Depletion of HPV16 early genes induces autophagy and senescence in a cervical carcinogenesis model, regardless of viral physical state

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Abstract

In cervical carcinomas, high-risk human papillomavirus (HR-HPV) may be integrated into host chromosomes or remain extra-chromosomal (episomal). We used the W12 cervical keratinocyte model to investigate the effects of HPV16 early gene depletion on in vitro cervical carcinogenesis pathways, particularly effects shared by cells with episomal versus integrated HPV16 DNA. Importantly, we were able to study the specific cellular consequences of viral gene depletion by using short interfering RNAs known not to cause phenotypic or transcriptional off-target effects in keratinocytes. We found that while cervical neoplastic progression in vitro was characterized by dynamic changes in HPV16 transcript levels, viral early gene expression was required for cell survival at all stages of carcinogenesis, regardless of viral physical state, levels of early gene expression or histology in organotypic tissue culture. Moreover, HPV16 early gene depletion induced changes in host gene expression that were common to both episome-containing and integrant-containing cells. In particular, we observed up-regulation of autophagy genes, associated with enrichment of senescence and innate immune-response pathways, including the senescence-associated secretory phenotype (SASP). In keeping with these observations, HPV16 early gene depletion induced autophagy in both episome-containing and integrant-containing W12 cells, as evidenced by the appearance of autophagosomes, punctate expression of the autophagy marker LC3, conversion of LC3B-I to LC3B-II, and reduced levels of the autophagy substrate p62. Consistent with the reported association between autophagy and senescence pathways, HPV16 early gene depletion induced expression of the senescence marker beta-galactosidase and increased secretion of the SASP-related protein IGFBP3. Together, these data indicate that depleting HR-HPV early genes would be of potential therapeutic benefit in all cervical carcinogenesis pathways, regardless of viral physical state. In addition, the senescence/SASP response associated with autophagy induction may promote beneficial immune effects in bystander cells.

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Introduction

There are approximately 500 000 new cases of cervical carcinoma per annum [1]. The majority are squamous cell carcinomas (SCCs), which arise from precursor squamous intra-epithelial lesions (SILs). While most carcinomas are characterized by integration of high-risk human papillomavirus (HR-HPV) DNA into the host genome, the virus remains extra-chromosomal (episomal) in ~15% of cases, equivalent to ~75 000 new carcinomas per annum [2].

An important early event in the development of cervical SCCs is uncoupling of HR-HPV oncogene expression from the differentiation state of the infected squamous cell (keratinocyte), leading to deregulated expression of the viral oncogenes E6 and E7 in proliferating basal cells [3,4]. There is in vitro evidence that such deregulation occurs in both integrant-associated and episome-associated routes of cervical carcinogenesis, albeit by different mechanisms [5]. Targeted depletion of E6 and E7 may therefore be a promising treatment for cervical carcinoma, providing the carcinoma cells depend on sustained HR-HPV oncogene expression for their survival. Depletion of E6 and E7 in cells containing integrated HR-HPV, using short-interfering RNA (siRNA) [6–10] or the bovine papillomavirus encoded transcriptional repressor E2 [11], caused widespread...
HPV oncogene depletion induces autophagy

In the present study, we depleted HPV16 early genes (including the oncogenes E6 and E7) in representative W12 series cells using a potent and specific siRNA, E7-141. In a recent thorough analysis of a range of siRNAs targeting HPV16 E7, we showed that E7-141 was the only siRNA that produced no significant off-target effects (OTEs) in keratinocytes from the cervix and skin [15]. This siRNA was therefore a uniquely suitable reagent for identifying specific effects of viral early gene depletion in HPV16-positive keratinocytes. Using E7-141 and the W12 model, we show that both integrant- and episome-associated cervical neoplastic progression depend on sustained HPV16 early gene expression. We also demonstrate that viral early gene depletion leads to autophagy as well as senescence and induces secretory phenotypes that are likely to produce beneficial bystander effects in neighbouring cells. These consequences are seen regardless of the physical state of HPV16 in the infected cells.

