Title

E5 can be expressed in anal cancer and leads to epidermal growth factor receptor-induced invasion in a human papillomavirus 16-transformed anal epithelial cell line

Permalink

https://escholarship.org/uc/item/00h4775g

Journal

Journal of General Virology, 99(5)

ISSN

0022-1317

Authors

Wechsler, Erin Isaacson
Tugizov, Sharof
Herrera, Rossana
et al.

Publication Date

2018-05-01

DOI

10.1099/jgv.0.001061

Peer reviewed

E5 can be expressed in anal cancer and leads to epidermal growth factor receptor-induced invasion in a human papillomavirus 16-transformed anal epithelial cell line

Erin Isaacson Wechsler, Sharof Tugizov, Rossana Herrera, Maria Da Costa and Joel M. Palefsky*

Abstract

We detected the first human papillomavirus (HPV)-16-immortalized anal epithelial cell line, known as AKC2 cells to establish an *in vitro* model of HPV-16-induced anal carcinogenesis. Consistent with detection of E6, E7 and E5 expression in anal cancer biopsies, AKC2 cells expressed high levels of all three HPV oncogenes. Also, similar to findings in anal cancer biopsies, epidermal growth factor receptor (EGFR) was overexpressed in AKC2 cells. AKC2 cells exhibited a poorly differentiated and invasive phenotype in three-dimensional raft culture and inhibition of EGFR function abrogated AKC2 invasion. Reducing E5 expression using E5-targeted siRNAs in AKC2 cells led to knockdown of E5 expression, but also HPV-16 E2, E6 and E7 expression. AKC2 cells treated with E5-targeted siRNA had reduced levels of total and phosphorylated EGFR, and reduced invasion. Rescue of E6/E7 expression with simultaneous E5 knockdown confirmed that E5 plays a key role in EGFR overexpression and EGFR-induced invasion.

INTRODUCTION

Human papillomaviruses (HPVs) are small 8 kb DNA viruses that infect a wide range of epithelial sites including the anus, cervix and oral cavity. Infections with high-risk HPVs (e.g. HPV-16 and HPV-18) can lead to the development of anal high-grade squamous intraepithelial lesions (HSIL), the anal cancer precursor lesion, of which a subset will progress to invasive cancers. Currently the incidence of anal cancer is increasing by 2.2 % per year in both men and women in the general population [1]. The largest increase in incidence of anal cancer is in the HIV-positive population, even after the introduction of antiretroviral therapy, and among HIV-positive men who have sex with men the incidence of anal cancer has been shown to be as high as 131/100 000 [2]. In total, 70 % of anal carcinomas are associated with HPV-16 infection [3, 4].

Compared with other HPV-associated cancers, including cervical cancer, the pathogenesis of anal cancer is poorly understood. This is partly due to a lack of immortalized HPV-positive anal epithelial cell lines that can be used to model anal cancer progression.

It has been shown that E5 expression enhances cellular proliferation and genome amplification in the productive phase of the HPV-16 and HPV-31 life cycles [5–8]. However, its role as an oncogene in HPV-associated cancers, where the epithelium is poorly differentiated, thus constituting an abortive life cycle, is less understood. To date there have been no studies that investigate its expression in anal cancer. Although it was previously believed that E5 expression is lost as a result of HPV genome integration into the host chromosome, several studies have also shown that E5 transcripts can be detected in HPV-positive cervical and oral cancer biopsies [9–12]. A recent study showed that E5 mRNA and E5 translated protein can both be detected in the CaSki cell line, a cervical cancer cell line that contains integrated copies of the full-length HPV-16 genome [13].

Past studies have also shown that up to 30 % of HPV-associated cancers contain a mixture of integrated and episomes with a small percentage exclusively containing viral episomes [14–16]. Therefore, it is also possible that E5 expression could occur from a mixture of integrated HPV genomes and/or viral episomes.

One of the most established roles of E5 is its ability to enhance epidermal growth factor receptor (EGFR) ligand-receptor activation. Several studies show that E5 increases EGFR ligand-receptor activation primarily through preventing lysosomal degradation of EGFR, resulting in an increase in EGFR recycling to the cell membrane. E5 may do so by

interfering with endosomal acidification preventing the passage of EGFR to lysosomes [17]. In addition, E5 may inhibit fusion of early endosomes that contain EGFR to late endosomes preventing their degradation [18]. It may also interfere with c-Cbl ubiquitin ligase, preventing ubiquitination and degradation of EGFR [19].

EGFR overexpression has been reported in HPV-positive cervical and oral cancers [20–24]. More recently it has been shown that EGFR is overexpressed in the majority of anal cancer biopsies [25, 26].

Only a few studies have investigated the role of E5-mediated increase in EGFR activation in the context of HPV-associated cancer progression. *In vitro* studies have shown that HPV-16 E5-induced proliferation and anchorage-independent growth are increased in the presence of the EGF ligand [27–29]. In addition, *in vivo* studies in mice suggest that EGFR expression is necessary for E5-induced hyperplasia [30]. It is not known if E5/EGFR plays a more significant role in anal cancer progression when co-expressed with E6 and E7.

Here we present a novel *in vitro* model of anal cancer pathogenesis using the first HPV-16-transformed anal epithelial cell line, known as AKC2 cells. Similar to our findings in HPV-16-positve anal cancer biopsies, AKC2 cells expressed all three HPV-16 oncogenes (E5, E6 and E7). We showed that reducing E5 expression with E5-targeted siRNAs in AKC2 cells led to 99 % reduction of all three oncogenes as well as the E2 replication gene. Rescue of E6 and E7 expression confirmed that E5 plays a major role in driving EGFR overexpression/activation and EGFR-mediated invasion of AKC2 cells. Coupled with detection of E5 expression in HPV-16-positive anal cancers, we conclude that E5 likely plays an important role in anal cancer progression and may be a good therapeutic target for treatment of HPV-16-associated anal HSIL or cancer.

RESULTS

HPV-16-positive anal squamous cell carcinoma (SCC) biopsies contain transcripts for E5, E6 and E7

To date there have been no studies that characterize viral oncogene expression in HPV-16-positive anal biopsies. To determine if all three viral oncogenes were expressed in HPV-16-positive anal SCC biopsies, we extracted total RNA from formalin-fixed sections of four HPV-16-positive anal SCCs. We performed HPV-specific genotyping of anal biopsies as described previously [31] (data not shown). We also extracted RNA from a HPV-18 and HPV-33-positive anal SCC, which were included as negative controls for detection of HPV-16-specific transcripts. We measured HPV-16 E5, E6 and E7 transcripts as well as an internal control, RPLP0 using the qPCR Sybr green method. We detected strong HPV-16 E5, E6 and E7 transcription in all four HPV-16-positive anal SCCs but not in the HPV-18 or 33-positive SCC (Fig. 1a).

Establishment and characterization of a novel HPV-16-positive (E5, E6 and E7) anal epithelial cell line

HPV-16-associated anal pathogenesis has been largely understudied due to a lack of permanent HPV-positive anal cell lines that phenotypically model anal cancer progression. We established the AKC2 cell line, the first known permanent HPV-16-(E5, E6 and E7)-positive anal cell line, by transfecting normal HPV-negative primary anal epithelial cells with the entire pEF3-99 plasmid that contains the full-length 8 kB HPV-16 W12 genome with a single disruption in the L1 ORF. Since our goal was to obtain a HPV-16-positive cell line that stably expressed E5, E6 and E7, we found this approach allowed for more rapid isolation of cell populations with integrated genomes.

HPV-negative anal epithelial cells from a single donor were previously isolated from a normal anal biopsy taken from a HIV-positive individual. We did not detect any HPV DNA in the cells prior to transfection of the 8 kB HPV 16 genome (data not shown). We originally established AKC2 cells as a population of HPV-16-transfected anal parental cells following G418 selection. To date we have passaged AKC2 cells over 400 times and we are now able to culture AKC2 cells in standard media (DMEM/10% FBS) without supplemented growth factors or an irradiated feeder layer.

Monolayer AKC2 cells exhibit an epithelial-like morphology with predominantly cobblestone-like colonies (Fig. 1b) and are positive for pan-keratin staining (Fig. 1b). We found that HPV copy number was stable at early (p10), mid (p105) and later (p455) passages. AKC2 cells contain approximately ten copies of E5 per cell, four copies of E6 per cell and nine copies of E7 per cell (Fig. 1c). This small variation in E6 and E7 copy number has also been previously reported for CaSki and SiHa cell lines [32, 33]. We did not detect L1 expression in AKC2 cells by qPCR analysis (data not shown).

