Interplay between NF- κ B/interferon signaling and the genome replication of HPV

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HPV infection can persist within the infected epithelium for years. The viral persistence is primarily attributed to the ability of the virus to maintain its genome as nuclear episomes in the basal cells. Recent studies have revealed that HPV induces DNA damage response to facilitate productive amplification of the viral genome. DNA damage response comprises a part of the cellular defense mechanism against viral infection and its activation can result in induction of innate immune responses. The activation of NF- κ B and interferon (IFN) signals has been shown to suppress the genome replication of HPV while the viral proteins inhibit NF- κ B/IFN signaling. This review intends to focus on illustrating the interplay between NF- κ B/IFN signaling and HPV genome replication in the HPV life cycle.

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HPV infection & cervical cancers

Papillomaviruses (PVs) are a large family of nonenveloped, small DNA viruses with an approximately 8-kbp double-stranded circular genome that exclusively replicate in the stratified squamous epithelium at various anatomical sites, such as skin, anogenital organ and oral cavity. To date, more than 180 types of HPVs and over 100 types of animal PVs have been cloned [1]. Based on DNA sequence, HPVs are divided into five genera (α , β , γ , μ and ν) with a preference of infection to either cutaneous or mucosal surfaces. HPV infection can be asymptomatic or cause hyperproliferative epithelial lesions, warts or papillomas, which can persist and occasionally progress to cancers, depending on the sites of infection and types of HPVs. The mucosa-tropic HPVs from the α -genus are further divided into low- and high-risk types, according to their association with cancers. Low-risk HPVs (LR-HPVs) that include HPV6 and 11 are associated more with benign anogenital warts or condylomata acuminata. High-risk (HR-HPVs) or oncogenic HPVs that include at least 12 HPV types (HPV16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58 and -59) are recognized as group 1 carcinogens for several cancers including nearly all cases of cervical cancers and a majority of anogenital and oropharyngeal cancers. Altogether, HR-HPVs are responsible for approximately 5% of all cancers worldwide [2].

Genital HPV infection is common among sexually active women and men and nearly 90% of infections are cleared by the host immune system within 3 years. However, approximately 10% of the infection results in persistent epithelial lesions that can progress to cervical cancer through a series of neoplastic changes. Low-grade cervical intraepithelial lesions (CINs)

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support the productive life cycle of HPVs, while high grade CINs no longer do so [3]. A key event in HPV-associated neoplastic progression is dysregulation of the virus gene expression, leading to increased expression of viral oncogenes, E6 and E7, in basal cells of the epithelium (Figure 1). Several mechanisms to cause dysregulation of HPV gene expression have been reported, such as integration of the virus genome into host chromosomes and DNA methylation of the viral genome. Herfs *et al.* recently reported that a majority of cervical cancers arises from discrete population of single-layered, cuboidal epithelial cells, called squamocolumnar junction cells, which are localized between ectocervical squamous epithelium and endocervical glandular epithelium. Thus, a certain cell type may have a propensity to develop cancer when infected with HR-HPVs [4]. Viral proteins of HR-HPVs have been shown to actively inhibit innate and adaptive immunities, contributing the viral persistence and disease progression [5,6]. On the other hand, recent evidence also suggests

that the maintenance of HPV genome in basal cells is controlled by interplay with innate immunity.

The productive life cycle of HPVs

The life cycle of HPVs is co-ordinated with the differentiation status of the host keratinocytes and can be divided into three stages, establishment, maintenance and productive stages (Figures 1 & 3) [2]. A stratified epithelium consists of sheets of multilayered keratinocytes in which the outer surface of the tissue is sloughed off and constantly replaced by cells below it. In the stratified squamous epithelium of adults, dividing cells are located mostly in the basal layer (basal cells) including stem cells, as well as transient amplifying cells that can divide limited times. When these cells divide, one daughter cell remains in the basal layer, while other migrates upward and begin to differentiate. As the cells pushed toward the tissue surface, they undergo terminal differentiation processes that involve a series of dynamic changes in gene expressions and cell morphology [8]. HPVs

Figure 1. The HPV life cycle in association with cancer progression. HPV infects basal cells via microlesions and establishes its genome as nuclear episomes through initial amplification. While the copy numbers of the viral genome are maintained constant in the basal cells (stable maintenance), it is drastically increased in a differentiating compartment of the epithelium (productive amplification). The dysregulation of the viral gene expression, increase of E6 and E7 levels and decrease of E2 level, is a key event of neoplastic progression of the epithelial lesions with high-risk HPV infection. The productive life cycle is supported in low-grade cervical intraepithelial lesions (CIN1) but is aborted in high-grade cervical intraepithelial lesions (CIN2–3). Adapted with permission from [7].

