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***Human papillomavirus (HPV) downregulates the expression of IFITM1 to resist the anti-proliferative effects of IFN $\gamma$  and TNF $\alpha$***

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*Submitted*

## ABSTRACT

Basal keratinocytes (KCs) are responsible for renewal of the epithelium and are the target cells for high risk human papilloma viruses (hrHPVs) which may cause KCs to become transformed. The immune system has developed means to counteract infections through several mechanisms, including the suppression of viral spread through the proliferation of infected cells via the production of the effector cytokines IFN $\gamma$  and TNF $\alpha$ . These two cytokines are known to synergize in the suppression of KC proliferation. Using an unique system for freshly established or persistent hrHPV infection, we show that hrHPV renders KCs resistant to the growth inhibitory effects of these cytokines. Furthermore, a comparative analysis of marker expression for senescence (*GLB1*), apoptosis (*BAX* and *BCL2*) and proliferation (*RARRES1* and *PCNA*) showed that hrHPV specifically resists the induction of an anti-proliferative state induced by IFN $\gamma$  in KCs. hrHPV accomplished this by targeting the expression of the anti-proliferative gene *IFITM1*, the expression of which was down-regulated already at 48 hours after hrHPV infection. Knock-down of *IFITM1* in uninfected KCs confirmed its role in providing resistance to the anti-proliferative effects of IFN $\gamma$  and TNF, whereas the analysis of *IFITM1*, *RARRES1* and *PCNA* in cells isolated from clinical samples of HPV-positive and – negative (pre-)malignant vulvar cells underlined the relevance of our finding. Thus, our study revealed that hrHPV targets *IFITM1* in order to evade the anti-proliferative effects of IFN $\gamma$  and TNF $\alpha$ .

## **IMPORTANCE**

A persistent infection with high-risk human papillomavirus (hrHPV) may cause cancer. In order to combat viruses the immune system has developed several mechanisms to counteract infections. One such a mechanism is the production of two cytokines, called interferon-gamma and tumor necrosis factor-alpha, which can prevent the proliferation of infected cells and as such can suppress viral spread. However, hrHPV has developed ways to evade the host's immune response for sustained periods of time. We showed that hrHPV accomplishes this by downregulating the expression of a negative regulator of cell growth called interferon-induced transmembrane protein 1 (IFITM1). When the cytokines of the immune system activate IFITM1 in non-infected cells a cellular program is started that stops cell proliferation. Downregulation of IFITM1, allows hrHPV infected cells to evade the anti-proliferative effects of the immune system on hrHPV-infected cells, thus promoting viral spread and the ability of hrHPV-induced lesions to progress.

## INTRODUCTION

High-risk human papillomaviruses (hrHPVs) infect undifferentiated keratinocytes (KCs) of squamous epithelia. Persistent infections may lead to cancers of the anogenital region as well as of the head and neck [1]. Studies in healthy individuals, immunosuppressed patients and in patients with spontaneously or vaccine-induced regressions revealed an important role for a strong type 1 (IFN $\gamma$  and TNF $\alpha$ )-associated HPV early antigen-specific T cell response in the control of HPV [2].

IFN $\gamma$  is a pleiotropic cytokine that affects immune regulation, immune surveillance, inflammation, tumor suppression, and has antiviral as well as anti-proliferative properties. Binding of IFN $\gamma$  to its receptor (IFN $\gamma$ R) leads to JAK1/2-mediated STAT1 phosphorylation, dimerization and nuclear translocation, resulting in interferon-stimulated gene expression [3]. TNF $\alpha$  also regulates immune and cell death mechanisms. It activates NF $\kappa$ B and MAP kinase pathways and induces the formation of cell death complexes [4]. hrHPV attenuates immune signalling of the STAT1 [5-8], IRF and NF $\kappa$ B pathways [9-15], resulting in suppressed innate and adaptive antiviral responses.

IFN $\gamma$  and TNF $\alpha$  are known to synergize in the suppression of KC proliferation [16]. IFN $\gamma$  induces growth arrest and differentiation [17,18]. TNF $\alpha$  also induces growth arrest but there are conflicting data concerning its capacity to induce cell death of primary KCs [16,19]. In unstimulated KCs, HPV can regulate cell growth via its early (E) proteins. E6 and E7 promote proliferation by directly modulating p53 and p21, however, their expression is regulated by E2, which can also induce apoptosis. E5 can both protect and induce apoptosis. E1<sup>E4</sup> expression results in growth arrest [20,21]. Previously, it was shown that retrovirus-mediated expression of E6 and/or E7 in KCs resulted in downregulation of IFN $\gamma$  responsive genes and the upregulation of genes associated with cellular proliferation [7,22]. However, the ability of HPV-infected KCs to resist the effects of IFN $\gamma$  and/or TNF $\alpha$  on proliferation as well as the underlying mechanisms are not well understood.