To characterize the physical status of the HPV-16 genome we conducted an amplification of papillomavirus oncogene transcripts (APOT) PCR which identified transcripts derived from a fully intact or fragmented early HPV-16 region. These data showed that integration of the HPV-16 genome was an early event and the integration pattern remained stable at passages 10, 105 and 455. Fig. 1(d) shows a representation of this band pattern in AKC2 at passage 455. Similar to what Klaes *et al.* reported in the CaSki line [34], Southern analysis of AKC2 using E7- and E4-specific probes identified a dominant product of approximately 650 bp, suggesting that portions of E4 ORF were retained following viral integration (Fig. 1d). In addition, Southern analysis of AKC2 using E7- and E4-specific probes also identified a minor band of approximately 1000 bp suggesting that transcription also occurred from a fully intact early HPV-16 region (Fig. 1d). The E7 probe also hybridized to a 250 bp band in both AKC2 and CaSki, however, Klaes *et al.* confirmed that this product was a result of the (dT) primer mis-annealing to an adenosine-rich sequence. Due to the
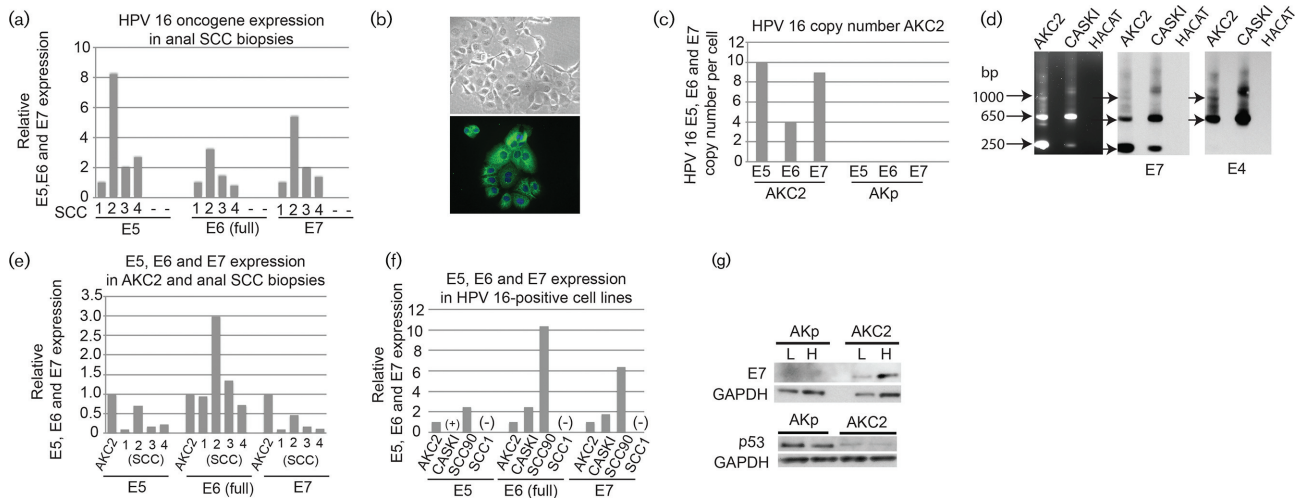
Fig. 1. HPV-16 E5 oncogene expression in anal SCC biopsies and a novel HPV-16-positive anal cell line, AKC2. (a) Relative HPV-16 E5, E6 and E7 expression in HPV-16-positive anal SCC biopsies was determined by qPCR. Negative control SCC biopsies marked (-) were positive for either HPV-18 or HPV-31. Columns represent the average relative fold change in HPV-16 E5, E6 and E7 expression, which was normalized to the housekeeping gene RPLPO. The $2^{-\Delta\Delta ct}$ method was used to calculate relative fold expression. (b) Morphology of AKC2 by phase contrast ($\times 20$ magnification); pankeratin expression (green) and DAPI nuclear stain nuclei (blue) ($\times 40$ magnification). (c) HPV-16 E5, E6 and E7 DNA copy number per cell in AKC2. (d) APOT PCR analysis of AKC2, CaSki (positive control) and HaCat (negative control). PCR products were separated on a 1.2 % gel and blotted on to a Biodyne membranes. Southern analysis using E7- and E4-specific probes was carried out to detect HPV-16-specific gene products. (e) Relative HPV-16 E5, E6 and E7 expression in AKC2 and HPV-16-positive anal SCC biopsies was determined by qPCR. Columns represent the average relative fold change in HPV-16 E5, E6 and E7 expression, which was normalized to the housekeeping gene RPLPO. The $2^{-\Delta\Delta ct}$ method was used to calculate relative fold expression. (f) Relative HPV-16 E5, E6 and E7 expression in integrated HPV-16-positve cell lines [i.e. AKC2 (anal), CaSki (cervical), SCC90 (oral) and SCC1 (oral-HPV-16-negative)] was determined by qPCR. Columns represent the average relative fold change in HPV-16 E5 expression from triplicate wells, which were normalized to the RPLPO housekeeping gene. CaSki cells marked with (+) were positive for E5 oncogene expression. The $2^{-\Delta\Delta ct}$ was used to calculate relative fold expression. (g) Western blots of HPV-16 E7 protein and p53 protein from HPV-16-negative parental anal keratinocytes (AKp) and HPV-16-positive AKC2 lysates. Lower (L=15 µg) and higher (H=25 µg) levels of protein were loaded to detect E7 expression.

---

fact that AKC2 is an expanded population, it is possible that the multiple band pattern detected in the APOT assay represents different integration patterns contained in individual cells. This could also explain the differences in E5, E6 and E7 genome copy numbers.

Similar to the HPV-16-positive anal SCC biopsies, we detected expression of HPV-16 E5, E6 and E7 in AKC2 cells as well as E2 expression by Sybr green qPCR methods. We then compared E5, E6 and E7 expression levels in AKC2 cells to expression levels in the anal SCC biopsies (Fig. 1e). AKC2 cells had generally higher HPV-16 E5 and E7 expression levels relative to the anal SCC biopsies. Full-length E6 expression levels were similar, if not slightly higher, in the biopsies compared with AKC2 cells. We also detected E5, E6 and E7 in two positive control cell lines [i.e. CaSki cell line (cervical) and the UPCI SCC90 cell line (oral)] but not in the HPV-negative oral cancer line SCC1 (Fig. 1f).

Finally, we detected E7 protein in AKC2 cells (Fig. 1f) but not in the AKp anal parental cells. p53 expression was reduced in AKC2 cells relative to AKp, consistent with E6 protein expression (Fig. 1g).

AKC2 cells have a poorly differentiated and invasive phenotype in three-dimensional raft culture

Since AKC2 cells expressed E5, E6 and E7, similar to the anal cancer biopsies, we used these cells to model HPV-16-associated anal carcinogenesis using three-dimensional raft culture. We investigated the anal parental (AKp) and AKC2 raft culture phenotypes from passages before (i.e. passage 58) and after media adaption (i.e. passage 256) through haematoxylin and eosin (H&E) staining and through markers of terminal differentiation (i.e. K10/K13) and E7 oncogene expression [i.e. minichromosome maintenance (MCM) and p16] [35, 36]. AKp HPV-negative parental cells produced only a few layers in raft culture where H&E staining showed evidence of more terminally differentiated cells throughout the upper layers (Fig. 2a). AKp raft cultures also showed evidence of terminal differentiation in the upper layers as indicated by positive K10/13 staining (Fig. 2c). The AKp raft cultures were negative for both MCM and p16 staining.

In contrast, HPV-16-positive AKC2 raft cultures of both early (p58) and late passages (p256) resembled a high-grade lesion containing poorly differentiated layers and also
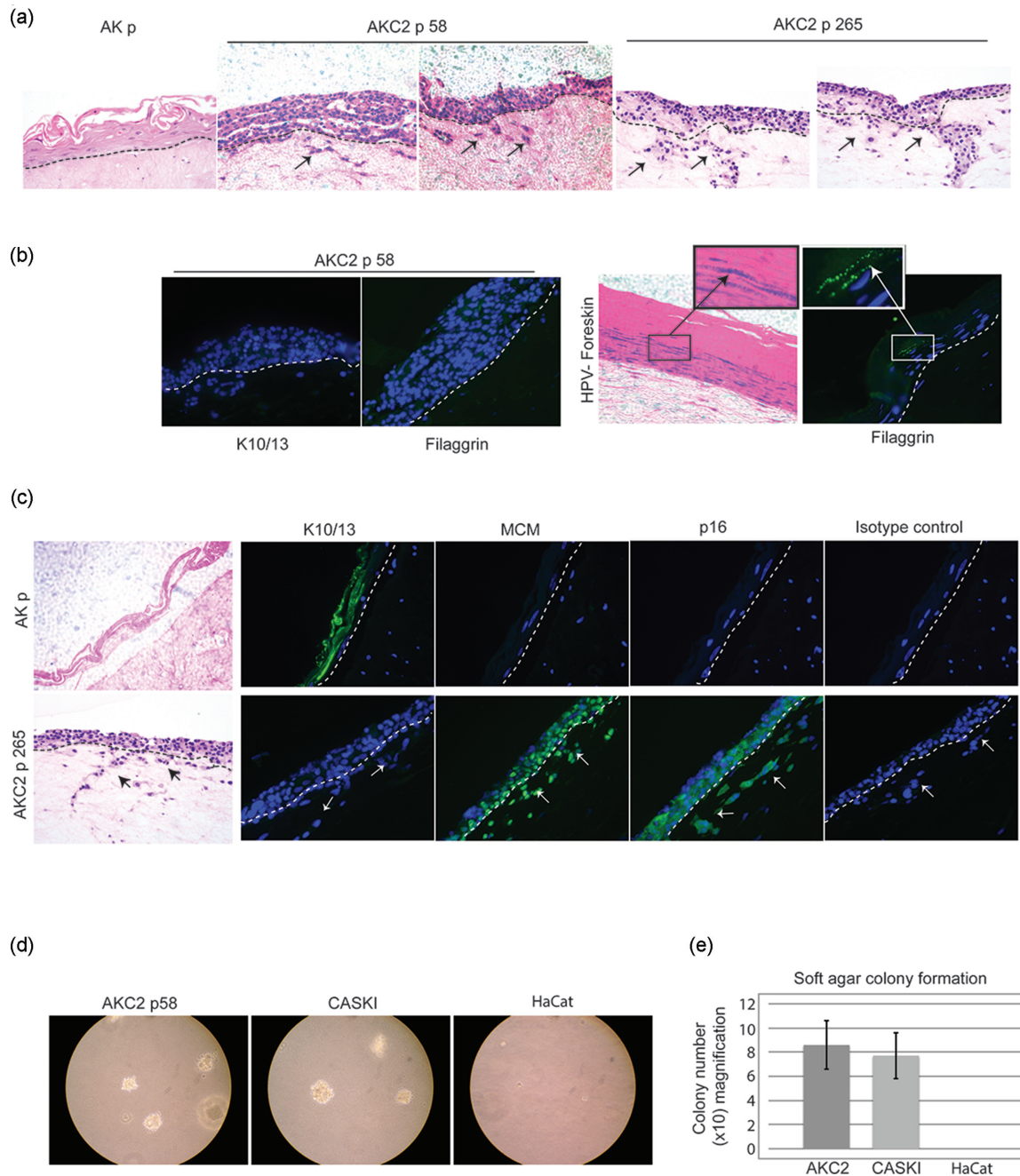
Fig. 2. AKC2 cells are invasive in three-dimensional organotypic raft cultures. (a) Cultures of HPV-16-negative AK parental (AKp) and HPV-16-positve AKC2 (passages 58 and 265) were differentiated on collagen-based dermal equivalents at the air–liquid interface for 15 days. AKp and AKC2 raft cultures were both stained with H&E. Black dashed lines identify the barrier between the basal layer and collagen dermal equivalent. Black arrows indicate invading cells into the collagen dermal equivalent. (b) Early passage AKC2 raft cultures (p 58) were stained for K10/13 or filaggrin (green) and DAPI nuclear stain (blue). HPV-negative foreskin raft cultures were used as a positive control to identify the granular layer by H&E and filaggrin expression (green). Both expanded boxes identify the granular layer in H&E-stained and filaggrin-stained foreskin raft cultures. White dashed lines identify the barrier between the basal layer and collagen dermal equivalent. (c) Raft cultures of AKp and AKC2 (passage 265) were stained with markers of late differentiation (K10/K13 (green), markers of E7 overexpression [MCM and p16 (green) and DAPI nuclear stain (blue)]. White dashed lines identify the barrier between the basal layer and collagen dermal equivalent. White arrows indicate invading cells into the collagen dermal equivalent. (d) Growth of HPV-16-positive AKC2 (anal), CaSki (cervical) and HPV-16-negative HaCat (foreskin) in semi-solid media (×20 magnification). (e) Quantification of colony growth in semi-solid media. Columns represent the average colony number obtained from six fields of view (×10 magnification) from triplicate wells. Error bars represent the SD.

