Induction of Interferon Kappa in Human Papillomavirus 16 Infection by Transforming Growth Factor Beta-Induced Promoter Demethylation

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ABSTRACT Persistent high-risk human papillomavirus (HPV) infection is the major causal factor in cervical and other anogenital cancers. Because there are currently no therapeutics capable of preventing neoplastic progression of HPV infections, understanding the mechanisms of HPV-mediated persistence, including immune evasion, is a major research priority. The multifunctional growth factor transforming growth factor beta (TGF β) has been shown to inhibit expression of early viral transcripts from cells harboring integrated HPV genomes or cells infected with retroviruses expressing HPV oncoproteins. However, the mechanism of TGF β -induced inhibition has not been fully defined. In this study, we have observed a previously uncharacterized ability of TGF β to repress the differentiation-induced upregulation of late HPV16 gene expression. In addition, interferon kappa (IFN- κ), a keratinocyte-specific, constitutively expressed cytokine suppressed by differentiation, can be transcriptionally induced by TGF β 1. TGF β -mediated IFN- κ transcription only occurs in cells containing HPV16, and this is due to TGF β 1-mediated reversal of HPV-induced methylation of the IFN- κ promoter through active DNA demethylation mediated by thymine DNA glycosylase (TDG). This novel interaction between growth factor and innate immune signaling may shed light on the mechanisms of HPV persistence and how the virus manipulates both immune and growth factor signaling to promote its life cycle.

IMPORTANCE Persistent infection by high-risk HPVs is the primary risk factor for development of HPV-induced cancers. Persistence involves viral evasion of the immune response, including the IFN response. HPV is also known to suppress TGF β signaling, which inhibits viral gene expression. Here, we show that the TGF β and IFN pathways are interrelated in the context of HPV16 infection through the upregulation of IFN- κ by TGF β . The ability of TGF β to induce IFN- κ promoter demethylation and transcriptional activation provides a new explanation for why HPV has evolved mechanisms to inhibit TGF β in infected cells.

KEYWORDS interferon, papillomaviruses, transforming growth factor-beta, differentiation, innate immunity, keratinocytes, methylation, transcriptional regulation

Human papillomaviruses (HPVs) infect cells of stratified squamous epithelia. Most HPV infections are benign and can be cleared within 18 to 24 months; however, some infections may persist for years without inducing a strong immune response needed for clearance (1, 2). Infections with high- and low-risk HPV types can each cause benign growths, but persistent infection by high-risk types (such as HPV16) can lead to cervical intraepithelial neoplasia (CIN), which can progress in severity and eventually become a carcinoma (1). Current vaccines are effective in preventing infection of the most medically significant types, but they have no effect on existing infections (1, 3). Long-term persistence of high-risk HPV types is the greatest risk factor for the development of anogenital malignancies because it allows the accumulation of host genetic

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changes which enable cancer development and progression (1–3). Therefore, it is essential that we understand how HPV subverts host immune responses to escape detection and persist in the host.

The life cycle of HPV is associated with differentiation of the infected cell (1, 4). HPV infects cells in the basal epithelial layer that become exposed through micro-wounds. Infected basal cells express only low levels of HPV antigens. Differentiation of HPV-positive cells induces the productive phase of the viral life cycle by upregulating viral gene expression, resulting in viral genome amplification and virion morphogenesis (1, 4). By restricting viral antigen synthesis to differentiated keratinocytes of the epidermis, HPV life cycle organization promotes viral persistence through facilitating immune evasion (1, 5, 6). In addition, activities of HPV oncoproteins E5, E6, and E7 can actively inhibit innate and adaptive immune responses, thereby promoting viral persistence (6, 7).

Keratinocytes, the targets of HPV, are sometimes called immune sentinels because they constitutively express low levels of cytokines, such as interferons (IFNs), that are upregulated during infection by pathogens (8). Type I IFNs, which include IFN- α , IFN- β , and IFN- κ , were originally identified for their ability to interfere with viral replication. Secreted type I IFNs bind to the type I interferon alpha/beta receptor (IFNAR) and induce the phosphorylation and dimerization of STATs, which in turn induce transcription of IFN-stimulated genes (ISGs) (9). ISGs engage in a wide variety of antiviral functions (9), including some that impact the HPV replication cycle (10–13). IFN- κ is a 180-amino-acid cytokine belonging to the type I IFN family (14). Although it shares ~30% homology with other type I IFNs, IFN- κ is produced specifically by keratinocytes and constitutively expressed, in contrast to IFN- α/β , which are expressed in many cell types and only at low levels in the absence of stimulation (15, 16). IFN- κ has previously been shown to suppress HPV31 transcripts (10); however, HPV has been shown to suppress IFN- κ expression through HPV oncoprotein E6-induced hypermethylation of one of two CpG islands in its promoter (15, 16). HPV can also suppress IFN- κ using the viral transcription factor E2 (17). IFN- κ suppression is also seen in HPV-induced pre-cancerous and cancerous lesions (17, 18).

Another regulator produced by keratinocytes is transforming growth factor beta (TGF β). TGF β 1 and TGF β 2 are expressed in epithelial cells and induce signaling by binding to TGF β receptors (19, 20). Activated TGF β receptor kinases stimulate signaling by multiple independent routes, including the canonical SMAD-dependent pathway (21). In the SMAD-dependent pathway, activated TGF β receptor 1 phosphorylates receptor-regulated SMAD2/3, which can then associate with co-SMAD4, translocate to the nucleus, and bind to SMAD binding elements in the promoters of target genes to activate transcription (21). Through these signaling pathways, TGF β controls a variety of cell processes, including immune functions (19, 20). TGF β can suppress or alter the activation, maturation, and differentiation of both innate and adaptive immune cells, including natural killer cells, dendritic cells, macrophages, and T cells (21–23). TGF β is also needed for the maturation and maintenance of Langerhans cells in the epidermis (21, 23, 24).

