Published in final edited form as:

Biochem Biophys Res Commun. 2010 September 3; 399(4): 617–622. doi:10.1016/j.bbrc.2010.07.125.

The HPV-16 E5 protein represses expression of stress pathway genes XBP-1 and COX-2 in genital keratinocytes

Sawali R. Sudarshan, Richard Schlegel*, and Xuefeng Liu

Department of Pathology, Georgetown University Medical School, 3900 Reservoir Road, NW, Washington, DC 20057

Abstract

The HPV-16 E5 protein resides in membranes of the endoplasmic reticulum (ER) and modulates cell growth and viral replication. In order to help define its biological activities, we analyzed E5-induced changes in human keratinocyte gene expression. Our studies identified the downregulation of spliced XBP-1 transcripts, a key player in the ER stress response, as a biochemical marker of E5 expression. IRE1 α , the endoribonuclease responsible for XBP-1 RNA splicing, was also downregulated. Furthermore, cDNA microarray analysis revealed the repression of COX-2, another member of the ER stress pathway. In contrast, these genes were not altered either by the low-risk HPV-6b E5, or a C-terminal HPV-16 E5 mutant, in which the histidine and alanine residues (conserved in high-risk HPVs) were replaced with tyrosine and isoleucine (conserved in low-risk HPVs). HPV-16 E5 was also able to lower COX-2 mRNA levels in cells co-expressing E6/E7, suggesting that it might exert similar activity during viral replication. Interestingly, the E6/E7 genes were independently able to lower COX-2 transcripts compared to vector cells, indicating that multiple pathways of COX-2 repression exist. COX-2 downregulation by E5 could be overcome by thapsigargin or tunicamycin treatments, which initiate ER stress via calcium fluxes and abnormal protein glycosylation respectively, making it unlikely that E5 specifically tempers these pathways. Overall, our data indicate that E5 represses the cellular ER stress response and suggest a potential role for E5 during productive HPV infection.

Keywords

Human papillomavirus type 16; E5 oncoprotein; COX-2; IRE1 α ; XBP-1; ER stress response; unfolded protein response

Introduction

Cervical cancer is the second most common cancer in women worldwide [1] and the high-risk human papillomaviruses (HPVs) are responsible for nearly all of these malignancies [2]. Approximately 60% of HPV-induced cervical cancers are caused by the high-risk type 16, which encodes three transforming proteins: E5, E6, and E7. The E6 and E7 proteins cooperate to immortalize primary keratinocytes and target several key cell regulatory proteins including p53, Rb, Myc, hTERT, and a significant number of proteins with PDZ domains [3;4;5]. In contrast, HPV-16 E5 is a weak transforming protein, which localizes

*corresponding author: schleger@georgetown.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

predominantly to the ER [6]. While E5 alone cannot immortalize human primary cells, it can induce anchorage-independent growth of immortalized rodent cells in soft agar [7] and enhance cell immortalization by E6/E7 [8]. In addition, estrogen-treated transgenic mice expressing HPV-16 E5 in addition to E6 and E7 developed a larger number of tumors than mice expressing E6 and E7 alone [9]. Most recently, our laboratory reported that E5 induces koilocytosis in collaboration with E6 [10]. The mechanism behind these E5-induced phenotypes is unknown. However, the ability of E5 to bind several cellular proteins, including the 16-kDa subunit of the vacuolar H⁺-ATPase [11;12;13], BAP31 [14], HLA [15;16], and ErbB4 [17] might account for some of its biological activities.

We recently reported that the ER-localized E5 protein of canine familiaris papillomavirus (CfPV2) alters XBP-1 splicing in keratinocytes [18]. XBP-1 is part of the ER stress response pathway, and alteration of its splicing has been shown to assist viruses establish persistent infections and promote the viral life cycle [19;20;21]. In this study, we examined the effect of the high-risk HPV-16 E5 protein on XBP-1 splicing. In addition to analyzing the XBP-1/IRE1 α pathway, we also used a gene expression microarray approach so that we would capture any additional alterations in the ER stress pathway. The only previous microarray analysis of the effect of HPV16-E5 expression was performed in HaCat cells, a spontaneously immortalized keratinocyte line with mutant p53 [22]. Consequently, analysis is temporally limited following the induction of E5 and is potentially confounded by the genetic changes present in these cells. Rather than study immortalized cells, we examined gene expression changes in primary genital keratinocytes which are the host cells of HPV infection.