Figure 3. Potential interplay between NF-κB/IFN signaling and three phases of the viral genome replication in the viral life cycle. HPV genome undergoes three phases of the replication, initial amplification, stable maintenance and productive amplification. NF-κB/IFN can be activated downstream of pathogen recognition receptor signals, as well as DDR. Although immediate cell response to HPV infection is poorly understood, recent studies suggested that NF-κB is activated presumably through a pathogen recognition receptor upon HPV infection. E1 and E7 of high-risk HPVs have been shown to activate DDR, which can result in the activation of NF-κB. The activation of NF-κB/IFN signaling during the establishment stage may trigger transition from initial amplification to stable maintenance as illustrated. On the other hand, the activation of DDR is shown to be necessary for productive amplification. Viral proteins, such as E6 and E7, may prevent the activation of NF-κB/IFN from being sustained during the maintenance, as well as productive stages, to support the viral genome replication.

DDR: DNA damage response; IFN: interferon.

infect basal cells via microlesions and establish its genome as nuclear episomes at low copy numbers (the establishment stage). In basal cells, the viral genome is maintained as nuclear episomes at constant copy numbers and the viral early genes are expressed at limited levels (the maintenance stage). As infected cells move to an upper differentiating compartment of the epithelium, the levels of viral early genes are elevated and the viral genomes are amplified at a large scale. The viral late genes, L1 and L2, major and minor capsid proteins, respectively, are expressed and assembled into progeny virions in the uppermost layers. The progeny

virions are released as the cells containing the infectious virions slough off from the epithelial surface (the productive stage). The replication of HPV genome relies on host machineries for DNA synthesis. However, cells in a differentiating compartment are no longer mitotically active in a normal stratified epithelium. E7 in co-operation with E6 and E5 have been shown to sustain or induce cells active for DNA synthesis mainly through inactivating RB family members in a differentiating compartment, thus creating intracellular environment that supports the productive amplification of the viral genomes.

Figure 2. The structure of the HPV16 genome. Early region encodes seven open reading frames (ORFs), E1, E2, E4, E5, E6, E7 and E8. Late region encodes L1 and L2 ORFs. URR contains *cis* elements involved in regulation of the viral transcription and replication. ORFs in green (E1, E2 and E8) encode viral proteins involved in the viral replication and transcription. ORFs in pink (E5, E6 and E7) encode viral oncoproteins. ORFs in blue encode capsid proteins. LCR: Long control region; URR: Upper regulatory region. Reproduced with permission from [7].

The structure of viral genome & viral proteins

PV genome can be divided into three major regions, early and late regions and upper regulatory region (URR) or also called long control region (LCR) (Figure 2). The early region encodes six major open reading frames (ORFs), E1, E2, E4, E5, E6 and E7 though some papillomaviruses lack E5. The late region encodes two ORFs, L1 and L2. URR contains several recognition sites for both viral and host transcription factors and the origin of replication (*ori*) and regulates viral gene transcription, genome replication and cellular tropism. HPV transcription occurs from one strand of DNA and is primarily derived from two major promoters, the early promoter (p97 for HPV16) located immediate upstream of E6ORF and the late promoter (p670 for HPV16) located within E7ORF. The viral early promoter directs expression of viral early proteins (E1, E2, E5, E6 and E7) and is constitutively active throughout the viral life cycle. The viral late promoter is activated in a differentiation-dependent manner and drives the expression of viral late proteins, as well as some of the early proteins. The gene product

of E4ORF is an E1^{E4} fusion protein in which a small peptide of N-terminal E1ORF is fused to E4ORF by splicing. As its expression is detected only in a differentiating compartment of the infected epithelium, the E1^{E4} is considered to be a viral late protein. In addition, the E8ORF has been proven to encode a functional protein, a spliced E8^{E2C} fusion protein in which E8ORF is fused to a C-terminal half of E2ORF for some PVs. Although the regulation of the viral gene expression has not been well understood, it appears under control of several distinct mechanisms such as binding of viral and cellular transcription factors, alternative splicing, mRNA stability and chromatin modification [9,10].

Regulation of the genome replication in the viral life cycle

The viral genome undergoes three phases of the replication, initial amplification, stable maintenance or maintenance replication and productive amplification or vegetative amplification, corresponding to establishment, maintenance and productive stages, respectively, in the viral life cycle (Figures 1 & 3). Although a precise mechanism responsible for switching

the genome replication from one phase to another has not been entirely elucidated, recent evidence revealed association of DNA damage response (DDR) with the replication of the viral genome. This section will briefly describe recent findings implicating DDR in three phases – replication of the viral genome [11,12].

• A basic molecular mechanism of the viral genome replication

Minimal components necessary for the replication of the viral genome are the ori located in the URR and two viral proteins, E1 and E2, along with the host DNA replication machinery [13]. The E1 is an ATP-dependent DNA helicase and the E2 is a viral transcription factor that regulates the viral gene expression by binding specific palindromic sequences, called E2 binding sites (E2BSs) in URR. E2 also plays essential roles for the replication and segregation of the viral genome [14,15]. Initiation of the viral genome replication begins with co-operative binding of E1 and E2 dimers to ori. Following the binding of E1 and E2 to ori, E1 trimer intermediates are formed and E2 is released upon ATP binding. E1 trimers are then converted to double hexamers encircling on each strand of the viral DNA and unwind viral DNA by power of ATP hydrolysis. E1 also recruits host cell replication factors, such as DNA polymerase α , topoisomerase I and RPA. In addition, *in vitro* studies have shown that the viral genome replication requires RFC, PCNA and DNA polymerase δ . As two replication forks containing the E1 hexamers progress in opposite directions from the viral origin, bidirectional replication via θ structure has been considered as a basic mode of HPV DNA replication.