Early in tumorigenesis, TGF β acts as a tumor suppressor by inhibiting epithelial cell proliferation (19). In later stages of cancer development, TGF β acts as a tumor promoter by fostering angiogenesis, suppressing antitumor immune responses, and inducing reactive stroma (19). Interestingly, cervical cells undergoing malignant progression exhibit a progressive loss of cell cycle arrest upon TGF β treatment, and downregulation of TGF β 1, TGF β 2, and SMAD2 has been observed in HPV-induced lesions as lesion severity increases (19, 25). Tumors do not usually acquire genetic defects in TGF β signaling pathways; rather, epigenetic mechanisms of TGF β inactivation, such as promoter methylation, have been proposed (26–28).

HPV has been previously shown to suppress TGF β signaling through receptors and downstream signaling components using oncoproteins E7 and E5, although the consequences of this suppression for immune responses to HPV have not been investigated (29–32). Studies showing interactions between TGF β signaling and HPV have

primarily used cells harboring integrated genomes or cells infected with retroviruses encoding HPV oncoproteins rather than episomally replicating virus with its full complement of viral proteins (30, 31, 33–35). In our *in vitro* model of HPV infection, human foreskin keratinocytes (HFKs) are immortalized by HPV16 genomes maintained episomally, cultured in either monolayer (which maintains undifferentiated conditions where viral replication and transcription are low) or suspended in methylcellulose (which promotes differentiation to activate viral late transcription) (36–38). These cells are capable of producing infectious virus upon growth in organotypic raft culture (36, 39), and this allows us to study host-virus interactions throughout the viral life cycle (40).

Studies of host genes regulated by HPV using microarray analysis have found that the IFN system and TGF β are both transcriptionally suppressed by HPV (41, 42) (J. M. Bodily, unpublished data). In this study, we sought to determine whether there was a connection between the IFNs and TGF β in HPV infection and whether the suppressive effect of TGF β on the viral life cycle could be due to cross talk with the IFN pathway. We found that TGF β 1 can upregulate the mRNA and protein levels of IFN- κ and that it can repress late HPV transcripts induced upon differentiation by episomal viral genomes. The ability of TGF β 1 to induce IFN- κ transcription depends on the presence of the virus: TGF β 1 reverses HPV-induced methylation of the IFN- κ promoter through an active demethylation mechanism involving thymine DNA glycosylase (TDG). These findings show that the TGF β and IFN signaling pathways cross talk with one another in the host cell response to HPV and suggest an important evolutionary rationale for the suppression of TGF β by HPV.

RESULTS

TGF β signaling suppresses viral transcripts. IFN- κ is specifically produced by keratinocytes, is constitutively expressed by uninfected keratinocytes (15–17), and has been observed to suppress early HPV31 HPV transcripts (10). Previous studies indicate that TGF β signaling can regulate type I IFN induction (43, 44), and microarray and other studies have shown that HPV regulates both IFN and TGF β signaling (30–32, 41, 42, and J. M. Bodily, unpublished). In an effort to better understand how the virus manipulates innate immune responses, we wished to determine whether there is cross talk between these two important signaling systems. We first measured the levels of type I IFNs in uninfected keratinocytes (HFKs) under undifferentiated (monolayer) or differentiated (methylcellulose) conditions. In sharp contrast to the other type I IFNs, IFN- κ is dramatically decreased upon differentiation, when HPV late genes are upregulated, whereas IFN- α/β levels are increased (Fig. 1a) (1). Cells containing episomally replicating HPV16 had reduced levels of IFN- κ transcripts compared to those of uninfected HFKs (Fig. 1b), consistent with findings of others (15–17). Also consistent with previous work, E6 represses levels of IFN- κ (15, 16). Keratinocytes immortalized by E7 or by ectopic expression of hTERT (NOK cells) did not show suppressed levels of IFN- κ transcripts, indicating that immortalization *per se* does not select for IFN- κ expression (Fig. 1b; see also Fig. S1a in the supplemental material). We next observed a decrease in TGF β 1 transcripts in HPV16-containing cells compared to those of HFKs, in agreement with the work of others on keratinocytes with integrated genomes (Fig. 1c) (25). Curiously, TGF β 2 transcripts were increased in HPV16-containing cells, showing that episomal HPV differentially regulates TGF β isoforms (Fig. 1c). We observed similar effects on protein levels of IFN- κ , TGF β 1, and TGF β 2 in HFKs versus those in HPV16-containing cells using Western blotting (Fig. 1d).

Previous studies have found that TGF β 1 and TGF β 2 can inhibit transcription of early genes E6 and E7 from integrated HPV genomes (33, 35). To ascertain the effects of TGF β on transcription of viral episomes, HPV16-containing cells were grown in monolayer or methylcellulose and treated with recombinant TGF β 1 or TGF β 2 for 48 h. We found that TGF β 1 modestly reduced levels of early E6-E7 transcripts under both differentiated and undifferentiated conditions, whereas TGF β 2 treatment led to increased viral transcripts in monolayer with no effect in methylcellulose (Fig. 2a). We found that both TGF β 1 and TGF β 2 treatment suppressed late E1[^]E4 transcripts upon differentiation, and TGF β 1 blocked E1[^]E4 transcript upregulation to a greater degree than TGF β 2 (Fig. 2b). When

FIG 1 HPV16 regulates IFN-κ and TGFβ. (a) RT-qPCR analysis of IFN-α/β/κ transcripts in uninfected human foreskin keratinocytes (HFKs) grown in monolayer or methylcellulose and normalized to the cyclophilin housekeeping gene, with monolayer set to 1. (b) RT-qPCR analysis of IFN-κ transcript levels in HFKs, HPV16-containing cells, or HFKs immortalized with either E6 or E7 alone grown in monolayer culture and normalized to the cyclophilin housekeeping gene, with HFK set to 1. (c) RT-qPCR analysis of TGFβ1 and -2 in HFK- and HPV16-containing cells normalized to the cyclophilin housekeeping gene, with HFK set to 1. (d) Immunoblot analysis of protein levels of IFN-κ, TGFβ1, TGFβ2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in HFKs and HPV16-containing cells. ***, $P < 0.01$; NS, not significant.