showed foci of invasion (Fig. 2a). Since AKC2 cells of later passage (p265) were previously adapted from low-calcium full-growth factor supplemented media to high-calcium non-growth factor supplemented media (i.e. DMEM/10% FBS), we wanted to further confirm that changes in growth media did not affect the raft culture phenotype. One previous study showed that HPV-16-positive cells acquired resistance to differentiation after being adapted to high-calcium non-growth factor supplemented media [37]. However, we found that non-adapted AKC2 cells (p 58) were negative for K10/13 as well as filaggrin, a marker of late terminal differentiation (Fig. 2b). We used HPV-negative foreskin raft cultures that have a well-defined granular layer as a positive control for late differentiation and filaggrin staining (Fig. 2b). We therefore concluded that AKC2 cells did not acquire resistance to differentiation due to the media change and was therefore a true biological phenotype of the cell line.

AKC2 raft cultures also displayed similar E7 oncogene expression patterns previously found in high-grade lesions [35]. Here we detected MCM and p16 expression throughout the entire AKC2 raft culture and in invading cells (Fig. 2c).

Since invasion can be indicative of transformation and tumourigenic potential we measured the anchorage-independent growth of AKC2 cells in soft agar. For comparison, we performed similar assays using CaSki cells and HaCat cells, a HPV-16-negative non-cancerous epithelial cell line. AKC2 cells as early as passage 58 produced colonies in soft agar that were comparable to CaSki cells, both in size and number, whereas we were unable to detect colony growth in HaCat cultures (Fig. 2d, e). Several attempts to grow AKC2 tumours beyond 14–16 days in NOD SCID gamma (NSG) mice, were unsuccessful (data not shown) with both early and late passaged AKC2 cells. These data suggest that AKC2 cells are immortalized and partially transformed but not fully tumourigenic and may phenotypically represent an early transition phase from anal precancer to anal cancer.
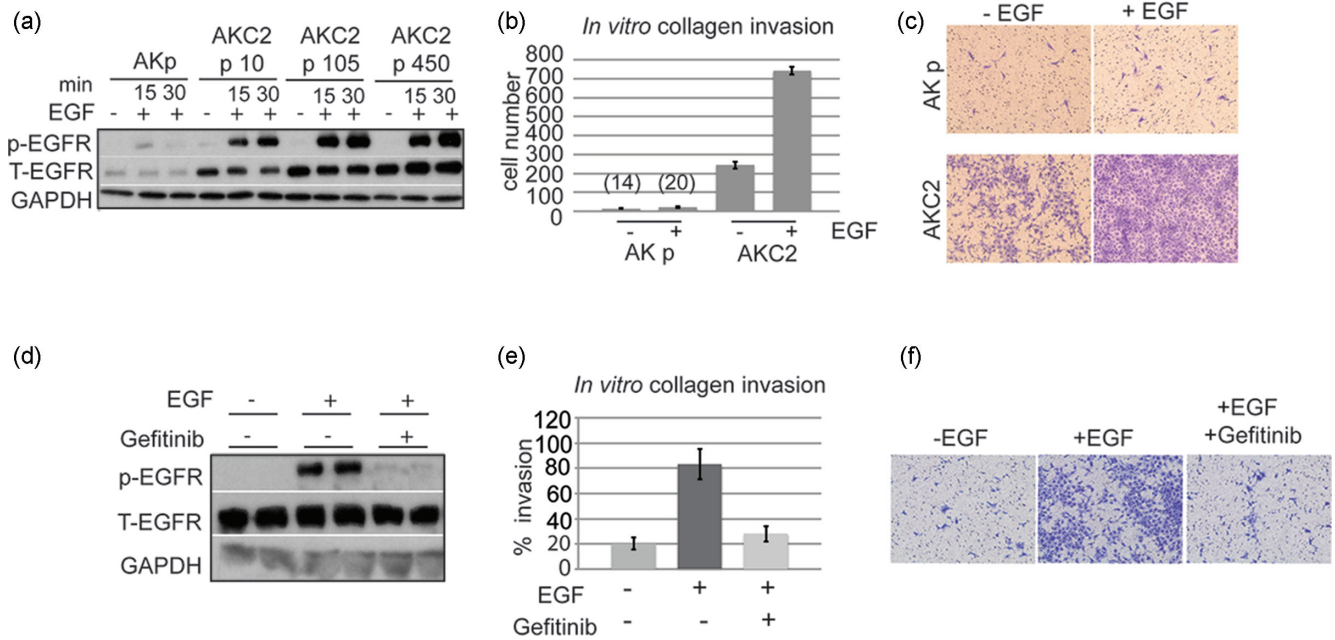
EGFR overexpression promotes EGF/EGFR-induced invasion of AKC2 cells

Recent studies have shown that EGFR can be overexpressed in HPV-associated anal cancer biopsies. E5, E6 and E7 expression have all been linked to EGFR overexpression. We therefore determined if total EGFR (T-EGFR) and the active phosphorylated form of EGFR (p-EGFR) were overexpressed in AKC2 cells and if EGFR overexpression contributed to their invasive phenotype. We serum-starved the AKp anal parental cells and early (p10), mid (p105) and late (p 450) passage AKC2 cells for 24 h and then stimulated them with 10 ng ml^{-1} EGF for 15 and 30 min without the addition of serum or additional growth factors. We included AKC2 cells of early passage (p10), which similar to AKp cells were maintained in low-calcium full-supplemented growth factor media. These were compared with AKC2 cells that were maintained in high-calcium non-growth factor

supplemented media to show that changes in EGFR expression were due to HPV-16 and not due to differences in cell culture media. Unstimulated AKC2 cells of all three passages contained approximately five (in AKC2 p10 and p105) to seven (in AKC2 p450) times more T-EGFR compared with AKp cells (Fig. 3a). We also found that p-EGFR expression levels decreased in AKp cells from 15 to 30 min EGF treatment whereas p-EGFR increased from 15 to 30 min in all three AKC2 cell passages (Fig. 3a). Our data indicate that: (1) the addition of HPV-16 even at early passage led to an overall increase in T-EGFR above that of AKp, which is consistent with the known mechanism of E5 to increase EGFR turnover to the cell membrane [17, 29]. Increases in T-EGFR would therefore result in increased EGF ligand/EGFR interactions and p-EGFR expression levels. (2) The increase in p-EGFR level in AKC2 from 15 to 30 min is consistent with previous work showing that E5 interferes with the Cbl E3 ubiquitin ligase, which is responsible for EGFR/p-EGFR degradation [19]. A recent study showed that Cbl mutants lose the ability to degrade p-EGFR products leading to increases in p-EGFR expression levels over time [17, 38].

To next determine if EGF alone (i.e. without the addition of serum or other growth factors) could induce invasion of AKC2 cells, we measured the invasive potential of the AKp and AKC2 cells using an *in vitro* collagen invasion assay. We seeded equal numbers of previously serum-starved cells, AKp or AKC2, onto collagen-coated membranes and measured invasion levels 24 h post-seeding. AKC2 cells had a higher basal level of invasion compared with AKp cells (Fig. 3b, c). There was a substantial increase in the number of invasive AKC2 cells compared with the AKp following exposure to 10 ng ml^{-1} EGF, whereas we did not detect a significant increase in AKp invasion (Fig. 3b, c).

Finally, to show that overexpression of p-EGFR played a role in AKC2 invasion we treated AKC2 cells with the EGFR tyrosine kinase inhibitor, gefitinib. Treatment of AKC2 cells with 1 µM of gefitinib for 24 or 48 h did not decrease cell viability (data not shown) and reduced p-EGFR expression levels of AKC2 close to basal levels (Fig. 3d). To determine if treatment with gefitinib also decreased EGF/EGFR-induced invasion we pre-treated AKC2 cells with 1 µM gefitinib for 24 h during serum starvation and then seeded an equal number of non-treated and treated cells on both control non-coated inserts and *in vitro* collagen-coated inserts. Non-coated inserts were used to control for changes in proliferation or other effects during seeding that might confound the collagen invasion results. Using EGF as a chemoattractant, we calculated percent invasion comparing the number of cells that invaded the collagen-coated insert after 24 h of incubation with the number of cells that grew on the control membrane. Treatment of AKC2 cells with gefitinib decreased the percent invasion close to basal levels (Fig. 3e, f) consistent with a role for total EGFR/p-EGFR overexpression in AKC2 invasion.

