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# Human papillomavirus type 16 E6 and E 7 proteins alter NF-kB in cultured cervical epithelial cells and inhibition of NF-kB promotes cell growth and immortalization

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ABSTRACT

The NF-kB family of transcription factors regulates important biological functions including cell growth, survival and the immune response. We found that Human Papillomavirus type 16 (HPV-16) E7 and E6/E7 proteins inhibited basal and TNF-alpha-inducible NF-kB activity in human epithelial cells cultured from the cervical transformation zone, the anatomic region where most cervical cancers develop. In contrast, HPV-16 E6 regulated NF-kB in a cell type- and cell growth-dependent manner. NF-kB influenced immortalization of cervical cells by HPV16. Inhibition of NF-kB by an IkB alpha repressor mutant increased colony formation and immortalization by HPV-16. In contrast, activation of NF-kB by constitutive expression of p65 inhibited proliferation and immortalization. Our results suggest that inhibition of NF-kB by HPV-16 E6/E7 contributes to immortalization of cells from the cervical transformation zone.



## Introduction

The major risk factor for cervical cancer is infection with a subset of high risk human papillomaviruses (HPV) including HPV type 16 (HPV-16). The HPV-16 E6 and E7 genes are important as they are selectively retained and expressed in most cervical cancers and they are sufficient to immortalize human epithelial cells (Munger et al., 1989). The E7 protein inactivates the retinoblastoma tumor suppressor protein and promotes cell cycle progression (Jones et al., 1997). E6 stimulates degradation of the p53 tumor suppressor protein (Scheffner et al., 1990) and contributes to immortalization by activating hTERT, the catalytic subunit of telomerase (Klingelhutz et al., 1996). In addition to effects on cell proliferation, HPV E6 and E7 proteins interfere with specific components the innate immune response (Chang and Laimins, 2000; Georgopoulos et al., 2000; Hasan et al., 2007; Nees et al., 2001; Ronco et al., 1998; Woodworth and Simpson, 1993) which may contribute to persistent HPV infection. These interactions are important because persistent infection with high risk HPVs and immortalization of epithelial cells are early events in the development of cervical cancer.

The NF-kB family of transcription factors regulates multiple biological functions. NF-kB serves a major role in the inflammatory and innate immune responses by stimulating expression of cytokines, cytokine receptors, and histocompatibility genes (Hayden et al., 2006). Several viruses have evolved mechanisms to regulate NF-kB, and activation or inhibition contributes to virus persistence, replication, or transformation of infected cells (Hiscott et al., 2006). NF-kB is active in epithelial cells of the cervix (Nees et al., 2001), and epithelial cells are an important component of the innate immune system.

NF-kB can function as an oncogene through its ability to stimulate cell proliferation and survival. NF-kB is constitutively activated in several human cancers (Karin, 2006; Li et al., 2005), including cancer of the cervix (Branca et al., 2006; Nair et al., 2003; Prusty et al., 2005). Activation of NF-kB promotes malignant development and progression in several animal models (Erez et al., 2010; Greten et al., 2004; Pikarsky et al., 2004) and NF-kB has been proposed to be an important link between chronic inflammation and cancer (Karin, 2009). Inhibition of NF-kB is a potential target for cancer therapy and chemoprevention (Baud and Karin, 2009). However, NF-kB can also act as a tumor suppressor in epidermal squamous cell carcinoma (Dajee et al., 2003; van Hogerlinden et al., 1999) and liver cancer (Maeda et al., 2005). We are interested in whether HPV infection alters NF-kB activation in human cervical epithelial cells and whether alterations in NF-kB contribute to cervical carcinogenesis.

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The HPV-16 E6 and E7 proteins regulate NF-kB, but conflicting evidence exists as to whether they stimulate (Hussain et al., 2011; James et al., 2006; Nees et al., 2001; Xu et al., 2010) or suppress activation (Havard et al., 2002, 2005; Huang and McCance, 2002; Perea et al., 2000; Spitkovsky et al., 2002). In this regard, the NF-kB activation pathway is dependent of the type of cell and context of the signal. Our first goal was to clarify how HPV-16 E6 and E7 proteins regulate NF-kB in epithelial cells cultured from the cervical transformation zone. These cells are the natural target for HPV infection and the progenitors for most cervical cancers (Burghardt and Ostor, 1983). The importance of NF-kB in the regulation of growth and immortalization of HPV-16 infected cervical cells is unclear. Our second goal was to determine whether inhibition or activation of NF-kB regulates cell proliferation or immortalization of epithelial cells from the cervical transformation zone by HPV-16.