we knocked down TGFβ1 in HPV16-containing cells using lentiviral short hairpin RNA (shRNA) transduction (TGFβ1 KDs) and grew these cells in monolayer or methylcellulose, levels of E1^ΔE4 transcripts in differentiation were increased in TGFβ1 KDs compared to those of nontarget controls (NT) (Fig. 2c and d). A simple explanation for the ability of TGFβ to inhibit differentiation-induced E1^ΔE4 transcript upregulation would be that TGFβ represses cellular differentiation; however, when we examined transcript levels of differentiation markers in TGFβ-treated cells, we observed upregulation of differentiation markers by suspension in methylcellulose, as expected, and no significant negative effect of TGFβ treatment (Fig. S1b). These results show that TGFβ1 inhibits late gene transcription from episomal HPV16 genomes and suggest that the role of TGFβ in inhibiting viral gene expression is not simply an effect on differentiation.

TGFβ signaling increases IFN transcript levels in HPV-containing cells. Since we observed suppression of late viral gene transcripts by TGFβ (Fig. 2b), we hypothesized that the antiviral effect of TGFβ is mediated through IFNs, since cross talk between these pathways has been previously reported (43, 44), and microarray and other studies have shown that HPV regulates both pathways (30–32, 41, 42, and Bodily, unpublished). We examined the effect of recombinant TGFβ1 or TGFβ2 on levels of IFN transcripts in HPV16-containing cells and observed a striking increase in IFN-κ transcript levels compared to those of the other type I IFNs with 24 h of TGFβ treatment (Fig. 3a and b). After 48 h of TGFβ treatment, levels of IFN-κ transcripts were further increased compared to those from 24 h of TGFβ treatment (Fig. 3a and c). Upregulation of IFN-κ transcripts was observed in both monolayer and methylcellulose, although IFN-κ

FIG 2 TGF β suppresses HPV transcripts in differentiation. RT-qPCR analysis of E6-E7 (a) or E1^{E4} (b) transcript levels in HPV16-containing cells that were untreated (none), treated with TGF β 1, or treated with TGF β 2 under undifferentiated (monolayer [Mono]) or differentiated conditions (methylcellulose [MC]) and normalized to the cyclophilin housekeeping gene, with untreated samples set to 1. (c) Immunoblot analysis of TGF β 1 and GAPDH protein levels in HPV-containing cells, nontarget control cells (NT), or TGF β 1 knockdown (KD) in HPV-containing cells. (d) E1^{E4} transcripts in NT or TGF β 1 KD HPV-containing cells in monolayer or MC and normalized to cyclophilin, with the NT monolayer sample set to 1. ***, $P < 0.01$; *, $P < 0.05$; NS, not significant.

transcripts overall were still reduced in differentiated cells compared to those in monolayer cultures (Fig. 3a and c). Induction of the other type I IFNs was weak at 24 h but stronger at 48 h, indicating that IFN- κ was induced in response to TGF β signaling prior to and may drive the induction of the other IFNs (Fig. 3b and d). TGF β 1 was more potent than TGF β 2 in promoting IFN- κ transcription at both time points (Fig. 3a and c). Increases in IFN- κ protein levels in response to 48 h of TGF β treatment in HPV16-containing cells were also observed and were statistically significant based on densitometry analysis of multiple blots (Fig. 4a). Transcript levels of IFN- κ in TGF β 1 KD cells showed a significant reduction (Fig. 4b), suggesting that TGF β supports basal IFN- κ transcript levels in HPV16-containing cells. We were unable to detect a difference at the protein level in TGF β 1 KD cells, since IFN- κ protein levels are already quite low in HPV16-containing cells. These findings show that TGF β drives increased levels of IFN- κ in cells containing episomal HPV16.

Since we observed increases in IFN- κ levels in HPV-containing cells treated with TGF β , we wanted to determine if increased IFN- κ levels would be reflected in the antiviral effectors of the IFN response. IFNs induce a signaling cascade that results in the transcription of interferon-stimulated genes (ISGs) (9). Transcripts for several ISGs were reduced in TGF β 1 KD cells (Fig. 4b). On the other hand, consistent with the suppressive activity of TGF β , higher levels of ISGs previously shown to have antiviral activity against HPV were observed in HPV-containing cells treated with TGF β 1 and, to a lesser degree, in cells treated with TGF β 2 (10–13) (Fig. 4d). Increased levels of transcripts for other canonical ISGs were observed as well (Fig. 4e). These data show that TGF β upregulates IFN signaling in HPV16-containing cells, including production of anti-HPV ISGs, which could contribute to the suppressive effect of TGF β on viral gene expression.

In order to determine if IFN signaling is involved in the suppression of viral transcripts by TGF β , we used the selective JAK1/2 inhibitor ruxolitinib (45, 46). HPV16-containing cells treated with TGF β 1 for 48 h showed an increase in both total STAT1 and STAT1 phosphorylated on tyrosine 701 (activated STAT1), confirming that IFN signaling activity in these cells is increased upon TGF β 1 treatment (Fig. 4f). When cells

FIG 3 TGF β increases IFN- κ transcript levels in HPV16-containing cells. RT-qPCR analysis of levels of IFN- κ transcripts in HPV16-containing cells left untreated, treated with TGF β 1, or treated with TGF β 2 for 24 h (a) or 48 h (c) under undifferentiated (monolayer) or differentiated (MC) conditions and normalized to cyclophilin, with untreated monolayer samples set to 1. RT-qPCR analysis of levels of IFN- α / β transcripts in HPV16-containing cells left untreated, treated with TGF β 1, or treated with TGF β 2 for 24 h (b) or 48 h (d) under undifferentiated (monolayer) or differentiated (MC) conditions and normalized to cyclophilin, with untreated monolayer samples set to 1. ***, $P < 0.01$; *, $P < 0.05$; NS, not significant.

were treated with ruxolitinib for 1 h and then treated with TGF β for 48 h, we observed that ruxolitinib suppressed both baseline and TGF β -induced STAT1 phosphorylation (Fig. 4f). We also observed that ruxolitinib treatment blunted the ability of TGF β to suppress late E1^ΔE4 transcription upon differentiation (Fig. 4g). These findings suggest that a portion of the suppressive effect of TGF β 1 on viral E1^ΔE4 transcripts is mediated through the IFN/JAK/STAT pathway.