• Initial amplification

Following the binding to basal cells, HPV virions are internalized by endocytic mechanisms, trafficked through endosomes where uncoated and the L2-viral genome complex enters nucleus [16]. It is generally assumed that the viral genomes undergo rapid amplification (initial amplification) following the expression of viral early genes in nucleus upon infection and are established as nuclear episomes at low copy numbers in the basal cells. Transient transfection experiments of viral DNA have shown that HPV genomes defective for E1 or E2 are not established as episomes, indicating

the initial amplification is dependent of E1 and E2 [17,18]. The replication of the viral genome is multirounds per cell cycle and continues until the viral copy numbers reach ‘optimal,’ ranging from 50 to 200 copies per cell. These numbers are based on copy numbers of the viral genome maintained in cultured cell lines, such as W12 cells and CIN612 cells, cell lines isolated from low-grade CINs infected with HPV16 and 31, respectively.

Reinson *et al.* recently reported that viral DNA replication initiates during S phase and continues into G2 phase of cell cycle initially after transfection but occurs only during S phase once the viral DNA become the stable episomes in U2OS cells. Overexpression of E1 and E2 proteins changed the replication time from S only to both the S and G2 phases in cells that maintain viral genomes as episomes, indicating that the duration of HPV genome replication is controlled by the levels of E1 and E2 [19]. Thus, reduction of the E1 and E2 levels appears essential to switch the initial amplification to the stable maintenance. Indeed, it was shown that E8⁺E2C, a viral transcriptional repressor, negatively regulates the initial amplification of HPV16, 18 and 31 by repressing the viral transcription from the early promoter [20,21]. However, since the E8⁺E2C defective HPVs are still able to enter stable maintenance, some other mechanisms are also involved in the termination of the initial amplification.

• Stable maintenance

In the maintenance stage, the replication of the viral genome is limited to on average once in S phase along with chromosomal DNA and the replicated genomes are segregated into two daughter cells during mitosis [22,23].

One mechanism proposed for S phase limited replication of the viral genome is regulation of nuclear localization and/or activity of E1 through post-translational modifications such as phosphorylation and sumoylation [14,24]. The N-terminal region of E1 contains nuclear localization signal (NLS) and nuclear export signal (NES). The phosphorylation of specific amino acids around NLS and NES have been shown to regulate nuclear accumulation of E1. It was shown that the nuclear import of HPV11 E1 was promoted by MAPK-mediated phosphorylation. On the other hand, the phosphorylation by CDK2, which is active during

S phase, facilitated nuclear export of E1 of HPV11 and 31. The HPV31 genome expressing the E1 mutant containing phosphomimic amino acids substituted for CDK2 target sites was not maintained as episomes even though it could replicate transiently. These results suggest that the phosphorylation dependent-nuclear export of E1 is essential for the maintenance stage [25]. Indeed, recent studies showed that nuclear accumulation of E1 induces DDR and strongly inhibits cell proliferation [26–28]. Thus, suppression of E1 level and/or activity appears important for HPV-containing basal cells to propagate during the maintenance phase.

Other mechanism proposed is that the stable maintenance of the viral genome is independent of E1. We recently reported that the E1-defective mutant of HPV16 can be maintained as nuclear episomes with the same efficiency as the wild-type after established in the presence of E1 expressed from a heterologous promoter [17]. These results were consistent with an earlier study using the temperature-sensitive E1 helicase mutant of BPV-1 genome [29]. Although mechanisms for the E1-independent replication is not understood, the best possible candidate performing the replication of the viral genome for the stable maintenance would be the host DNA helicase complex, consisting of MCMs and origin recognition complex (ORC), similar to the case for a latent replication of Epstein–Barr virus (EBV) [30].

Hoffmann *et al.* reported that HPV16 genome replicates in an ordered once-per-S-phase manner in W12 cells and exogenous expression of E1 converts the replication mode to a random choice mechanism in which individual copies of the viral genome can replicate more than once per cell cycle. In theory, both types of replication can maintain the copy number of the viral genomes overall constant. Based on these results, they proposed that there are two modes of genome replication at least for HPV16 and that the mode of the genome replication is determined by the presence or the level of E1 during the maintenance stage [31].

E2 has been shown to tether the viral genome to mitotic chromosomes through its binding to E2BSs, ensuring proper partitioning of the viral genomes into two daughter cells [15,23]. Several cellular proteins have been suggested to mediate segregation of the viral

genomes by interacting with E2, including Brd4 for BPV-1 and TopBP1 for HPV16 and 31 [32]. E6 and/or E7 of HPVs has been shown to play a role of the establishment and/or maintenance of HPV genomes. The mutant genomes of HPV16, 18, 31 and 11 defective for E6 or E7 expression failed to establish and/or maintain viral genomes as nuclear episomes in human keratinocytes. Recent studies showed that expression of a dominant negative mutant of p53 rescued the establishment and/or maintenance of the E6 defective HPV16, 18 and 31, indicating that the ability of E6 to inactivate p53 is necessary for the establishment and/or maintenance of the viral genome [33,34].