In this study, we analyzed the influence of HPV on the IFN $\gamma$  and TNF $\alpha$ -

mediated cell growth inhibition of KCs by functional and biochemical analyses. Here we show that hrHPV presence renders KCs more resistant to the anti-proliferative effects of *IFN $\gamma$*  and *TNF $\alpha$* , via the downregulation of *IFITM1*. Ex-vivo analysis of KCs isolated from clinically obtained control tissue and HPV-induced (pre)malignancies of the vulva confirmed our observations *in situ*.

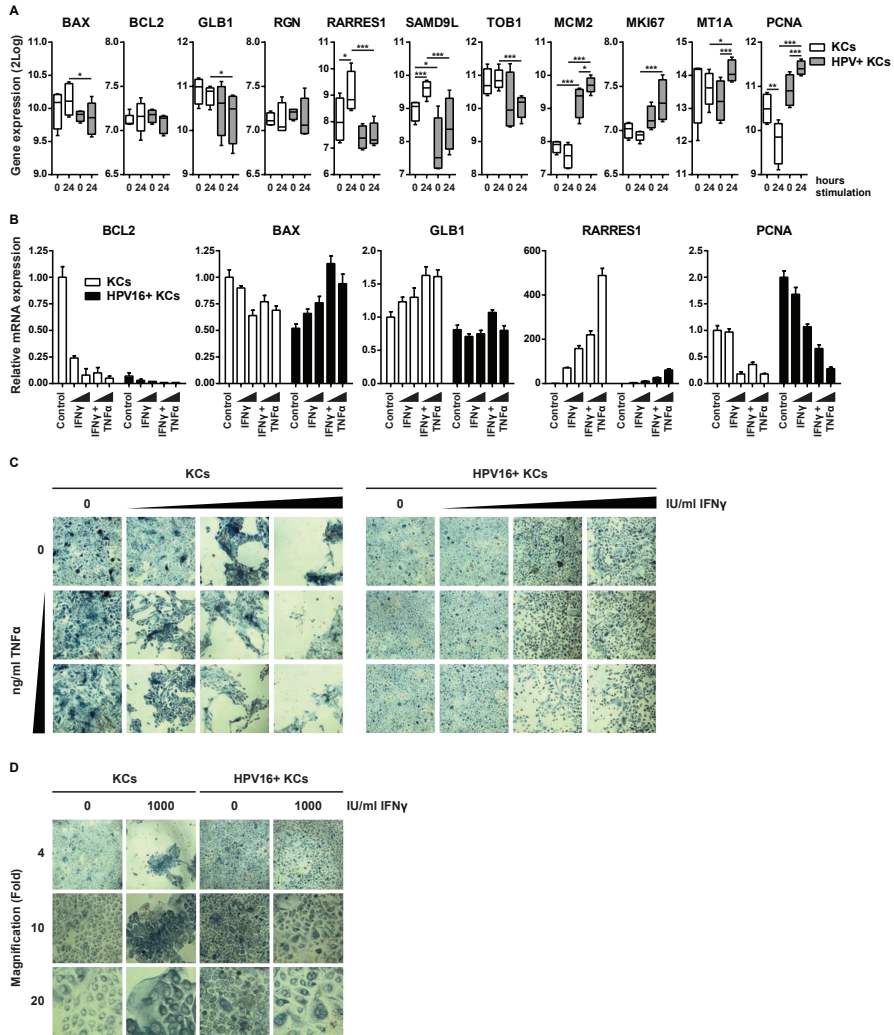
## RESULTS

### HPV hampers the anti-proliferative state of KCs upon IFN $\gamma$ and/or TNF $\alpha$ treatment

We previously reported that hrHPV suppresses the IFN $\gamma$  and TNF $\alpha$ -induced immune response of KCs. Since IFN $\gamma$  and TNF $\alpha$  are also known to synergize in the suppression of KC proliferation by inducing growth arrest and differentiation [16-19], we studied the influence of HPV herein. First, our previously reported validated microarray, in which uninfected KCs of four different donors and four different hrHPV-infected KCs were pre-stimulated with IFN $\gamma$  for 72 hours, and subsequently treated with control or IFN $\gamma$  for another 24 hours [15], was re-analysed for gene expression of markers indicative for apoptosis, senescence, or proliferation (Figure 1A). Genes indicative for apoptosis (*BAX*, *BCL2*) and senescence (*GLB1*, *RGN*) were not differentially expressed, whereas genes indicative for anti-proliferation (*RARRES1*, *SAMD9L*, *TOB1*) were downregulated and pro-proliferative genes (*MCM2*, *MKI67*, *MT1A*, *PCNA*) upregulated in hrHPV+ KCs compared to KCs after stimulation.

