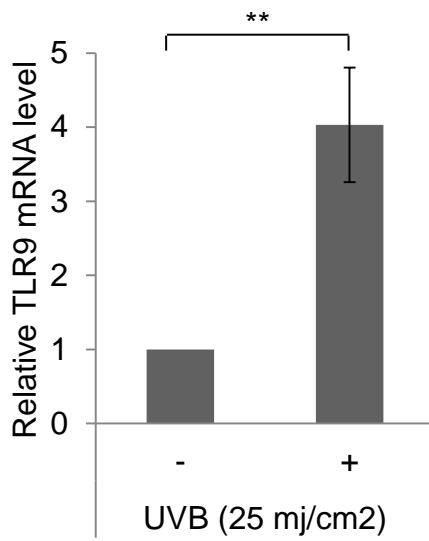
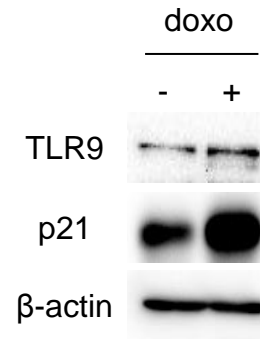
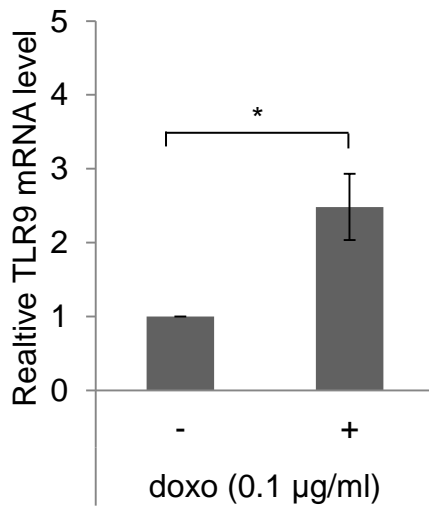
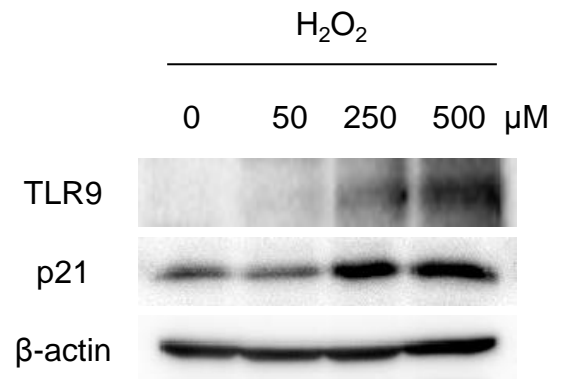
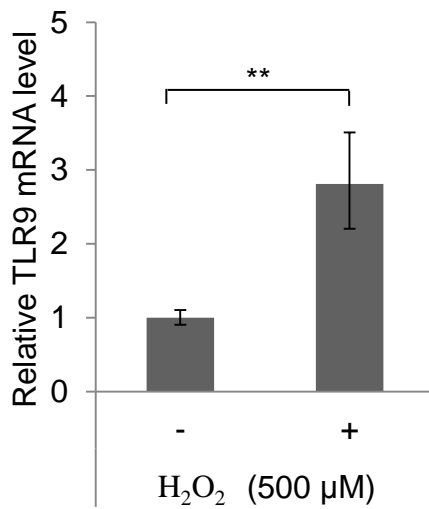
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Figure 1. Pacini et al.