## Results

### *Immortalization decreases NF-kB activity*

We examined activation of NF-kB in human epithelial cells isolated from three anatomic regions of the cervix including ectocervix, endocervix and the transformation zone (Fig. 1A). Most cervical cancers arise within the transformation zone (Burghardt and Ostor, 1983) where the columnar endocervical epithelium is replaced by metaplastic squamous epithelium. Cells cultured from each region demonstrated different morphology (Fig. 1B) and grew well in monolayer culture. We used a reporter gene assay to compare NF-kB activity in the normal cells and five HPV-16-immortalized cervical cell lines (three from transformation zone cells, two from endocervical cells) that were derived after transfection with the complete HPV-16 genome. NF-kB activity was significantly higher ($p \leq 0.05$) in early passage, unimmortalized cells from endocervix or the transformation zone than in ectocervical cells (Fig. 2A). NF-kB activity decreased significantly ($p \leq 0.05$) in each of the five HPV-16-immortalized cell lines compared to their respective normal cell type. We also examined whether expression of several NF-kB-responsive genes was down regulated in a subset of the immortalized cervical cell lines that had reduced NF-kB. RNAs for beta-defensins 1, 2, 3, and 5 were significantly

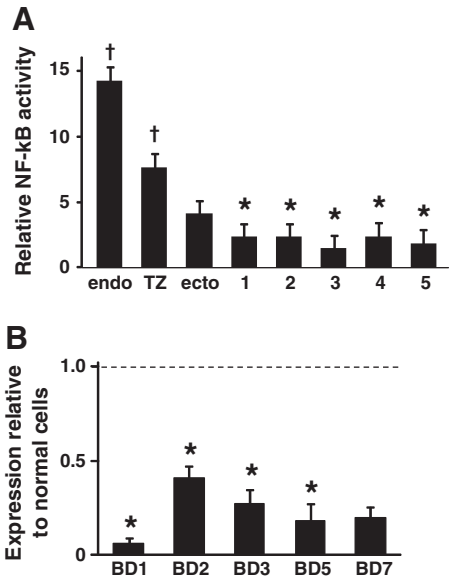


**Fig. 2.** Immortalization by HPV-16 decreases NF-kB activity and expression of β-defensins in cervical cells. A. Relative NF-kB activity in confluent cultures of primary cells derived from: endocervix (endo), transformation zone (TZ), ectocervix (ecto), and five immortal cell lines derived from either TZ (1, 2, and 3) or endocervix (4 and 5). Each bar represents the mean of at least three reporter gene experiments ± standard error. Asterisks are values that differ statistically ($p \leq 0.05$) from TZ cells (1, 2, 3) or endocervical cells (4 and 5). Crosses are values that differ significantly from ectocervical cells. B. Real time PCR analyses of expression of RNAs for β-defensins in confluent HPV-immortalized cervical cells with reduced NF-kB activity (average of lines 2 and 3 from TZ and lines 4 and 5 from endocervix) expressed as percentage of normal cervical cells (average of five normal cultures). Each bar represents the mean of 4 different immortal cell lines ± standard error. Asterisks show values that differ statistically ($p \leq 0.05$) from normal cells.

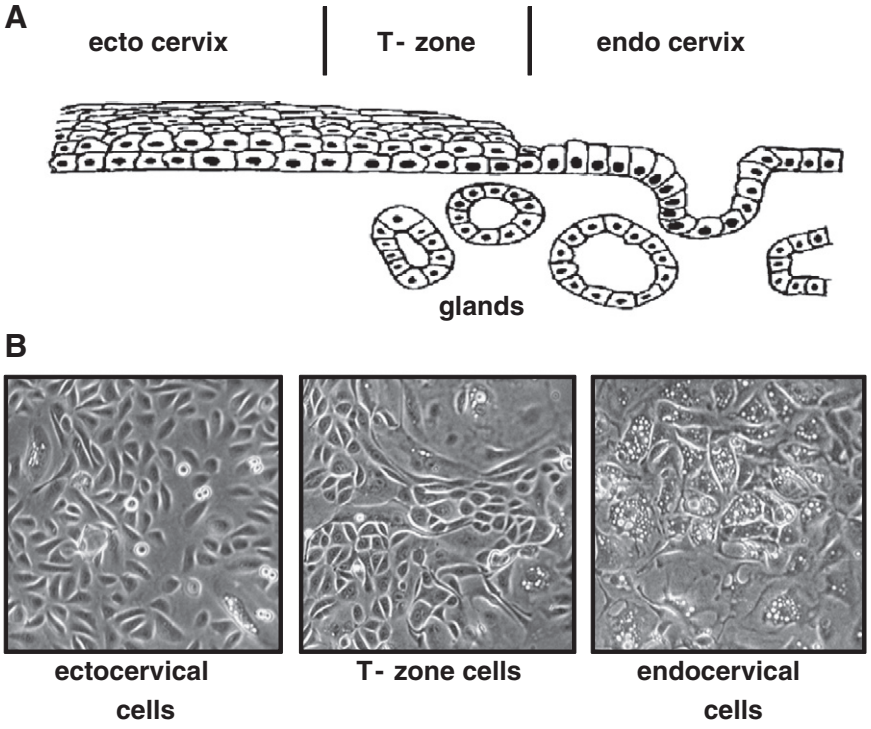


**Fig. 1.** Origin and morphology of cervical cell cultures. A. Schematic illustration showing the histology of the ectocervix, transformation zone (T-zone), and endocervix. B. Phase contrast micrograph of cervical cells from each region growing in monolayer culture.

decreased ($p \leq 0.05$) in immortal cells (two from transformation zone and two from endocervix) with respect to normal cervical cells (Fig. 2B). Thus, immortalization of cervical cells decreased NF-kB activity and expression of specific NF-kB-responsive genes.