To validate the microarray data and investigate the additive role of TNF $\alpha$ , KCs and HPV+ KCs were harvested after 24 hours of IFN $\gamma$  and TNF $\alpha$  stimulation and the gene expression of markers indicative for apoptosis, senescence, or proliferation were determined by RT-qPCR (Figure 1B). In this setting, uninfected KCs displayed a slight increase in the expression of the senescence marker beta-Galactosidase (*GLB1*), a strong reduction in the expression of the anti-apoptosis marker *BCL2* while the expression level of *BAX* was marginally affected (Figure 1B). Importantly, this analysis confirmed the anti-proliferative state of IFN $\gamma$ - and TNF $\alpha$ -stimulated KCs since the expression level of *RARRES1*, a marker for anti-proliferation [23,24], was highly upregulated and in parallel, the level of the proliferation marker *PCNA* was decreased.

In hrHPV-positive KCs, the expression levels of *BCL2*, *GLB1* and *BAX* mirrored that of non-infected KCs, albeit that the basal level of the anti-apoptotic gene *BCL2* was lower in hrHPV+ KCs. Analysis of the genes involved in proliferation revealed that the basal levels of *PCNA* were higher in hrHPV-positive KCs than in uninfected KCs. In contrast to non-infected KCs stimulated with IFN $\gamma$  and/or TNF $\alpha$ , the hrHPV+ KCs displayed only a marginal



**Figure 1: hrHPV resists IFN $\gamma$  and TNF $\alpha$ -induced growth inhibition**

(A) Microarray intensities for BAX, BCL2, GLB1, RGN, RARRES1, SAMD9L, TOB1, MCM2, MKI67, MT1A and PCNA in 72 hours IFN $\gamma$  (50 IU/ml) pre-treated four independent KCs and four independent hrHPV+ KCs, stimulated with IFN $\gamma$  (50 IU/ml) for 0 or 24 hours, represented in a box plot. The box contains the 1st quartile up to the 3rd quartile, the median is represented as a line, whiskers represent the values of the outer 2 quartiles. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

(B) RT-qPCR of BCL2, BAX, GLB1, RARRES1 and PCNA in 24 hours Control, IFN $\gamma$  (50 or 250 IU/ml) or IFN $\gamma$  and TNF $\alpha$  (50 IU/ml IFN $\gamma$  + 50 ng/ml TNF $\alpha$  or 250 IU/ml IFN $\gamma$  + 250 ng/ml TNF $\alpha$ )-treated KCs and HPV16+ KCs. Gene expression was normalized using GAPDH as the calibrator gene. Fold changes over control-stimulated undifferentiated KCs were calculated and depicted.



(C) Microscopy pictures (4x magnification) of 72 hours IFN $\gamma$  (0, 50, 250 or 1000 IU/ml) and/or TNF $\alpha$  (0, 50 or 250 ng/ml)-treated KCs and HPV16+ KCs.

(D) Microscopy pictures (4x, 10x and 20x magnifications) of 72 hours IFN $\gamma$ -treated KCs and HPV16+ KCs.

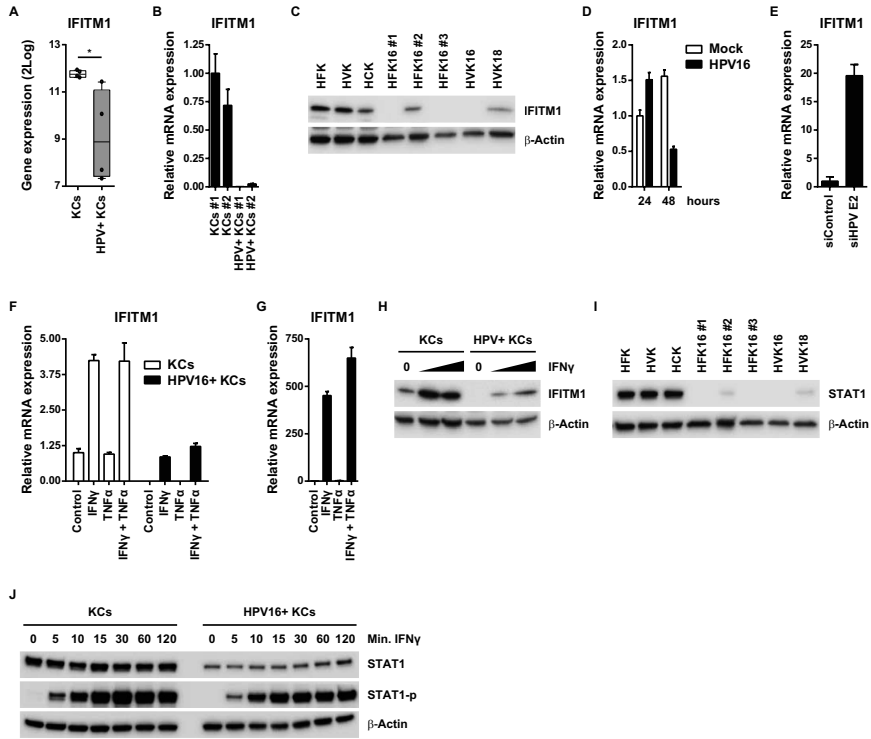
Representative results of three independent experiments.

increase in *RARRES1* expression and the levels of *PCNA* were only reduced to the levels observed in uninfected KCs upon stimulation with a combination of the highest doses of IFN $\gamma$  and TNF $\alpha$ .