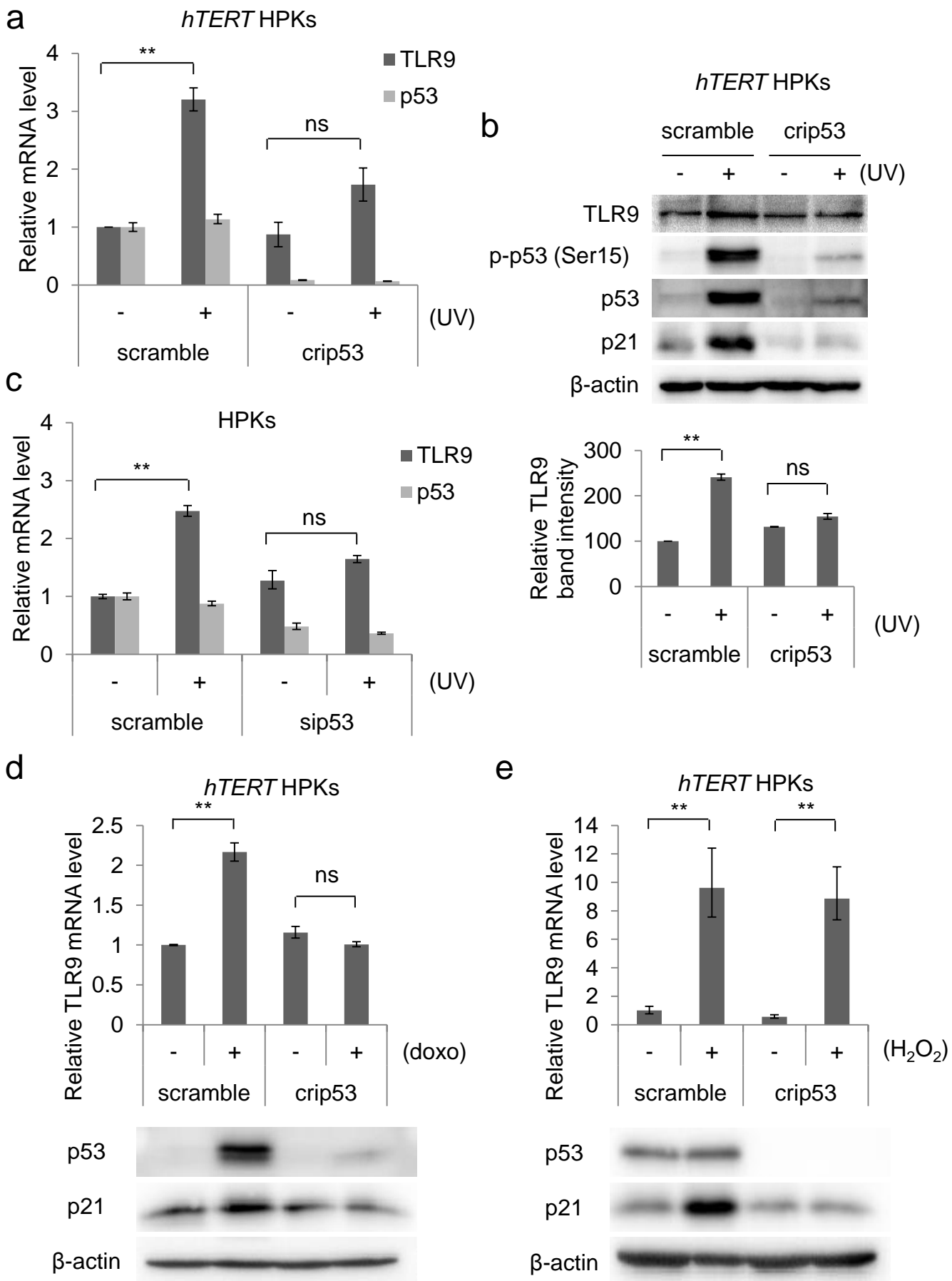
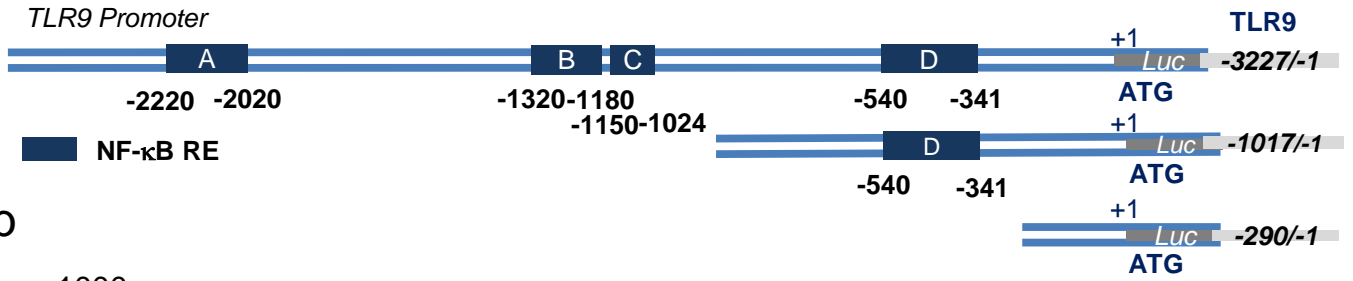


Figure 2. Pacini et al.

