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Human Papillomavirus Promotes Epstein-Barr Virus Maintenance and Lytic Reactivation in Immortalized Oral Keratinocytes

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Abstract

Epstein-Barr virus and human papillomaviruses are human tumor viruses that infect and replicate in upper aerodigestive tract epithelia and cause head and neck cancers. The productive phases of both viruses are tied to stratified epithelia highlighting the possibility that these viruses may affect each other's life cycles. Our lab has established an in vitro model system to test the effects of EBV and HPV co-infection in stratified squamous oral epithelial cells. Our results indicate that HPV increases maintenance of the EBV genome in the co-infected cells and promotes lytic reactivation of EBV in upper layers of stratified epithelium. Expression of the HPV oncogenes E6 and E7 were found to be necessary and sufficient to account for HPV-mediated lytic reactivation of EBV. Our findings indicate that HPV increases the capacity of epithelial cells to support the EBV life cycle, which could in turn increase EBV-mediated pathogenesis in the oral cavity.

Keywords

HPV; EBV; organotypic culture; latency; lytic; life-cycle; E6; E7

Introduction

Epstein-Barr virus (EBV) and human papillomaviruses (HPVs) are human tumor viruses that cause head and neck cancers. EBV is an enveloped, double-stranded DNA virus with tropism for epithelial cells and resting B lymphocytes. EBV was the first virus to be

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associated with human cancers; it is associated with several lymphoid and epithelial cancers including Burkitt's lymphoma, Hodgkin disease, nasopharyngeal carcinoma, and gastric cancers. HPVs are small, non-enveloped, double-stranded DNA viruses with a tropism for epithelial cells. High-risk HPVs are associated with 5% of human cancers including cervical and other anogenital cancers as well as a growing fraction of head and neck cancers, all epithelial in origin.

HPV and EBV both infect and replicate in upper aerodigestive tract epithelia [1, 2]. EBV infects the oral epithelia causing oral hairy leukoplakia on the tongue which is characterized by its productive infection [3] in addition to its association with nasopharyngeal cancers [4]. Likewise, HPV infects oral keratinocytes and the high-risk subtypes are associated with an increasing percentage of oropharyngeal cancers [5, 6]. The life cycles of these two tumor viruses also share a relationship with the stratified epithelium in that the productive phase of the HPV life cycle and lytic reactivation of EBV are both induced upon differentiation of epithelial cells. The productive phase of the HPV life cycle, which includes late protein expression and genome amplification, occurs in the suprabasal compartment of stratified epithelium [7]. Similarly, EBV immediate-early protein expression and genome amplification, two hallmarks of EBV lytic reactivation, occur in suprabasal epithelial layers [8, 9].

Given the tropism of both HPV and EBV for epithelial cells and the parallels in regard to their life cycles being tied to the differentiation of stratified epithelium, the question arises as to whether these viruses influence each other's life cycles under conditions in which co-infection may occur in the upper aerodigestive tract. We have established an in vitro model system for studying the influence of high-risk HPV and EBV on each other in monolayer oral epithelial cells as well as in stratified oral epithelial cells using organotypic (raft) cultures harboring these viruses alone and together. We found that the presence of HPV18 stabilizes the long-term maintenance of EBV genomes in monolayer culture and promotes EBV lytic reactivation in the suprabasal compartment of raft cultures. In addition, HPV oncogenes E6 and E7 are necessary and sufficient to promote HPV-mediated EBV lytic reactivation in suprabasal layers of oral stratified epithelium.

Results

HPV18 and EBV can co-exist in human oral epithelial cells

HPV and EBV both infect and replicate in upper aerodigestive tract epithelia [1, 2]. However, no in vitro model has been developed to study EBV and HPV co-infections in stratified epithelium. While it is known that the EBV life cycle is supported in NOKs cells, a tert-immortalized normal oral keratinocyte cell line, [8, 10] it was unknown whether NOKs cells support the HPV life cycle prior to this study. To determine if NOKs cells can stably harbor HPV18, a high-risk HPV subtype associated with human oral infections and human head and neck cancers, we transfected HPV18 genomes into NOKs cells and performed Southern blot analysis. Briefly, total genomic DNA was isolated from NOKs cells from three individual cell populations at passage 0 and passage 6 post-transfection with re-circularized HPV18 genomes. The DNA was double-digested with an enzyme that linearizes the HPV18 genome (Nco1) and an enzyme that cuts only unreplicated input DNA (Dpn1). NOKs cells

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stably harbored the HPV18 genome upon transfection and genome maintenance was observed over at least 6 passages (Fig 1A).