To confirm that hrHPV-positive KCs are indeed less sensitive to the IFN $\gamma$  and/or TNF $\alpha$ -induced arrest in proliferation, uninfected KCs and hrHPV-positive KCs, seeded into 96 well plates, were treated for four days with increasing doses of IFN $\gamma$  and/or TNF $\alpha$ . Cell confluence was monitored by phase-contrast microscopy as a measure of proliferation since we and others [25] had observed that KC proliferation can not be quantified via usual proliferation assays (data not shown). As expected, the growth of uninfected KCs was greatly affected by increasing doses of IFN $\gamma$ . In contrast, hrHPV-positive KCs were much more resistant (Figure 1C). TNF $\alpha$  in itself appeared not to affect the growth of uninfected or HPV-infected KCs, but when combined with IFN $\gamma$  augmented the reduction in cell density (Figure 1C). We observed that the remaining KCs after treatment displayed a senescence-like morphology [26] following IFN $\gamma$  stimulation (Figure 1D), fitting well with the upregulated expression of *GLB1* in the uninfected KCs. All together, these data confirm that IFN $\gamma$  or IFN $\gamma$  and TNF $\alpha$  affect the growth of uninfected KCs by arresting their proliferation and skewing them to a senesced, pro-apoptotic state. Moreover, our data clearly indicate that hrHPV alters the IFN $\gamma$  and TNF $\alpha$ -regulated proliferative pathway in KCs by resisting the induction a proliferative arrest.

### **HPV downregulates the expression of IFITM1**

The interferon-induced transmembrane protein 1 (IFITM1) plays an essential role in the anti-proliferative action of IFN $\gamma$  [27], making it a potential target for hrHPV. Indeed, re-analysis of the data from one of our earlier validated microarrays, in which the basal expression of genes measured in different uninfected and hrHPV infected KCs was compared [28], showed that *IFITM1* expression is downregulated in HPV-positive KCs (Figure 2A).



**Figure 2: HPV downregulates IFITM1 expression**

(A) Microarray intensities for IFITM1 in four independent KCs and four independent hrHPV+ KCs represented in a box plot. The box contains the 1st quartile up to the 3rd quartile, the median is represented as a line, whiskers represent the values of the outer 2 quartiles. \*  $p < 0.05$ .

(B) RT-qPCR of IFITM1 expression in two independent KCs and two independent HPV16+ KCs cultures.

(C) Western blot of IFITM1 protein levels in three independent KC, four independent HPV16+ KC, and one HPV18+ KC cultures.

RT-qPCR of IFITM1 expression in KCs infected with mock or HPV16 for 1 or 2 days, as indicated (D), and siControl and siHPV16 E2-transfected HPV16+ KCs (E).

(F) RT-qPCR of IFITM1 expression in 24 hours IFN $\gamma$  (50 IU/ml) and/or TNF $\alpha$  (50 ng/ml)-stimulated KCs and HPV16+ KCs. Fold changes over control-stimulated undifferentiated KCs were calculated and depicted.

(G) RT-qPCR of IFITM1 expression in 24 hours IFN $\gamma$  (50 IU/ml) and/or TNF $\alpha$  (50 ng/ml)-stimulated HPV16+ KCs. Fold changes over control-stimulated HPV16+ KCs were calculated and depicted.

(H) IFITM1 protein levels in KC and HPV18+ KC stimulated with IFN $\gamma$  (0, 100 or 1000 IU/ml).

(I) STAT1 protein levels in three independent KC, four independent HPV16+ KC, and one HPV18+ KC cultures.

(J) STAT1 and phosphorylated STAT1 protein levels in KCs and HPV16+ KCs stimulated with IFN $\gamma$  (50 IU/ml) as indicated.

*Representative results of at least two independent experiments.*

This was confirmed by RT-qPCR (Figure 2B) and western blot (Figure 2C) in different independent hrHPV-positive primary KC cultures. To show that the expression of *IFITM1* was genuinely altered by the presence of hrHPV in KCs, undifferentiated KCs were infected with native HPV16 virions resulting in a reduced expression of *IFITM1* two days after infection (Figure 2D). Reciprocally, the knock-down of total HPV16 early gene expression by introduction of siRNA against HPV16 E2 in HPV-positive KCs [14], resulted in the upregulation of *IFITM1* (Figure 2E).