*The HPV-16 E7 protein decreases NF-kB activity*

The HPV-16 E6 and E7 proteins are necessary and sufficient for immortalization of primary epithelial cells. We examined whether E6, E7 or both were required for down regulation of NF-kB in epithelial cells cultured from the cervical transformation zone. Cultures of normal human cervical cells were infected with retroviruses encoding HPV-16 E6, E7, E6/E7 or vector only (control) and examined for NF-kB activity using a reporter gene assay. The results from a typical experiment are shown in Fig. 3. NF-kB activity was significantly higher ($p \leq 0.05$) in slowly growing confluent cultures than in rapidly growing sub confluent cultures. Treatment with TNF-α for 24 h induced NF-kB under both conditions (Fig. 3). The E7 and E6/E7 proteins significantly inhibited baseline NF-kB as well as TNF-α-induced NF-kB activation. However, the effects of E6 were variable (see below).

Because the activity of NF-kB varied in cells cultured from different individuals and in different experiments, we performed four independent experiments using cells from the cervical transformation zones of different donors. Although our work focused on transformation zone cells, other cell types (endocervical, ectocervical, foreskin and oral epithelial cells) were used for comparison and to examine cell-type specific affects on NF-kB activation. The results of each experiment were normalized with respect to the internal control (vector only = 1). In both foreskin and cervical cells, the E7 protein reduced baseline NF-kB as well as TNF-α-inducible activity compared to the control (Fig. 4). To examine the mechanism for down regulation of NF-kB, we used an HPV-16 E7 mutant that is defective in the ability to bind the retinoblastoma (Rb) protein (Edmonds and Vousden, 1989). Infection of cervical transformation zone cells with retroviruses encoding this mutant did not decrease basal or TNF-α-inducible NF-kB (Fig. 5), indicating that the integrity of the Rb binding site on E7 is necessary for its ability to inhibit NF-kB.

*The HPV-16 E6 protein regulates NF-kB in a cell-type and cell-growth dependent manner*

In contrast to HPV-16 E7, the E6 protein did not significantly alter NF-kB activity in confluent cultures of cells from the cervical transformation zone or foreskin (Fig. 4, left). However, E6 increased baseline and TNF-α-inducible NF-kB by two- to three-fold in rapidly growing foreskin cells (Fig. 4, bottom right). The effect of E6 in proliferating

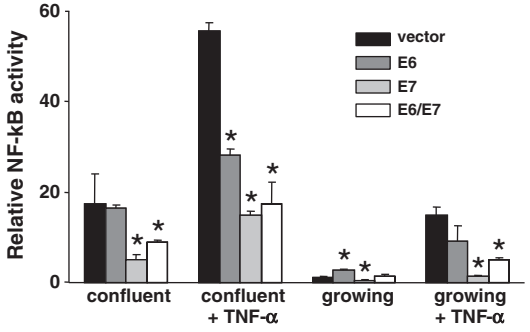


**Fig. 3.** HPV-16 E6 and E7 proteins regulate baseline and TNF-α-inducible NF-kB in cells from cervical transformation zone. Typical reporter gene experiment showing relative NF-kB activity in cells infected with HPV-16 retroviruses and maintained under different culture conditions including 95% confluence (confluent) or less than 50% confluence (growing) ± 1.0 nM TNF-α for 24 h. Bars equal the mean of 3 replicate wells ± standard error and asterisks are values that differ statistically ($p \leq 0.05$) from the control (vector) within each of the four culture conditions.

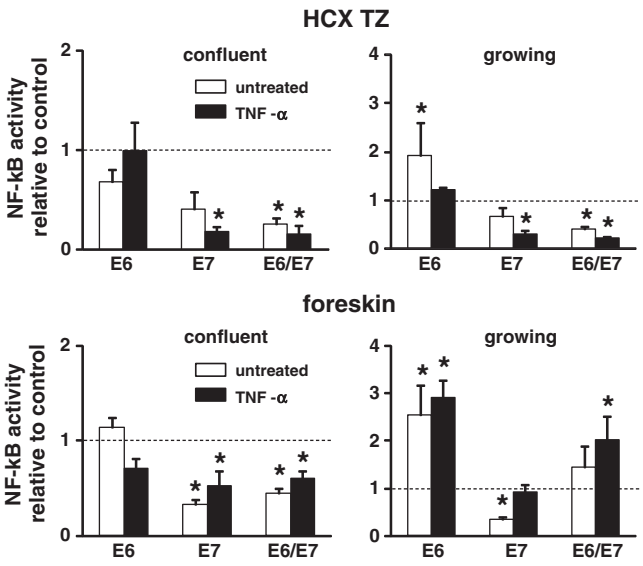


**Fig. 4.** HPV-16 E6 and E7 genes regulate NF-kB differently in cervical transformation zone cells (TZ) and foreskin cells maintained as growing or confluent cultures. NF-kB activation is shown relative to control (vector = 1) in cultures of cervical cells (top) and foreskin keratinocytes (bottom) that were 95% confluent (growing slowly) or growing rapidly (less than 50% confluent) ± 1.0 nM TNF-α for 24 h. Bars indicate the mean ± standard error of at least 3 experiments using cells from different donors, and asterisks are values that differ statistically ($P \leq 0.05$) from the control (vector).

foreskin cells was dominant over E7 because E6/E7 together also increased NF-kB (Fig. 4, bottom right). In addition, the effect of E6 was dependent on cell type because E6 or E6/E7 did not significantly induce NF-kB in cells from the cervical transformation zone (Fig. 4, top).