IFN- κ sensitivity to TGF β requires HPV. The straightforward interpretation of the ability of TGF β to upregulate IFN- κ is that IFN- κ is simply a TGF β -responsive gene. However, when we treated uninfected HFKs with TGF β , we failed to observe increased levels of IFN- κ transcripts (compare Fig. 3 to Fig. 5a) and protein (Fig. 4a), in sharp contrast to HPV16-containing cells, suggesting that IFN- κ sensitivity to TGF β is seen only in the presence of the virus. Effects of TGF β on other type I IFNs in uninfected HFKs were weak and inconsistent (Fig. 5b). However, phosphorylated SMAD2 could be detected in both HFKs and HPV16-containing cells treated with TGF β (Fig. 5c), indicating that TGF β can induce canonical SMAD-dependent signaling in both cell types, even though IFN- κ is only induced in HPV16-containing cells.

TGF β reverses HPV-induced hypermethylation of the IFN- κ promoter. We next considered the mechanism for this unusual regulation of IFN- κ by TGF β . SMAD binding elements were not detected in the IFN- κ promoter by computational methods (data not shown), and a luciferase reporter containing the IFN- κ promoter sequence was only weakly inducible by TGF β in HPV-containing cells and not inducible at all in HFKs (data not shown). These observations suggest that TGF β does not activate IFN- κ by simply inducing binding of SMADs to SMAD binding elements in the IFN- κ promoter. Instead, alterations in epigenetic modifications in response to TGF β signaling may be important for the ability of IFN- κ to respond to TGF β in HPV-containing cells. Previous studies showed that HPV suppresses IFN- κ in part by inducing methylation of the promoter in a manner dependent on E6 (15, 16). To confirm that methylation of the IFN- κ promoter

FIG 4 TGF β induces interferon-stimulated genes in HPV16-containing cells. (a) Immunoblot analysis of protein levels of IFN- κ in HFKs or HPV16-containing cells left untreated, treated with TGF β 1, or treated with TGF β 2 for 48 h under undifferentiated (monolayer) conditions. Upregulation was statistically significant based on densitometry using TGF β 1 ($P = 0.013$) and TGF β 2 ($P = 0.007$). (b and c) RT-qPCR analysis of levels of IFN- κ (b) or IFI6, IFI27, and Sp100 (c) transcripts in NT or TGF β 1 KD HPV16-containing cells under undifferentiated (monolayer) conditions and normalized to cyclophilin, with NT set to 1. (d and e) RT-qPCR analysis of Sp100, IFIT1/p56, IFI16 (d), or other ISG (e) transcripts in HPV16-containing cells left untreated (U), treated with TGF β 1, or treated with TGF β 2 for 48 h under undifferentiated (monolayer) conditions and normalized to cyclophilin, with untreated monolayer samples set to 1. (f) Immunoblot analysis of total STAT1 and Y701-phosphorylated STAT1 in HPV16-containing cells treated with TGF β 1 with or without a 1-h pretreatment with the JAK inhibitor ruxolitinib. (g) RT-qPCR analysis of levels of E1^{A4} transcripts in HPV16-containing cells under undifferentiated or MC conditions, treated with TGF β 1 with or without 1 h of pretreatment with ruxolitinib and normalized to cyclophilin, with NT set to 1. ***, $P < 0.01$; *, $P < 0.05$; NS, not significant.

suppresses gene expression during HPV infection, we treated HPV16-containing cells with 5-aza-2'-deoxycytidine (5-aza-dc), which inhibits DNA methylation, and observed an increase in levels of IFN- κ transcripts (Fig. 6a), supporting previous results (15, 16).

TGF β has been previously shown to increase expression of some target genes via demethylation of CpG sequences, even in genes that do not contain SMAD binding elements (47–49). The IFN- κ promoter contains a CpG-rich island close to the transcriptional start site (TSS) that is preferentially hypermethylated through the activity of HPV E6 (15). Therefore, we hypothesized that TGF β induces IFN- κ expression by reversing E6-mediated promoter methylation, a mechanism that would only operate in cells expressing E6. In order to determine if TGF β could mediate demethylation of the IFN- κ promoter, we treated HPV-containing cells with recombinant TGF β and collected DNA for bisulfite sequencing analysis to assess the methylation status of the IFN- κ CpG island closest to the TSS. In agreement with previous work by others (15), we observed that the IFN- κ promoter was methylated at four of five CpGs in HPV-containing cells (Fig. 6b). Furthermore, TGF β 1 induces significant demethylation of the IFN- κ promoter, while TGF β 2 was unable to do so (Fig. 6b). These results indicate that TGF β 1 upregulates IFN- κ in HPV16-containing cells by reversing the promoter hypermethylation induced by HPV.

FIG 5 TGFβ does not induce IFN-κ in HFK cells. RT-qPCR analysis of levels of IFN-κ transcripts in uninfected HFKs left untreated, treated with TGFβ1, or treated with TGFβ2 for 48 h under undifferentiated (monolayer) or differentiated (MC) conditions and normalized to cyclophilin, with untreated monolayer samples set to 1. (b) RT-qPCR analysis of levels of IFN-α/β transcripts in uninfected HFK cells left untreated, treated with TGFβ1, or treated with TGFβ2 for 48 h under undifferentiated conditions and normalized to cyclophilin, with untreated monolayer samples set to 1. (c) Immunoblot analysis of phosphorylated SMAD2 (pSMAD2), total SMAD2, and GAPDH in HFKs or HPV16-containing cells left untreated, treated with TGFβ1, or treated with TGFβ2 for 48 h under undifferentiated (monolayer) conditions. NS, not significant compared to values for untreated samples.