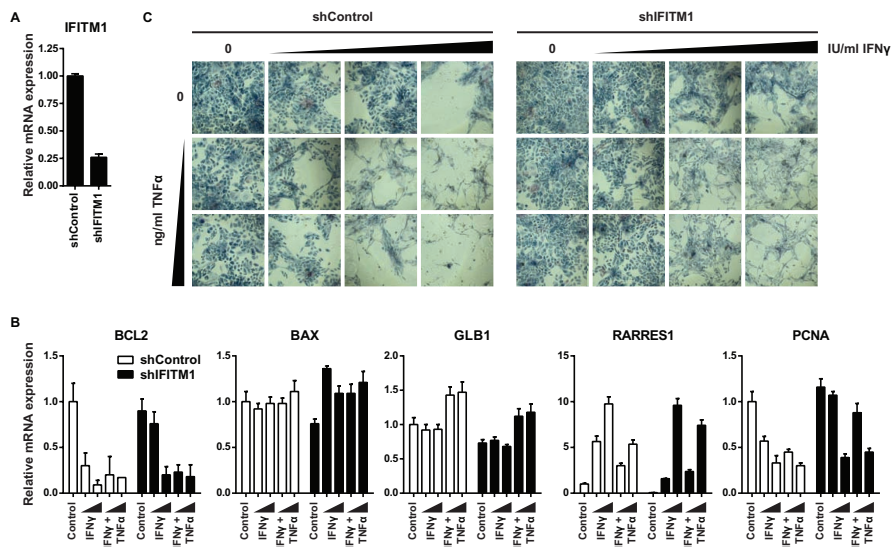
IFN $\gamma$  induces *de novo* synthesis of *IFITM1* for which STAT1 is required [29-32]. Indeed, IFN $\gamma$  stimulation of uninfected KCs resulted in approximately 4-fold increase in *IFITM1* after 24 hours (Figure 2F). Strikingly, IFN $\gamma$  stimulation of hrHPV+ KCs resulted in a much stronger relative increase of *IFITM1* levels (Figure 2G), albeit that these levels still remained lower than those measured in uninfected KCs (Figure 2F). *IFITM1* protein levels in IFN $\gamma$ -stimulated KCs and hrHPV+ KCs confirmed the gene expression data (Figure 2H). These data indicated that hrHPV predominantly regulates the expression of *IFITM1* at the basal level but less at the level of IFN $\gamma$ -mediated induction of *IFITM1* gene expression. It has been reported that HPV can lower *STAT1* mRNA and protein levels in KCs [5-8], and this was also detected in the hrHPV+ KCs analyzed at the protein level (Figure 2I). Interestingly, the HPV+ KCs with the highest basal *IFITM1* protein expression (Figure 2C) also showed the highest *STAT1* levels (Figure 2I). Concomitant with the induction of *IFITM1* expression, IFN $\gamma$  stimulation also stimulated the phosphorylation of *STAT1* (Figure 2J). Together this indicates that HPV represses the basal levels of *STAT1* but does not interfere with *STAT1* signalling in our persistently hrHPV infected KCs. Furthermore, it explains why IFN $\gamma$  is able to stimulate the expression of *IFITM1*. TNF $\alpha$  did not influence *IFITM1* expression (Figure 2F-G).

### **IFITM1 downregulation helps to overcome the anti-proliferative effects of IFN $\gamma$ and TNF $\alpha$**

To study the effects of *IFITM1* on KC proliferation in a setting where all additional influences of HPV are ruled out [20,21], *IFITM1* was knocked-down in uninfected KCs (Figure 3A). The KCs were stimulated with IFN $\gamma$  or a combination of IFN $\gamma$  and TNF $\alpha$ . *IFITM1* knock-down KCs displayed a less pronounced downregulation of *BCL2* and *PCNA* upon IFN $\gamma$  stimulation

(Figure 3B). The expression of *RARRES1* was lower at the basal level when *IFITM1* was knocked-down and its *IFN $\gamma$* -induced expression was only affected when KCs were stimulated with a low but not with a higher concentration of *IFN $\gamma$* . Importantly, *IFITM1* knock-down KCs were more resistant to the anti-proliferative effects of *IFN $\gamma$*  and the combination of *IFN $\gamma$*  and *TNF $\alpha$*  than control shRNA and non-transduced KCs (Figure 3C).

Thus, HPV is able to resist *IFN $\gamma$* -mediated arrest of proliferation by lowering the basal levels of *IFITM1*.



**Figure 3: IFITM1 downregulation helps to overcome the anti-proliferative effects of *IFN $\gamma$*  and *TNF $\alpha$***

(A) RT-qPCR of *IFITM1* expression in control and *IFITM1* knock-down KCs.