• Productive amplification

Upon differentiation of the cells maintaining HPV genomes, levels of viral early genes are elevated and the viral genomes are amplified to more than a three log increase [35]. The productive amplification of the viral genome is dependent of E1 and E2. DDR plays essential roles for the productive amplification by inducing post-translational modification of E1 and recruiting DNA repair factors to the viral DNAs.

Moody *et al.* reported that the productive amplification but not the stable maintenance of HPV31 is abrogated in the presence of a specific inhibitor of ATM, a key regulator of DDR [36]. As E7 of HPV16 and 31 and E1 of various HPVs have been shown to induce activation of ATM, the increased expression of E1 and E7 likely contributes to ATM activation during the productive stage. The activation of ATM in differentiating cells resulted in the caspase dependent-cleavage of an N-terminal 50-amino acid peptide from E1. The productive amplification of the HPV31 expressing the E1 mutant resistant to the caspase-dependent cleavage was much reduced compared with the wild-type, indicating that the removal of N-terminal E1 promotes the productive amplification. These results also imply that N-terminal region of E1 contains an inhibitory domain to restrict E1 activity to low level during the maintenance stage though a nature of this inhibitory domain has not been determined [37]. On the contrary, N-terminal truncation of HPV31 E1 rather reduced the viral DNA replication in a transient replication assay [38].

The MRN complex (MRE11-Rad50-NBS1) functions as a DNA sensor, inducing activation

of ATM upon double-stranded breaks and mediates DNA repair by homologous recombination (HR) [39]. Recently, it was reported that the MRN complex colocalizes with the viral genome together with E1 and E2, forming nuclear foci in keratinocytes containing HPV16 or 31 genomes upon differentiation [40,41]. Depletion of NBS1 by siRNA resulted in dissociation of the MRN complex from the viral genome and inhibited the productive amplification but not ATM activation. Thus, it is proposed that the productive amplification is recombination-based replication mediated by HR factors, rather than bidirectional replication. Flores and Lambert previously proposed that the productive amplification is supported by rolling circle replication based on the results of two dimensional gel electrophoresis showing that the intermediate structures of the viral DNA replication become consistent with unidirectional replication during the productive stage [42]. Since both types of replication can give rise to multicopies of viral DNAs with a single initiation event, it could be beneficial for HPVs to produce large quantities of the viral DNAs with a limited supply of the DNA replication machinery in differentiating cells. Rolling circle and recombination-based replication are proposed to be involved in lytic replication of herpes viruses, such as Herpes simplex virus-1 (HSV-1) and EBV [30,43].

Interplay between NF- κ B/IFN signaling & HPV infection

NF- κ B comprises a family of transcription factors that govern immune and inflammatory processes in response to injury and pathogen invasion. Currently, five members of the family including RelA (p65) p50/p105, p52/p100, RelB and c-Rel are identified in mammals and they form homo- or hetero-dimers. In the resting state, the activity of NF- κ Bs is negatively regulated primarily through its cytoplasmic retention by binding to I κ Bs including I κ B α . Upon stimulation, the phosphorylation-dependent degradation of I κ Bs by an ubiquitin proteasome pathway liberates NF- κ Bs into the nucleus. The transcriptional activity of NF- κ Bs is also modulated by post-translational modifications, such as phosphorylation, acetylation and ubiquitination [44,45].

Upon virus infection, pattern recognition receptors (PRRs), such as TLRs, RIG-like receptors (RLRs), NOD-like receptors and

DNA sensors, recognize pathogen-associated molecular patterns (PAMPs) and lead to activation of the downstream transcription factors, IRFs, NF- κ B and AP-1. These transcription factors cooperate to induce transcription of type I IFNs, as well as proinflammatory cytokines. Secreted IFNs then bind to their cognate receptors on the surface of the infected cells, as well as uninfected neighboring cells, resulting in induction of IFN-stimulated genes (ISGs), most of which encode the gene products with antiviral effects. The secretion of proinflammatory cytokines such as IL-1 β , IL-6 and TNF- α induces inflammation and the activation and recruitment of the adaptive immune cells including T and B cells [46]. NF- κ Bs are also activated by those proinflammatory cytokines, which can induce gene expression involved in control of cell cycle and apoptosis.