*The HPV-16 E6/E7 proteins decrease NF-kB in different types of epithelial cells*

HPV-16 infects epithelia from different anatomic sites. Therefore, we compared effects of HPV-16 E6/E7 proteins on cells derived from different types of epithelia including ectocervical, foreskin, oral, and transformation zone epithelium. Infection with E6/E7 reduced NF-kB activity consistently in confluent cultures of oral epithelial cells, foreskin cells and transformation zone cells (Fig. 6) but not in

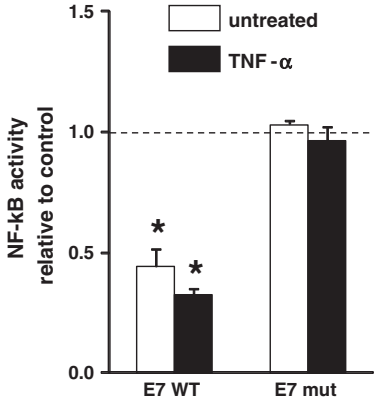


**Fig. 5.** Down regulation of NF-kB by HPV-16 E7 depends upon inactivation of the Rb tumor suppressor protein. NF-kB activity relative to control (vector = 1) in confluent cervical cells from the transformation zone after infection with HPV-16 retroviruses that encode either wild type E7 protein or the p24gly E7 mutant (E7 mut) that is defective for binding the retinoblastoma tumor suppressor protein. Each bar represents the mean ± standard error of at least 3 experiments using cells from different donors. Asterisks represent values that are statistically different ($p \leq 0.05$) than the control (vector = 1).

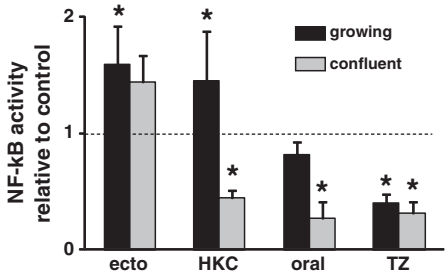


**Fig. 6.** HPV-16 E6/E7 expression alters NF-kB activity relative to control (vector = 1) in cultures of growing or confluent cells from ectocervical epithelium (ecto), foreskin epithelium (HKC), oral epithelium, or the transformation zone (TZ). Bars indicate the mean ± standard error from at least 3 independent experiments and asterisks are values that differ statistically ($p \leq 0.05$) from control.

cultures derived from ectocervical epithelium. In contrast, E6/E7 stimulated NF-kB in growing cultures of foreskin and ectocervical cells. Cells from the transformation zone were unique in that E6/E7 inhibited NF-kB under both confluent and growing conditions (Fig. 6). Cells cultured from ectocervix or the transformation zone grew at approximately the same rate with a doubling time of 36 to 42 h.

*Regulation of clonal growth by NF-kB*

NF-kB regulates a variety of pathways that control cell growth, survival, and gene expression. Because NF-kB is known to promote development and progression of cancer (Karin, 2006; Li et al., 2005), we hypothesized that reduced NF-kB would inhibit cell growth and immortalization of cervical transformation zone epithelial cells by HPV-16. To examine this hypothesis, we used a dominant negative mutant of IkBα (IkBαm) to inhibit activation (Van Antwerp et al., 1996). IkBαm lacks sites for phosphorylation by IkB kinase so it is not ubiquitinated and degraded. Therefore, active NF-kB subunits do not accumulate in the nucleus. We also used an expression vector for p65 to increase NF-kB activation. Primary cultures from the cervical transformation zone were cotransfected with the complete HPV-16 genome plus an expression vector for either IkBαm, p65 or the empty vector (pLZRS) as a negative control. Cells were selected for resistance to the antibiotic G418 (the HPV-16 plasmid contains the neomycin resistance gene) and then plated at clonal density to measure colony formation. We used a reporter gene assay to confirm that p65 stimulated NF-kB activity and that IkBαm inhibited NF-kB relative to the control pLZRS (Fig. 7A).

Unexpectedly, cells that were cotransfected with IkBαm plus HPV-16 formed larger and more numerous colonies than cells transfected with HPV-16 plus the pLZRS vector (Fig. 7B). In contrast, cells cotransfected with HPV-16 plus the p65 expression vector formed fewer and smaller colonies, and cells grew slowly. In six independent experiments, IkBαm stimulated colony formation by two-fold and p65 inhibited colony formation by 70% (Fig. 7C, HPV-16). To check our results, we used two additional assays involving (1) infection of normal cervical transformation zone cells with retroviruses encoding the HPV-16 E6/E7 genes and subsequent transfection with p65 or IkBαm and (2) transfection of seven different HPV-16 immortalized cell lines with p65 or IkBαm. In each assay, p65 expression significantly inhibited colony formation ($p \leq 0.05$) and IkBαm significantly stimulated colony formation (Fig. 7C).