Rincon-Orozco et al. previously reported that the IFN-κ promoter is hypermethylated in HPV16 E6-expressing cells but unmethylated in both uninfected keratinocytes and E7-expressing cells (Fig. 1b) (15). If the mechanism by which TGFβ induces IFN-κ transcription is through inducing promoter demethylation, TGFβ treatment should upregulate IFN-κ in cells expressing E6 only but not in cells expressing E7 only. When we treated cells expressing either E6 or E7 alone with TGFβ, we found that only the cells expressing E6 showed appreciable IFN-κ induction (Fig. 6c). These findings suggest that TGFβ induces IFN-κ transcription in HPV16-containing cells through reversing E6-mediated promoter methylation.

TGFβ1 induces active demethylation of the IFN-κ promoter. DNA demethylation can occur through either passive or active mechanisms (50). In passive demethylation, DNA methyltransferase 1 (DNMT1), which mediates methylation during replication, is not recruited to promoter CpGs of hemimethylated DNA during DNA replication, resulting in loss of maintenance of methylation over subsequent rounds of DNA

FIG 6 TGF β induces demethylation of the IFN- κ promoter. (a) RT-qPCR analysis of IFN- κ transcript levels in HPV16-containing cells left untreated or treated with 5-aza-2'-deoxycytidine (5 nM) for 96 h and normalized to cyclophilin, with untreated sample set to 1. (b, top) Schematic of CpG positions relative to the transcriptional start site (TSS) in the IFN- κ promoter. (Bottom) Bisulfite sequencing of the IFN- κ promoter in HPV16-containing cells left untreated, treated with TGF β 1, or treated with TGF β 2 for 48 h under undifferentiated conditions. Each row represents the methylation pattern of a single sequenced PCR product, and each column represents a different CpG site in the promoter-proximal CpG island. Black circles indicate the presence of a methylated CpG, and white circles indicate the presence of an unmethylated CpG. (c) RT-qPCR showing levels of IFN- κ transcripts in keratinocytes expressing either E6 or E7 alone from retroviral vectors, treated with TGF β 1 or TGF β 2. ***, $P < 0.01$; *, $P < 0.05$ compared to untreated samples; NS, not significant.

synthesis (50). In active demethylation, methylated CpGs are enzymatically modified to or replaced with unmethylated cytosines in a replication-independent manner (50). In uninfected immortalized keratinocytes, TGF β has previously been shown to mediate active DNA demethylation of the p15^{ink4a} promoter through a mechanism involving the recruitment of the DNA glycosylase enzymes thymine DNA glycosylase (TDG) and methyl-binding domain 4 protein (MBD4) to methylated CpGs (49). These enzymes, which are normally involved in excision repair, can modify methylated CpGs, creating an abasic site that is recognized by the base excision repair machinery and replaced with unmethylated cytosines, resulting in DNA demethylation (51). The kinetics of this mechanism are relatively fast, occurring within 90 min (49), compared to passive methylation, which takes on the order of days. In order to determine whether TGF β promotes passive or active demethylation in HPV16-containing cells, we performed a time course of TGF β 1 treatment and were able to detect an increase in IFN- κ transcripts rapidly, within 2 h (Fig. 7a). These rapid kinetics suggest that TGF β uses an active demethylation mechanism to induce IFN- κ promoter demethylation.

Since knockdown of DNA glycosylases TDG and MBD4 was previously observed to prevent TGF β -mediated active demethylation in keratinocytes (49), we knocked down these enzymes using short interfering RNA (siRNA) in HPV16-containing cells and then treated the cells with TGF β 1 to determine if TGF β 1 could still mediate demethylation of the IFN- κ promoter. Partial knockdown of DNA glycosylase TDG resulted in partial loss of TGF β -mediated induction of IFN- κ transcripts (Fig. 7b and c). However, knockdown of MBD4 was unable to prevent increased IFN- κ transcription in response to

FIG 7 TGF β induces active demethylation. (a) RT-qPCR analysis of IFN- κ transcript levels in HPV16-containing cells left untreated or treated with TGF β 1 for the indicated time and normalized to cyclophilin, with untreated sample set to 1. (b) RT-qPCR analysis of levels of thymine DNA glycosylase (TDG) transcripts in HPV16-containing cells transfected with 100 nM nontarget (NT) control or TDG siRNA and normalized to cyclophilin, with the NT sample set to 1. (c) RT-qPCR analysis of IFN- κ transcript levels in HPV16-containing cells transfected with 100 nM NT control or TDG siRNA, left untreated, or treated with TGF β 1 for 24 h and normalized to cyclophilin, with untreated sample set to 1. (d) RT-qPCR analysis of MBD4 transcripts in HPV16-containing cells transfected with 10 nM NT control or MBD4 siRNA and normalized to cyclophilin, with NT sample set to 1. (e) RT-qPCR analysis of IFN- κ transcript levels in HPV16-containing cells transfected with 10 nM NT control or MBD4 siRNA, left untreated, or treated with TGF β 1 for 24 h and normalized to cyclophilin, with the TGF β 1-treated sample set to 1. ***, $P < 0.01$; NS, not significant.

TGF β (Fig. 7d and e). These data indicate that TGF β treatment requires TDG but not MBD4 in order to activate IFN- κ , supporting the idea that TGF β 1 induces the active DNA demethylation of the IFN- κ promoter. TDG transcript levels did not change upon TGF β treatment in either HFKs or HPV-containing cells (not shown).