Materials and Methods

Constructs

The codon-optimized, AU1-tagged 16E5 construct in the pLXSN vector was described previously [23]. E5 mutant constructs were generated by Celtek Biosciences (Nashville, TN). All constructs were made with an N-terminal AU1 tag (DTYRYI), a Kozak sequence (CTCGAG) 5' of the start codon, and restriction sites flanking the E5 open reading frame (EcoRI and XhoI at the 5' end; BamHI and SalI at the 3' end). EcoRI and BamHI sites were used to clone the construct into the pLXSN vector for stable expression (Clontech, Mountain View, CA). All mutant DNA sequences were confirmed by sequencing.

Cells and Cell Culture

Human foreskin keratinocytes (HFKs) were prepared from human foreskins donated by Georgetown University Hospital. Human ectocervical cells (HECs) were obtained from tissue procured from hysterectomies following benign uterine disease, and immortalized with retroviruses encoding HPV-16 E6 and E7 followed by puromycin selection [24;25]. For treatment with chemical inducers of ER stress, HFKs were exposed for 4 hours at 37 degrees Celsius with KGM containing either thapsigargin (300 nM, 30 nM, or 3 nM; Sigma Aldrich) or tunicamycin (3 μ g/ml, 0.3 μ g/ml, or 0.03 μ g/ml, Sigma Aldrich) prior to harvesting RNA using the RNAqueous-4PCR kit (Ambion) as per manufacturer's instructions.

Microarray

HPV-16 E5 or the pLXSN vector was stably expressed in three different pools of HFKs. Cells were grown on 100 mm tissue culture dishes (BD Falcon) to 80% confluence before harvesting RNA with 1 mL TRIzol Reagent according to manufacturer's protocol. DNase treatment was done according to the manufacturer's protocol for the RNAqueous-4PCR Kit (Ambion, Austin, TX). RNA was sent to MOGene, LC (St. Louis, MO) for microarray analysis. Each E5 cell line was run against the corresponding pLXSN cell line on a two-

color Agilent whole human genome slide with a $4 \times 44K$ format. A total of six comparative arrays were run, with each of the three biological replicates run in duplicate for dye swap. RNA was amplified using the Agilent Low Input Linear Amplification kit (Agilent Technologies, Santa Clara, CA), and then labeled with either cyanine-5 or cyanine-3 using the ULS aRNA Fluorescent Labeling Kit (Kreatech Biotechnology, Amsterdam, The Netherlands) according to manufacturer's instructions. 825 ng each of labeled c-DNA was hybridized overnight at $65^{\circ}C$ in an ozone-free room to protect the label. All washes and hybridization conditions followed were consistent with the Agilent processing manual (protocol version 4.0). Arrays were scanned using Agilent scanner (G2505B) and extracted using the Agilent Feature Extraction software (Agilent Technologies, Santa Clara, CA). Initial data analysis was performed by MOGene using the GeneSpring software (Agilent). The Bioinformatics and Biostatistics Shared Resource at the Georgetown University Lombardi Comprehensive Cancer Center (Washington, DC) performed pre-processing and differential analysis, including calculating average fold change and p-values, using Rosetta Resolver (Rosetta Biosoftware, Microsoft).