*HPV-16 induced immortalization is regulated by NF-kB*

We examined whether immortalization of cells by the complete HPV-16 genome or the E6/E7 genes was influenced by NF-kB. Cells from the cervical transformation zone were (1) cotransfected with

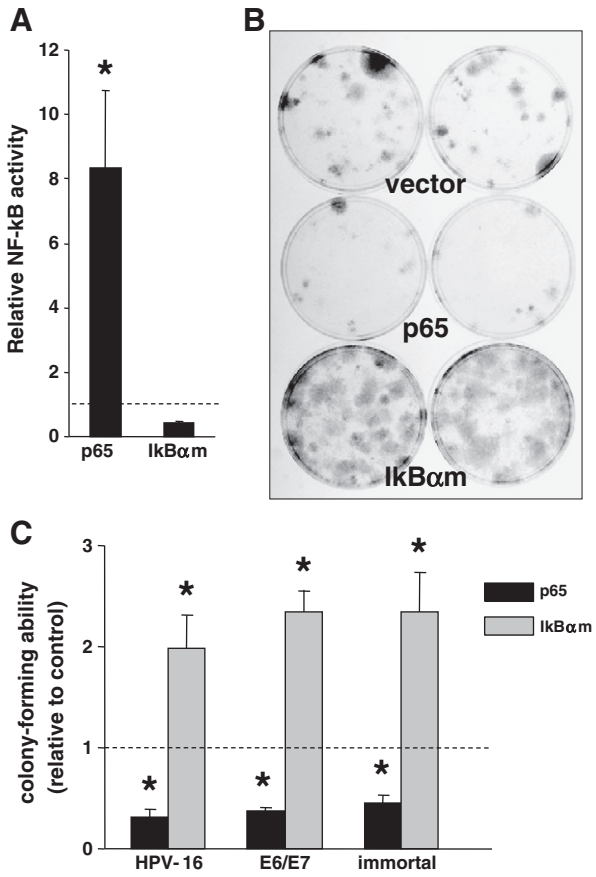


**Fig. 7.** Genetic manipulation of NF-kB influences growth of HPV-16 expressing cervical transformation zone cells. A. Transfection of cervical cells with a p65 expression construct or the IkBαm super repressor mutant activates or inhibits relative NF-kB activity, respectively. B. Cotransfection of P65 or IkBαm with HPV-16 DNA inhibits or stimulates colony formation, respectively in normal cervical cells from the transformation zone. C. Measurement of colony forming ability relative to control (vector = 1) after cotransfection of normal cells with HPV-16 DNA (HPV-16) and either vector, p65 or IkBαm, after cotransfection of E6/E7 retrovirus-infected cells (E6/E7), or after cotransfection of seven different HPV-16-immortalized cell lines (immortal). Each bar represents the mean ± standard error of at least 3 independent experiments and asterisks are values that differ statistically ($P \leq 0.05$) from control (vector).

the complete HPV-16 genome plus either p65, IkBαm or the vector, or (2) infected with HPV-16 E6/E7 retroviruses then cotransfected with the puromycin resistance gene plus either IkBαm, p65 or vector. The negative control for immortalization consisted of cells that did not receive HPV-16 or E6/E7. Transfected or infected/transfected cultures were maintained in medium containing G418/puromycin to select for stably transduced cells. The resulting colonies were pooled and subcultured repeatedly (usually two to three times) before the negative controls without HPV-16 became senescent.

In three independent experiments using cells from different individuals, IkBαm increased the percentage of cultures that contained immortal cells (Table 1). This was true for immortalization by the complete HPV16 genome or retrovirus infection with E6/E7. In contrast, p65 inhibited immortalization by HPV-16 or the E6/E7 genes. To examine whether the effects of NF-kB on immortalization were specific for HPV-16, we also performed immortalization assays after transfection with Simian Virus 40 (SV40) DNA (Woodworth et al., 1986). SV40 is a DNA tumor virus that resembles HPV-16 because both inactivate the retinoblastoma and p53 tumor suppressor proteins. In three independent experiments, expression of IkBαm significantly increased immortalization of cervical transformation zone cells by SV40 and the p65 expression vector decreased immortalization (Table 1). When data for HPV-16, E6/E7 retrovirus

**Table 1**
Effect of p65 or IkBαm on the frequency of immortalization of human cervical cells by the complete HPV-16 genome, HPV16 E6/E7 genes, or SV40.

| Experimental group | Exp-1 | Exp-2 | Exp-3 | total |
|---|---|---|---|---|
| HPV-16 + vector | 3/6[1] | 3/5 | 2/5 | 8/16 |
| HPV-16 + p65 | 0/5 | 2/6 | 1/6 | 3/17 |
| HPV-16 + IKBα | 5/6 | 4/4 | 4/6 | 13/16 |
| Retrovirus E6/E7 + vector | 4/5 | 4/6 | 2/4 | 10/15 |
| Retrovirus E6/E7 + p65 | 2/5 | 1/6 | 1/6 | 4/17* |
| Retrovirus E6/E7 + IKBα | 6/6 | 6/6 | 6/6 | 18/18* |
| SV40 + vector | 4/6 | 2/4 | 2/6 | 8/16 |
| SV40 + p65 | 1/5 | 2/6 | 0/5 | 3/16* |
| SV40 + IKBα | 4/6 | 5/5 | 4/5 | 13/16* |

[1] Number of dishes with immortal cells/total number of dishes. Each column is an independent experiment using cells from the cervical transformation zone of different individuals. Asterisks indicate values that were statistically different ($p \leq 0.05$) than the vector control.

infection, and SV40 were combined, p65 inhibited immortalization strongly ($p \leq 0.001$) and IkBαm stimulated immortalization strongly ($p \leq 0.001$) relative to the vector-only control. These results indicate that activation of NF-kB inhibits immortalization, but that inhibition is not specific for HPV-16.