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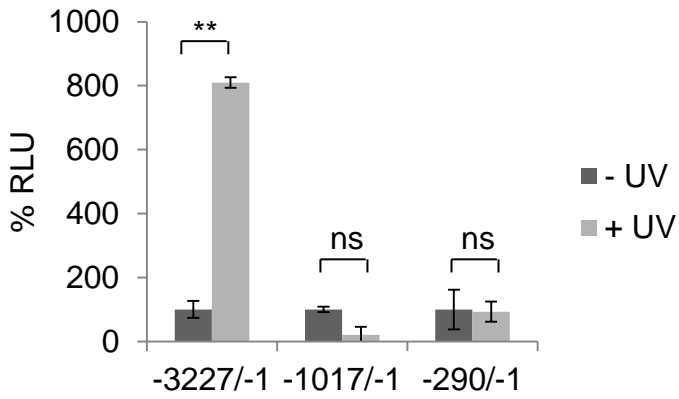


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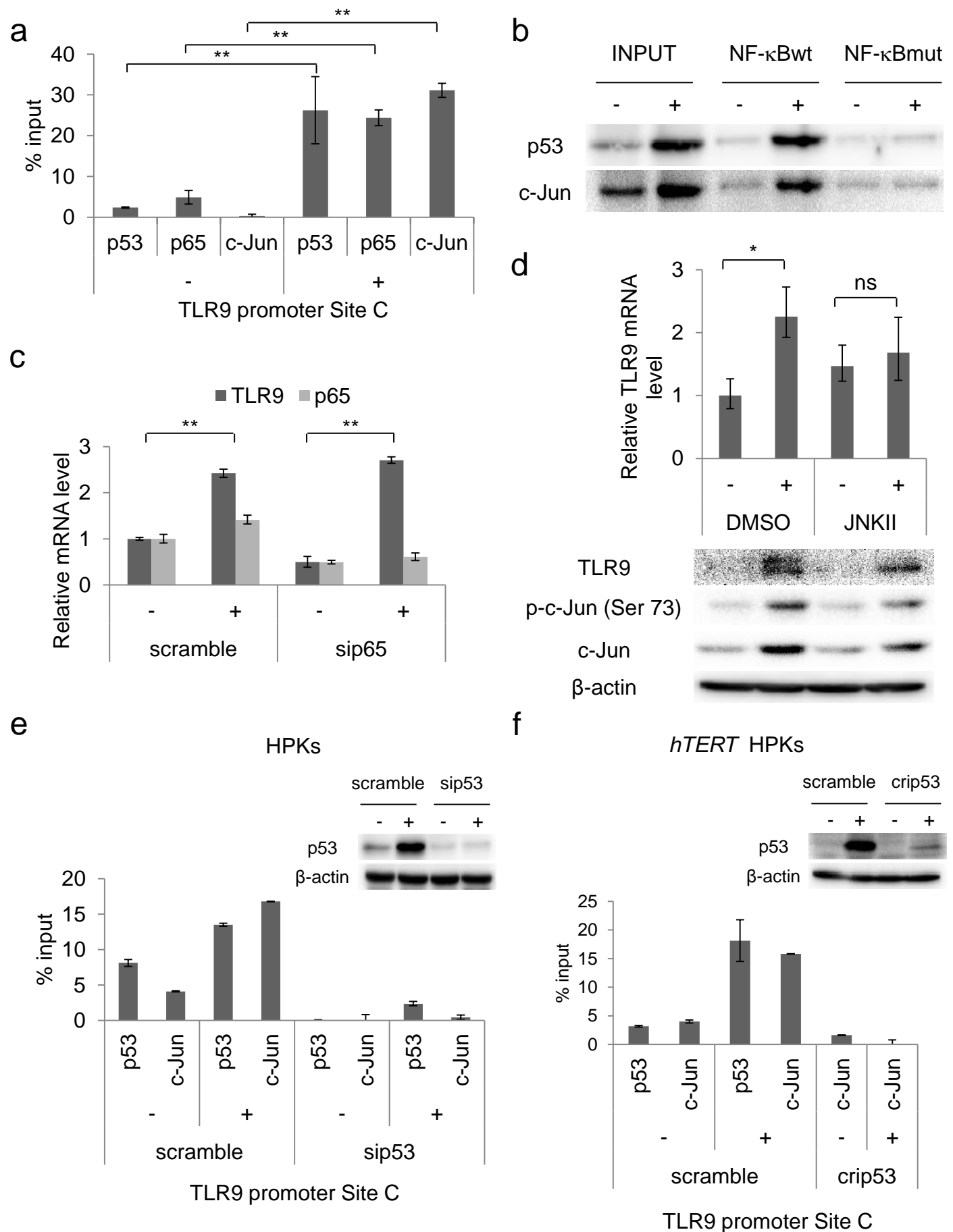