To determine if HPV18 and EBV could co-habit in oral epithelial cells in vitro, NOKs cells harboring EBV were transfected with recircularized HPV18 genomes. HPV18-specific Southern analysis demonstrated that NOKs cells harboring EBV support the establishment and maintenance of HPV18 genomes over 6 passages (Fig 1A). In both NOKs and NOKs-EBV cells, the HPV18 genome was retained as an extrachromosomal nuclear plasmid (S1 Fig). Likewise, EBV-specific FISH indicated that EBV could be maintained in HPV18-positive NOKs cells over multiple passages following HPV18 transfection (Fig 1B). These results indicate that the two viruses can be maintained simultaneously in NOKs cells rendering NOKs cells a model in which to study EBV and HPV18 co-infection in oral epithelial cells.

It is known that cultured cells lose EBV genomes unless EBV provides its host cells a selective advantage [11–13]. We therefore asked if HPV has any impact on the maintenance of the EBV genome in NOKs cells in the absence of continued selection. We quantified the number of EBV genomes per cell in 100 independent EBV-infected NOKs cells grown with or without G418 selection for neomycin resistance as the EBV genome expresses the neomycin resistance gene and with or without HPV18 (Fig 1C–F) for 12 passages. EBV-positive NOKs with HPV18 grown with G418 selection dually harbor both viruses as few or no cells lack EBV when selected with G418. As predicted without selection, the average number of EBV genomes per cell decreased from 21.8 to 4.8 in NOKs-EBV cell populations grown in the absence of selection (Fig 1C,D), indicating that oral epithelial cells also lose EBV genomes unless they are grown under selection. This shift in distribution likely reflects the inherent inefficiencies in the synthesis of EBV DNA in S phase and the equal partitioning of viral genomes to daughter cells during mitosis [13]. In HPV18-positive cells, i.e. in NOKs-EBV-HPV18 cells (Fig 1E,F), the shift in the distribution of cells harboring lower numbers of EBV genomes in the absence of selection for 12 passages (average of 12.5 EBV genomes per cell) was less pronounced than that seen in the absence of HPV18 (Fig 1D). Comparing NOKs-EBV minus selection (Fig 1D) to NOKs-EBV-HPV18 minus selection (Fig 1F) indicated a highly significant shift in copy number distribution conferred by the presence of HPV18 ($p = 3.4 \times 10^{-5}$, two-sided Wilcoxon rank sum test). This observation indicates that the presence of HPV18 helps to maintain the EBV genome in NOKs cells grown without selection for EBV. Collectively, these results demonstrate that NOKs cells can support the maintenance of EBV and HPV18 alone and together, and that HPV18 stabilizes EBV in these cells.

The productive phase of the HPV18 life cycle is supported in oral epithelial cells and some aspects of this productive phase are inhibited by co-infection with EBV

NOKs cells support the lytic phase of the EBV life cycle [8]. The HPV life cycle has been well characterized in human foreskin-derived keratinocytes, however, little is known about the HPV life cycle in human oral keratinocytes. We analyzed biomarkers for HPV18's productive phase in the context of organotypic (raft) cultures generated from HPV18-positive NOKs cells with and without EBV. Raft cultures of EBV-negative NOKs cells