Since DNA methylation is an epigenetic mark transmitted to daughter cells (50), we wanted to determine if the demethylation of the IFN- κ promoter in response to TGF β resulted in permanently high levels of IFN- κ transcripts after removal of TGF β . We

FIG 8 Suppression of IFN- κ following removal of TGF β occurs slowly. (a) RT-qPCR analysis of IFN- κ transcript levels in HPV16-containing cells left untreated or treated with TGF β 1 for 48 h, washed, and incubated for an additional 48, 96, and 144 h and normalized to cyclophilin, with the TGF β 1-treated sample set to 1. ***, $P < 0.01$.

treated HPV-containing cells with recombinant TGF β 1 for 48 h, washed the cells to remove the exogenous growth factor, and continued to incubate the cells for various lengths of time. We observed that levels of IFN- κ transcripts were gradually lost over a period of days with a half-life of approximately 61 h, with some upregulation still persisting even 144 h following TGF β 1 removal (Fig. 8). These slow kinetics suggest that the ability of HPV to suppress IFN- κ transcripts through promoter methylation of the IFN- κ promoter involves a passive mechanism that requires cellular replication.

DISCUSSION

Our findings support a model for TGF β -induced IFN- κ transcription illustrated in Fig. 9. In uninfected keratinocytes, IFN- κ is constitutively expressed because the promoter is unmethylated (Fig. 9a) (15). Rincon-Orozco et al. showed that HPV induces hypermethylation of the IFN- κ promoter through activity of E6, perhaps involving DNMT1, resulting in reduced transcription (Fig. 9b). Our findings suggest that treatment of HPV-containing cells with TGF β induces a signaling cascade resulting in TDG-dependent active demethylation of the promoter and reexpression of IFN- κ (Fig. 9c). TGF β treatment thus has no effect on inducing transcription of IFN- κ in HFKs because the promoter is already unmethylated (Fig. 5a) (15). The specific signaling pathway and DNA modification mechanism used by TGF β remains unclear, but our findings demonstrate a connection between growth factor and IFN signaling regulated by HPV.

For almost 30 years, researchers have known that TGF β can inhibit early HPV viral gene

FIG 9 Model of TGF β -induced demethylation of the IFN- κ promoter. (a) IFN- κ is constitutively transcribed in HFKs because of low levels of CpG methylation in its promoter. (b) HPV E6 induces methylation of the IFN- κ promoter, suppressing its expression. (c) TGF β -induced signals trigger TDG-mediated promoter demethylation, increasing IFN- κ transcription.

expression in cells containing integrated viral genomes, which has been attributed to the effects of TGF β on the cell cycle and reduced transcription factor binding to the viral genome (33, 35, 52). In this study, we have found that TGF β can repress late gene expression from the viral episome through a previously uncharacterized mechanism involving upregulated IFN signaling. These observations suggest that suppression of TGF β signaling, which is correlated with increased severity in clinical HPV-induced lesions (19, 25) and targeted by multiple viral proteins (29–32), is required for the viral life cycle in order to evade innate immune responses and maintain a persistent infection.

Why is IFN- κ different from other IFNs? Classical type I IFNs are strongly induced by viral infection, which activates pattern recognition receptors (9). In contrast to other type I IFNs, IFN- κ is constitutively expressed in a cell type-specific manner and only moderately induced by pattern recognition receptor pathways (14–16, 18) (B. L. Woodby and J. M. Bodily, unpublished data). Other type I IFNs also lack the CpG islands observed in the IFN- κ promoter, presumably to enable quick induction upon infection (15). These observations support the idea that IFN- κ is regulated in a very different manner from the other IFNs, suggesting that it has separate functions. Furthermore, although all type I IFNs bind the same receptor complex, interactions with the receptor or induction of signaling pathways can vary, influencing biological responses (53). How IFN- κ signaling may differ from that of the other IFNs and what effect that has on HPV is not yet known. IFN- κ transcripts were dramatically reduced upon differentiation in methylcellulose, just as viral late genes are induced. It is possible that reduction in IFN- κ upon differentiation contributes to the activation of late viral events upon differentiation.

IFN upregulation contributes to TGF β suppressive activity. Baldwin et al. previously reported that TGF β 1 treatment inhibits early viral transcripts in cells containing integrated HPV by decreasing NF1-Ski interactions and NF1 binding to the HPV16 enhancer (52). While modest effects on viral early transcripts by TGF β 1 were observed in our studies, TGF β 1 could effectively interfere with the increase in late viral transcripts induced upon differentiation by episomal viral genomes (Fig. 2b) (1). A potential explanation for the ability of TGF β to repress late viral gene expression is that TGF β simply represses differentiation; however, we did not observe large differences in the fold upregulation of differentiation markers in TGF β -treated cells compared to those of untreated cells (see Fig. S1b). Instead, recombinant TGF β 1 or TGF β 2 caused increases in IFN- κ and ISG transcripts in both monolayer and methylcellulose in HPV16-containing cells (Fig. 3 and 4a to e). We also observed that inhibiting IFN signaling through use of ruxolitinib partially interfered with the ability of TGF β to suppress late viral transcripts (Fig. 4g). These findings indicate that TGF β signaling interfaces with the IFN pathway, which is responsible for a portion of TGF β 's suppressive effects on HPV16. Which specific ISGs are responsible for the suppressive effects of TGF β and how IFN-related mechanisms relate to other TGF β -induced suppressors remain to be determined.

Although we have shown that TGF β signaling increases IFN- κ transcript levels through a mechanism involving active demethylation of the IFN- κ promoter (Fig. 6 and 7), the signaling pathways responsible for the interplay of TGF β and IFN in the context of keratinocyte differentiation remain to be worked out. TGF β stimulates signaling by at least two independent routes: the SMAD-dependent canonical pathway and several SMAD-independent or noncanonical pathways, including the ERK, c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) pathways (54). TGF β can also cross talk with the epidermal growth factor signaling pathway through coordinating actions between SMAD-dependent signaling and Erk1/2 activation (20, 55). Whether SMAD-dependent or -independent pathways are involved in upregulation of IFN- κ by TGF β in HPV-containing cells remains undetermined.