Fig. 3. EGFR is overexpressed in AKC2 cells and leads to EGF/EGFR-induced invasion. (a) Western blot of total EGFR (T-EGFR) and phosphorylated EGFR (p-EGFR) expression in AK p and AKC2 cells following 24 h serum starvation and induction with EGF (10 ng ml^{-1}, 15 or 30 min). (b) *In vitro* collagen invasion assay of AKp and AKC2. AKp and AKC2 were both serum/growth factor starved for 24 h prior to seeding on collagen-coated inserts. EGF (10 ng ml^{-1}) was used as a chemoattractant and invasion was measured 24 h later. Columns represent average cell numbers calculated from three fields of view (×10 magnification) per well and from triplicate wells. Cell numbers for Akp are located above each bar. Error bars represent the SD. (c) Images of the collagen-coated inserts following EGF-induced invasion of AKp and AKC2 cells (×10 magnification). (d) Western blot of T-EGFR and p-EGFR protein expression in AKC2 following 24 h treatment with gefitinib, serum starvation and EGF induction (10 ng ml^{-1}, 30 min). (e) *In vitro* collagen invasion assay of AKC2 treated with gefitinib (24 h) compared to non-treated cultures. EGF (10 ng ml^{-1}) was used as a chemoattractant and percent invasion was measured 24 h later. Percent invasion was calculated as the percentage of cells that invaded collagen-coated inserts to the number of cells that grew on non-coated inserts. Columns represent average percent invasion calculated from triplicate inserts of both non-coated and collagen-coated inserts. Error bars represent the SD. (f) Images of the *in vitro* collagen inserts. AKC2 cells are detected by the purple stain (×10 magnification).

Reducing E5 expression by transfecting E5-targeted siRNAs leads to knockdown of multiple HPV-16 genes and normalizes EGFR expression levels

Having established that EGFR overexpression plays an important role in AKC2 invasion we wanted to determine which HPV-16 oncogenes contributed to EGFR overexpression. To examine the individual roles of E5, E6 and E7 in EGFR overexpression in AKC2 cells we transfected AKC2 cells with individual targeted siRNAs to knock down each viral oncogene. Consistent with previous studies, our initial attempts to knock down E6 and E7 individually were unsuccessful since HPV-16 contains a polycistronic genome. We then used a cocktail of E6- and E7-targeted siRNAs to ensure a high knockdown efficiency of both oncogenes. In addition, we transfected individual cultures with equal concentrations of E5-targeted siRNAs as well as a scrambled control siRNA. We harvested total RNA from each well 72 h post-transfection and measured HPV-16 E5, E6 and E7 expression using Sybr Green qPCR methods.

Transfection with E6/E7 siRNAs resulted in a 83 % decrease in both E6 and E7 expression (Fig. 4a). In addition, transfection of E6/E7-specific siRNAs also resulted in a 65 % decrease in E5 expression and a 84 % decrease in E2 expression, relative to controls (Fig. 4a). Transfection with E5-targeted siRNAs also resulted in a global knockdown of E2, E5, E6 and E7 expression and with a knockdown efficiency of 99 % in all four genes relative to controls (Fig. 4a). We detected a larger increase in p53 protein (Fig. 4b) (i.e. a direct measurement of E6 protein loss of function), a larger decrease in E7 protein (Fig. 4b), and lower proliferation rates in cells transfected with E5-targeted siRNAs, compared with cells transfected with control or E6/E7-targeted siRNAs (Fig. 4c).

To determine if reduction in E6/E7 or E5 expression resulted in a decrease in total EGFR and p-EGFR, we transfected AKC2 with either control, E6/E7- or E5-targeted siRNAs. We harvested lysates 72 h post-transfection following 24 h of serum starvation and then 30 min of induction with 10 ng ml^{-1} EGF. We did not detect a decrease in total
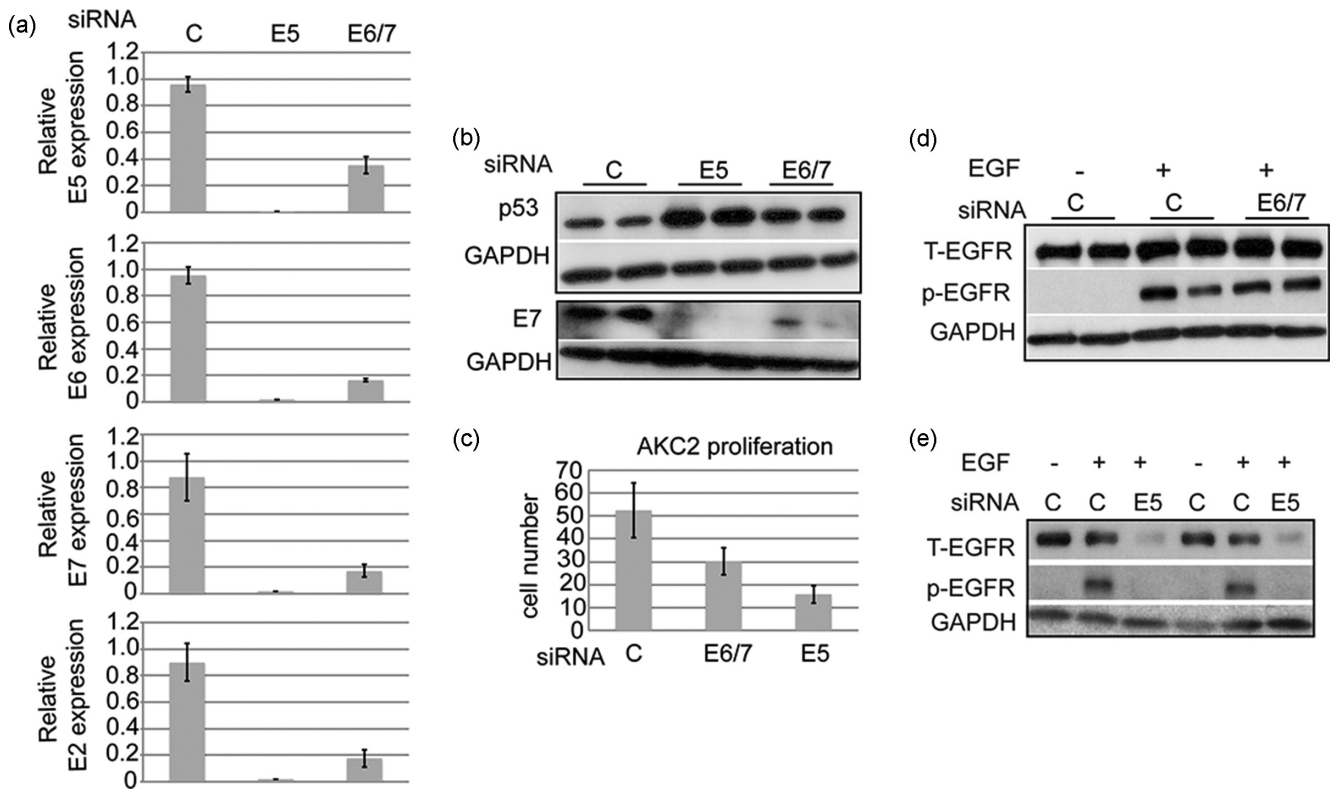
Fig. 4. Transfection of HPV-16 E5-specific siRNAs leads to reduction of E2, E5, E6 and E7 as well as a reduction in EGFR expression in AKC2 cells. (a) AKC2 cells were transfected with control, E5 or a cocktail of E6 and E7 siRNAs. Relative HPV-16 gene expression was determined by qPCR 72 h post-transfection. Columns represent average relative fold changes in HPV-16 E5, E6, E7 and E2 expression from triplicate wells. HPV-16 gene expression was normalized to the beta-actin housekeeping gene. The $2^{-\Delta\Delta ct}$ was used to calculate relative fold expression. Error bars represent the SD. (b) Western blots of HPV-16 E7 and p53 protein expression taken from AKC2 lysates 72 h post-transfection of control, E5 or E6/E7 siRNAs. (c) AKC2 proliferation following transfection of control, E5 or E6/E7 siRNAs. Columns represent the average cell number calculated from triplicate wells 72 h post-transfection. Error bars represent the SD. (d) Western blot of total EGFR (T-EGFR) and phosphorylated EGFR (p-EGFR) expression levels in AKC2 cells, 72 h post-transfection of control, or E6/E7 siRNAs. AKC2 cells were serum-starved for 24 h, followed by induction with EGF (10 ng ml^{-1}, 30 min). (e) Western blot of T-EGFR and p-EGFR expression levels in AKC2 cells, 72 h post-transfection of control or E5 siRNAs. AKC2 cells were serum-starved for 24 h, followed by induction with EGF (10 ng ml^{-1}, 30 min).

EGFR or p-EGFR expression levels in EGF-induced cultures transfected with the lower-efficient E6/E7-targeted siRNAs relative to controls (Fig. 4d). However, transfection with the higher-efficient E5-targeted siRNAs reduced total EGFR and p-EGFR expression to control levels (Fig. 4e). Although transfecting AKC2 with E6/E7-targeted siRNAs led to a decrease in all three HPV-16 oncogenes it is possible that the lower efficiency of E6/E7-targeted siRNAs in reducing E5, E6 and E7 expression was not sufficient to reduce total EGFR or p-EGFR expression levels.

Although this did not pinpoint which specific HPV oncogenes play a role in EGFR regulation, this confirmed an overall contribution from HPV in EGFR overexpression following transfection of E5-targeted siRNAs. This also showed that targeting E5 could be a highly efficient way to eliminate E5, E6, E7 as well as E2, also leading to normalization of EGFR.