cDNA and Quantitative Real Time PCR

RETROScript kit (Ambion) was used to perform reverse-transcriptase PCR (RT-PCR). RNA was denatured for three minutes at $80^{\circ}C$ with Oligod(T) and random hexamers. This was followed by the reverse-transcriptase step consisting of 60 minutes at $45^{\circ}C$ and 10 minutes at $92^{\circ}C$. cDNA samples were diluted to 75 ng/ μ l for use in real-time RT-PCR. Real time reactions for XBP-1 and GAPDH were 20 μ l, and contained 0.8 μ l cDNA at 75 ng/ μ l, 10 μ l 2x Bio-Rad IQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), 0.125 20uM forward primer, 0.125 20uM reverse primer, and 8.95 μ l dH₂O. Other real time reactions (performed using primers ordered from realtimeprimers.com) were also 20 μ l, but contained 0.8 μ l cDNA at 75 ng/ μ l, 10 μ l 2x Bio-Rad IQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), 0.125 20uM primer mix (forward and reverse primers), and 9.08 μ l dH₂O. Reactions were annealed and analyzed using a Bio-Rad iCycler and accompanying software (Bio-Rad Laboratories). Primer sets used include total, spliced, and unspliced XBP-1 [18], as well the following

COX-2/PTGS2-F: 5' TCT GAA ACC CAC TCC AAA CA 3'

COX-2/PTGS2-R: 5' AAG GCT TCC CAG CTT TTG TA 3'

IRE1 α /ERN1-F: 5' GGC GAA CAG AAT ACA CCA TC 3'

IRE1 α /ERN1-R: 5' TCA CTG TCC ACA GTC ACC AC 3'

MED26-F: 5' AGC ATC CAT GAC CTG AAG AG 3'

MED26-R: 5' AAG CTC TCT GGA CTC CCA CT 3'

UBE2E1-F: 5' GCA AAC CGA GAA AGA AAC AA 3'

UBE2E1-R: 5' GGC CCT AGA ATG GTT GAT CT 3'

GPR135-F: 5' AGG GCT ACC GGA CTA GGA AT 3'

GPR135-R: 5' TTA GGC TGT TTG GTC ACT GC 3'

Results

Downregulation of spliced XBP-1 mRNA is specific to high-risk HPV-16 E5

We have previously published that the low-risk HPV-6b E5 protein did not significantly alter the splicing of XBP-1 mRNA [18]. To determine if this was also true for an ER-localized, high-risk HPV E5 protein, we stably transduced HFKs with 16E5 or control

vector (pLXSN). Using real-time RT-PCR, we quantified the ratio of spliced to unspliced XBP-1 transcripts in both cell types (Figure 1a) as well as the levels of spliced, unspliced, and total XBP-1 mRNA (Figure 1b). These experiments were repeated using three independent preparations of HFKs. We found that level of spliced XBP-1 was significantly reduced in E5-expressing HFKs when compared to vector-expressing controls, either when normalized to the unspliced transcript (Figure 1a) or to GAPDH (Figure 1b).

We then generated several E5 mutants to define the protein domains that might account for the biological differences between the low- and high-risk E5 proteins. First, an alignment of the E5 amino acid sequences from several low and high-risk HPV types was performed (Supplement Figure S2). Mutations were made in regions of the E5 protein that were highly conserved in the high-risk E5 proteins, but not conserved in the low-risk types. Four mutants were constructed with point substitutions to alanine: L32A, S35AS37A, Y39A, and Y63A. In the fifth mutant, H77YA78I, the histidine and alanine residues (conserved in high-risk HPVs) were replaced with tyrosine and isoleucine (conserved in low-risk HPVs). Immunofluorescence was used to confirm the expression and localization pattern of mutant constructs. All mutant E5 proteins merged with the ER-marker calnexin in both transfected COS-1 cells and stably-expressing HECs (Supplemental Figure S1). This pattern is similar to the previously published localization of the wild-type 16E5 protein [6]. XBP-1 levels were measured in HFKs stably expressing these mutant proteins. Two mutants, mutant H77YA78I and mutant Y63A, were defective for reducing the spliced:unspliced ratio of XBP-1 mRNA by real-time PCR (Figure 2A). Only mutant, H77YA78I, was unable to reduce the levels of spliced XBP-1 mRNA normalized to GAPDH (Figure 2B). These findings suggest that the ability of a high-risk E5 protein to reduce XBP-1 splicing is dependent upon two highly-conserved C-terminal amino acids.