Materials and methods

Cell culture

We used two groups of W12 cells, all of which have been described in detail previously [5,12]. The cells were:

a W12 long-term culture series 4 (W12Ser4) and its derivative series 4B (W12Ser4B) [5]. W12Ser4 cells were characterized by episome-associated progression to SCC, with dynamic changes in HPV16 early gene expression. Maximal levels of viral oncogenes were seen at passage (p) 31 and were associated with acquisition of the malignant phenotype. Further phenotypic progression was characterized by reductions in oncogene transcript levels (presumably to limit deleterious genomic instability), which reverted to levels seen in ‘parental’ polyclonal W12 cells by p85 [5]. In the present study, we examined the effects of HPV16 oncogene depletion in W12Ser4p31-33 and W12Ser4p86-87 cells in order to test whether there were differences in oncogene dependence in cells with different levels of episome-derived oncogene expression. W12Ser4B was derived directly from W12Ser4 but was characterized by episome clearance and emergence of cells containing an integrant at 8q24.21 [5]. We studied W12Ser4Bp83-84 cells in order to compare the effect of HPV16 oncogene depletion in genetically similar integrant-containing (W12Ser4B) versus episome-containing (W12Ser4) cells;

b isogenic clones of integrant-containing cells derived from W12 long-term culture series 2. The clones were derived under non-competitive conditions in order to investigate the range of HPV16 integration sites that occur during early cervical carcinogenesis [12]. We selected three clones (A5, G2, and H), in which the number of HPV16 DNA copies per cell ranged from 1 to 4. Despite this low template copy number, levels of viral oncogene expression per cell were similar to those in episome-containing cells, in which there are 150 copies per cell [5] (Supplementary Figure 1A). Across the clones, levels of viral oncogene expression per DNA template (measured by qRT-PCR) varied by approximately six-fold (Supplementary Figure 1B). These cells therefore allowed us to study differences in the degree of HPV16 oncogene dependence in isogenic clones that differed only by levels of viral oncogene expression.

The W12 cells were grown in organotypic culture as previously described [5]. The resulting ‘rafts’ were stained using haematoxylin and eosin (H&E) and immunohistochemistry (see below) and examined by multiple observers, including a consultant histopathologist (NC). Across the W12 populations studied, the reformed epithelia represented a wide range of histological stages in cervical carcinogenesis. Clone A5 reformed an L-SIL, while clone G2 reformed an H-SIL (Supplementary Figure 1C). The W12Ser4p31, W12Ser4p86, and W12Ser4Bp83 cells each reformed an SCC [5], thus modelling epismal-associated and integrant-associated malignancies, respectively.

In addition to the various W12 populations, we used the cervical SCC cell line CaSki [15]. All cells were cultured in monolayers as previously described [5,15]. Growth curves were generated as previously described...
Figure 1. Potency of E7-141 siRNA in the six W12 populations tested. (A) Depletion of mRNAs for HPV16 (i) E7 (top row), (ii) E6 (middle row), and (iii) E2 (bottom row) following E7-141 treatment of W12 cells containing HPV16 episomes (left column) or integrated HPV16 DNA (right column). (B) Depletion of the HPV16 proteins E7 (top row), E6 (middle row), and E2 (bottom row) following E7-141 treatment of representative W12 cells containing HPV16 episomes (left and middle columns) or integrated HPV16 DNA (right column). The middle row also shows levels of the HPV16 E6 target protein p53. Tubulin was used as a loading control for E7 and E6, while the nuclear protein p84 was used for E2. For effects of E7-141 on HPV protein levels in the other W12 populations tested, see Supplementary Figure 2.

[15], with growth inhibition being determined from the percentage change in the area under the curve in cells treated with E7-targeting siRNAs, compared with cells treated with non-targeting control (NTC) siRNAs (Dharmacon/Thermo Scientific, Waltham, MA, USA).

siRNA treatment
In order to minimize the possibility of non-specific observations, we deliberately used an HPV16 E7-targeting siRNA (E7-141; sequence 5’-GGACAGAGCCAUUACAAU-3’) that we had demonstrated not to cause significant OTEs in keratinocytes [15]. In our previous study, this reagent was shown to deplete HPV16 E7 transcripts with high potency, while not producing phenotypic or transcriptional OTEs in HPV-negative, phenotypically normal or near-normal keratinocytes from the cervix (normal cervix; NCx) and skin (HaCaT). This was in contrast to the multiple other E7-targeting siRNAs tested, which did induce phenotypic and transcriptional OTEs [15]. In the absence
of any other potent E7-targeting siRNA known not to cause OTEs, we used E7-141 for all experiments. Cells were transfected at 20–30% confluency in Lipofectamine RNAiMAX (Invitrogen, Paisley, UK) as previously described [15], using E7-141 or NTC siRNAs, each at 37 nM. Untreated cells were also used as additional negative control samples.