E5 plays a key role in p-EGFR overexpression and EGF/EGFR-induced invasion

Since E5-targeted siRNAs led to reduced expression of E5, E6 and E7, we next sought to determine their relative importance in EGFR overexpression and downstream EGF/EGFR-induced invasion. We therefore performed E6/E7 rescue experiments in AKC2 cells, while also knocking down E5 expression. We first transfected AKC2 cells with either a control promoter-less plasmid (pGL3-Basic) or the pB-actin E6/E7 expression plasmid. Then, 24 h later we transfected either a scrambled control or E5-targeted siRNA. We harvested total RNA 72 h post-plasmid transfection and 48 h post-siRNA transfection, and measured E5, E6 and E7 expression by Sybr Green qPCR methods. Transfection with the E6/E7 plasmid followed by control siRNA increased both E6 and E7 expression levels relative to cells transfected with control plasmid and control siRNA. Transfection of the E6/E7 plasmid did not lead to a change in E5

expression levels (Fig. 5a). Transfection of E6/E7 plasmid followed by E5 siRNA successfully rescued E6/E7 expression relative to cultures transfected with control plasmid and E5 siRNA. We also found that transfection of E6/E7 plasmid rescued E7 protein levels in cultures that were also transfected with E5 siRNAs (Fig. 5b). Since the Western blot gradient gels only permitted us to load small amounts of AKC2 lysate, we were only able to detect E7 protein in AKC2 cells that were transfected with the HPV-16 E6/E7 overexpression plasmid (Fig. 5b). We were unable to detect E6 protein due to lack of a suitable E6 antibody. However, since E6 and E7 RNA expression levels were almost identical following transfection of the E6/E7 expression plasmid and it is known that E6 and E7 are translated with equal efficiency, we assumed that their protein levels were also similar.

To next determine if E5 played a role in EGFR overexpression/p-EGFR activation during E6/E7 rescue we performed the identical rescue transfection protocol and we harvested AKC2 lysates 72 h post-plasmid transfection. We found that overexpression of E6/E7 did not restore p-EGFR expression levels to control levels in cultures with reduced E5 expression levels (E6/E7/E5) (Fig. 5b), consistent with an important role for E5 in p-EGFR overexpression. Since the E6/E7 rescue experiment could only be performed using a staggered (plasmid/siRNA) transfection leaving a 48 h E5-knockdown phase, we only observed a slight decrease in total EGFR expression levels in both E5-knockdown (C/E5) and E6/E7 rescue cultures (E67/E5) (Fig. 5c). However, reductions in activated p-EGFR were easily observed as early as 48 h post-E5 siRNA transfection. We observed a more pronounced reduction in total EGFR protein at 72 h post-siRNA transfection, likely due to an extended protein degradation time (Fig. 5d).

To determine if E5 played a role in EGF/EGFR-induced invasion we performed an identical rescue transfection experiment using our invasion model and measured percent invasion into collagen over 24 h. Overexpression of E6/E7 in cultures with reduced E5 expression (E6/7/E5) did not increase EGF/EGFR-induced invasion above control E5 knockdown cultures (C/E5) (Fig. 6a, b) consistent with an
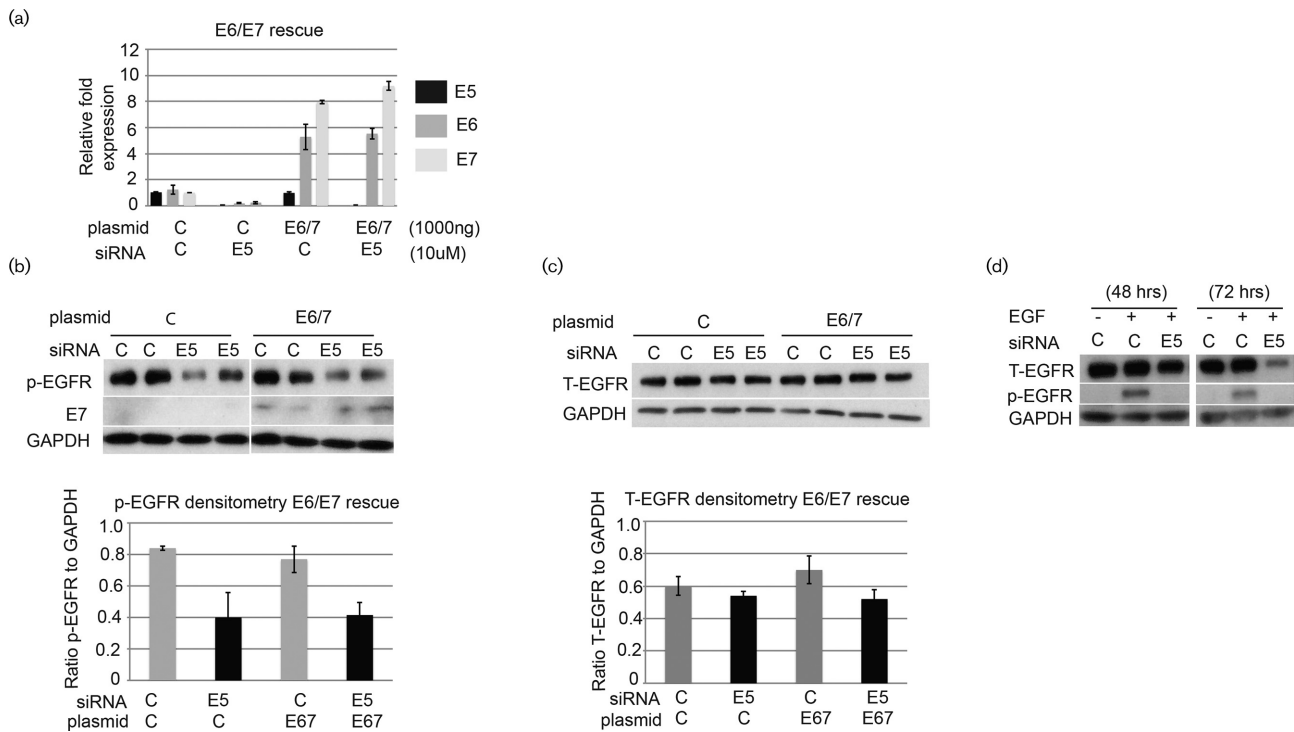
Fig. 5. HPV-16 E5 plays a key role in EGFR overexpression. (a) E5-mediated knockdown of E5, E6 and E7 accompanied by HPV-16 E6/E7 rescue. AKC2 cells were transfected with control or a E6/E7 expression plasmid, followed by control or E5 siRNAs 24 h later. Relative E5, E6 or E7 expression was measured by qPCR 72 h post plasmid transfection. Columns represent the average relative fold expression calculated from triplicate wells. HPV-16 gene expression was normalized to the beta-actin housekeeping gene. The $2^{-\Delta\Delta ct}$ was used to calculate relative fold expression. Error bars represent the SD. (b) Western blot of p-EGFR and HPV-16 E7 expression in AKC2 cells following HPV-16 E5-mediated knockdown of E5, E6 and E7 accompanied by HPV-16 E6/E7 rescue. Columns represent the average densitometry measurements from duplicate lanes calculated as the ratio of p-EGFR to GAPDH. (c) Western blot of T-EGFR expression in AKC2 cells following HPV-16 E5 mediated knockdown of E5, E6 and E7 accompanied by HPV-16 E6/E7 rescue. Columns represent the average densitometry measurements from duplicate lanes calculated as the ratio of T-EGFR to GAPDH. (d) Western blot of T-EGFR and p-EGFR expression levels in AKC2 cells following transfection of control or E5 siRNAs at 48 h and 72 h post-transfection.

important role for E5 in modulation of EGFR overexpression and EGFR-mediated invasion.

We also repeated the collagen invasion assay using a second E5-targeted siRNA, which targeted a different region of the E5 ORF. Transfection of this second E5-targeted siRNA led to an 80 % decrease in E5, E6 and E7 (Fig. S1a, available in the online version of this article), also accompanied by a substantial decrease in cellular proliferation when compared to control cultures (data not shown). Results following transfection with the second E5-targeted siRNA, using similar E6/E7 rescue conditions, were also consistent with a role for E5 in EGFR-induced invasion. Here a fourfold increase in E6 and E7 expression, above control levels, accompanied by a 80 % reduction in E5 expression, did not restore EGFR-induced invasion back to control levels (Fig. S1b).

One limitation to this study is that we were unable to perform the same rescue for E5 expression due to the high potency of E5-targeted siRNAs. Although it is possible that E5 alone plays a role in EGFR overexpression and EGFR-induced invasion, we cannot definitively exclude some contribution from E6 and E7. In addition, it is likely that E6/7 plays a role in cellular invasion via other mechanisms [39]. However, our data show that E5 is a key contributor too in EGFR overexpression and EGFR-induced invasion.

DISCUSSION

In this study, we detected co-expression of HPV-16 E5, E6 and E7 in HPV-16-positive anal SCC biopsies. To date there have been no studies that characterize the complete range of viral oncogene expression in HPV-16-positive anal SCC biopsies and few studies have described E5 expression in biopsies from non-cervical HPV-associated epithelial lesions. Few studies have investigated the contribution of E5 to HPV-16-associated carcinogenesis when endogenously co-expressed with E6 and E7. Herein we describe the first HPV-transformed anal epithelial cell line, AKC2 cells, and

Fig. 6. HPV-16 E5 plays a key role in EGF/EGFR-induced invasion of AKC2 cells. (a) *In vitro* collagen invasion assay following E5-mediated knockdown of E5, E6 and E7 accompanied by E6/E7 rescue. AKC2 cells were transfected with control or E6/E7 expression plasmid, followed by control or E5 siRNAs 24 h later. Transfected cells were seeded onto collagen-coated inserts 48 h post siRNA transfection. EGF (10 ng ml^{-1}) was used as a chemoattractant and percent invasion was measured 24 h later. Percent invasion was calculated as the percentage of cells that invaded collagen-coated inserts to the number of cells that grew on non-coated inserts. Columns represent average percent invasion calculated from triplicate inserts of both non-coated and collagen-coated inserts. Error bars represent the SD. (b) Images of the *in vitro* collagen inserts from the collagen invasion assay. AKC2 cells are detected by the purple stain (×10 magnification).

use these cells to show how E5 may contribute to the HPV-16-associated anal cancer pathogenesis.

Earlier studies have suggested that E5 is lost during viral integration and is therefore missing in most HPV-associated cancers. However, it is now clear that several HPV-16-positive tumour-derived cell lines including the oral cancer line SCC90 and CaSki cells contain E5 mRNA and translated protein [13, 32].

The AKC2 line was established by transfecting the entire pEF399 plasmid containing the HPV-16 W12 genome into primary HPV-negative anal epithelial cells derived from a normal anal biopsy. We detected HPV-16 E2, E5, E6 and E7 expression in early and late passage AKC2 cells. Over long-term passage AKC2 developed into a robust, rapidly growing, HPV-16-positive cell line and *in vitro* model to investigate HPV-16-associated anal cancer pathogenesis.