The regression of the epithelial lesions with HPV infection is mostly attributable to cell-mediated immune response against viral early proteins, such as E2 and E6. Massive infiltration of mononuclear cells, such as CD4⁺ and CD8⁺ during the regression, has been shown for natural infection of animal PVs and HPVs [5,6]. On the other hand, initial immune response to HPV infection is poorly understood due to difficulty in recapitulating the differentiation-dependent production of infectious virions and poor infectivity of pseudovirions to keratinocytes. Recently, Karim *et al.* reported that the activation of NF- κ B and IFN production is detected in keratinocytes at 24 h postinfection of HPV16 virions isolated from raft cultures and that the activation of NF- κ B and IFN production is suppressed by 48 h postinfection. These results suggest that HPVs cannot completely avoid the immune detection by PRRs upon infection, though the expression of the viral early proteins prevents the activation of NF- κ B/IFN signaling from being sustained [47]. Indeed, E2, E5, E6 and E7 of HR-HPVs have been shown to target several components of NF- κ B/IFN signaling to interrupt their signal cascades through multiple independent mechanisms [48]. Many viruses have evolved different strategies to modulate the NF- κ B/IFN signaling to evade antiviral responses or to utilize it for their own benefits [49,50]. Interestingly, some components of NF- κ B/IFN signaling are shown to augment the viral transcription and replication of HPVs.

Table 1. Summary for interaction between viral proteins and NF- κ B, interferon and DNA damage response signals.

Viral proteins	Interaction with NF- κ B-activating signal cascades	Interaction with IFN signals	Interaction with DDR
E6	<ul style="list-style-type: none"> – Upregulation of p100 and p105 – Downregulation of RIG1, MDA5 and TLR3 through suppression of IFN-κ expression – Inhibition of NF-κB-dependent activation of IL-8 promoter by binding coactivator p300/CBP – Inhibition of TNF-α-induced apoptosis through interaction with TNFR and FADD 	<ul style="list-style-type: none"> – Inhibition of IRF3-dependent IFN induction – Inhibition of STAT1/2 phosphorylation by binding Tyk2 – Downregulation of STAT1 – Downregulation of ISGs 	<ul style="list-style-type: none"> – Inactivation of p53 – Inactivation of DNA repair proteins, such as BRCA and XRCC
E7	<ul style="list-style-type: none"> – Downregulation of TLR9 – Inhibition of TNF-α-dependent NF-κB activation by binding IKK complex – Inhibition of NF-κB dependent activation of IL-8 promoter by binding coactivator, KATB2 	<ul style="list-style-type: none"> – Inhibition of STING by direct binding – Inhibition of IRF1-dependent transcription – Inhibition of nuclear translocation of IRF9 – Downregulation of ISGs 	<ul style="list-style-type: none"> – Stabilization of p53 – Activation of ATM through STAT-5 – Induction of FANCD2 – Inactivation of RB, p21 and p27 to bypass cell cycle arrest by DDR
E5	<ul style="list-style-type: none"> – Inhibition of NF-κB-dependent transcription by inducing deacetylation of RELA – Induction of NF-κB-dependent COX2 expression by increasing RELA and p50 levels 	<ul style="list-style-type: none"> – Induction of IFN-β through upregulation of IRF1 	<ul style="list-style-type: none"> – Suppression of p21 and p27
E1	<ul style="list-style-type: none"> – Activation of NF-κB through induction of ATM/ATR activation 	<ul style="list-style-type: none"> – Localization adjacent to ND10 together with E2 and the viral DNA – Inhibition of helicase activity by IFIT1 binding 	<ul style="list-style-type: none"> – Activation of ATM/ATR pathways
E2	<ul style="list-style-type: none"> – Potentiation of TNF-α-dependent NF-κB activation through TRAF5/6 interaction 	<ul style="list-style-type: none"> – Downregulation of STING, IFN-κ – Localization adjacent to ND10 together with E1 and the viral DNA 	<ul style="list-style-type: none"> – Interaction with p53

CBP: CREB-binding protein; DDR: DNA damage response; IFN: Interferon.

Taken together, these studies have revealed the interplay between NF- κ B/IFN signaling and the viral genome replication in the HPV life cycle. **Table 1** summarizes the interaction between viral early proteins and NF- κ B, IFN and DDR signals.

• Suppression of PRR & inflammatory cytokine signals by viral early proteins of HR-HPVs

Upon recognition of PAMPs, PRRs induces kinase complexes through adaptors, which subsequently recruit TRAF2, 3 and 6. The activated kinase complex, IRAK complex or TBK1-IKK ϵ , then phosphorylates IRF7 or IRF3, respectively. TRAFs also induce activation of MAPK and the IKK complex, resulting in activation of AP-1 and NF- κ B, respectively. All TLRs except for TLR3 signal through an adaptor, MyD88 to induce the IRAK complex consisting of IRAK1, 2 and 4, and recruits TRAF3 and 6. TLR3 and 4 signal through an alternative pathway mediated

by TIRIF, recruiting TRAF3 that induces TBK1-IKK ϵ complex. Cytosolic RNA sensors via MAVS, DNA sensors via STING, induce TBK1-IKK ϵ to activate IRF3. The induction of type I IFNs requires sequence specific binding of the activated transcription factors in its promoter. IFN- β promoter contains four positive regulatory domains that are occupied by transcription factor complexes, IRF3, IRF7, AP-1 and NF- κ B. The induction of IFN- α requires binding of IRF7 but not AP-1 and NF- κ B. Other members of IRFs, IRF1, 3, 4, 5 and 8 have been shown to induce IFN- α in a cell-type specific manner [46,50].