Analysis of HPV16 gene expression and copy number
RNA was extracted and reverse-transcribed as previously described [15]. Quantitative PCR (qPCR) was performed using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich, Dorset, UK), with the primers listed in Supplementary Table 1 (Sigma-Aldrich, unless otherwise specified). Expression levels were normalized against the mean of three housekeeping genes, as described elsewhere [16]. Copy numbers of the HPV16 E7 and E6 genes were determined using SYBR Green qPCR of genomic DNA, as previously described [5]. Levels were referenced to the control genes TLR2 and IFNβ (previously shown not to undergo copy number change in multiple W12 populations and series) and adjusted for cell ploidy, as previously described [5].

Microarray expression profiling
Microarray expression profiling of E7-141-treated, NTC-treated, and untreated cells was carried out as described previously [15], using Illumina WG-GX Beadchips HumanHT-12 v4. The raw intensities files (.txt) are available at ArrayExpress (Accession: EMTAB-1331).

Expression data were analysed using the bioinformatic algorithm Sylamer, as previously described [15]. Sylamer assesses for enrichment and/or depletion of nucleotide words of specific length, complementary to elements of the seed region of miRNAs/siRNAs, in the 3′UTRs of genes within ranked lists [17]. In addition, the lists of up- and down-regulated genes in E7-141-treated versus NTC-treated cells from each W12 population were used for Gene Ontology (GO) analysis (\(-1 > \log_{2} FC > 1\) and adj.P.val < 0.01). GO term enrichment was performed using the Bioconductor GOstats package [18], and the mappings of GO ‘Biological Process’ terms were obtained from the Bioconductor illuminaHumanv4.db [19] annotation library. GO terms with adjusted P-value less than 0.05 were considered significant.

In order to understand the relationships between differentially expressed genes and potentially biologically important pathways, we performed KEGG pathway analysis using the Bioconductor Geneanswers package [20]. We also used this package to represent gene-pathway networks visually and to look for overlap between genes differentially expressed (\(-0.5 > \log_{2} FC > 0.5\) and adj.P.val < 0.01) in the E7-141-treated versus untreated cell comparison and the NTC-treated versus untreated cell comparison. This exercise was performed for each W12 population separately, as well as for all four W12 populations combined. To focus on apoptosis and senescence, we extracted eight manually curated gene set enrichment analysis (GSEA) sets relating to genes specifically up- and down-regulated in apoptosis or senescence (Supplementary Table 2), and also examined the specific canonical KEGG pathways. The mapping of entrez IDs to gene symbols and KEGG pathways was obtained from the org.Hs,eg.db annotation library (http://www.bioconductor.org/packages/release/data/annotation/html/org.Hs.es.db.html). The KEGG pathways with FDR <10% were considered significant.

Western immunoblotting and immunohistochemistry
Western immunoblotting detection was carried out as previously described [15]. The primary antibodies used were against HPV16 E7 (ED17; Santa Cruz Biotechnology, CA, USA), HPV16 E6 (1E-6F4; Euromedex, Souffelweyersheim, France), beta-tubulin (ab6046; Abcam, Cambridge, UK), HPV16 E2 (TVG261; Cancer Research Technology, London, UK), p53 (sc-126; Santa-Cruz), LC3B (#2775; Cell Signaling Technology, Danvers, MA, USA) and p62 (610832; BD Transduction Laboratories, Oxford, UK). Immunohistochemistry (IHC) of organotypic cell culture rafts was performed as described elsewhere [21], using primary antibodies against MCM2, a marker of cell cycle entry [22] (in-house clone D1 12 A3; 1 : 40), and the squamous cell differentiation markers cytokeratins 10/13 (K10/13) [23] [clone DE-K13 (Dako, Ely, UK); 1 : 100].

Analysis of cell phenotype
Senescent cells were detected by beta-galactosidase histochemistry, using Senescent Cell Staining reagents (Sigma-Aldrich, Dorset, UK). Apoptotic cells were detected using the Caspase-Glo 3/7 assay (Promega, Southampton, UK) and Synergy HT Multi-Mode Microplate Reader (Biotek, Vermont, USA). Positive control cells were G3T3 fibroblasts treated with 1 μM staurosorpin. Immunofluorescence was performed as previously described [24], in cells treated with siRNAs for 96 h. We used primary mouse monoclonal anti-LC3 antibody (clone 5F10; Nanotools Antikörpertechnik, Teningen, Germany; 1 : 50), detected using Alexa-546-conjugated anti-mouse secondary antibody (Life Technologies, Paisley, UK), with a DAPI counter-stain. Cells were processed for electron microscopy using standard protocols and examined using an FEI Tecnai G2 electron microscope operated at 120 kV, using an AMT XR60B digital camera running Deben software. Levels of secreted IGFBP3 protein were measured by ELISA (Quantikine human IGFBP3 ELISA; R&D Systems, Abingdon, UK), using the Synergy HT Multi-Mode Microplate Reader (Biotek, Winooski, VT, USA). The IGFBP3 concentration was normalized to
cell number, with levels in E7-141-treated cells being referenced to those in NTC-treated cells.