HPV-16 E5 downregulates COX-2 and IRE1 α in HFKs

Given the consistency with which 16E5-expressing cells demonstrated a reduction in spliced XBP-1 levels, we examined whether E5 altered upstream or downstream components of XBP-1 splicing in the ER stress signaling pathway. IRE1 α , immediately upstream of XBP-1 in the ER stress response pathway, is the endoribonuclease responsible for the cleavage of unspliced XBP1 mRNA into its more stable spliced form. We found that IRE1 α transcript levels were also significantly downregulated in E5-expressing keratinocytes as compared to those expressing the vector-control (Figure 3A). This finding suggested that a spectrum of gene expression changes, including perhaps changes in other ER stress response genes, may occur after expression of 16E5. To address this possibility, we performed a cDNA microarray study to identify E5-induced changes in gene expression. To ensure that these changes were reproducible, we performed the microarray assays in triplicate, using three different donor pools of foreskin keratinocytes that were separately transduced with either E5 or pLXSN and selected with G418. These biological replicates included a dye swap to further validate the findings. Surprisingly, less than 25 genes were consistently regulated in the arrays (>1.5 fold change in each array, p-value < 0.01). Real-time RT-PCR was used to confirm the downregulation of four genes (Figure 3B). One of these was COX-2/PTGS2, which has been shown to be a part of the ER stress response pathway [26]. COX-2 could possibly be both downstream of XBP-1 and IRE1 α , as well as independently activated through another arm of the ER stress response [26]. Additionally, consistent with the pattern seen with XBP-1 repression, 6bE5-expressing cells and H77YA78I E5-expressing cells were unable to repress COX-2 mRNA levels (Figure 3B). Thus, our data indicated that the high-risk HPV16 E5 protein specifically altered these ER stress related genes in HFKs.

Discussion

HPV-16 E5 is a small, hydrophobic, ER-localized viral protein whose biological function has remained elusive. In this study we examined primary human keratinocytes expressing either 16E5 for changes in gene expression using real-time RT-PCR and cDNA microarray. By analyzing multiple pools of primary keratinocytes, we aimed to identify gene changes that were reproducible in different donor cells.

To date, no gene expression studies have been performed on primary keratinocytes that are stably expressing 16E5. The only other gene expression study on 16E5 was conducted in HaCat cells using a dexamethasone-inducible promoter [22]. That study found that 179 genes were significantly altered (no fold change cutoff, $p < 0.01$) by 16E5 expression, including lamin A/C, PKC- γ , and PI3K. E5 was suggested to inhibit apoptosis by affecting pathways involved in cell adhesion, motility, and mitogenic signaling. In contrast, our analysis indicated that a far smaller subset of genes (~25) were consistently affected (fold change >1.5 , p -value < 0.01) in three independent isolates of primary keratinocytes. The difference between our data and the above study may be due to site origins or genetic background (foreskin vs adult trunk keratinocytes), cell status (primary vs immortalized), or gene expression level (stable expression vs transient inducible expression).

Our study indicates that HPV-16 E5 suppresses 3 key players in the ER stress pathway: COX-2, XBP-1 and IRE1 α . Numerous other viral proteins have been reported to cause alterations of the ER stress response pathway, including the canine papillomavirus E5 protein (Cfpv2 E5) [18;20;21;27]. It is noteworthy that hepatitis C virus (HCV) suppresses the XBP-1/IRE1 α pathway and may therefore promote the expression of HCV proteins and aid in viral persistence [20]. Persistent viral infection appears to be a major contributory factor to the development of cancer, especially in the case of infection by the high-risk HPVs [28]. It is interesting to note that the substitution of just two amino acids conserved in high-risk HPV E5 protein with amino acids conserved in low-risk E5 protein is enough to completely abrogate COX-2 suppression. In fact, in contrast to the high-risk HPV-16 E5, the low-risk HPV-6b E5 is unable alter XBP-1 [18] and may even increase levels of COX-2, although this was not statistically significant. Indeed, previous studies have shown increased levels of COX-2 in recurrent respiratory papillomatosis (RRP) lesions which are caused by low-risk HPV type 6b and 11 [29;30]. However, the COX-2 phenotype described in RRP has not yet been linked to a specific HPV protein. Our findings suggest that change of COX-2 levels by low risk HPVs may be modulated by a single viral gene.