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supported the productive HPV18 life cycle as evidenced by the following: induction of suprabasal DNA synthesis as measured by BrdU-incorporation (Fig 2A), induction of expression of the E2F-responsive protein, MCM7, in suprabasal cells (Fig 2B), amplification of the HPV18 genome as measured by fluorescence in situ hybridization (FISH; Fig 2C), and expression of the HPV productive phase protein E1^E4 (0.31% of total nuclei; Fig 2D). We next asked if the presence of EBV influences the productive phase of the HPV life cycle. Rafts of NOKs cells harboring either EBV alone or EBV and HPV18 had similar increases in suprabasal DNA synthesis. Thus, the effect of EBV and HPV18 on suprabasal DNA synthesis was not synergistic (Fig 2A,E). Similarly, raft cultures harboring either EBV alone or EBV and HPV18 had positive suprabasal expression of MCM7 indicating that EBV alone can induce E2F-responsive protein expression, albeit to a lesser extent than HPV18 (Fig 2B). Raft cultures harboring both EBV and HPV18 contained fewer cells with FISH-positive signals for amplified HPV18 genomes, and we detected no E1^E4 expression in raft cultures harboring both viruses (Fig 2C,D). These findings indicate that NOKs cells can support the productive phase of the HPV18 life cycle in the context of raft cultures, EBV alone induces suprabasal DNA synthesis and expression of an E2F-responsive protein which is not synergistic with HPV18, and EBV appears to block amplification of HPV18 and late gene expression, two hallmarks of late stages of the productive phase of the HPV18 life cycle.

HPV18 induces EBV lytic reactivation in suprabasal layers of oral epithelial organotypic raft cultures

Prior studies indicate that lytic reactivation of EBV occurs upon induction of differentiation in NOKs [8]. Given that the productive phase of the HPV life cycle is also induced upon differentiation of NOKs and is associated with reprogramming of differentiated cells to support DNA synthesis (Fig 2), we asked if HPV18 influences lytic reactivation of EBV. NOKs cells containing EBV and HPV18 or EBV alone were grown in raft cultures, and resulting tissue sections were analyzed for markers of lytic reactivation of EBV. Using immunofluorescence staining, we found a significant increase ($p < 1x10^{-6}$) in the number of cells expressing the EBV immediate-early Z protein in EBV-positive raft cultures harboring HPV18 (9.88%) compared to raft cultures without HPV18 (0.57%; Fig 3A and 3C). EBV-specific FISH showed that EBV genomes were present in all cells in EBV-positive raft cultures represented by small green foci in every cell. In addition, the number of cells containing intense FISH signals for EBV, representing EBV genome amplification, significantly increased ($p < 1x10^{-6}$) in EBV-positive raft cultures harboring HPV18 (3.27%) compared to raft cultures without HPV18 (0.67%; Fig 3B and 3D). Notably, the increase in signals for both immediate early Z protein and FISH for amplified EBV genomes occurred primarily in the suprabasal layers. Taken together, these results indicate that HPV18 promotes EBV lytic reactivation in suprabasal layers of NOKs raft cultures.

Promotion of EBV lytic reactivation by HPV does not correlate with an ability of HPV18 to re-engage the differentiation program of NOKs

EBV lytic reactivation in NOKs occurs when cells are induced to differentiate ([8] and Fig 3). However, it is also known that EBV inhibits epithelial cell differentiation [8, 14]. We therefore asked if HPV promotes the EBV lytic phase by overcoming the inhibition of differentiation imposed by EBV. We analyzed hallmarks of differentiation in NOKs raft

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cultures harboring EBV, HPV18 or both. Hematoxylin and eosin (H&E) staining indicated that the presence of EBV causes irregular morphology and invasion into the dermal equivalent, and these effects were not completely reversed by the presence of HPV18 (Fig 4A). Immunofluorescence staining showed that expression of the differentiation marker cytokeratin-10 (K10) decreased dramatically in rafts harboring EBV compared to rafts with or without HPV18 (Fig 4B). In addition, expression of two cellular proteins that promote terminal differentiation (KLF4 and BLIMP1) was decreased in rafts harboring EBV compared to rafts with or without HPV18 (Fig 4C,D). Thus, the induction of lytic reactivation of EBV by HPV in raft cultures of NOKs cells does not correlate with an ability of HPV18 to re-engage the differentiation program of these oral keratinocytes based on morphology and K10, KLF4, and BLIMP1 staining of raft cultures.