Proinflammatory cytokines, such as IL-1 β and TNF- α , induces activation of NF- κ B through their receptors, IL-1R and TNFR1, respectively, expressed on the cell surface in autocrine as well as paracrine manner. IL-1R recruits MyD88, leading to the formation of IRAK1 and 4 and TRAF6 complex and TNFR1 activates TRADD, inducing the complex

formation consisting of RIP1–TRAF2–cIAP, that results in the IKK-dependent phosphorylation of I κ B α .

Keratinocytes express most of PRRs including TLR 1–6 on the cell surface and TLR3 and 9 in the endosomes. TLR7 expression in the endosomes can be induced by stimulating TLR3. The expression of RLRs such as RIG-1 and MDA5, the DNA sensors, such as AIM2 and IFI16, was also detected in cytoplasm of keratinocytes. Several studies have indicated that PRRs and ISGs are globally downregulated in human keratinocytes harboring viral episomes of HR-HPVs [51,52]. E6 and E7 of HR-HPVs are shown to downregulate PRRs including RIG1, MDA5 and TLR3 and 9. It was reported that E7 of HPV16 inhibits expression of TLR9 by inducing an inhibitory complex containing NF- κ B, ER α , HDAC1 and JARID1B, histone demethylase, to TLR9 promoter [53]. E6 of HPV18 was shown to reduce expression of RIG1, MDA5 and TLR3 by inhibiting the expression of IFN- κ [52]. E2 of HPV16 and 18 was also shown to downregulate IFN- κ and STING [54]. E6 of HPV16 inhibits transcriptional activities of IRF3, suppressing the expression of IFN- β [55]. In addition, viral early proteins have been shown to modulate NF- κ B activity by several mechanisms, such as inhibiting or potentiating TNFR signals and increasing levels of NF- κ Bs (Table 1) [48].

• NF- κ B & HPV genome replication

Genotoxic stress can result in the activation of NF κ B and some of the proteins in DDR pathways including MRN complex have been proposed to function as a PRR [56,57]. It has been shown that ATM activates NF- κ B by inducing phosphorylation-dependent degradation of I κ B α through activating IKK complex. We recently reported that the activation of DDR by HPV16 E1 results in the activation of NF- κ B via degradation of I κ B α and the NF- κ B activation inhibits the E1- and E2-dependent replication of the viral genome by promoting degradation of E1. Based on these results, we proposed a model that E1 and NF- κ B form a negative feedback loop and this negative feedback loop is involved in the transition from initial amplification to stable maintenance in the establishment stage (Figure 4) [58]. Interestingly, the E1-dependent activation of NF- κ B was induced by activation of ATR, a key signal inducer of DDR,

consistent with the study indicating that HPV18 E1 induces ATR activation during the initial amplification following transfection of the viral genome in U2OS cells [28]. URR of several HPVs including HPV16 has been shown to contain an NF- κ B binding site and the binding of NF- κ B represses the viral early promoter of HPV16 [59]. Therefore, it is also possible that NF- κ B suppresses initial amplification by repressing the viral gene expression. Weak but constitutive increase of NF- κ B activity was detected in human keratinocytes maintaining HPV16 and 31 as episomes and expression of the degradation resistant mutant of I κ B α slightly increased the copy numbers of the viral genomes. Conversely, the activation of NF- κ B by TNF- α resulted in reduction of the viral DNA. These results imply that the change in the cytokine levels within infected tissues could affect the stable maintenance of HPVs.

• IFNs & HPV genome replication

Several studies showed that treatment of IFNs reduces the copy numbers of HPVs in the cells maintaining the viral genome as episomes, suggesting that IFN signals are not completely disrupted in the presence of the viral early genes. Time course for the reduction of the viral episomes with treatment of IFNs suggests that

Figure 4. A negative feedback between E1 and NF- κ B. E1 induces activation of NF- κ B through DNA damage response and NF- κ B in turn limits E1- and E2-dependent replication of the viral genome by promoting degradation of E1. Reproduced with permission from © American Society for Microbiology (2015) [58].

loss of the viral episomes occurs as a result of active process rather than an indirect result of antiproliferative effects of IFNs. In case of BPV-1 genome in mouse fibroblasts, C127 cells, the viral episomes were completely eliminated and the biological characters of the cells were reverted to that of normal C127 cells. On the other hand, the treatment of IFNs resulted in outgrowth of an IFN-resistant cell population that contains only integrated copies of HPV16 or 31 genome after rapid loss of the viral episomes was observed in W12 cells or CIN612 cells [22,60]. It was reported that spontaneous loss of the viral episomes of HPV16 correlates with increased expression of ISGs in W12 cells [61]. These studies indicate that extrinsic, as well as intrinsic, activation of IFN signaling inhibits the replication of the viral genome in the maintenance stage. Although a nature of intrinsic activation of IFN signaling has not been elucidated, DDR activation could elicit production of IFNs. Large T antigen (LT) of simian virus 40 (SV40) is a viral DNA helicase that has been shown to share homology of functional domains with HPV E1. Interestingly, it was reported that LT induces activation of ATR and IRF1, resulting in IFN- β production [62].