Results

HPV16 oncogene depletion

As with our previous findings in CaSki cervical SCC cells [15], E7-141 caused potent depletion of HPV16 early gene expression in all W12 cells tested (Figure 1 and Supplementary Figure 2). Across the six populations examined, maximal levels of E7 mRNA depletion ranged from 76.4% to 90.3% (Figure 1A). In keeping with the polycistronic nature of HPV16 transcripts, levels of E6 mRNA showed similar reductions, with maximal levels of depletion ranging from 77.6% to 88.3% (Figure 1A). In all cells, expression of E7, E6, and E2 proteins fell in parallel to the mRNA levels, with the reductions in E6 being mirrored by increased levels of the E6 target p53 (Figure 1B and Supplementary Figure 2). There were no differences in the levels of gene depletion achieved in episome-containing versus integrant-containing cells, nor in cells with high versus low baseline gene expression levels. Levels of depletion were similar in cells that reformed L-SIL, H-SIL or SCC in organotypic tissue culture. In both episome- and integrant-containing W12 cells, maximal depletion of early gene transcripts was seen 48 h after E7-141 transfection. After 7 days, mRNA and protein levels had recovered to baseline in the integrant-containing W12 cells, but remained depleted in the episome-containing cells (Figures 1A and 1B). This prolonged depletion of HPV16 transcript levels in episome-containing cells was not related to reductions in DNA template copy number (Supplementary Figure 3).

Effects on host gene expression

We used microarray analysis to identify and compare the effects of HPV16 early gene depletion on host mRNA expression profiles in episome-containing and integrant-containing W12 cells. We examined cells at the peak of the siRNA effects (48 h), examining the episome-containing cells W12Ser4p31 and W12Ser4p86, as well as the integrant-containing cells W12Ser4Bp83 and G2p13 (a representative integrant-containing clone).

We first used Sylamer to exclude direct microRNA-like effects of E7-141, by interrogating genes down-regulated in E7-141-treated cells versus NTC-treated or untreated cells. For all four W12 populations examined, we found no evidence in these comparisons of enrichment of mRNAs in which the 3′ UTR contained sequences complementary to the 2–7 nucleotide seed of E7-141 (Supplementary Figure 4 and Supplementary Table 3). These findings showed that E7-141 produced no direct transcriptional OTEs in the treated W12 cells and supported our previous findings of a lack of any significant E7-141 OTEs in HPV-negative keratinocytes from the cervix and skin [15]. In addition, none of 345 conserved human microRNA seeds analysed showed enrichment for the complementary sequence in the 3′ UTR of mRNAs down-regulated in E7-141-treated versus NTC-treated cells ( Supplementary Figure 4). In E7-141-treated versus untreated cells, there was enrichment for the sequence complementary to the miR17-92 cluster in the 3′ UTR of down-regulated genes. However, the absence of this effect in E7-141-treated versus NTC-treated cells indicated that it was a non-specific consequence of siRNA transfection (Supplementary Figure 4 and Supplementary Table 3). Taking these data together, we concluded that any gene expression changes seen in E7-141-treated cells, but not in NTC-treated or untreated cells, would represent specific effects of HPV16 early gene depletion.

Our detailed analyses of the gene expression changes seen following HPV16 viral oncogene depletion in episome-containing W12 (Ser4p31 and Ser4p86), integrant-containing W12 (Ser4Bp83 and G2p13), and in the combined data sets are given in Supplementary Figure 5 and Supplementary Tables 4–31. Figure 2 summarizes the pathways that were significantly enriched in up-regulated (Figure 2A) and down-regulated (Figure 2B) genes across all W12 populations treated with E7-141 siRNA, compared with the NTC-treated and untreated control cells. As the W12 populations contained either episomal or integrated HPV16, the genes in these pathways represented common effects of HPV16 oncogene depletion, regardless of viral physical state or cell line histology in organotypic tissue culture. The up-regulated genes were enriched for several anti-cancer pathways, including autophagy, senescence, innate immune response, cell matrix adhesion, and anti-apoptotic pathways (Figure 2A), while the down-regulated genes showed enrichment of cell cycle and apoptosis pathways (Figure 2B).