Our data indicate that the ability to suppress XBP-1, COX-2, and IRE1 α is unique to the high-risk 16E5, suggesting that these genes may be used as biochemical markers of HPV-16 E5 expression. The mechanism by which this downregulation occurs remains unknown, although 16E5's inability to interfere with ER stress induced by tunicamycin and thapsigargin suggests that it cannot alter the calcium or glycosylation pathways (Supplemental results and Figure S4). However, it is possible that 16E5 may be able to exert an effect on ER stress induced by expression of viral proteins. In our primary keratinocyte model, data indicate that while E6 and E7 induce XBP-1, they are also able to suppress COX-2. Together, E5, E6, and E7 exhibit an additive effect on COX-2 suppression (supplemental results and Figure S3).

When studied in different cell systems, it appears that several HPV proteins can alter COX-2 expression. For example, E5, E6, and E7 have been shown to induce COX-2 expression in spontaneously immortalized lines (HaCat cells and HEK cells), and cervical cancer lines (C33A and SiHa) [31;32]. COX-2 levels were also upregulated in esophageal epithelial cells immortalized with E6, E7, and hTERT [33]. Clinically, overexpression of COX-2 was

inversely related to HPV-16 infection in esophageal squamous cell carcinomas [34], and inversely related to HPV load in patients with cervical intraepithelial neoplasia [35]. However, other studies showed that COX-2 protein levels did not correlate with the disease severity of HPV-induced cervical lesions [36], or with HPV-positivity in primary and metastatic cervical cancer tissues [37;38]. It is possible that the observed phenotype in the above studies varies depending on factors such as cell origin, immortalization and transformation status, and the relative expression of the HPV early proteins.

In primary genital keratinocytes, our data show a very specific and consistent downregulation of ER stress response genes by 16E5, and suggest a potential role for E5 in repressing the cellular ER stress response following HPV infection (Figure 4). Although in vivo studies are not possible, one could speculate that the downregulation of this stress pathway would be favorable to viral replication and persistence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by an R01-CA053371 grant from the National Cancer Institute awarded to R.S.

References

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin.* 2005; 55:74–108. [PubMed: 15761078]
2. Munoz N, Bosch FX, de Sanjose S, Herrero R, Castellsague X, Shah KV, Snijders PJ, Meijer CJ. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *N Engl J Med.* 2003; 348:518–27. [PubMed: 12571259]
3. Howie HL, Katzenellenbogen RA, Galloway DA. Papillomavirus E6 proteins. *Virology.* 2009; 384:324–34. [PubMed: 19081593]
4. McLaughlin-Drubin ME, Munger K. The human papillomavirus E7 oncoprotein. *Virology.* 2009; 384:335–44. [PubMed: 19007963]
5. Liu X, Dakic A, Zhang Y, Dai Y, Chen R, Schlegel R. HPV E6 protein interacts physically and functionally with the cellular telomerase complex. *Proc Natl Acad Sci U S A.* 2009; 106:18780–5. [PubMed: 19843693]
6. Supryniewicz FA, Disbrow GL, Krawczyk E, Simic V, Lantzky K, Schlegel R. HPV-16 E5 oncoprotein upregulates lipid raft components caveolin-1 and ganglioside GM1 at the plasma membrane of cervical cells. *Oncogene.* 2007
7. Straight SW, Hinkle PM, Jewers RJ, McCance DJ. The E5 oncoprotein of human papillomavirus type 16 transforms fibroblasts and effects the downregulation of the epidermal growth factor receptor in keratinocytes. *J Virol.* 1993; 67:4521–32. [PubMed: 8392596]
8. Stoppler MC, Straight SW, Tsao G, Schlegel R, McCance DJ. The E5 gene of HPV-16 enhances keratinocyte immortalization by full-length DNA. *Virology.* 1996; 223:251–4. [PubMed: 8806560]
9. Genter Williams SM, Disbrow GL, Schlegel R, Lee D, Threadgill DW, Lambert PF. Requirement of epidermal growth factor receptor for hyperplasia induced by E5, a high-risk human papillomavirus oncogene. *Cancer Res.* 2005; 65:6534–42. [PubMed: 16061632]
10. Krawczyk E, Supryniewicz FA, Liu X, Dai Y, Hartmann DP, Hanover J, Schlegel R. Koilocytosis: a cooperative interaction between the human papillomavirus E5 and E6 oncoproteins. *Am J Pathol.* 2008; 173:682–8. [PubMed: 18688031]
11. Conrad M, Bubb VJ, Schlegel R. The human papillomavirus type 6 and 16 E5 proteins are membrane-associated proteins which associate with the 16-kilodalton pore-forming protein. *J Virol.* 1993; 67:6170–8. [PubMed: 7690419]