IFIT1, also known as ISG56 or p56, is an ISG downregulated in human keratinocytes harboring HR-HPVs and its expression can be reactivated by IFN- β treatment. It is shown that IFIT1 binds to C-terminus of HPV18 E1 through its N-terminal tetratricopeptide repeat 2 domain and inhibits helicase activity, E2 binding and ori binding of the E1 [63]. Thus, the expression of IFIT1 may mediate IFN induced loss of the viral episomes though the effect of IFIT1 expression on the viral episome in keratinocytes has not been demonstrated.

It has been shown that type I IFN can induce p53 expression and that p53 transcriptionally activates some of ISGs [64]. Therefore, the inability of the E6 defective genome of HR-HPVs to establish and/or maintain the viral episomes could be attributable to p53 dependent activation of ISGs [33].

In addition, Lace *et al.* recently reported that the reduction of the viral episomes by IFN- γ was abrogated in the presence of a proteasome inhibitor, MG132, suggesting that a proteasome dependent mechanism may also be involved [60].

• STATs & HPV genome replication

E6 and E7 of HR-HPVs have been shown to downregulate expression of STAT-1, a major transcription factor inducing expression of ISGs downstream of IFN receptors (IFNRs). Restoration of STAT-1 levels in CIN612 cells was shown to induce loss of the HPV31 episomes and outgrowth of cell population with an integrated viral DNA [65]. It was also shown that E6 of HPV18 hamper phosphorylation of STAT-1 and 2 by TyK2, a signal transducer of IFNRs, and that E7 prevents nuclear translocation of IRF9, a member of the IFN-stimulated gene factor 3 complex (ISGF3) with STAT-1 and -2, which induces ISG expression [66]. On the other hand, Hong *et al.* recently reported that HPV31 E7 activates STAT-5 through increasing its phosphorylation and the activated STAT-5 promotes productive amplification by activating ATM [67].

• IRFs & HPV genome replication

IRFs are a family of transcription factors that activate expression of type I IFNs downstream of PRRs. It was reported that IRF1 and IRF2 bind to an IFN-responsible element (IRE) located immediate upstream of p97 promoter of HPV16 and the binding sites of IRF1 and IRF2 are also present in some other HPVs [68]. Low-dose IFN- β enhanced replication of the viral genome through an IRF-1-dependent upregulation of the viral early promoter upon transfection. When the IRF-1 binding to IRE in the viral promoter was abrogated, the IFN treatment resulted in loss of the viral episomes of HPV16 and 31, suggesting that IFN reduces the viral episomes in an IRF-1-independent manner. Thus, it appears that the initial amplification of the viral genome could be facilitated by initial, low production of IFN through IRF-1 and it is later counteracted by higher dose of IFNs. IRF-2 has been shown to positively regulate p97 promoter of HPV16 in the absence of IFN [69]. Muto *et al.* recently reported that E5 of HPV16 upregulates IRF-1 to induce IFN- β expression [70]. On the other hand, E7 of HPV16 has been shown to inhibit IRF-1 mediated transcription by recruiting HDAC to its promoter. E7 also negatively regulated IRF-2 promoter partly through inhibiting IRF-1 dependent activation of the IRF-2 promoter [69]. Thus, the IRF-1 dependent activation of viral genes expression could be under positive and negative feedback via E5

and E7, respectively, during the establishment and maintenance stages of the viral life cycle.

• ND10 & HPV genome replication

ND10s are nuclear domains originally characterized by their striking punctuate appearance when examined by immunofluorescence. PML is an organizer of ND10s that recruit many proteins upon stimulation. Permanent members of ND10s include the Sp100 and Daxx. IFNs have been shown to facilitate formation of ND10s by transcriptionally activating PML. Thus, ND10s are considered to play important roles in innate immunity. As many DDR associated proteins including p53 have been shown localized to ND10s upon DNA damage, ND10s are also linked to DDR [71,72]. The viral proteins and/or genomes of many viruses including HPVs are shown to localize at or adjacent to ND10s at some points of their life cycles and manipulate ND10s. Day *et al.* reported that BPV-1 genome together with L2 localizes at ND10s upon infection and the viral transcription is reduced in the absence of PML, indicating that ND10s or PML facilitate initial transcription of the viral genes [73]. A recent study showed that knockdown of Sp100 but not Daxx by siRNA in human keratinocytes enhances the transcription and replication of HPV18 genomes immediately following transfection. Consistent with the BPV-1 study, the knockdown of PML modestly reduced viral transcription and replication of HPV18. Interestingly, Sp100 mediated suppression was no longer observed in the cells stably maintaining HPV18 genome, suggesting that Sp100 negatively regulate initial amplification by repressing the viral gene expression in the establishment but not in the maintenance stage. It was presumed that Sp100 may distinguish incoming viral DNA from established viral DNA through epigenetic modifications [74]. Other study found that HPV18 and 11 genomes were recruited to ND10s upon transfection in U2OS cells. Knockdown of Daxx reduced the viral transcription and replication of HPV18 and 11 in U2OS cells, suggesting a positive role of Daxx in the establishment stage [75].