Phenotypic effects of HPV16 oncogene depletion

Consistent with the observed down-regulation of cell-cycle pathways, E7-141 treatment caused significant reductions in the growth of all six W12 populations analysed over a 6-day period (Supplementary Figure 6). There was no difference in the effects observed in episome-containing versus integrant-containing cells (Supplementary Figure 6).

In view of the induction of autophagy pathways after viral oncogene depletion, we used multiple complementary approaches [25] to test for phenotypic evidence of autophagy following E7-141 treatment of representative episome-containing W12Ser4p33 cells. By electron microscopy, we observed double-membranated vacuolar structures with the appearance of autophagosomes 4 days after E7-141 transfection (Figure 3A), but not in NTC-treated or untreated...
HPV oncogene depletion induces autophagy.

Figure 2. Pathway analysis of host gene expression changes following HPV16 oncogene depletion. The panels show pathways that were significantly enriched in the combined data for all four W12 populations analysed. Up-regulated genes are shown in A, while down-regulated genes are in B. In each panel, the genes differentially expressed in E7-141-treated cells but not in NTC-treated or untreated cells are shown as red dots. Genes differentially expressed in both E7-141-treated and NTC-treated cells but not in untreated cells are shown as green dots. There were no genes that were differentially expressed in NTC-treated cells but not in the E7-141-treated or untreated cells.
Figure 2. Continued
cells. Immunofluorescence staining for the autophagy marker LC3 showed punctate staining in 85% of W12Ser4p33 cells 4 days after E7-141 transfection (Figures 3B and 3C and Supplementary Figure 7), compared with 25% of NTC-treated cells and 6% of untreated cells. We used western blotting to measure the protein levels in all six W12 populations of LC3B and p62, substrates of autophagy that are present on autophagosomes but degraded in autolysosomes. Following E7-141 treatment of both episome-containing and integrant-containing cells (but not in NTC-treated or untreated cells), there was conversion of LC3B-I to the lipidated form LC3B-II and a reduction in levels of p62 (Figure 3D and Supplementary Figure 8). Together, these data indicated that E7-141 treatment up-regulated autophagic flux in W12 cells, regardless of the HPV16 physical state, with no evidence of any downstream block in the autophagy pathway. These findings were supported by evidence of conversion of LC3B-I to LC3B-II and reduced levels of p62 in CaSki cervical SCC cells treated with E7-141 for 72 h [15] (Supplementary Figure 8).

Autophagy has been linked with the induction of senescence [26], and our microarray data as well as previous studies by others [8] demonstrated up-regulation of senescence-associated genes following HPV16 oncogene depletion. At 7 days following E7-141 transfection, the senescence marker beta-galactosidase was expressed by a mean of 75.6% of cells (range 61–98%) across all W12 series tested (Figures 4A and 4B and Supplementary Figure 9), compared with 2.2% of NTC-treated cells and 1.0% of untreated cells. Interestingly, the senescence phenotype was present in integrant-containing cells at day 7 (Figures 4A and 4B), despite the recovery of viral oncogene levels in these cells (Figures 1A and 1B).

Our microarray data also demonstrated that apoptosis was down-regulated after viral oncogene depletion.
Figure 4. Quantification of senescence and apoptosis following HPV16 oncogene depletion. (A) Expression of beta-galactosidase 7 days after siRNA treatment. Data are shown for all six W12 populations tested, as well as HPV-negative control HaCaT cells, comparing E7-141-treated cells with NTC-treated and untreated cells. (B) Images from the experiment quantified in A, showing representative episome-containing cells (top row) and integrant-containing cells (bottom row). Beta-galactosidase is stained blue. Images for the other W12 populations are shown in Supplementary Figure 9. (C) Levels of caspase 3/7 activity 7 days after siRNA treatment, comparing the E7-141-treated cells with NTC-treated cells. The positive-control cells were G3T3 fibroblasts treated with staurosporin. The horizontal dotted line represents a two-fold up-regulation of caspase 3/7 activity.