Despite having similar functions, TGF β 2 has a weaker affinity for TGF β receptor type II (TGF β RII) than TGF β 1, and optimal signaling by TGF β 2 actually requires another receptor (TGF β RIII) to enhance its binding to TGF β RII (21). The observation that TGF β 2

is less potent than TGF β 1 is also supported by our data, since TGF β 2 induces less phosphorylation of SMAD2 than TGF β 1 regardless of HPV infection (Fig. 5c). In accordance with the strong signaling capacity of TGF β 1, we observed that TGF β 1 could promote demethylation of the IFN- κ promoter and induce IFN- κ transcripts and protein levels in HPV-containing cells (Fig. 3 and 6). Since TGF β 2 is a less robust signal transducer, we found that TGF β 2 was unable to significantly promote demethylation of the IFN- κ promoter and detected only a modest increase in IFN- κ transcripts and protein in HPV-containing cells treated with TGF β 2 (Fig. 3 and 6). Whether this modest effect is due to low levels of demethylation undetected in our assays or to a separate pathway is not clear. The reduced ability of TGF β 2 in upregulating IFN- κ and repressing viral gene expression compared to TGF β 1 may explain why TGF β 1 is specifically repressed by HPV and TGF β 2 is increased (Fig. 1c and d). HPV may, in fact, promote increased TGF β 2 expression in order to promote higher levels of E6-E7 transcription (Fig. 2a). We repeatedly failed to achieve stable knockdown of TGF β 2 in HPV-containing cells, suggesting that TGF β 2 provides a growth-promoting effect. It is also possible that HPV-mediated upregulation of TGF β 2 helps the virus remodel its surrounding microenvironment, such as by driving generation and differentiation of regulatory T cells or reactive stroma (56).

TGF β 1 and active DNA demethylation. Thillainadesan et al. previously showed that TGF β 1 induces active demethylation of the p15^{ink4a} promoter by recruiting enzymes such as DNA glycosylases TDG and MBD4 and base excision repair components (49). In this study, we have identified active DNA demethylation as the mechanism involved in TGF β -mediated upregulation of IFN- κ ; however, the exact sequence of events in this pathway remains unknown. In most studies of DNA demethylation, 5-methyl-cytosine (5mC) is an unfavorable substrate for cleavage by TDG and MBD4 (50). Instead, 5mC is thought to be oxidized by the Ten Eleven Translocation (TET) proteins 1 to 3 to form 5-hydroxymethylcytosine (5hmC) (50). After oxidation, 5hmC may then be further modified and cleaved by TDG and/or MBD4 (50). TET proteins may be recruited to the IFN- κ promoter, since we observed that MBD4 knockdown was unable to prevent TGF β 1-mediated induction of IFN- κ , in contrast to TDG knockdown (Fig. 7); TDG is the only glycosylase so far shown with activity against 5hmC intermediates, unlike MBD4 (57). It remains to be determined whether/how TGF β signaling can induce the recruitment of TET proteins or TDG to CpGs in the methylated IFN- κ promoter.

Interestingly, data from Thillainadesan et al. also suggested that TGF β plays a role in globally decreasing DNA methylation using dot blot analysis of bulk genomic DNA with anti-5mC antibodies, which was reversed after TDG knockdown. It is possible that IFN- κ promoter demethylation is a consequence of global DNA demethylation in TGF β 1-treated cells. However, Thillainadesan et al. also showed that TGF β 1 induces the recruitment of SMADs to SMAD-binding elements on the p15^{ink4a} promoter, which can then recruit TDG and MBD4 (49). Interactions of SMADs with interferon response factors (IRFs) or AP-1 have been reported previously (28, 43, 44, 58, 59), and we have observed putative IRF and AP-1 binding sites at CpGs 4 and 5 in the IFN- κ promoter (data not shown). Therefore, it is possible that TGF β treatment induces interactions between SMADs and IRFs or AP-1 at the IFN- κ promoter, bringing along active demethylation machinery. It is also possible that TGF β 1 promotes the binding of NF1 (52) to the IFN- κ promoter, since we did predict a binding site for NF1 between CpGs 4 and 5, although promoter binding studies will be necessary to explore this idea.

Both HPV oncoproteins E6 and E7 have previously been shown to regulate DNA methyltransferases (60–62), but only E6 has been shown to induce hypermethylation of the IFN- κ promoter in keratinocytes (15). The mechanism of E6-induced hypermethylation has not been determined. DNA methylation can occur actively or passively. Active DNA methylation involves the activity of *de novo* DNA methyltransferases (DNMTs) DNMT3A and DNMT3B on CpG sites (50). DNA methylation can also occur passively when newly synthesized DNA is modified through activity of DNMT1, which associates with the replication fork (50). Our data suggest that HPV promotes passive methylation

of the IFN- κ promoter because TGF β -mediated upregulation of IFN- κ transcripts persisted several days after drug removal (Fig. 8). These kinetics are consistent with a mechanism requiring cellular DNA replication.

It is possible that targeting active DNA demethylation is a means to reverse hypermethylation of tumor suppressor genes or antiviral genes, including IFN- κ , in HPV-induced cancers (63, 64). In addition, the ability of TGF β to induce promoter demethylation in HPV-containing cells could be exploited for both research and therapeutic purposes. Future studies may expand on the roles of oncoproteins in regulating specific methyltransferases and growth factor signaling to prevent innate immune responses and investigate the mechanism of IFN- κ -specific ISG inhibition of the viral life cycle. These studies shed light on the antagonism between HPV and TGF β and open the door to potential strategies to manipulate the TGF β signaling pathway for therapeutic purposes.