12. Adam JL, Briggs MW, McCance DJ. A mutagenic analysis of the E5 protein of human papillomavirus type 16 reveals that E5 binding to the vacuolar H⁺-ATPase is not sufficient for biological activity, using mammalian and yeast expression systems. *Virology*. 2000; 272:315–25. [PubMed: 10873774]
13. Andresson T, Sparkowski J, Goldstein DJ, Schlegel R. Vacuolar H(+) -ATPase mutants transform cells and define a binding site for the papillomavirus E5 oncoprotein. *J Biol Chem*. 1995; 270:6830–7. [PubMed: 7896830]
14. Regan JA, Laimins LA. Bap31 is a novel target of the human papillomavirus E5 protein. *J Virol*. 2008; 82:10042–51. [PubMed: 18684816]
15. Ashrafi GH, Haghshenas M, Marchetti B, Campo MS. E5 protein of human papillomavirus 16 downregulates HLA class I and interacts with the heavy chain via its first hydrophobic domain. *Int J Cancer*. 2006; 119:2105–12. [PubMed: 16823848]
16. Gruener M, Bravo IG, Momburg F, Alonso A, Tomakidi P. The E5 protein of the human papillomavirus type 16 down-regulates HLA-I surface expression in calnexin-expressing but not in calnexin-deficient cells. *Virol J*. 2007; 4:116. [PubMed: 17971213]
17. Chen SL, Lin ST, Tsai TC, Hsiao WC, Tsao YP. ErbB4 (JM-b/CYT-1)-induced expression and phosphorylation of c-Jun is abrogated by human papillomavirus type 16 E5 protein. *Oncogene*. 2007; 26:42–53. [PubMed: 16819515]
18. Condjella R, Liu X, Suprynowicz F, Yuan H, Sudarshan S, Dai Y, Schlegel R. The canine papillomavirus E5 protein signals from the endoplasmic reticulum. *J Virol*. 2009
19. Ku SC, Lee J, Lau J, Gurumurthy M, Ng R, Lwa SH, Lee J, Klase Z, Kashanchi F, Chao SH. XBP-1, a novel human T-lymphotropic virus type 1 (HTLV-1) tax binding protein, activates HTLV-1 basal and tax-activated transcription. *J Virol*. 2008; 82:4343–53. [PubMed: 18287238]
20. Tardif KD, Mori K, Kaufman RJ, Siddiqui A. Hepatitis C virus suppresses the IRE1-XBP1 pathway of the unfolded protein response. *J Biol Chem*. 2004; 279:17158–64. [PubMed: 14960590]
21. Yu CY, Hsu YW, Liao CL, Lin YL. Flavivirus infection activates the XBP1 pathway of the unfolded protein response to cope with endoplasmic reticulum stress. *J Virol*. 2006; 80:11868–80. [PubMed: 16987981]
22. Kivi N, Greco D, Auvinen P, Auvinen E. Genes involved in cell adhesion, cell motility and mitogenic signaling are altered due to HPV 16 E5 protein expression. *Oncogene*. 2008; 27:2532–41. [PubMed: 17982485]
23. Disbrow GL, Sunitha I, Baker CC, Hanover J, Schlegel R. Codon optimization of the HPV-16 E5 gene enhances protein expression. *Virology*. 2003; 311:105–14. [PubMed: 12832208]
24. Baeye AC, Berger A, Schlegel R, Veldman T, Schlegel R. Cervical epithelial cells transduced with the papillomavirus E6/E7 oncogenes maintain stable levels of oncoprotein expression but exhibit progressive, major increases in hTERT gene expression and telomerase activity. *Am J Pathol*. 2002; 160:1251–7. [PubMed: 11943710]
25. Pear WS, Nolan GP, Scott ML, Baltimore D. Production of high-titer helper-free retroviruses by transient transfection. *Proc Natl Acad Sci U S A*. 1993; 90:8392–6. [PubMed: 7690960]
26. Hung JH, Su JJ, Lei HY, Wang HC, Lin WC, Chang WT, Huang W, Chang WC, Chang YS, Chen CC, Lai MD. Endoplasmic reticulum stress stimulates the expression of cyclooxygenase-2 through activation of NF-kappaB and pp38 mitogen-activated protein kinase. *J Biol Chem*. 2004; 279:46384–92. [PubMed: 15319438]
27. Su HL, Liao CL, Lin YL. Japanese encephalitis virus infection initiates endoplasmic reticulum stress and an unfolded protein response. *J Virol*. 2002; 76:4162–71. [PubMed: 11932381]
28. Schiffman M, Castle PE. The promise of global cervical-cancer prevention. *N Engl J Med*. 2005; 353:2101–4. [PubMed: 16291978]
29. Wu R, Abramson AL, Shikowitz MJ, Dannenberg AJ, Steinberg BM. Epidermal growth factor-induced cyclooxygenase-2 expression is mediated through phosphatidylinositol-3 kinase, not mitogen-activated protein/extracellular signal-regulated kinase kinase, in recurrent respiratory papillomas. *Clin Cancer Res*. 2005; 11:6155–61. [PubMed: 16144915]