HPV oncogenes E6 and E7 are necessary and sufficient to induce HPV mediated EBV lytic reactivation in suprabasal layers of organotypic raft cultures

HPV oncogenes E6 and E7 are viral oncogenes that have multiple roles in the HPV life cycle and HPV-induced carcinogenesis [reviewed respectively in 15, 16]. Since EBV lytic reactivation occurs in the same compartment of the stratified epithelium as the productive phase of the HPV life cycle, and the latter depends upon HPV oncogenes E6 and E7, we asked if E6 and/or E7 are necessary to promote EBV lytic reactivation in NOKs raft cultures. NOKs cells containing EBV and either HPV18 wild-type, HPV18 E6-null (contains a stop codon to prevent expression of E6 while remaining genome is intact), or HPV18 E7-null (contains a stop codon to prevent expression of E7 while remaining genome is intact) genomes were grown in raft cultures, and resulting tissue sections were analyzed for markers of EBV lytic reactivation. We found that in raft cultures harboring E6-null or E7-null genomes, the percentage of cells expressing EBV immediate-early Z protein (0.60% and 0.69%, respectively) and containing amplified EBV genomes (0.49% and 0.33%, respectively) was similar to levels observed in raft cultures with no HPV present (0.57% Z-positive nuclei and 0.67% nuclei with amplified EBV genomes; Fig 5). These data indicate that E6 and E7 are both necessary to induce HPV mediated EBV lytic reactivation in NOKs raft cultures. We next asked if E6 and E7 are sufficient to induce EBV lytic reactivation. We transduced two populations of NOKs cells containing EBV (clone 1 and clone 2) with a pBabe recombinant retrovirus expressing HPV16 E6 and E7 (pBabe E6E7) and subsequently grew cells in raft cultures. We observed a higher baseline percentage of cells expressing EBV immediate-early Z protein in the suprabasal compartment of raft cultures containing parental NOKs EBV clone 2 cells (3.94% Z-positive nuclei in rafts with parental cells and 3.33% Z-positive nuclei in rafts with vector-only transduced cells) than in those containing parental NOKs EBV clone 1 cells (0.32% Z-positive nuclei in rafts with parental cells and 0% Z-positive nuclei in rafts with vector-only transduced cells). However, the percentage of cells expressing Z protein increased in raft cultures of both EBV clones containing E6 and E7 transduced cells (2.23% Z-positive nuclei in rafts with EBV clone 1 and 11.02% Z-positive nuclei in rafts with EBV clone 2) compared to raft cultures with their respective parental cells (Fig 6). These results indicate that E6 and E7 are both necessary and sufficient to promote HPV mediated EBV lytic reactivation in raft cultures.

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Discussion

EBV and HPVs share a number of characteristics regarding their infection of oral epithelial tissue. Both DNA viruses infect and replicate in tissue from the upper aerodigestive tract epithelia, and epithelial differentiation induces both the productive phase of HPV's life cycle and the lytic phase of EBV's life cycle. We asked if these viruses affect each other's life cycles in oral epithelial cells. We found that high-risk HPV stabilizes the EBV genome in normal oral keratinocytes and induces EBV lytic reactivation in differentiating epithelial cells, suggesting that co-infection with HPV may increase EBV-mediated pathogenesis in the oral cavity.

The EBV life cycle in epithelial cells remains poorly understood despite the discovery of EBV replication in suprabasal layers of epithelial cells in the tongues of oral hairy leukoplakia patients in 1985 [3]. The ability of EBV to infect and spread within oral keratinocytes has recently been confirmed in vitro using organotypic culture [9]. Another recent study indicated that the virion glycoprotein profile of EBV shed in the saliva of normal patients is consistent with EBV produced in epithelial cells [17] suggesting that EBV can replicate in normal oral epithelia. Furthermore, periodontal gingival epithelial cells of normal patients were found to commonly act as a latently-infected reservoir for EBV [2]. Our findings indicate that HPV and EBV can be maintained alone or together in normal oral epithelial cells, NOKs, rendering NOKs a suitable model for studying co-infection of these two viruses in the oral cavity.