Figure 4. Pacini et al.

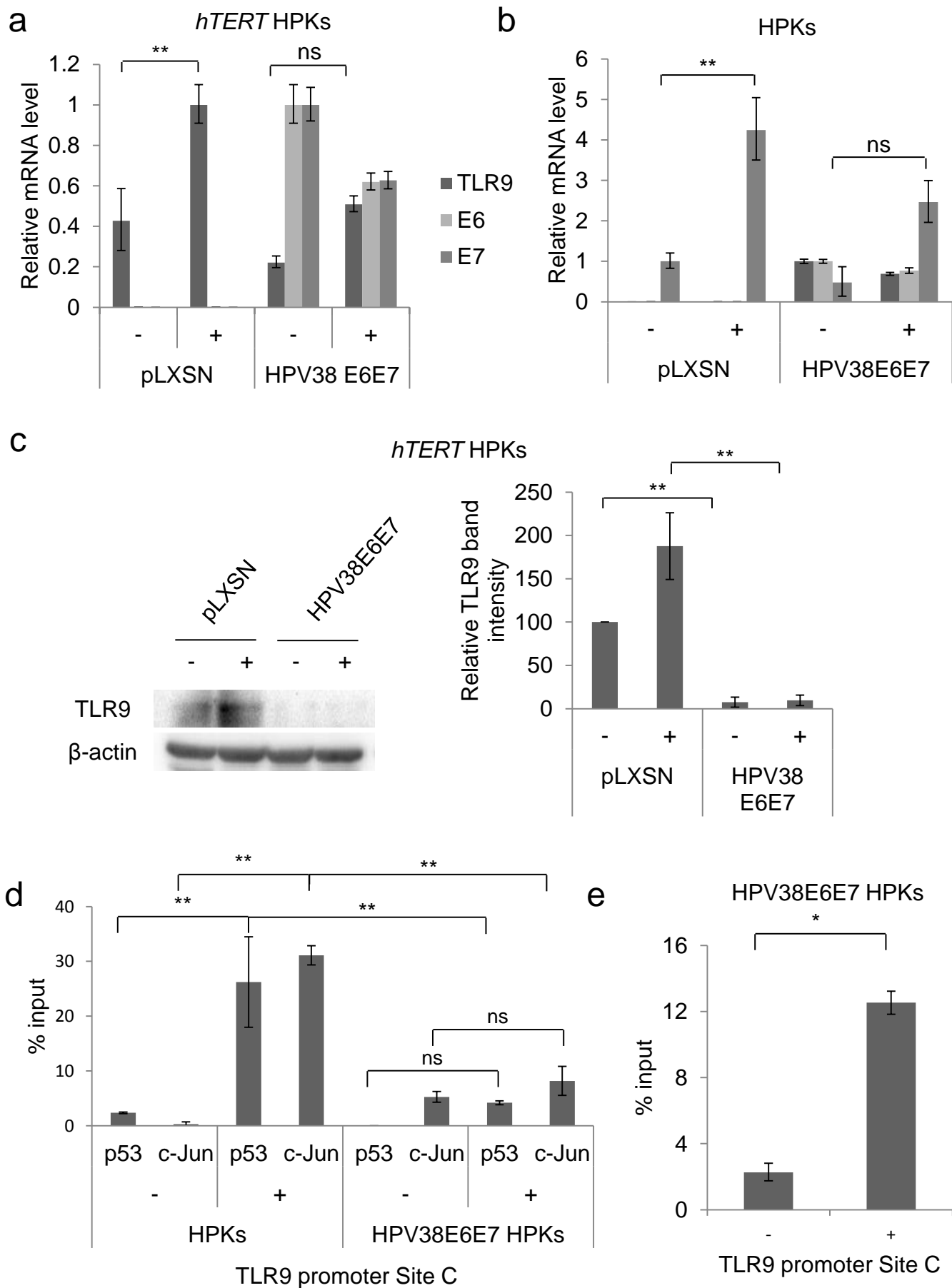


Figure 5. Pacini et al.

Table 1. Sequences of different siRNA and CRISPR/Cas9 vectors used for gene silencing

Target	siRNA sequence or description (source)
Scrambled (negative control)	5'-GGUGGAAGAGGUGGUGAGC-3'
p65	siGenome SMART pool M-003533-02-0005, human RELA, NM_021975 (Thermo Scientific)
p53	5'-CAAUGGUUCACUGAAGACCUU-3'
p53 vector #1	F: 5'-TCCATTGCTTGGGACGGCAAGTTTT-3'
	R: 5'-TTGCCGTCCCAAGCAATGGACGGTG-3'
p53 vector #2	F: 5'-CCATTGTTCAATATCGTCCGGTTTT-3'
	R: 5'-CGGACGATATTGAACAATGGCGGTG-3'
p53 vector #3	F: 5'-CTCGGATAAGATGCTGAGGAGTTTT-3'
	R: 5'-TCCTCAGCATCTTATCCGAGCGGTG-3'
p53 vector #4	F: 5'-CACTTTTCGACATAGTGTGGGTTTT-3'
	R: 5'-CCACACTATGTCGAAAAGTGCGGTG-3'
Scrambled vector	F: 5'-GGATGGACGGTAGAGGTGGGTTTT-3'
	R: 5'-CCACCTCTACCGTCCATCCCGGTG-3'

Table 2. Sequences of primers used for RT-qPCR analyses, ChIP, oligonucleotide pulldown

Promoter or gene	Primer sequence ^a
TLR9 promoter NF-κB site C	F: 5'-GAGAGCACTCAGGGGAACAG-3'
	R: 5'-GGTCACATTCAGCCCCTAGA-3'
GAPDH	F: 5'-AAGGTGGTGAAGCAGGCGT-3'