Consistent with this, we observed minimal change in levels of caspase 3/7 cleavage in E7-141-treated W12 cells, compared with NTC-treated controls. This was seen in both the episome-containing populations (1.1-fold decrease to 1.6-fold increase) and the integrant-containing cells (1.4- to 2.0-fold increase) (Figure 4C). Such changes were significantly less than in positive-control G3T3 fibroblast cells treated with staurosporin, in which the levels of caspase 3/7 cleavage rose 20-fold (P-value < 0.0001).

In view of our evidence of senescence induction, we interrogated our microarray data to examine for up-regulation of the senescence-associated secretory phenotype (SASP) [27], which can recruit innate immune cells able to clear senescent cells [28,29]. At 7 days following E7-141 treatment (compared with NTC-treated cells), we observed up-regulation of SASP-related mRNAs, such as IGFBP3 and SERPINE1 [30], as well as factors associated with an innate immune response, including cytokines (eg IL1β) and interferon-stimulated genes (eg IFI27 and MX1) (Supplementary Figure 10A and 10B). By ELISA analysis of cell culture media, we observed increased secretion of IGFBP3 in E7-141 treated cells, compared with NTC-treated cells (Supplementary Figure 10C), in both the episome-containing and the integrant-containing W12 populations. Interestingly, the changes in IGFBP3 mirrored the effects of E7-141 on HPV16 oncogene depletion. While levels were greatest in the episome-containing cells at day 7, in the integrant-containing cells they were greater at day 2 than at day 7 (Supplementary Figure 10C), reflecting the recovery of viral oncogene levels by day 7 in the integrant-containing cells (Figures 1A and 1B).

Discussion

We show that specific depletion of HPV16 early genes in W12 causes common cellular effects, regardless of the viral physical state (episomal or integrated), baseline levels of early gene expression or histology of the epithelium reformed in organotypic tissue culture. These effects included up-regulation of autophagy genes, together with senescence and innate immune
response pathways. The phenotype induced by HPV16 E6/E7 depletion in vitro included autophagy (demonstrated using a range of complementary assays) as well as senescence, including up-regulation of the SASP response.

The siRNA used in this study, E7-141, is the only one known to combine on-target potency with an absence of phenotypic or transcriptional OTEs in keratinocytes [15]. Various previous studies have described the consequences of depleting viral oncoproteins in cervical carcinoma cells containing integrated HR-HPV [6–10]. The effects observed have included growth inhibition, senescence, and apoptosis. However, no study has previously excluded the possibility of OTEs caused by the siRNAs used. Indeed, significant OTEs were caused by almost all of the siRNAs targeting HPV16-E7 tested in our previous study, with the exception of E7-141 [15]. The present investigation is therefore the first to allow confident identification of the specific consequences of HPV16 early gene depletion in cervical keratinocytes.

We demonstrate for the first time that depletion of HPV16 early genes in cervical keratinocytes induces autophagy. This is likely to be due to loss of a dominant anti-autophagic effect of HPV16 E6 [31,32], which counteracts the pro-autophagic consequences of HPV16 E7-induced metabolic stress [33]. Our data argue against the notion of using autophagy inhibitors as a treatment for cervical carcinoma [34], as HPV16-infected W12 cells are not addicted to autophagy for survival and indeed induction of autophagy is associated with cell senescence. In our experiments, the senescence phenotype induced by HPV16 oncogene depletion was associated with up-regulation of SASP and innate immune-response genes, which would be beneficial in vivo, by inducing a field effect and promoting an anti-HPV16 adaptive immune response. The down-regulation of apoptosis that we observed suggests that specific depletion of HPV16 oncogenes in cervical carcinoma cells in vivo would not lead to the generation of apoptotic cells, which have been described as having potential transforming properties [35].

We observed that the consequences of HPV16 oncogene depletion were the same in W12 cells that contained either episomal or integrated viral DNA. This is the first demonstration that episo-mediated cervical carcinogenesis requires sustained HR-HPV early gene expression for cell survival, as is the case for integrand-associated carcinogenesis. Moreover, similar anti-tumour effects were seen in cells from both early and late time-points in the episomal-associated and integrand-associated W12 progression series, when levels of viral early gene expression varied substantially. We conclude that while HPV16-associated cervical carcinogenesis in vitro is characterized by dynamic changes in viral oncogene expression, cells at all stages of neoplastic progression remain dependent on viral early genes for their survival.