Early and late passage AKC2 cells resembled a HSIL/invasive cancer when propagated in three-dimensional raft culture. AKC2 raft cultures also phenotypically resembled HSIL/invasive cancer based on their H&E appearance and expression patterns of established HPV-associated biomarkers including MCM (E7 surrogate marker) and p16, which are both associated with E7 overexpression [35, 40]. Previous studies have shown that raft cultures of HPV-16-positive cell lines can mimic different lesion grades (i.e. LSIL, HSIL and cancer) when stained with antibodies specific to MCM and p16 [41].

Few studies of anal cancer pathogenesis have characterized the mechanisms by which HPV oncogenes and cellular partners contribute to the transformation process. The EGF/EGFR pathway may play an important role in HPV-associated anal, cervical and oral cancer pathogenesis, and several recent studies have shown that similar to HPV-associated cervical and oral-squamous cell cancers, EGFR is often overexpressed in HPV-associated anal lesions and increases with lesion severity [25, 26, 42, 43].

Increased EGFR activity has been widely linked to cellular invasion in many cell types [44–46]. In our study, we show that EGFR overexpression/activation plays an important role in anal carcinogenesis through enhancing invasion. Furthermore, treatment with gefitinib, an FDA-approved EGFR tyrosine kinase inhibitor, led to a decrease in EGFR phosphorylation and prevented AKC2 invasion in the presence of EGF ligand.

Previous studies investigating EGFR overexpression in anal biopsies did not explore the role of HPV genes in inducing EGFR expression. However, they found that EGFR overexpression staining did not correlate with EGFR gene copy number or EGFR mutations [42, 43]. These data suggested that a HPV-16 gene such as E5 could be responsible for the observed overexpression possibly through its established mechanism of reducing EGFR turnover.

To investigate the role of E5 in EGFR overexpression and EGFR-induced invasion we used E5-targeted siRNAs to knock down E5 expression. We found that targeting E5 in AKC2 led not only to knock down HPV-16 E5 gene expression, but also to reduction of expression of E2, E6 and E7 by 99 %. HPV-16 contains polycistronic transcripts that code for multiple HPV genes. Previous studies have shown that targeting E7 with E7-targeted siRNAs led to a decrease in E6 and E2 expression [47].

Because of its effects on expression of E6 and E7, targeting E5 with E5-targeted siRNAs did not allow us to investigate its individual role in EGFR overexpression and EGF/EGFR-induced invasion. Although the observed reduction of total EGFR was consistent with a role for E5 in increased EGFR turnover, previous studies have also shown that E6/7 may affect total EGFR expression levels through increasing EGFR-specific mRNA levels or EGFR promoter activity [48, 49]. However, we found that E6/7 overexpression in AKC2 did not increase EGFR-specific mRNA levels (data not shown) nor did it lead to an increase in total or phosphorylated EGFR expression levels. Furthermore, rescue of E6/7 expression simultaneously with E5 knockdown did not result in restoration of phosphorylated EGFR expression levels or EGFR-induced invasion levels.

In addition to its role in EGFR-related pathogenesis it is also possible that E5 plays a role in regulating E6/7 expression, and this may also occur through its effects on EGFR expression. Previous studies have shown that E6/7 mRNA levels and alternate splicing of HPV-16 E6 are coupled to EGF/EGFR signalling [50, 51]. It is therefore possible that at least some of the reduction in E6/7 expression observed when knocking down E5 expression could be related to a loss in EGFR signalling. Further studies are needed to establish this, but if confirmed, however this would be another important mechanism by which E5 contributes to HPV-16-associated carcinogenesis and provides further rationale to target E5 for therapeutic purposes.

In summary, we have shown that E5 is expressed in HPV-16-positive anal SCCs consistent with an important role for this protein in anal carcinogenesis. We have established the first permanent HPV-16-positive anal cell line. This cell line, which expresses E2, E5, E6 and E7, provides a robust model for studying the role of E5 and other HPV oncogenes in HPV-associated pathogenesis, including anal cancer pathogenesis. Using this model, we found that E5 plays a key role in EGFR overexpression and EGFR-induced invasion. Targeting E5 using an siRNA approach leads to knockdown of at least four HPV-16 early genes including both E6 and E7, and this may represent a powerful approach to HPV-specific therapy for HPV-associated cancers including anal cancer.

METHODS

Anal cancer biopsies

Anal cancer biopsies were obtained from participants in the AIDS Malignancy Consortium (AMC)-045 protocol evaluating the effects of the addition of cetuximab to inhibit EGFR to standard chemoradiation therapy for anal cancer.

All samples were obtained prior to initiation of therapy and were analysed with institutional review board permission.

Establishment of the AKC2 cell line: isolation of normal anal primary epithelial cells and anal fibroblasts

Primary anal epithelial cells were isolated from clinically normal-appearing anal tissue taken from a HIV-positive donor. The biopsy was divided immediately after collection with part placed in formalin and paraffin-embedded for histopathological examination, which confirmed normal histology. The other part of the biopsy was cut into several 1 mm-sized explants, which were placed on Matrigel-coated inserts (Costar). The explants were cultured in small airway epithelial growth medium (SAGM) (Lonza) for 12 days. Epithelial cells that grew out of the explants were then trypsinized, and passaged to standard cell culture flasks in SAGM for expansion. Anal fibroblasts were isolated from stromal tissue taken from a normal anal biopsy from a different donor. To isolate anal fibroblasts the stromal tissue was first minced and then incubated in 100 U ml^{-1} collagenase (Invitrogen) in Hank's balanced salt solution containing 3 mM Ca^{2+} for 1 h at 37 $^{\circ}$C, with vigorous shaking. Fibroblasts were expanded in Dulbelco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin.

AKC2 cell line generation and monolayer cell culture

The AKC2 cell line was established by co-transfecting the primary anal epithelial cells with the entire pEF399 plasmid (a kind gift from Paul Lambert), which contained the 8 kb HPV16-W12 genome, and the pcDNA3.1 V5-HIS/lacZ plasmid, which contained a neomycin resistance gene. Transfections were carried out using the Lipofectamine LTX reagent (Invitrogen). The transfected cells underwent selection in 1 mg ml^{-1} G418 (Life Technologies) for the first three days and then in 0.4 mg ml^{-1} G418 for an additional four days. AKC2 cells were initially expanded as a population after selection in a 24-well plate. AKC2 cells were initially grown in serum-free SAGM (Lonza) without an irradiated fibroblast layer and then were adapted to standard DMEM-high glucose containing 10 % FBS and 1 % penicillin-streptomycin.

Total genomic DNA extraction and HPV 16 oncogene copy number determination

Total genomic DNA was extracted from monolayer epithelial cells using the QiAmp DNA Blood Mini Kit (Qiagen). DNA for HPV-16-W12 genes or housekeeping genes was amplified using Power SYBR Green PCR Master Mix (Life Technologies-Applied Biosystems) and was quantified using an AB 7900HT machine (Life Technologies-Applied Biosystems). Copy number determination for each HPV-16 oncogene was based on standard curve equations for HPV-16 E5, E6, E7 and beta-actin housekeeping gene primers. Primer sets for HPV-16 E5, E6, E7 and beta-actin are listed below.

RNA extraction from biopsies and AKC2 cells, reverse transcription and quantitative PCR

Total RNA was extracted from 10-micron sections of formalin-fixed paraffin embedded anal cancer biopsies using the Recover All Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific). Total RNA was isolated from all monolayer epithelial cells using the RNeasy mini kit (Qiagen). The remaining DNA was removed using the TURBO DNA-free kit (Ambion) and the RNA was then reverse transcribed using the High Fidelity cDNA synthesis kit (Roche). All cDNA for HPV-16-W12 genes or housekeeping genes were amplified using Power SYBR Green PCR Master Mix (Life Technologies-Applied Biosystems) and were quantified using an AB 7900HT machine (Life Technologies-Applied Biosystems). Relative transcript levels were calculated from triplicate wells using the $2^{-\Delta\Delta ct}$ method. The following primer sets were used to amplify the HPV-16-W12 genes and cellular endogenous controls:

HPV-16-W12 primer sequences
E2 F 5′-GCCAACACTGGCTGTATCAA-3′
E2 R 5′-CATCCTGTTGGTGCAGTTAAA-3′
E5 F 5′-CCACAACATTACTGGCGTGC-3'
E5 R 5′-GCAGAGGCTGCTGTTATCCAC-3′
E6 F 5′-CTGCAATGTTTCAGGACCCA-3′
E6 R 5′-TCATGTATAGTTGTTTGCAGCTCTGT-3′
E7 F 5′-ACCGGACAGAGCCCATTACA-3′
E7 R 5′-GCCCATTAACAGGTCTTCCAAA-3′
L1 F 5′-CGTTATGACATACATACATTCTATGAATTCC-3′
L1 R 5′-GATGTTACAAACCTATAAGTATCTTCTAAGTGT-3′
Endogenous control primer sequences
Beta actin F 5′-TCACCCACACTGTGCCCATCTAC-GA-3′
Beta actin R 5′-CAGCGGAACCGCTCATTGCCAATGG-3′
RPLPO F 5′-GCACCATTGAAATCCTGAGTGA-3′
RPLPO R 5′-CGCTGGCTCCCACTTTGT-3′

APOT PCR and Southern blot analysis

HPV oncogene transcripts were amplified as previously described using identical primer sequences and cycling conditions [34]. Briefly total RNA (1 µg) was reverse transcribed using 25 µM oligo-(dT) primer coupled to a linker sequence, with 200 U of SuperScript III Reverse Transcriptase at 50 degrees Celsius for 60 min, followed by inactivation at 70 degrees Celsius for 15 min. PCR products were separated on a 1.2 % agarose gel and blotted on to a Biodyne membrane (Pall Corporation) overnight, using 20× SSC. Membranes were subsequently incubated at 80 degrees Celsius, for 1 h. Membranes were then hybridized to biotin-labelled E7 5′-TCGTACTTTGGAAGACCTGTTAATG-3′ or E4-specific 5′-GAAGAAACACAGACGACTATCCAG-3′ probes and detection was carried out using a HRP-conjugate streptavidin (Thermo Fisher) and ECL chemiluminescent (GE Healthcare) substrate as previously described [52].