30. Wu R, Coniglio SJ, Chan A, Symons MH, Steinberg BM. Up-regulation of Rac1 by epidermal growth factor mediates COX-2 expression in recurrent respiratory papillomas. *Mol Med.* 2007; 13:143–50. [PubMed: 17592548]
31. Subbaramaiah K, Dannenberg AJ. Cyclooxygenase-2 transcription is regulated by human papillomavirus 16 E6 and E7 oncoproteins: evidence of a corepressor/coactivator exchange. *Cancer Res.* 2007; 67:3976–85. [PubMed: 17440114]
32. Kim SH, Oh JM, No JH, Bang YJ, Juhn YS, Song YS. Involvement of NF-kappaB and AP-1 in COX-2 upregulation by human papillomavirus 16 E5 oncoprotein. *Carcinogenesis.* 2009; 30:753–7. [PubMed: 19321801]
33. Zhuang ZH, Tsao SW, Deng W, Wang JD, Xia HH, He H, Feng HC, Wang LD, Gu Q, Lam SK, Lin MC, Kung HF, Wong BC. Early upregulation of cyclooxygenase-2 in human papillomavirus type 16 and telomerase-induced immortalization of human esophageal epithelial cells. *J Gastroenterol Hepatol.* 2008; 23:1613–20. [PubMed: 18717758]
34. Liu WK, Jiang XY, Zhang MP, Zhang ZX. The relationship between HPV16 and expression of cyclooxygenase-2, P53 and their prognostic roles in esophageal squamous cell carcinoma. *Eur J Gastroenterol Hepatol.* 2010; 22:67–74. [PubMed: 19730383]
35. Song SH, Lee JK, Hur JY, Kim I, Saw HS, Park YK. The expression of epidermal growth factor receptor, vascular endothelial growth factor, matrix metalloproteinase-2, and cyclooxygenase-2 in relation to human papilloma viral load and persistence of human papillomavirus after conization with negative margins. *Int J Gynecol Cancer.* 2006; 16:2009–17. [PubMed: 17177839]
36. Sarian LO, Derchain SF, Yoshida A, Vassallo J, Pignataro F, De Angelo Andrade LA. Expression of cyclooxygenase-2 (COX-2) and Ki67 as related to disease severity and HPV detection in squamous lesions of the cervix. *Gynecol Oncol.* 2006; 102:537–41. [PubMed: 16537091]
37. Kim MH, Seo SS, Song YS, Kang DH, Park IA, Kang SB, Lee HP. Expression of cyclooxygenase-1 and -2 associated with expression of VEGF in primary cervical cancer and at metastatic lymph nodes. *Gynecol Oncol.* 2003; 90:83–90. [PubMed: 12821346]
38. Singh A, Sharma H, Salhan S, Gupta SD, Bhatla N, Jain SK, Singh N. Evaluation of expression of apoptosis-related proteins and their correlation with HPV, telomerase activity, and apoptotic index in cervical cancer. *Pathobiology.* 2004; 71:314–22. [PubMed: 15627842]