MATERIALS AND METHODS

Cell culture. Human foreskin keratinocytes (HFKs) were isolated from discarded and deidentified neonatal foreskins. HFKs containing HPV16 genomes (W12 strain) were created by transfection and selection as previously described (36). HFKs expressing individual HPV oncogenes were created by retroviral transduction as described previously (36). HFKs and keratinocyte-derived cell lines were cultivated in E medium with 5% fetal bovine serum (FBS) in the presence of mitomycin C-treated NIH 3T3 J2 fibroblast feeders (36, 65). Normal oral keratinocytes immortalized by retroviral transduction of hTERT were described previously (66). Cell lines derived from at least three donors were used in separate experiments, and data were compiled from at least three individual experiments. Low-passage-number HFKs (fewer than 10) and low-passage-number HFKs containing HPV16 genomes (fewer than 12 passages postselection) were used in each experiment. Episomal maintenance of the viral DNA was confirmed by Southern blotting as previously described (65). Differentiation was induced by suspending cells in E medium containing 1.6% methylcellulose (MC) for 24 or 48 h, followed by washing with phosphate-buffered saline (PBS) (65). TGF β 1 or TGF β 2 was reconstituted in sterile 4 mM HCl containing 1 mg/ml bovine serum albumin per the manufacturer's instructions and added to methylcellulose or to monolayer medium at a concentration of 5 ng/ml at the time of cell seeding (see Table S1 in the supplemental material for a list of drugs and antibodies used). 5-Aza-2'-deoxycytidine was reconstituted in dimethyl sulfoxide (DMSO) per the manufacturer's instructions and added to cells in monolayer at a concentration of 5 nmol/liter. Medium was changed daily with fresh drug added for a total treatment time of 96 h. Ruxolitinib was reconstituted in DMSO per the manufacturer's instructions and added to cells in monolayer and methylcellulose at a concentration of 10 μ M 1 h prior to TGF β treatment.

Lentivirus preparations. For TGF β 1 knockdown experiments, shRNA lentiviruses were produced by transfecting human embryonic kidney (HEK293T) cells with pLKO.1 TRC vectors (Dharmacon nontarget control TRC shRNA or TGF β 1 TRCN000005803), together with psPAX2 packaging plasmid and pMD2.G envelope plasmid (kind gifts from Jeremy P. Kamil), using polyethylenimine (Polysciences). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and antibiotics. At 48 h posttransfection, virus-containing supernatant medium was filtered through 0.22- μ m filters. Virus stocks were frozen at -80°C until used.

Two donor genetic backgrounds of HPV16 cells were infected with either nontargeting control shRNA lentivirus particles or particles targeting TGF β 1 along with Polybrene (5 μ g/ml). Feeder fibroblasts were added 6 h after infection, followed by puromycin (2 μ g/ml) selection 24 h later. Following selection and expansion, cells were seeded in monolayer medium or methylcellulose for 24 h and analyzed for TGF β 1 protein levels using Western blotting. Cell lines with less than 50% TGF β 1 were analyzed for HPV transcript and genome levels.

siRNA transfection. TDG was targeted with ON-TARGETplus SMARTpool L-003780-01-0005 and MBD4 with ON-TARGETplus SMARTpool L-011554-00-0005, and the negative control was D-001810-10-05 (all from Dharmacon). Cells were plated in 24-well plates at a cell density of 100,000 cells per well in E medium with 5% FBS. Twenty-four hours later, cells were washed with PBS and then transfected at concentrations of 10 nmol (MBD4) or 100 nmol (TDG) using DharmaFECT1 at a concentration of 5 μ l/ml in DMEM with FBS according to the manufacturer's protocol. After 6 h of transfection, we changed the medium to E medium with FBS and treated cells with TGF β 1 at 24 h (MBD4) or 72 h (TDG) posttransfection, depending on when we were able to detect knockdown. Cell lines derived from at least three donors were used in each experiment.

RNA extraction, qPCR, and Western blotting. Total RNA was isolated using RNA-STAT 60 (TelTest, Inc.), digested with RNase-free DNase (Promega), phenol-chloroform extracted, and reverse transcribed using qScript (Quanta) as described previously (67). Quantitative PCR (qPCR) was performed using the PerfeCTa SYBR green SuperMix ROX (Quanta) on an Applied Biosystems StepOne Plus real-time PCR machine using the primers listed in Table S2. Western blotting was performed as reported previously, using antibodies listed in Table S1 (68), and imaged using near-IR secondary antibodies on a Li-Cor Odyssey infrared imager.

DNA extraction, bisulfite treatment, and CpG methylation analysis. Total DNAs were isolated using phenol-chloroform extraction as described previously (67). Bisulfite treatment was performed with the Zymo EZ DNA methylation direct kit as directed by the manufacturer, using 200 ng of purified DNA.

CpG methylation was evaluated by PCR amplification with IFN- κ promoter bisulfite-specific primers previously established (same PCR conditions as those in reference 15) with AmpliTaq Gold 360 master mix. PCR amplicons were TA cloned into the pCR4-TOPO@TA vector (Thermo Fisher) and sequenced by Genewiz. Sequences from bisulfite-treated DNA were then aligned to the promoter region of human IFN- κ genomic sequence [NM_020124.2](#) (NCBI reference sequence) using the web-based tool Quantification Tool for Methylation Analysis (QUMA) (69). To control for sequence quality, bisulfite-treated sequences that contained more than 10 base mismatches and/or gaps after alignment with the genomic sequence were excluded (69). To control for incomplete bisulfite conversion, bisulfite-treated sequences that contained more than 5 unconverted CpGs (CpA, CpC, and CpT) and/or bisulfite-treated sequences with a percent conversion (number of converted CpGs divided by the total number of CpGs) lower than 95% were also excluded (69). Data were compiled from two independent TGF β -treated bisulfite modifications and included sequences from 7 different clones each. Percent methylation was determined by dividing the number of given sequences in a sample with methylated CpGs by the total number of sequences included.

Statistics. To determine the statistical significance of our methylation data, we used the QUMA tool to perform a statistical analysis between the methylation profiles of the different groups (69). This program determines the statistical significance of the difference between two bisulfite sequence groups at each CpG site using Fisher's exact test and the entire set of CpG sites with the Mann-Whitney U test. Significance of other experiments was calculated using Welch's unequal-variance *t* test.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JVI.01714-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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