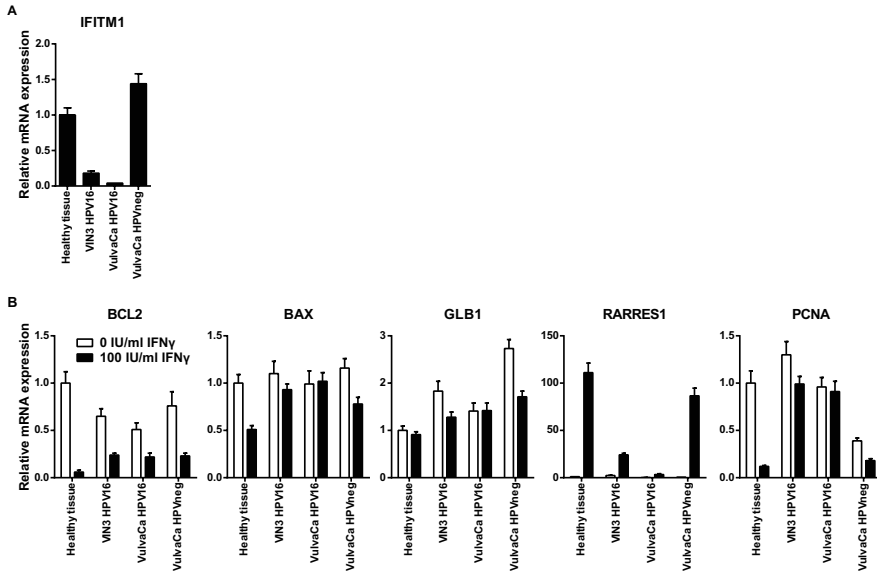
(B) RT-qPCR of *BCL2*, *BAX*, *GLB1*, *RARRES1* and *PCNA* in 24 hours Control, *IFN $\gamma$*  (50 or 250 IU/ml) or *IFN $\gamma$*  and *TNF $\alpha$*  (50 IU/ml *IFN $\gamma$*  + 50 ng/ml *TNF $\alpha$*  or 250 IU/ml *IFN $\gamma$*  + 250 ng/ml *TNF $\alpha$* )-treated control and *IFITM1* knock-down KCs.

(C) Microscopy pictures (4x magnification) of 72 hours *IFN $\gamma$*  (0, 50, 250 or 1000 IU/ml) and/or *TNF $\alpha$*  (0, 50 or 250 ng/ml)-treated control and *IFITM1* knock-down KCs.

Representative results of three independent experiments.

**IFITM1 expression is down in HPV-induced premalignant and malignant vulvar cells.**

In order to study the relevancy of these results we analysed the five genetic markers for apoptosis, senescence and proliferation in cells isolated from clinical biopsies taken from women suffering from different stages of HPV-induced (pre)malignancies. The isolated cells were treated with 0 or 100 IU/ml IFN $\gamma$  for 24 hours and gene expression was analyzed by RT-qPCR. Basal *IFITM1* expression was lower in the cells isolated from a HPV16-induced vulvar intraepithelial neoplasia (VIN) and a HPV16-induced vulvar carcinoma when compared to that in the KCs obtained from control tissue or an HPV-negative vulvar carcinoma (Figure 4A). The levels of *GLB1* and *BAX* were similar between control and HPV16+ vulvar cells, both at the basal level as after IFN $\gamma$  stimulation. The expression of *BCL2* was lower in the HPV16+ vulvar cells than in controls, but similar to control KCs, the HPV16+ cells displayed a decreased *BCL2* expression upon stimulation with IFN $\gamma$  (Figure 4B), albeit that control KCs show a stronger decrease. The levels of *PCNA* and *RARRES1* were similar in control cells and HPV16+ vulvar cells, however upon stimulation with IFN $\gamma$  only the control cells showed a strong decrease in *PCNA* expression and a stronger increase in *RARRES1* when compared to the HPV16+ vulvar cells (Figure 4B). Interestingly, the cells isolated from an HPV-negative vulvar carcinoma reacted more or less similar as control cells, pointing out the HPV-specific component in these analyses. These results indicate that also *in situ* hrHPV+ cells display a reduced expression of *IFITM1* and a concomitant resistance to IFN $\gamma$ -induced arrest of proliferation.



**Figure 4: IFITM1 expression is decreased in HPV-induced VIN lesions**

(A) RT-qPCR of IFITM1 expression in KCs derived from clinical biopts.

(B) RT-qPCR of BCL2, BAX, GLB1, RARRES1 and PCNA in 24 hours control or IFN $\gamma$  (100 IU/ml)-stimulated KCs derived from clinical biopts.

## DISCUSSION

Using a unique *in vitro* model we here show that hrHPV infection renders KCs resistant to IFN $\gamma$  and TNF $\alpha$ -induced arrest of cell growth. Analysis of the expression of markers representative for senescence, apoptosis and proliferation of KCs showed that HPV specifically counteracts the arrest in cell proliferation of KCs when stimulated by IFN $\gamma$ . The resistance of hrHPV+ cells to an IFN $\gamma$ -mediated proliferative arrest was associated with a strong downregulation in the basal expression of the negative regulator of cell growth *IFITM1* and an impaired IFN $\gamma$ -mediated increase in the expression of the anti-proliferative *RARRES1* gene. Notably, this basal and IFN $\gamma$ -stimulated gene profile was also found *ex vivo*, in cells isolated from biopsies of HPV-induced (pre-)malignant vulvar lesions.