Figure 1. HPV-16 E5 reduces spliced XBP-1 mRNA

Splicing of XBP-1 is a marker of an ongoing ER stress response in cells. Primary HFKs were stably transduced with either 16E5 or LXSN control vector, real-time RT-PCR was used to measure mRNA levels. **(A)** The ratio of spliced to unspliced transcripts of XBP-1. **(B)** Levels of XBP-1 transcripts relative to GAPDH. n=3. Bars represent means $\pm$ SEM. ** indicates p value < 0.01 as determined by a paired student's t-test.

Figure 2. An HPV-16 E5 mutant, H77YA78I, fails to reduce levels of spliced XBP-1 mRNA

HPV-16 E5 mutants were made in regions of the protein that were highly conserved amongst high-risk HPVs but not in low-risk HPVs (see supplement Figure 2). All mutants contain alanine substitutions except for mutant H77YA78I, where amino acids histidine and alanine (highly conserved in high-risk HPVs) were swapped for tyrosine and isoleucine (highly conserved in low-risk HPVs). **(A)** The ratio of spliced to unspliced transcripts of XBP-1 in mutant constructs. **(B)** Levels of XBP-1 transcripts relative to GAPDH in mutant constructs. $n=3$. Bars represent means $\pm$ SEM. ** indicates p value < 0.01 as determined by a paired student's t -test. * indicates p value < 0.05 by student's t -test.

Figure 3. HPV-16 E5 reduces mRNA levels of IRE1 α (the endoribonuclease responsible for XBP-1 splicing) and COX-2

(A) Real-time RT-PCR was used to measure IRE1 α mRNA transcripts relative to GAPDH. $n=3$. Bars represent means $\pm$ SEM. * indicates p value < 0.05 as determined by a paired student's t -test. (B). HPV-16 E5 downregulates COX-2 mRNA. cDNA microarray was performed on three different donor pools of primary foreskin keratinocytes which were stably transduced with 16E5 or LXSN. Four genes were chosen for confirmation by real-time RT-PCR. 6bE5 did not cause significant changes in expression of these genes. In addition, 16E5 H77AY78I is also defective for reduction in these genes. Of note is COX-2/PTGS2, also a member of the ER stress response pathway, which was specifically repressed by 16E5.

Figure 4. Potential model of 16E5 ER stress repression

Occasionally, cells are exposed to various environmental conditions that result in a disruption of ER function and buildup of unfolded proteins. The ER stress response, or unfolded protein response, is the cell's reaction to such insults. For a non-lytic virus, cell survival is essential for viral replication. It is possible that viral proteins may attempt to curb their own stimulation of such a host response, inducing one arm of the ER stress response while simultaneously suppressing another. E6 and E7 induce XBP-1, but are able to suppress COX-2. E5 alone in keratinocytes is able to suppress IRE1α, XBP-1, and COX-2. Together, E5, E6, and E7 exhibit an additive effect on COX-2 suppression.