*P65 inhibits expression from the HPV-16 long control region (LCR)*

Continuous expression of the HPV-16 E6 and E7 genes is required for immortalization (von Knebel Doeberitz et al., 1992), and expression of E6/E7 is regulated by promoters and enhancers located within the LCR, a short sequence of DNA that is upstream of E6. We used a reporter gene assay to examine whether cotransfection with p65 or IkBαm regulated expression of a luciferase reporter gene driven by the HPV-16 LCR. Transfection of normal cervical transformation zone cells with p65 stimulated NF-kB strongly and inhibited expression from the HPV-16 LCR by 60% (Fig. 8). Transfection with IkBαm decreased NF-kB but it did not alter HPV-16 LCR activity. These results suggest that p65 inhibits immortalization in part by decreasing HPV gene expression, but they do not provide evidence that IkBαm stimulates immortalization by increasing HPV expression.

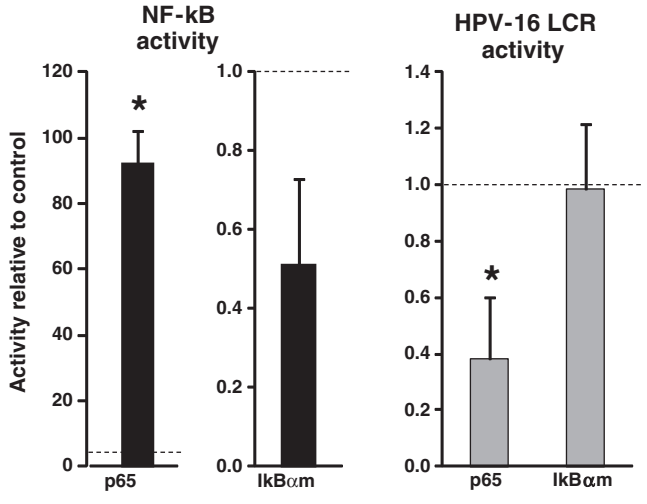


**Fig. 8.** Stimulation of NF-kB activity by transfection with p65 causes decreased expression from the HPV-16 LCR. Normal cervical transformation zone cells were cotransfected with p65, IkBαm or vector (control) plus a reporter gene for either NF-kB (left) or the HPV-16 LCR (right). Bars represent the mean ± standard error of 3 experiments and asterisks are values that differ statistically ($P \leq 0.05$) from control (vector = 1).

## Discussion

Persistent infection with high-risk HPVs and immortalization of epithelial cells are important early events in the development of cervical cancer. We found that HPV-16 E6 and E7 proteins inhibited NF-kB activity in cells cultured from the cervical transformation zone, the region where most cervical carcinomas develop. NF-kB serves an important role in the host immune response. Down regulation of NF-kB might represent a unique mechanism by which HPV interferes with innate immunity and promotes persistent infection. We also found that inhibition of NF-kB stimulated HPV-16-induced cell proliferation and immortalization of cells from the transformation zone. Thus, NF-kB has the potential to act as a tumor suppressor in cervical cells.

The HPV-16 E7 and E6/E7 proteins inhibited basal and TNF-α-inducible NF-kB activity in cells from the transformation zone. Furthermore, immortalization by HPV-16 decreased expression of several β-defensins that are NF-kB-inducible cationic peptides that contribute to innate immunity (Diamond et al., 2008). HPV-16 is known to interfere with specific NF-kB-responsive pathways that are important for immunity including expression of major histocompatibility genes (Georgopoulos et al., 2000), Toll-like receptor-9 (Hasan et al., 2007), and interferons (Nees et al., 2001; Ronco et al., 1998). The ability of HPV-16 E6/E7 to decrease NF-kB activation is a common denominator that might contribute to each of these responses. Our observation that inhibition NF-kB was dependent on the Rb binding domain of E7 is consistent with the fact that Rb is important for NF-kB activation and that adenovirus E1A inhibits NF-kB in an Rb-dependent manner (Cook et al., 2002; Takebayashi et al., 2003).

In contrast, we observed that HPV-16 E6 altered NF-kB in a cell type- and cell growth-dependent manner. E6 and E6/E7 stimulated activation in foreskin and ectocervical cells that were rapidly growing. However, E6 and E6/E7 were less effective in cells from the transformation zone or in slowly growing cultures. Our results are consistent with the fact that NF-kB regulation is often dependent on cell-type and context (Hinata et al., 2003). Conflicting evidence exists about whether HPV-16 stimulates (Hussain et al., 2011; James et al., 2006; Nees et al., 2001; Xu et al., 2010) or suppresses activation of NF-kB (Huang and McCance, 2002; Perea et al., 2000; Spitkovsky et al., 2002). Our observations help to clarify these results by showing that growth rate and cell type are important determinants of whether HPV-16 E6 or E6/E7 stimulate or inhibit NF-kB. The mechanism by which E6 activates NF-kB is incompletely understood. The E6 protein reduces levels of the p53 tumor suppressor protein (Scheffner et al., 1990), and p53 decreases NF-kB activation (Webster and Perkins, 1999). In addition, the ability of the E6 protein to activate the transcriptional regulator NFX1-91 or to target PDZ domains may also be important (James et al., 2006; Xu et al., 2010).