The HPV oncoproteins, E6 and E7, were found to be necessary and sufficient to induce the HPV-mediated lytic reactivation of EBV in the suprabasal compartment of oral epithelia (Fig 5 and Fig 6, respectively). These oncoproteins are multifunctional [reviewed in 15, 16]. It remains to be learned what activities contribute to the lytic reactivation of EBV. KLF4 is known to contribute to lytic reactivation of EBV in NOKs [8]. HPV has been reported to decrease levels of miR-145 [18], a microRNA that down-regulates KLF4, and therefore, HPV could contribute to an increase in KLF4 levels. However, we failed to see any induction of KLF4 by HPV18 in the context of HPV/EBV infected NOKs (Fig 4). Thus, the mechanism by which HPV E6 and/or E7 oncoproteins cause lytic reactivation of EBV remains unknown.

We found that EBV increased suprabasal DNA synthesis in raft cultures (Fig 2A) which is consistent with the findings of Scholle et al. [14] using raft cultures of HaCaT cells expressing an EBV latent membrane protein LMP2A. We also found that EBV alone could induce E2F-responsive protein expression in suprabasal layers as evidenced by MCM7 immunohistochemistry (Fig 2B), suggesting that EBV reprograms terminally differentiating cells to create an environment favorable for cell cycle progression. HPV's productive phase normally occurs in suprabasal epithelial cells that are reprogrammed to support cell cycle progression by HPV oncogenes, and we observed both suprabasal DNA synthesis and E2F-responsive protein expression in raft cultures harboring HPV. The effects were not synergistic in rafts harboring both EBV and HPV, suggesting that HPV does not increase the efficiency of EBV replication by simply providing a better environment supportive of DNA synthesis. Interestingly, we saw no evidence of HPV's productive cycle in suprabasal

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epithelial layers when EBV is present in rafts (Fig 2C and 2D), which suggests that EBV is preventing HPV's productive cycle despite both viruses' ability to reprogram terminally differentiating cells. The inhibition of epithelial cell differentiation by EBV that we and others [8, 14] have demonstrated could explain the inability of HPV to complete its full life cycle, as the HPV life cycle is intimately tied to epithelial differentiation.

Whether HPV and EBV co-exist within the same head and neck cancers is highly controversial. Some studies have detected the presence of both viral genomes in oral squamous cell carcinomas [19, 20] and nasopharyngeal carcinomas [21], and Jiang et al. found a significant association of the presence of EBV and HPV together in oropharyngeal cancers of the tonsil and base of tongue [20]. In addition, Shimabuku et al. [22] found that co-expression of EBV latent membrane protein-1 and HPV 16 E6 in primary mouse fibroblasts induced anchorage-independent growth and caused tumor formation in nude mice. However, there remains no compelling experimental evidence that HPV and EBV actually co-exist in the same cells within head and neck cancers. The fact that the vast majority of people are persistently infected with EBV, and EBV-positive B cells likely are present within epithelial tissues including epithelial cancers, could account for the above-cited co-associations. Nevertheless, co-infections by these two viruses could contribute to head and neck cancers in several ways that do not require that the two viruses co-exist in the actual cancers that arise, as discussed below.

Considering the results of our study, we propose several potential mechanisms to explain how co-infection with HPV and EBV may contribute to oral cancer. First, our findings indicate that HPV stabilizes EBV in oral epithelial cells (Fig 1 C–F). Increased persistence of EBV in epithelial cells of the oral cavity could contribute to the likelihood of malignancy in this tissue. Although the mechanism is currently unclear, the presence of HPV may increase the efficiency of EBV genome synthesis during S phase and/or increase the efficiency of equal partitioning of EBV DNA during mitosis as inefficiencies in both of these processes contribute to the loss of EBV from cultured cells [13]. Secondly, by promoting EBV lytic reactivation in suprabasal layers of the oral epithelium, HPV could potentially contribute to an increase in EBV viral yield and therefore increase in the number of subsequent infection events in the oral cavity both within and among individuals. Thirdly, the induction of the lytic cycle in a fraction of cells can increase expression of viral and cellular cytokines and growth factors, such as vascular endothelial growth factor, which, in turn can enhance proliferation of neighboring cells [23, 24]. Finally, an "abortive" lytic cycle has been proposed in which lytic EBV enhances instability of the cellular genome and then EBV switches back to its latent cycle [25, 26]. Determining the mechanisms by which co-infection with HPV and EBV increase susceptibility to oropharyngeal cancers remains an area of ongoing research.