Western blot analysis of AKC2 cells

Cellular protein lysates were extracted by cell scraping into standard RIPA lysis buffer (Cell Signaling) containing Complete EDTA-free Total Protease Inhibitor Cocktail (1×) (Sigma). PhosSTOP Phosphatase Inhibitor Cocktail (1×) (Roche) was added to the lysis buffer when detecting protein phosphorylation products. All samples were separated on a 4–20 % gradient Tris Glycine denaturing gel (Novex). Proteins were transferred to immunoblot 0.45 µm (high molecular weight products) or 0.22 µm (low molecular weight products) PVDF membranes (GE Healthcare). Transferred membranes were blocked in 5 % milk in TBS-0.1% Tween20 and were probed with the appropriate primary antibodies. The following primary antibodies were used: E7-ED17 (Santa Cruz Biotechnologies) and mouse anti-E7-8C9 (Invitrogen), mouse anti-p53-DO1 (Santa Cruz Biotechnologies), rabbit anti-EGFR (Cell Signaling) and rabbit anti-phosphorylated EGFR Y1173 (Cell Signaling).

Immunofluorescence of AKC2 cells

Monolayer cells were grown on glass coverslips and were fixed with 4 % paraformaldehyde for 15 min and then were permeabilized with 0.1 % Triton-×100 for 5 min at room temperature. The cells were blocked with 10 % normal goat serum (NGS) or normal horse serum (NHS) for 1 h and then primary antibodies were incubated in 5 % NGS or NHS, overnight at 4 °C. Keratins were stained using a rabbit anti-pan-keratin antibody (Invitrogen). DAPI was used as a nuclear counterstain (Life Technologies). Secondary antibodies included an anti-mouse IgG (DyLight 488) (Vector Labs). Immunostained cells were visualized using a Nikon Eclipse E400 fluorescent microscope.

Organotypic raft cultures of AKC2 cells

Organotypic raft cultures of the AK parental and AKC2 cell lines were established as described previously [53]. Briefly cells were seeded onto collagen dermal equivalents containing collagen type 1 from rat tail (Millipore) and anal fibroblasts. Raft cultures were differentiated for a total of 15 days and were then formalin-fixed as described previously [53]. Raft cultures were then paraffin-embedded and 5-micron-thick sections of each raft were placed onto plus-charged slides.

Immunohistochemistry of raft cultures

Sections of formalin-fixed and paraffin-embedded raft cultures were de-waxed in Citra-Solv twice for five minutes and were rehydrated in graded ethanol (95, 70 and 50 %) and water for 5 min each. Antigen retrieval was achieved by boiling the sections in 10 mM citric acid (pH 6.0) and .05 % Tween 20 for a total of 15 min. The sections were then equilibrated in PBS and were blocked with 10 % NGS for 1 h. Primary antibody staining was completed overnight at 4 °C. The following primary antibodies were used: mouse anti-Cytokeratin K10/K13 monoclonal (DE K13) (Santa Cruz Biotechnologies), mouse anti-MCM monoclonal (CDC47 Ab-2) (Neomarkers), mouse anti-p16 monoclonal (BD Biosciences). To remove unbound antibody the sections were then washed three times in PBS/.05% Tween 20 three times for 5 min each. The secondary antibody anti-mouse IgG (Dylight 488) (Vector Labs) was then incubated with DAPI for 45 min at room temperature followed by an identical wash method. Immunostained cells were visualized using a Nikon Eclipse E400 fluorescent microscope.

Soft agar assay of AKC2 cells

To investigate anchorage-independent growth in soft agar, a base agar containing 0.8 % agarose, DMEM and 10 % FBS was first added to individual wells of 6-well plates of AKC2 cells. The top agar containing 0.7 % agarose, 2.5×10^5 cells, DMEM and 10 % FBS was then added on top of the base layer of each well. Colony growth was measured 10 days later by phase microscopy.

Proliferation assay of AKC2 cells

AKC2 cells were trypsinized and stained with 0.4 % trypan blue (Gibco) to select for live cells. Standard cell counts were conducted using a hemocytometer.

HPV-16 siRNA transfection and E6/E7 rescue experiments

AKC2 cells were plated at 1.5×10^5 cells in 6-well plates and were transfected with 30 pmol of siRNA oligonucleotides using RNAi MAX reagent (Invitrogen). The following siRNA oligonucleotides were used for transfection: Control siRNA-A (Santa Cruz Biotechnologies) HPV-16 E6 (Santa Cruz Biotechnologies), HPV-16 E7 (Santa Cruz Biotechnologies), HPV-16 E5 target sequence: AATGGTATTACTA TTGTGGATAA (Eurofins MWG Operon) and HPV-16 E5 target sequence #2 CAACATTACTGGCGTGCTTTT (Dharmacon). RNA expression was measured 72 h post-siRNA transfection. To perform E6/E7 rescue experiments 1.5×10^5 AKC2 cells were first transfected with 1 µg of pB-actin E6/7 expression plasmid (a kind gift from Karl Munger) or the promoter-less pGL3 basic control plasmid (Promega) using Lipofectamine LTX and Plus reagent (Invitrogen). Then, 24h later the AKC2 cultures were transfected again with 30 pmol of the appropriate siRNA using RNAi MAX reagent (Invitrogen). cDNA or protein expression was measured 72 h post-plasmid transfection.

EGFR tyrosine kinase inhibition and EGFR induction assays

The EGFR tyrosine kinase inhibitor gefitinib (Iressa, Selleckchem) was added to cells at 1 µM for 24 h during serum starvation. EGFR induction was carried out post-serum starvation by adding 10 ng ml^{-1} EGF (Sigma) alone without serum, for 30 min. AKC2 lysates were then harvested for Western blot analysis.

Chemotactic *in vitro* collagen invasion assay and quantitation of invasion

The chemotactic *in vitro* collagen invasion assays were performed using the Collagen Cell Invasion Assay-Colorimetric (8 µM) (EMD Millipore) according to the manufacturer's protocol. Here cells move through a collagen-coated

membrane in response to chemoattractant stimulus. Briefly 5×10^4 cells were seeded onto individual collagen-coated inserts in DMEM only. DMEM alone or DMEM containing $10\,\text{ng}\,\text{ml}^{-1}$ EGF (i.e. chemoattractant) was added to the lower chamber. Cell invasion was measured 24 h later by counting individual cells that invaded the collagen inserts using light microscopy. To quantify invasion, equal cell numbers were seeded onto non-coated control inserts $0.8\,\mu\text{M}$ (Millipore) and collagen-coated inserts $0.8\,\mu\text{M}$. Percent invasion was used as a measure to control for cellular proliferation and migration following exposure to EGF. Percent invasion was calculated by expressing as a percentage of the number of cells that invaded the collagen inserts compared with the number of cells that grew on the non-coated control inserts following addition of DMEM only or DMEM containing $10\,\text{ng}\,\text{ml}^{-1}$ EGF.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
Anal cancer biopsies were obtained from participants in the AIDS Malignancy Consortium (AMC)-045 protocol evaluating the effects of addition of cetuximab to inhibit EGFR to standard chemoradiation therapy for anal cancer. All samples were obtained prior to initiation of therapy and were analysed with institutional review board permission.

References
1. **Brickman C, Palefsky JM.** Human papillomavirus in the HIV-infected host: epidemiology and pathogenesis in the antiretroviral era. *Curr HIV/AIDS Rep* 2015;12:6–15.

2. **Silverberg MJ, Lau B, Justice AC, Engels E, Gill MJ et al.** Risk of anal cancer in HIV-infected and HIV-uninfected individuals in North America. *Clin Infect Dis* 2012;54:1026–1034.

3. **Hoots BE, Palefsky JM, Pimenta JM, Smith JS.** Human papillomavirus type distribution in anal cancer and anal intraepithelial lesions. *Int J Cancer* 2009;124:2375–2383.

4. **Steinau M, Unger ER, Hernandez BY, Goodman MT, Copeland G et al.** Human papillomavirus prevalence in invasive anal cancers in the United States before vaccine introduction. *J Low Genit Tract Dis* 2013;17:397–403.

5. **Genther SM, Sterling S, Duensing S, Münger K, Sattler C et al.** Quantitative role of the human papillomavirus type 16 E5 gene during the productive stage of the viral life cycle. *J Virol* 2003;77:2832–2842.

6. **Fehrmann F, Klumpp DJ, Laimins LA.** Human papillomavirus type 31 E5 protein supports cell cycle progression and activates late viral functions upon epithelial differentiation. *J Virol* 2003;77:2819–2831.

7. **Regan JA, Laimins LA.** Bap31 is a novel target of the human papillomavirus E5 protein. *J Virol* 2008;82:10042–10051.

8. **Kotnik Halavaty K, Regan J, Mehta K, Laimins L.** Human papillomavirus E5 oncoproteins bind the A4 endoplasmic reticulum protein to regulate proliferative ability upon differentiation. *Virology* 2014;452-453:223–230.

9. **Chang JL, Tsao YP, Liu DW, Huang SJ, Lee WH et al.** The expression of HPV-16 E5 protein in squamous neoplastic changes in the uterine cervix. *J Biomed Sci* 2001;8:206–213.

10. **Cavuslu S, Starkey WG, Kell B, Best JM, Cason J.** Detection of human papillomavirus type 16 in microtitre plate based immunoenzymatic assays: use to determine E5 gene expression in cervical carcinomas. *Clin Diagn Virol* 1996;5:215–218.

11. **Shirasawa H, Tomita Y, Kubota K, Kasai T, Sekiya S et al.** Transcriptional differences of the human papillomavirus type 16 genome between precancerous lesions and invasive carcinomas. *J Virol* 1988;62:1022–1027.