Chronic inflammation and activation of NF-kB contribute to several types of human cancer (Karin, 2009). NF-kB is constitutively activated in high grade squamous intraepithelial neoplasia and cervical cancer (Branca et al., 2006; Nair et al., 2003; Prusty et al., 2005). However, we observed that expression of HPV-16 E6/E7 and cell immortalization inhibited NF-kB in cells from the cervical transformation zone. This suggests that activation of NF-kB during cervical carcinogenesis may not be autonomous for epithelial cells. Instead, activation may result from changes in the tumor microenvironment such as the presence of chronic inflammatory cells and activated fibroblasts (Greten et al., 2004; Pikarsky et al., 2004). The latter are known to promote inflammation and tumor formation in an NF-kB-dependent manner in an HPV-16 transgenic mouse model (Erez et al., 2010).

NF-kB stimulates cell growth and survival and can function as an oncogene. Inhibitors of NF-kB sensitize cervical cancer cells to apoptosis (Venkatraman et al., 2005). Therefore, we originally hypothesized that activation of NF-kB would stimulate cell growth and immortalization. However, we observed the opposite. We found

that activation of NF-kB in normal cervical cells inhibited clonal growth and immortalization by HPV-16. Similarly, inhibition of NF-kB by dominant negative IkBαm stimulated clonal growth and the frequency of immortalization. Stimulation was observed using the complete HPV-16 genome, the E6/E7 genes, or the tumor virus SV40. This indicates that effects of IkBαm and p65 are not specific for cells expressing HPV-16. Our results are consistent with the observation that p65 inhibits growth of normal human keratinocytes (Seitz et al., 1998) and that inhibition of NF-kB promotes epidermal carcinogenesis in mice (Dajee et al., 2003; van Hogerlinden et al., 1999). The apparent difference in NF-kB responsiveness between normal and cancerous cervical cells is potentially important, although our experiments did not address this question.

The mechanism(s) by which NF-kB inhibits cell growth and immortalization of normal cervical cells is incompletely understood. Expression of the HPV-16 E6 and E7 genes is required for continued growth of cervical carcinoma cells and HPV-immortal cell lines (von Knebel Doeberitz et al., 1992). The HPV-16 LCR contains a binding site for NF-kB (Fontaine et al., 2000) and we found that p65 inhibited expression from the HPV-16 LCR in a reporter gene assay. This suggests that one mechanism by which NF-kB activation might inhibit growth and immortalization is by decreasing HPV gene expression. However, we also found that the IkBαm did not stimulate HPV-16 LCR activity. Thus, decreased NF-kB does not appear to promote growth and immortalization by stimulating HPV-16 gene expression. Additional mechanisms might contribute to the ability of NF-kB to inhibit cell growth and immortalization. Activation of NF-kB signaling promotes cell senescence (Rovillain et al., 2011) and NF-kB RelA opposes epidermal proliferation driven by TNFR1 and JNK (Zhang et al., 2004).

## Conclusions

We show that HPV-16 E6 and E7 proteins inhibit basal and TNF-α-inducible NF-kB activity in cells of the cervical transformation zone where most cervical cancers develop. Decreased NF-kB activation might represent a unique mechanism for the virus to interfere with the host immune response and promote persistent infection. We also show that down regulation of NF-kB stimulates clonal growth and immortalization of cervical epithelial cells. NF-kB is a potential target for cancer chemoprevention and therapy. Our observations raise concerns about chronic suppression of NF-kB in patients who have an increased risk for cervical cancer.

## Materials and methods

### *Cell culture*

Samples of cervical and oral epithelial tissue were obtained from patients with non malignant disease by the Cooperative Human Tissue Network. Cell cultures from endocervix, ectocervix or the transformation zone were isolated and identified as described (Woodworth and Simpson, 1993). Foreskins were obtained from the nursery of the local hospital. All tissues were collected in RPMI medium with 10% fetal bovine serum plus antibiotics and used within 24 h. Epithelial cells were isolated using a two step enzymatic digestion (Woodworth and Simpson, 1993). Briefly, each tissue was digested for 16 h at 4 °C in dispase (BD Biosciences) and the epithelium was removed from the underlying connective tissue by scraping. The epithelial sheet was cut into 1 $mm^2$ pieces and digested in 0.25% trypsin at 37 °C for 10 min. Trypsin was neutralized by addition of fetal bovine serum and cells were collected by centrifugation at 2000xg. Normal epithelial cells, HPV-16 immortalized cells (Woodworth et al., 1990) and cervical carcinoma cell lines (Woodworth et al., 1995) were cultured in Keratinocyte Serum-Free Medium (KSFM, Invitrogen).