L2 is shown to localize to ND10s, indicating the L2 directs the L2-viral genome complex to ND10s after nuclear entry upon infection [73]. However, transient replication experiments of a naked viral DNA clearly indicated that HPV genomes can be recruited to ND10s in the absence of L2. Several studies have shown that E1, E2 and

viral genomes form nuclear foci and this replication foci are localized adjacent to ND10s, though E1 or E2 alone does not [76]. Increased numbers of ND10s was described in the cells maintaining HPV16 genomes as episomes [77]. As ND10s are shown to mediate DDR, increased ND10 formation and/or the recruitment of the viral genome to ND10s could be mediated by DDR induced cellular factors together with E1 and E2.

Conclusion & future perspective

Since there is no antiviral drug to treat HPV infection and diseases except for the TLR7 agonist, imiquimod used for condyloma acuminata, current therapies employ invasive methods including cryotherapy and surgical removal by knives, laser or loop electrosurgical excision procedures. Physical removal of the infected epithelial lesions can be effective in the short term but it often recurs even after multiple treatments [78]. Laser or loop electrosurgical excision procedures can increase risks of infertility and premature birth. Therefore, it is necessary to develop antiviral drug to treat HPV infection. To eradicate the viral genomes from basal cells, it is crucial to understand molecular mechanisms for the establishment and maintenance of the viral genome. As shown for other oncogenic viruses [79,80], HPV appears to have intricate relationships between the viral persistence and NF- κ B/IFN signaling at multiple levels. Further understanding of the interplay between NF- κ B/IFN signaling and the replication of the viral genome will help to find an effective target for antiviral therapies.

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EXECUTIVE SUMMARY**HPV infection & cervical cancers**

- Approximately 10% of the epithelial lesions with high-risk HPV infection develop persistence.
- The productive life cycle of high-risk HPV is supported in low-grade cervical intraepithelial lesions while it is aborted in high-grade cervical intraepithelial lesions.
- The increased expression of viral oncogenes, E6 and E7, facilitates epithelial transformation.
- A majority of cervical cancers arises from a specific type of cells in the squamocolumnar junction.

Productive life cycle of HPVs

- The life cycle of HPV is tightly linked to differentiation status of the host keratinocytes.
- The production of progeny virions occurs exclusively in a differentiating compartment of the stratified squamous epithelium.
- The viral genome is maintained as nuclear episomes at low-copy numbers with very limited expression of the viral early genes in basal cells.
- E7 modulates cell-cycle control to sustain or induce terminally differentiating cells mitotically active to support the productive amplification of the viral genome.

The structure of viral genome & viral proteins

- The viral genome can be divided into three regions, early and late regions and upper regulatory regions.
- The expression of viral early proteins is derived from the viral early promoter and is active throughout the viral life cycle.
- The expression of the late proteins is derived from the viral late promoter and is dependent on the differentiation of infected cells.

Regulation of the viral genome in the life cycle

- The viral genome undergoes three phases of the replication, initial amplification, stable maintenance and productive amplification.
- Minimal components necessary for HPV genome replication are E1, E2 and upper regulatory region.
- Initial and productive amplification are mediated by E1 and E2.
- The stable maintenance could be mediated by E1-dependent, as well as E1-independent, mechanisms.
- The expression levels and/or post-translational modification of E1 possibly determine the duration, timing and mode of viral genome replication.
- E2 plays essential roles for partitioning of the viral genomes during the maintenance stage.
- Activation of ATM is required for the productive amplification.
- ATM regulates productive amplification by at least two mechanisms, activation of caspase-dependent cleavage of E1 and the induction of MRN complex.
- Productive amplification could be mediated by recombination-based replication or rolling circle replication switched from bidirectional replication in the stable maintenance.

Interplay between NF- κ B/interferon signaling & HPV infection

- NF- κ B can be activated by pattern recognition receptors upon HPV infection and induces production of interferons.
- The expression of pattern recognition receptors and IFN-stimulated genes is globally downregulated in the cells containing HR-HPV genomes by expression of E6, E7 and E2.

EXECUTIVE SUMMARY (CONT.)

- The viral early proteins interrupt activation of NF- κ B/IFN signaling.
- E1-induced DNA damage response can result in the activation of NF- κ B.
- Activation of NF- κ B inhibits the E1- and E2-dependent replication by promoting degradation of E1 and/or suppressing the viral early promoter.
- IFNs reduce the viral episomes in the maintenance stage.
- IFIT1 may mediate IFN-induced loss of the viral episomes by inhibiting E1-dependent replication of the viral genome.
- IFN may facilitate the replication of the viral genome through IRF-1-dependent activation of the viral early promoter during the establishment stage.
- IRF-1 can be activated by E5 but inhibited by E7 of HPV16.
- Components of ND10 differentially modulate viral transcription and replication upon infection during the establishment stage.

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