The IFN $\gamma$ -induced increase in expression of both *IFITM1* and *RARRES1* depends on STAT1, which is downregulated by HPV E6 and E7 proteins [5-7]. Basal *IFITM1* expression is downregulated in HPV+ KCs, but *RARRES1* is not, which might be explained by the fact that the basal expression of *RARRES1* in uninfected KCs is already low. Our data confirm the hrHPV-mediated decrease in STAT1 protein levels but also show that hrHPV does not hamper IFN $\gamma$ -induced STAT1 activation, as reflected by STAT1 phosphorylation and increase in *RARRES1* and *IFITM1* expression in HPV+ KCs. Still, as total STAT1 levels are lower in HPV+ KCs, the amount of available STAT1 to phosphorylate and signal is lower in hrHPV+ KCs potentially explaining why the increase in *RARRES1* and *IFITM1* expression does not reach the levels observed in uninfected KCs. This is also demonstrated in our study showing that the effect of *IFITM1* knock-down on proliferation of uninfected KCs does not resemble the influence of hrHPV on KCs. Whilst the effect of *IFITM1* in uninfected KCs is apparent and anti-proliferative as indicated by the retained expression of *PCNA* and *RARRES1* in KCs stimulated with a low dose of IFN $\gamma$  when *IFITM1* was knocked-down, clearly the downregulation of STAT1 as well as the positive growth signals as delivered by hrHPV [20,21] are missing in these cells. Hence differences in IFN $\gamma$ -stimulated arrest of proliferation are less noticeable. Thus, whereas the decreased basal level of *IFITM1* is already providing resistance to the IFN $\gamma$ -stimulated arrest of proliferation, the downregulation of STAT1 is

likely to exaggerate this effect.

The downregulation of *IFITM1* clearly is advantageous to hrHPV as it allows infected KCs to expand. Mechanistically, *IFITM1* inhibits the phosphorylation of ERK and thus regulates mitogen-activated protein (MAP) kinase signalling [27]. Furthermore, *IFITM1* mediates the dephosphorylation of p53 at Thr55 resulting in increased p53 stability and transcriptional activity, as indicated by the upregulated expression of p21. Consequently, arrest occurs in cell cycle progression at the G1 phase and, hence, a halt in proliferation [27]. This is also reflected by the retained *PCNA* expression when *IFITM1* was knocked-down in low dose *IFN $\gamma$* -stimulated KCs. Conceivably, the effect of hrHPV on *IFITM1* in infected KCs extends to HPV-induced cancer cells as we found that cells isolated from an HPV16-induced vulvar tumor, but not cells isolated from a non-HPV induced vulvar tumor, displayed a strongly decreased level of *IFITM1* and a highly impaired response to *IFN $\gamma$*  stimulation with respect to the expression of *RARRES1* and *PCNA*.

In conclusion, hrHPV allows infected KCs to resist the *IFN $\gamma$* -induced anti-proliferative state by regulating the expression of (anti-)proliferative genes through regulation of STAT1 and *IFITM1*. This identifies *IFITM1* as one the proteins within the *IFN $\gamma$* -signalling pathway that is targeted by hrHPV to evade the anti-proliferative effects of the immune system on hrHPV-infected cells, thus promoting viral spread and the ability of hrHPV-induced lesions to progress.



## **MATERIALS & METHODS**

### **Ethics Statement**

The use of discarded human foreskin, cervical and vaginal keratinocyte tissues to develop cell lines for these studies was approved by the Institutional Review Board at the Pennsylvania State University College of Medicine and by the Institutional Review Board at Pinnacle Health Hospitals. The Leiden University Medical Ethic Committee approved our study on prospective collection of healthy control tissue and for keratinocyte isolation patients were enrolled in the Circle study, which investigates cellular immunity against HPV-induced neoplasia. All human samples were anonymized.

### **Cell culture**

Primary cultures of human epithelial keratinocytes (KCs) were established from foreskin, vaginal, vulva and cervical tissues as previously described [28] and grown in keratinocyte serum-free medium (K-SFM; Medium 154 supplemented with HKGS kit, Invitrogen, Breda, The Netherlands). KCs stably maintaining the full episomal HPV genome following electroporation (HPV-positive KCs) were grown in monolayer culture using E medium in the presence of mitomycin C (Sigma-Aldrich) treated J2 3T3 feeder cells [33,34] for two passages and were then adapted to K-SFM for one passage before experimentation. J2 3T3 mouse fibroblasts were cultured in Iscove's modified Dulbecco's medium supplemented with 8% fetal bovine serum, 2 mM l-glutamine and 1% penicillin-streptomycin (complete IMDM medium) (Gibco-BRL, Invitrogen, Breda, The Netherlands).

### **HPV16 knock-down in HPV16-positive KCs and infection of undifferentiated keratinocytes**

HPV16-positive KCs were transfected with 50 nM Control or HPV16 E2 siRNA for at least 72 hours as previously described [14]. Primary basal layer human foreskin keratinocytes were infected with native HPV16 at MOI 100 as previously described [14]. Cells were washed and harvested and target gene expression was assayed by RT-qPCR.