	R: 5'-GAGGAGTGGGTGTCGCTGTT-3'
TLR9	F: 5'-CGTCTTGAAGGCCTGGTGTGA-3'
	R: 5'-CTGGAAGGCCTTGGTTTTAGTGA-3'
p53	F: 5'-GATGAAGCTCCCAGAATGCC-3'
	R: 5'-CAAGAAGCCCAGACGGAAAC-3'
p65	F: 5'-GTCACCGGATTGAGGAGAAA-3'
	R: 5'-GCTCAGGGATGACGTAAAGG-3'
E6	F: 5'-TCTGGACTCAAGAGGATTTTG-3'
	R: 5'-CACTTTAAACAATACTGACACC-3'
E7	F: 5'-CAAGCTACTCTTCGTGATATAGTT-3'
	R: 5'-CAGGTGGGACACAGAAGCCTTAC-3'
Probes for oligonucleotide pulldown assay	F: 5'-Btn-GAGAGCACTCAGGGGAACAG-3'
	R: 5'-GGTCACATTCAGCCCCTAGA-3'

^aF, forward; R, reverse.

CONCLUSIONS AND PERSPECTIVES

V. CONCLUSIONS AND PERSPECTIVES

Approximately 20% of human cancers worldwide are attributed to viruses (1), representing a significant proportion of the global cancer burden. The link between mucosal HR HPV infection and cervical cancer was firstly reported in the early 1970s by Harald Zur Hausen (2). Over the last decades, the association between HPV and neoplastic transformation has been extensively investigated and, to date, nearly all cases of cervical cancer can be attributable to HR HPV infection.