Taken together, our results indicate that HPV infection in the oral cavity has the potential to stabilize EBV in latently-infected oral epithelial cells and increase EBV viral yield upon differentiation of the same cells. These HPV-associated effects could contribute to EBV-related pathologies of the oral cavity in co-infected epithelia. Future studies will dissect the mechanisms by which HPV affects EBV's life cycle.

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Materials and Methods

Cell Lines

Normal oral keratinocytes (NOKs) immortalized with human telomerase were a gift from Karl Munger (Tufts University) and are described in Piboonniyom et al. [27]. NOKs were maintained at subconfluence on mitomycin C-treated J2 3T3 feeder cells in complete F medium (3 parts F12 to 1 part Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 24 μg/ml adenine, 8.4 ng/ml cholera toxin, 10 ng/ml epidermal growth factor, 2.4 μg/ml hydrocortisone, 5 μg/ml insulin, and pen/strep). NOKs cells were infected with a recombinant version of the Akata strain of EBV that contains the neomycin resistance gene [28] by Bill Sugden, and these cells were maintained under the same conditions as NOKs cells not harboring EBV with the addition of G418 (500 μg/ml) to select for EBV-positive cells.

Plasmids

Sequences for pcDNA6 (Invitrogen) and pCL-10A1 (Imgenex) are described by their respective manufacturers. The plasmid carrying the HPV18 wild-type genome (a gift from Carl Baker) and the plasmid harboring the HPV18 E6stop mutation were both previously described in Lorenz et al. [29]. Briefly, HPV18 E6stop contains a G to T mutation at base pair 117 of the HPV18 sequence found in the papilloma virus genome database (http://pave.niaid.nih.gov) resulting in a stop codon at amino acid number 5 of E6. HPV18 E7stop contains a G to T mutation at base pair 596 of the HPV18 sequence found in the papilloma virus genome database resulting in a stop codon at amino acid number 3 of E7. The following primers were used for site directed mutagenesis to make the E7stop mutation: forward primer 5'-GTA TAA TAT TAA GTA TGC ATT GAC CTA AGG CAA CAT TGC AAG AC -3' and reverse primer 5'-GTC TTG CAA TGT TGC CTT AGG TCA ATG CAT ACT TAA TAT TAT AC -3'. The pBabe empty vector and pBabe 16E6E7 retrovirus plasmids were gifts from Dennis McCance (University of New Mexico) [30].

Transfections

HPV18 genomes (wild-type, E6stop, and E7stop) were recircularized by releasing the genomes from their bacterial vectors and religating with T4 DNA ligase. Transfections were performed as previously described [31] with the following modification. NOKs and NOKs EBV cells were transfected with recircularized HPV18 genomes along with a selection plasmid (pcDNA6) expressing resistance to blasticidin using FuGENE HD transfection reagent (Promega) according to the manufacturer's instructions. Transfections were performed in triplicate. Successfully transfected cells were selected using 7 μg/ml blasticidin, and cells were expanded and pooled.

Transductions

NOKs and NOKs EBV cells were transduced with pBabe E6E7 or pBabe empty vector to stably express HPV16 E6 and E7. To make retrovirus, pBabe E6E7 or pBabe vector along with pCL10-A1 packaging plasmid were transfected into 293FT cells using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific) according to the manufacturer's

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instructions. Retrovirus-containing medium was collected at 48 and 72 hours post-transfection. Parental NOKs and two clones of NOKs EBV (clone 1 and clone 2) were transduced by adding retrovirus-containing medium and 8 μg/ml of polybrene (Sigma) onto cells for 48 hours. Successfully transduced cells were selected using 1.5 μg/ml puromycin (Invitrogen).