### *Retrovirus infection with HPV-16 genes*

HPV genes were introduced into cultured cervical cells by infection with recombinant HPV-16 retroviruses (Halbert et al., 1991). Secondary cultures were infected with high-titer retroviruses as described (Woodworth et al., 1992) encoding HPV-16 E6, E7, or E6/E7 genes inserted into the vector pLXSN, which contains the neomycin resistance gene. Retroviruses containing the p24gly mutant (Edmonds and Vousden, 1989) have been described (Nees et al., 2001). Infection was performed for 3 h in medium with 10 ng/ml polybrene with rocking every 15 min. Subsequently, medium was changed and cells grew for 24 h before cultures were split 1:3. After 24 h, infected cells were selected by growth for 2 days in KSFM containing 200 μg/ml G418 and used immediately.

### *Dual luciferase reporter gene assay*

Cells were grown in 12 well plates and cotransfected with lipofectamine (Invitrogen) for 3 h using either 0.5 μg of the NF-kB responsive firefly luciferase reporter gene (Clontech) or the HPV-16 LCR firefly luciferase reporter gene (Khan et al., 1997), and 0.25 μg of a renilla luciferase reporter gene (Clontech). The latter promoter has weak activity and served as an internal control for transfection efficiency. Transfected cultures were maintained in KSFM for 24 h prior to measuring NF-kB activity using a dual luciferase assay (Promega). Each transfection was performed in triplicate, and transfection experiments were repeated at least 3 times using cells from different donors. Positive and negative controls for NF-kB activation included cotransfection with a p65 expression vector (Seitz et al., 1998) or a vector containing the IKBαm dominant negative mutant (Van Antwerp et al., 1996), respectively.

### *Immortalization assay*

Immortalization assays were performed after transfection with plasmid pMHPV16d containing the complete HPV-16 genome (Woodworth et al., 1988), the complete SV40 genome (Woodworth et al., 1986), or infection with HPV-16 E6/E7 retroviruses (Woodworth et al., 1992). For retrovirus-based assays, E6/E7-infected cells were subsequently cotransfected with the puromycin resistance gene (Clontech) plus either p65, IkBαm, or the empty vector pLZRS. After 24 h, cells were subcultured (6×60 mm dishes experimental group) in KSFM containing 10 ng puromycin for 2 days. Cells infected with retroviruses containing only the pLXSN vector (lacking HPV-16 genes) served as the negative control for immortalization. For transfection-based immortalization assays, cultures were cotransfected with pMHPV16d plus the neomycin resistance gene, and either p65, IkBαm, or pLZRS (vector). After selection for stably transfected cells using G418, cultures were fed with fresh KSFM every two days. Immortalization was measured by pooling the colonies in each dish and subculturing cells continually. Cells transfected with the neomycin resistance gene alone served as a negative control for immortalization. These cells became senescent after 2 or 3 additional passages. Cells transfected with a plasmid encoding the SV40 genome served as a control for determining specificity of effects for HPV-16.

### *Clonal growth assay*

Cells were stably transduced with HPV-16 DNA or E6/E7 retroviruses plus p65, IkBαm or pLZRS expression plasmids as described for immortalization assays. Cultures were maintained in KSFM for 14 days (retrovirus-infected) or 21 days (transfected). After colonies became visible, dishes were fixed with formalin and stained with Giemsa to identify colonies. A colony was defined as 50 or more cells.

*Real Time RT-PCR*

RNA was extracted from five different primary cervical cultures and four HPV-immortalized cell lines using Trizol (Invitrogen). Cellular DNA was removed using Turbo RNA-Free DNAse (Ambion) and RNA was reverse transcribed using Superscript First Strand kit (Invitrogen). No RT controls were included to confirm absence of cell DNA. Real-time PCR was performed using an iCycler IQ (Bio-Rad) using triplicate reactions (50 μl) containing 25 μl of 2x SYBR-Green Supermix, 0.4 μM of each forward and reverse primer, and 1 μl cDNA. Primers were: human β-defensin-1 forward GGGAGTGACCA GAAGAAATGA and reverse TTGGCCCAAAGGAGGTAT, human β-defensin-2 forward CTGGAACAAAATGCTGCAAA and reverse TGGTTTACATGTCGCACGTC, human β-defensin-3 forward TCCAAAG GAGGAACAGATCG and reverse GGCATTTCCACACTTTACAACA, human β-defensin-5 forward TGTCTGTGAGTCGTGCAAGC and reverse CTGCAGCAGAGAAAGTTCAGC, human β-defensin-7 forward AGGTCACTGTGAAGCCGAAT and reverse GCAGCCAGAGAATCTT CACC, β-actin forward GGACTTCGAGCAAGAGATGG and reverse AGCACTGTGTTGGCGTACCAG, and RPL38 forward CGAAAG GATGCCGGGTCTGT and reverse TCAGTTCCTTCACTGCCAA.

Reactions were performed for 40 cycles and melting temperature analysis was conducted on each sample to check specificity of product. Relative expression was calculated using the Pfaffl method (Pfaffl, 2001). Housekeeping genes (β-actin, and RPL38) were examined for normalization and β-actin was chosen because immortalization did not alter expression.

*Statistical analysis*

One way analysis of variance (ANOVA) was used to compare the means of multiple experimental groups when data was distributed normally, and the Kruskal-Wallis test was used for comparisons when the distribution was not normal. Tukey's multiple comparison test was used to compare differences between each pair of means.

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