In our experiments, recovery of HPV16 oncogene expression following E7-141 treatment was faster in integrant-containing W12 cells than in episome-containing cells. The sustained reduction in oncogene expression in the latter cells was not due to reduced levels of viral DNA template. The integrant-containing cells had a far lower copy number of HPV16 DNA templates than the episo-mem-contained cells (1–4 copies versus 500–600 copies) but expressed similar levels of viral transcripts, indicating a substantially higher rate of E2-independent viral transcription that was associated with faster recovery of expression levels following siRNA treatment. Further functional work is now required to understand the basis of the different kinetics of viral gene expression recovery that we observed.

Interestingly, the senescence phenotype observed in the integrant-containing W12 cells persisted to day 7 despite the recovery in E6/E7 levels, consistent with the essentially irreversible changes that occur when senescence pathways are activated [36]. Nevertheless, our data suggest that different dosing schedules may be required in order to achieve prolonged HR-HPV early gene depletion in integrant-containing versus episome-containing carcinoma cells.

These findings in W12 now need to be extended to other systems. Our observations in the cervical SCC cell line CaSki indicate that autophagy can be a common response to depleting E6/E7 in HPV16-containing cervical keratinocytes. It is not yet known whether the same consequences would occur in cells containing other HR-HPV types and similar experiments in cell systems analogous to W12 are required [37]. It will also be important to understand more of the functional significance of autophagy in inducing senescence/SASP and whether the mechanisms differ in episome-associated versus integrant-associated routes of cervical carcinogenesis.

Together, our findings indicate that depleting HPV16 early genes can offer therapeutic benefits in cervical carcinogenesis, including in cases where the virus remains episomal. There may be similar benefits at other anatomical sites, including HPV-associated squamous carcinogenesis in the head and neck [38]. It will be important to select siRNA reagents that lack OTEs, based on detailed analyses similar to those previously reported for E7-141 and other HPV16 E7-targeting siRNAs [15]. Our data suggest that E7-141 is a promising reagent for further pre-clinical development, and may also be of value in studies of aberrant autophagy and autophagy-mediated senescence [26].

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Author contribution statement

JH carried out all of the molecular biology work, contributed to the design of the study, was involved in all data analysis and interpretation, and co-wrote the manuscript. HS was involved in the design of the study, performed data analysis and interpretation, and drafted the manuscript. MM and MC performed some molecular biology work, helped coordinate the study, were involved in data analysis and interpretation, and helped draft the manuscript. SvD performed bioinformatic analysis involving Sylamer and contributed to the manuscript. DW provided assistance with molecular techniques and contributed to the manuscript. EB, CS, and IG contributed to the design of the study and to the manuscript. MS and AE were involved in data analysis, study coordination, and contributed to the manuscript. MP and NC conceived the study and participated in its design and in data analysis and interpretation. NC co-wrote the manuscript. All authors read and approved the manuscript.

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SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article.

Figure S1. Features of the W12 clones containing integrated HPV16.

Figure S2. Levels of HPV16 oncoproteins and p53 protein following siRNA treatment of integrant-containing W12 clones.

Figure S3. Relationship between HPV16 early gene depletion and viral DNA copy number in W12 cells.

Figure S4. Sylamer analysis of seed-dependent off-target effects (OTEs) following siRNA treatment.

Figure S5. Pathway analysis of genes differentially expressed following siRNA treatment.

Figure S6. Growth of the six W12 cell populations following E7-141 treatment.

Figure S7. Expression of LC3 following siRNA treatment.

Figure S8. Levels of the autophagy substrates LC3B and p62 following siRNA treatment.

Figure S9. Expression of beta-galactosidase following siRNA treatment.

Figure S10. Induction of senescence-associated secretory phenotype (SASP) and innate immune response genes following HPV16 oncogene depletion.

Table S1. Primers used for qRT-PCR.

Table S2. Curated pathways included in KEGG analysis.

Table S3. Single summed significance score P-value for assessment of significant enrichment of E7-141 SCR ‘ATTACAAA’ and miR17-92 SCR ‘GCCCTTTA’ in transcripts down-regulated by E7-141 in each W12 population tested.

Table S4. Significantly enriched (adjusted P-value < 0.05) GO terms for genes up-regulated in W12Ser4p31 following E7_141 siRNA transfection (compared with NTC-treated cells).

Table S5. Significantly enriched (adjusted P-value < 0.05) GO terms for genes down-regulated in W12Ser4p31 following E7_141 siRNA transfection (compared with NTC-treated cells).

Table S6. Significantly enriched (adjusted P-value < 0.05) GO terms for genes up-regulated in W12Ser4p86 following E7_141 siRNA transfection (compared with NTC-treated cells).