### **IFITM1 knock-down in undifferentiated KCs**

shRNA's were obtained from the MISSION TRC-library of Sigma-Aldrich (Zwijndrecht, The Netherlands). The MISSION shRNA clones are sequence-verified shRNA lentiviral plasmids (pLKO.1-puro) provided as frozen bacterial glycerol stocks (Luria Broth, carbenicillin at 100  $\mu$ g/ml and 10% glycerol) in *E. coli* for propagation and downstream purification of the shRNA clones. pLKO.1 contains the puromycin selection marker for transient or stable transfection. The construct against IFITM1 (NM\_003641) was TRCN0000057499: CCGGCCTCATGACCATTGGATTCAT CTCGAGATGAATCCAATGGTCATGAGGTTTTTG and the control was: SHC004 (MISSION TRC2-pLKO puro TurboGFP shRNA Control vector): CCGGCGTGATCTTCACCGACAAGATCTCGAGATCTTGTCGGTGAAGATCACGT TTTT. KCs at ~60% confluency were transduced with lentivirus at MOI 5-10 over night, after which medium was replaced. At least 72 hours post-transduction cells were harvested, washed and plated as indicated and allowed to attach overnight. Cell were stimulated as indicated and assayed accordingly.

### **Proliferation assay**

KC, HPV+ KCs, control shRNA-expressing KCs, or IFITM1 shRNA-expressing KCs were seeded 5,000 cell/well in 96-well plates and allowed to attach over night. Cells were cultured in presence of indicated concentrations of IFN $\gamma$  (Immunotools, Friesoythe, Germany) and/or TNF $\alpha$  (Invivogen, Toulouse, France) in 150  $\mu$ l for 96 hours. 15  $\mu$ l/well MTT (3-(4,5-dimethylthiazol-2-yl)-2,3-diphenyl-2H-tetrazolum bromide) stock solution (5 mg/ml in 0.1 M PBS) was added for 3 hours. When the purple formazan precipitate was clearly visible under the microscope, bright light pictures were made using an Olympus IX51 inverse fluorescence microscope (Olympus, Zoeterwoude, The Netherlands) and ColorView II Peltier-cooled charge-coupled device camera (Olympus), and archived using Cell<sup>F</sup> software (Olympus).

### **RNA expression analyses**

All microarray data is accessible in the Gene Expression Omnibus database. The microarray data of Karim *et al.* [28] (accession number GSE21260) compared four independent KC cultures with four independent HPV+ KCs cultures, whereas the microarray data of Tummers *et al.* [15]

(accession number GSE54181) compared four independent KC cultures with four independent HPV+ KCs cultures that were pre-stimulated with IFN $\gamma$  for 72 hours after which they were treated with IFN $\gamma$  in the presence of Control L-cells for 24 hours. Plots were generated using the webtool R2: microarray analysis and visualization platform (<http://r2.amc.nl>).

KC, HPV+ KCs, control shRNA-expressing KCs, or IFITM1 shRNA-expressing KCs were seeded 150,000 cell/well in 12-well plates and allowed to attach over night. Cells were cultured in presence of indicated concentrations of IFN $\gamma$  and/or TNF $\alpha$  in 1 ml for 24 hours. Total RNA was isolated using the NucleoSpin RNA II kit (Machery-Nagel, Leiden, The Netherlands) according to the manufacturer's instructions. Total RNA (0.5 – 1.0  $\mu$ g) was reverse transcribed using the SuperScript III First Strand synthesis system from Invitrogen. TaqMan PCR was performed using TaqMan Universal PCR Master Mix and pre-designed, pre-optimized primers and probe mix for *IFITM1*, *BAX*, *BCL2*, *GLB1*, *RARRES1*, *PCNA* and *GAPDH* (Applied Biosystems, Foster City, USA). Threshold cycle numbers (Ct) were determined using the CFX PCR System (BioRad, Veenendaal, The Netherlands) and the relative quantities of cDNA per sample were calculated using the  $\Delta\Delta$ Ct method using GAPDH as the calibrator gene. The error bars indicate standard deviations of triple PCR measurements.

### **Western blot analysis**

Polypeptides were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane (Bio-Rad, Veenendaal, The Netherlands). Immunodetection was achieved with anti-IFITM1 (1:1000, PA5-20989, Thermo Scientific) anti-STAT1 (1:1000, #9172, Cell Signaling Technology (CST)), anti-phospho-STAT1 (Tyr701, 1:1000, #9167, CST), b-actin (1:10,000, Sigma-Aldrich) primary antibodies, and HRP-coupled anti-mouse (1:5000, CST) and HRP-coupled anti-rabbit (1:5000, CST) secondary antibodies. Chemoluminescence reagent (Bio-Rad) was used as substrate and signal was scanned using the Chemidoc and accompanying Software (Bio-Rad).

### **Conflict of interest**

CM has received speaker honoraria from Merck, Quest Diagnostics, GSK,

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