12. **Um SH, Mundi N, Yoo J, Palma DA, Fung K et al.** Variable expression of the forgotten oncogene E5 in HPV-positive oropharyngeal cancer. *J Clin Virol* 2014;61:94–100.

13. **Sahab Z, Sudarshan SR, Liu X, Zhang Y, Kirilyuk A et al.** Quantitative measurement of human papillomavirus type 16 e5 oncoprotein levels in epithelial cell lines by mass spectrometry. *J Virol* 2012;86:9465–9473.

14. **Park JS, Hwang ES, Park SN, Ahn HK, Um SJ et al.** Physical status and expression of HPV genes in cervical cancers. *Gynecol Oncol* 1997;65:121–129.

15. **Cullen AP, Reid R, Campion M, Lörincz AT.** Analysis of the physical state of different human papillomavirus DNAs in intraepithelial and invasive cervical neoplasm. *J Virol* 1991;65:606–612.

16. **Matsukura T, Koi S, Sugase M.** Both episomal and integrated forms of human papillomavirus type 16 are involved in invasive cervical cancers. *Virology* 1989;172:63–72.

17. **Straight SW, Herman B, McCance DJ.** The E5 oncoprotein of human papillomavirus type 16 inhibits the acidification of endosomes in human keratinocytes. *J Virol* 1995;69:3185–3192.

18. **Suprynowicz FA, Krawczyk E, Hebert JD, Sudarshan SR, Simic V et al.** The human papillomavirus type 16 E5 oncoprotein inhibits epidermal growth factor trafficking independently of endosome acidification. *J Virol* 2010;84:10619–10629.

19. **Zhang B, Srirangam A, Potter DA, Roman A.** HPV16 E5 protein disrupts the c-Cbl-EGFR interaction and EGFR ubiquitination in human foreskin keratinocytes. *Oncogene* 2005;24:2585–2588.

20. **Schrevel M, Gorter A, Kolkman-Uljee SM, Trimbos JB, Fleuren GJ et al.** Molecular mechanisms of epidermal growth factor receptor overexpression in patients with cervical cancer. *Mod Pathol* 2011;24:720–728.

21. **Mathur SP, Mathur RS, Young RC.** Cervical epidermal growth factor-receptor (EGF-R) and serum insulin-like growth factor II (IGF-II) levels are potential markers for cervical cancer. *Am J Reprod Immunol* 2000;44:222–230.

22. **Soonthornthum T, Arias-Pulido H, Joste N, Lomo L, Muller C et al.** Epidermal growth factor receptor as a biomarker for cervical cancer. *Ann Oncol* 2011;22:2166–2178.

23. **Santini J, Formento JL, Francoual M, Milano G, Schneider M et al.** Characterization, quantification, and potential clinical value of the epidermal growth factor receptor in head and neck squamous cell carcinomas. *Head Neck* 1991;13:132–139.

24. **Kalyankrishna S, Grandis JR.** Epidermal growth factor receptor biology in head and neck cancer. *J Clin Oncol* 2006;24:2666–2672.

25. **Lê LH, Chetty R, Moore MJ.** Epidermal growth factor receptor expression in anal canal carcinoma. *Am J Clin Pathol* 2005;124:20–23.

26. **Ajani JA, Wang X, Izzo JG, Crane CH, Eng C et al.** Molecular biomarkers correlate with disease-free survival in patients with anal canal carcinoma treated with chemoradiation. *Dig Dis Sci* 2010;55:1098–1105.

27. **Leechanachai P, Banks L, Moreau F, Matlashewski G.** The E5 gene from human papillomavirus type 16 is an oncogene which enhances growth factor-mediated signal transduction to the nucleus. *Oncogene* 1992;7:19–25.

28. **Pim D, Collins M, Banks L.** Human papillomavirus type 16 E5 gene stimulates the transforming activity of the epidermal growth factor receptor. *Oncogene* 1992;7:27–32.

29. **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;67:4521–4532.

30. **Genther Williams SM, Disbrow GL, Schlegel R, Lee D, Threadgill DW et al.** Requirement of epidermal growth factor receptor for hyperplasia induced by E5, a high-risk human papillomavirus oncogene. *Cancer Res* 2005;65:6534–6542.

31. **Palefsky JM, Holly EA, Ralston ML, Jay N.** Prevalence and risk factors for human papillomavirus infection of the anal canal in human immunodeficiency virus (HIV)-positive and HIV-negative homosexual men. *J Infect Dis* 1998;177:361–367.

32. **Akagi K, Li J, Broutian TR, Padilla-Nash H, Xiao W et al.** Genome-wide analysis of HPV integration in human cancers reveals recurrent, focal genomic instability. *Genome Res* 2014;24:185–199.

33. **Wang-Johanning F, Lu DW, Wang Y, Johnson MR, Johanning GL.** Quantitation of human papillomavirus 16 E6 and E7 DNA and RNA in residual material from ThinPrep Papanicolaou tests using real-time polymerase chain reaction analysis. *Cancer* 2002;94:2199–2210.

34. **Klaes R, Woerner SM, Ridder R, Wentzensen N, Duerst M et al.** Detection of high-risk cervical intraepithelial neoplasia and cervical cancer by amplification of transcripts derived from integrated papillomavirus oncogenes. *Cancer Res* 1999;59:6132–6136.

35. **Middleton K, Peh W, Southern S, Griffin H, Sotlar K et al.** Organization of human papillomavirus productive cycle during neoplastic progression provides a basis for selection of diagnostic markers. *J Virol* 2003;77:10186–10201.

36. **Griffin H, Soneji Y, van Baars R, Arora R, Jenkins D et al.** Stratification of HPV-induced cervical pathology using the virally encoded molecular marker E4 in combination with p16 or MCM. *Mod Pathol* 2015;28:977–993.

37. **Pirisi L, Creek KE, Doniger J, Dipaolo JA.** Continuous cell lines with altered growth and differentiation properties originate after transfection of human keratinocytes with human papillomavirus type 16 DNA. *Carcinogenesis* 1988;9:1573–1579.

38. **Niemeyer CM, Kang MW, Shin DH, Furlan I, Erlacher M et al.** Germline CBL mutations cause developmental abnormalities and predispose to juvenile myelomonocytic leukemia. *Nat Genet* 2010; 42:794–800.

39. **Hu D, Zhou J, Wang F, Shi H, Li Y et al.** HPV-16 E6/E7 promotes cell migration and invasion in cervical cancer via regulating cadherin switch *in vitro* and *in vivo*. *Arch Gynecol Obstet* 2015;292: 1345–1354.

40. **Doorbar J.** Molecular biology of human papillomavirus infection and cervical cancer. *Clin Sci* 2006;110:525–541.

41. **Isaacson Wechsler E, Wang Q, Roberts I, Pagliarulo E, Jackson D et al.** Reconstruction of human papillomavirus type 16-mediated early-stage neoplasia implicates E6/E7 deregulation and the loss of contact inhibition in neoplastic progression. *J Virol* 2012;86: 6358–6364.

42. **Walker F, Abramowitz L, Benabderrahmane D, Duval X, Descatoire V et al.** Growth factor receptor expression in anal squamous lesions: modifications associated with oncogenic human papillomavirus and human immunodeficiency virus. *Hum Pathol* 2009;40:1517–1527.

43. **Alvarez G, Perry A, Tan BR, Wang HL.** Expression of epidermal growth factor receptor in squamous cell carcinomas of the anal canal is independent of gene amplification. *Mod Pathol* 2006;19: 942–949.

44. **Zuo JH, Zhu W, Li MY, Li XH, Yi H et al.** Activation of EGFR promotes squamous carcinoma SCC10A cell migration and invasion via inducing EMT-like phenotype change and MMP-9-mediated degradation of E-cadherin. *J Cell Biochem* 2011;112:2508–2517.

45. **Lu Z, Jiang G, Blume-Jensen P, Hunter T.** Epidermal growth factor-induced tumor cell invasion and metastasis initiated by dephosphorylation and downregulation of focal adhesion kinase. *Mol Cell Biol* 2001;21:4016–4031.

46. **Zhen Y, Guanghui L, Xiefu Z.** Knockdown of EGFR inhibits growth and invasion of gastric cancer cells. *Cancer Gene Ther* 2014;21: 491–497.

47. **Hanning JE, Groves IJ, Pett MR, Coleman N.** Depletion of polycistronic transcripts using short interfering RNAs: cDNA synthesis method affects levels of non-targeted genes determined by quantitative PCR. *Virol J* 2013;10:159.

48. **Akerman GS, Tolleson WH, Brown KL, Zyzak LL, Mourateva E et al.** Human papillomavirus type 16 E6 and E7 cooperate to increase epidermal growth factor receptor (EGFR) mRNA levels, overcoming mechanisms by which excessive EGFR signaling shortens the life span of normal human keratinocytes. *Cancer Res* 2001;61:3837–3843.

49. **Sizemore N, Choo CK, Eckert RL, Rorke EA.** Transcriptional regulation of the EGF receptor promoter by HPV16 and retinoic acid in human ectocervical epithelial cells. *Exp Cell Res* 1998;244:349–356.

50. **Peto M, Tolle-Ersü I, Kreysch HG, Klock G.** Epidermal growth factor induction of human papillomavirus type 16 E6/E7 MRNA in tumor cells involves two AP-1 binding sites in the viral enhancer. *J Gen Virol* 1995;76:1945–1958.

51. **Rosenberger S, de-Castro Arce J, Langbein L, Steenbergen RD, Rösl F.** Alternative splicing of human papillomavirus type-16 E6/E6* early mRNA is coupled to EGF signaling via Erk1/2 activation. *Proc Natl Acad Sci USA* 2010;107:7006–7011.

52. **Wheeler CM, Yamada T, Hildesheim A, Jenison SA.** Human papillomavirus type 16 sequence variants: identification by E6 and L1 lineage-specific hybridization. *J Clin Microbiol* 1997;35:11–19.

53. **Lambert PF, Ozbun MA, Collins A, Holmgren S, Lee D et al.** Using an immortalized cell line to study the HPV life cycle in organotypic "raft" cultures. *Methods Mol Med* 2005;119:141–155.