Table S7. Significantly enriched (adjusted P-value < 0.05) GO terms for genes down-regulated in W12Ser4p86 following E7_141 siRNA transfection (compared with NTC-treated cells).

Table S8. Significantly enriched (adjusted P-value < 0.05) GO terms for genes up-regulated in W12Ser4p83 following E7_141 siRNA transfection (compared with NTC-treated cells).

Table S9. Significantly enriched (adjusted P-value < 0.05) GO terms for genes down-regulated in W12Ser4p83 following E7_141 siRNA transfection (compared with NTC-treated cells).

Table S10. Significantly enriched (adjusted P-value < 0.05) GO terms for genes up-regulated in G2p13 following E7_141 siRNA transfection (compared with NTC-treated cells).

Table S11. Significantly enriched (adjusted P-value < 0.05) GO terms for genes down-regulated in G2p13 following E7_141 siRNA transfection (compared with NTC-treated cells).

Table S12. Significantly enriched (adjusted P-value < 0.05) GO terms for genes up-regulated in all E7-141-treated W12 populations (compared with NTC-treated cells).

Table S13. Significantly enriched (adjusted P-value < 0.05) GO terms for genes down-regulated in all E7-141-treated W12 populations (compared with NTC-treated cells).

Table S14. Significantly enriched (adjusted FDR P-value < 0.1) KEGG pathways for genes down-regulated in W12Ser4p31 following E7_141 siRNA transfection.

Table S15. Significantly enriched (adjusted FDR P-value < 0.1) KEGG pathways for genes up-regulated in W12Ser4p83 following E7_141 siRNA transfection.

Table S16. Significantly enriched (adjusted FDR P-value < 0.1) KEGG pathways for genes up-regulated in W12Ser4p86 following E7_141 siRNA transfection.

Table S17. Significantly enriched (adjusted FDR P-value < 0.1) KEGG pathways for genes up-regulated in W12Ser4p83 following E7_141 siRNA transfection.
Table S18. Significantly enriched (adjusted FDR $P$-value $< 0.1$) KEGG pathways for genes down-regulated in W12Ser4Bp83 following E7_141 siRNA transfection.

Table S19. Significantly enriched (adjusted FDR $P$-value $< 0.1$) KEGG pathways for genes up-regulated in G2p13 following E7_141 siRNA transfection.

Table S20. Significantly enriched (adjusted FDR $P$-value $< 0.1$) KEGG pathways for genes down-regulated in G2p13 following E7_141 siRNA transfection.

Table S21. Significantly enriched (adjusted FDR $P$-value $< 0.1$) KEGG pathways for genes up-regulated in all W12 populations following E7_141 siRNA transfection.

Table S22. Significantly enriched (adjusted FDR $P$-value $< 0.1$) KEGG pathways for genes down-regulated in all W12 populations following E7_141 siRNA transfection.

Table S23. Significantly enriched (adjusted FDR $P$-value $< 0.1$) KEGG pathways for genes down-regulated in W12Ser4p31 following E7_141 siRNA transfection.

Table S24. Significantly enriched (adjusted FDR $P$-value $< 0.1$) KEGG pathways for genes up-regulated in W12Ser4p86 following E7_141 siRNA transfection.

Table S25. Significantly enriched (adjusted FDR $P$-value $< 0.1$) KEGG pathways for genes down-regulated in W12Ser4p86 following E7_141 siRNA transfection.

Table S26. Significantly enriched (adjusted FDR $P$-value $< 0.1$) KEGG pathways for genes up-regulated in W12Ser4Bp83 following E7_141 siRNA transfection.

Table S27. Significantly enriched (adjusted FDR $P$-value $< 0.1$) KEGG pathways for genes down-regulated in W12Ser4Bp83 following E7_141 siRNA transfection.

Table S28. Significantly enriched (adjusted FDR $P$-value $< 0.1$) KEGG pathways for genes up-regulated in G2p13 following E7_141 siRNA transfection.

Table S29. Significantly enriched (adjusted FDR $P$-value $< 0.1$) KEGG pathways for genes down-regulated in G2p13 following E7_141 siRNA transfection.

Table S30. Significantly enriched (adjusted FDR $P$-value $< 0.1$) KEGG pathways for genes up-regulated in all W12 populations following E7_141 siRNA transfection.

Table S31. Significantly enriched (adjusted FDR $P$-value $< 0.1$) KEGG pathways for genes down-regulated in all W12 populations following E7_141 siRNA transfection.

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