The HPV oncoproteins E6 and E7 are the main players for cancer development. Indeed, they are able to deregulate many cellular events, such as cell cycle, differentiation, senescence, DNA repair and apoptosis, favouring the transformation of infected cells (3). In addition an efficient evasion of the immune system is a key event for cancer development, since only chronic HR HPV infections are associated with the disease. The E6 and E7 oncoproteins from mucosal HR HPV types are able to inhibit the innate and adaptive immune response (4), but very little is known about the impact of viral oncoproteins of beta HPV types on the immune surveillance. TLR9 is an important player in the innate immune response sensing viral or bacterial dsDNA containing highly conserved un-methylated CpG motif. Several studies have shown that well-established oncogenic viruses, such as HPV16, EBV, HBV and MCPyV, inhibit the expression of TLR9, proving the relevance of this event in viral infection (5–8).

Epidemiological and biological studies support a possible involvement of cutaneous beta HPV type in NMSC, in particular in synergism with other risk factors (9). *In vitro* and *in vivo* mouse experimental studies revealed that E6 and E7 from certain beta cutaneous HPV type (in particular HPV38 and 49) have the abilities, like their homologues from HR HPV types, to deregulate molecular pathways related to cell survival, proliferation and apoptosis (10–14). Moreover, experimental mouse models where HPV38 E6 and E7 are expressed under the

control of the keratinocyte-specific promoter support the evidence that beta HPV types significantly contribute to NMSC rendering keratinocytes more susceptible to UV-induced carcinogenesis (15).

Here we show that expression of E6 and E7 oncoproteins from beta HPV38 in HFK results in the formation of a transcriptional repressing complex that is recruited to the TLR9 promoter and inhibits its basal levels of expression. The formation of this complex is mainly driven by $\Delta Np73\alpha$, a well-characterized antagonist of p53. Most importantly, our data showed that two epigenetic enzymes, EZH2 and DNMT1, are part of the IKK β / $\Delta Np73\alpha$ complex. The same complex has been recently characterised by our group and it seems to be involved in the transcriptional repression of p53-regulated genes in HPV38 E6 and E7 HFK (16). Moreover, it has been shown that EZH2 and DNMT1 are often up-regulated in cancer (17–20), where they appear to target specific genes for methylation (21). These findings highlight some functional similarities between well-established oncogenic viruses and beta HPV38, providing further lines of evidence for cutaneous HPV types in human carcinogenesis.

We also observed that in primary human cells, TLR9 expression can be activated by different types of cellular stresses, i.e. UV irradiation, doxorubicin and H₂O₂ treatment. The TLR9 transcriptional activation induced by the first two stresses, UV irradiation and doxorubicin, is mediated by p53, while deletion of p53 gene did not hamper the TLR9 expression in cells exposed to H₂O₂. Little is known about differential DNA damage response to different stresses. However, it has been reported that differences in DNA damage removal are strictly related to the challenge insult (UV light or oxidative stress) and the differentiation state of the cells (22), indicating that different kinds of stress lead to distinct responses. Further studies will need to be done in this sense.

Our findings show that, in addition to p53, c-Jun is also recruited to the TLR9 promoter. Both transcription factors are part of a transcriptional regulatory complex recruited on a specific

NF- κ B RE on the TLR9 promoter. Finally as observed for p53, the inhibition of c-Jun abolished the UV-mediated activation of TLR9 expression.

Other studies provide evidence of a link between TLR9 and different types of genotoxic stresses (23,24). In 2012 Shatz et al. showed that exposure of different cancer derived cells to stresses, such as doxorubicin, 5-fluorouracil or UV and ionizing radiation, resulted in a strong up-regulation of TLR9 expression partly mediated by p53 activation (23). In addition, TLR9 expression is strongly activated *via* p53 in primary human blood lymphocytes and alveolar macrophages upon exposure to different types of DNA-damaging insults (24).

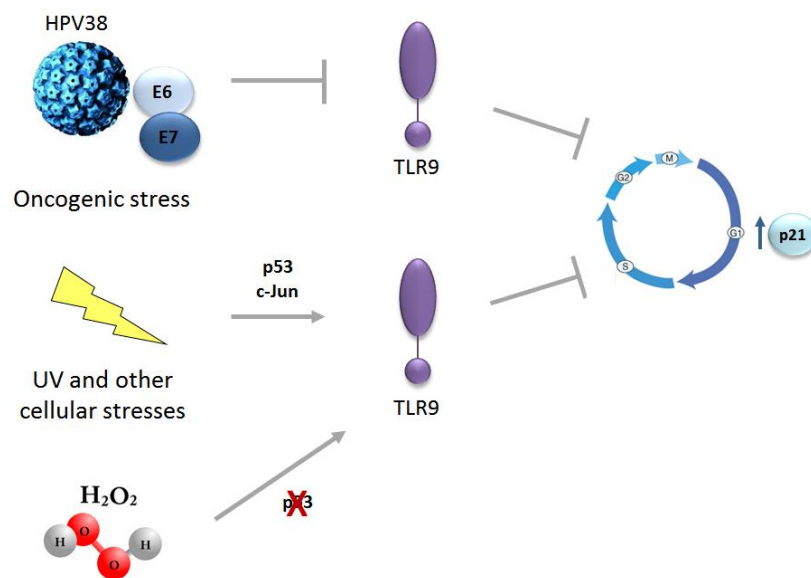
The biological significance of the stress-mediated TLR9 up-regulation is still unclear. Most likely, TLR9 is part of a cellular response that guarantees damage recovery or elimination of damaged cells. One plausible hypothesis is that different types of stress can lead to the accumulation of endogenous TLR9 ligands, activating specific signalling pathways. The physiological ligands which may induce TLR9 activation remain poorly characterized. We can speculate that the inhibitory role of TLR9 on cellular proliferation is may induced by DAMPs, which are normally released by damaged or stressed tissues to alert the immune system of tissue injury. It has been shown that hypoxia induced HMGB1 (High mobility group box 1) with released of mitochondrial DNA (mtDNA) lead to the activation of TLR9-mediated tumour growth in a model of hepatocellular carcinoma showed that (25). Similar to CpG ODN, mtDNA is rich in CpG motifs, and thus it is highly possible that TLR9 senses mtDNA in a similar way as CpG ODN and other nucleic acids.

In the contest of HPV infection, we may speculate that TLR9 expression is activated in response to the oncogenic stress. Thus, the virus has to inhibit this event in order to guarantee the survival and proliferation of the infected cell. In agreement with this hypothesis, TLR9 re-expression in HPV38 E6 and E7 HFK showed a significant decrease in cellular proliferation. This result correlated with an increase of protein levels of the cell cycle inhibitor p21^{WAF1/Cip1}.

Consistently, other groups showed that the loss of TLR9 expression may also disable its ability to control the cell cycle and events that may control transformation (26). These results highlight the association of TLR9 in cell cycle control in cancer cells. Finally, the importance of our findings has been corroborated in clinical studies showing that TLR9 expression was reduced in several tissue biopsies from an independent cohort of head and neck cancer patients (26).

All together these findings allowed us to hypothesize the working model illustrated below.

Working model.



Oncogenic viruses inhibit TLR9 expression by E6 and E7 oncoproteins. UV irradiation and other cellular stresses (doxorubicin and H₂O₂ treatment) induce TLR9 transcriptional activation by different mechanisms. In particular, UV irradiation induces TLR9 up-regulation through the recruitment of p53 and c-Jun on a specific NF-κB RE on the TLR9 promoter. On the other hand, the H₂O₂-dependent TLR9 activation appear to be a p53-independent event. TLR9 regulation may be involved in cellular events linked to cell cycle by inducing the accumulation of p21^{WAF1/Cip1}.

Together, our study describes a new mechanism of viral and stress mediated TLR9 regulation. Increasing evidences demonstrate that TLRs play an important role in the initiation and progression of cancer. Therefore, our results are of special significance for supporting further link between TLR9 deregulation and human carcinogenesis. Future work will be necessary to substantiate the broad significance of the mechanism elucidated here. The study of viral biological properties and their relation with the host will improve our knowledge of cellular biology and virus-mediated carcinogenesis.

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