Organotypic raft cultures

Cells were grown in organotypic (raft) cultures as described in Genther et al. [32]. Briefly, a dermal equivalent containing collagen (Wako Chemicals) and early passage human foreskin fibroblasts (EF-1-F) was plated onto transwells and incubated in fibroblast medium (F12 supplemented with 10% fetal bovine serum and pen/strep) for 6 days. The following cells (2.1×10^5) were plated onto the dermal equivalent: NOKs parental, NOKs EBV clone 1 parental, NOKs +/– EBV clone 1 harboring HPV18 wild-type, HPV18 E6stop, or HPV18 E7stop genomes (passage 0 post-HPV18 genome transfection), NOKs EBV clone 2 parental, as wells as NOKs parental, NOKs EBV clone 1 and NOKs EBV clone 2 transduced with empty vector or HPV16 E6E7. Rafts were then incubated in keratinocyte plating medium (3 parts F12 to 1 part Dulbecco's modified Eagle's medium supplemented with 0.5% fetal bovine serum, 24 μg/ml adenine, 8.4 ng/ml cholera toxin, 2.4 μg/ml hydrocortisone, 5 μg/ml insulin, 1.22 mM Ca^{2+} and pen/strep). Four days after plating of epithelial cells, rafts were lifted to the air-liquid interface and further cultured with cornification medium (3 parts F12 to 1 part Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 24 μg/ml adenine, 8.4 ng/ml cholera toxin, 2.4 μg/ml hydrocortisone, 5 μg/ml insulin, 1.22 mM Ca^{2+} and pen/strep) containing 10 μM C8:0. Rafts were harvested in 1% formalin 2% agar 11 days post-lifting. On the day of harvesting, 10 μM bromodeoxyuridine (BrdU) was added to the raft medium for 8 hours to label newly synthesized DNA for later detection using immunohistochemistry. Raft cultures were fixed in 10% neutral buffered formalin overnight, paraffin-embedded, and cut into 5-micron sections.

Southern blot analysis

Genomic DNA (gDNA) was extracted from three populations each of NOKs +/– EBV harboring HPV18 wild-type genomes 0 and 6 passages post-transfection using Qiagen's DNeasy Blood and Tissue kit according to the manufacturer's instructions. Double restriction digests were performed on 7 μg gDNA using Nco1 (a single cutter of the HPV18 genome) and Dpn1 (cuts only methylated DNA, i.e., replicated in mammalian cells) in a 200 μl final volume. A phenol:chloroform (1:1) extraction followed by an isopropanol precipitation was performed on digested gDNA, and 2.5 μg of gDNA was separated on a 0.8% agarose gel. DNA in the gel was depurinated, denatured, and neutralized by rocking the gel gently in the following: 0.25 M HCl for 10 minutes; 0.5 M NaOH/1.5 M NaCl for 2 x 45 minutes; and 1 M Tris pH7.4/1.5 M NaCl for 2 x 45 minutes. DNA was transferred overnight onto a Hybond N+ membrane (Amersham) using the upward capillary transfer method with 10X Saline Sodium Citrate (SSC). DNA was cross-linked to the membrane using a UV Stratalinker 2400 (Stratagene). The membrane was probed overnight at 47°C using a mix of 20 oligonucleotides spanning the HPV18 genome [29] and radiolabeled with gamma-ATP in Church hybridization buffer (250 mM Na_2HPO_4, 1% BSA, 245 mM SDS and 5 mM EDTA pH 8.0). The membrane was washed 5x at 52°C with Church wash buffer

(140 mM SDS, 80 mM Na_2HPO_4) and exposed to a storage phosphor screen. The storage phosphor screen was then scanned with the Typhoon 8610.

Fluorescence in situ hybridization (FISH)

For FISH performed on monolayer NOKs EBV +/− HPV18 for several passages, 3 x 10^6 cells were washed in 1X PBS and resuspended in 5 mls of hypotonic solution (0.075 M KCl) at 37°C for 20 minutes. Ten drops of 3:1 methanol: acetic acid fixative was added to the cells for 5 minutes. Cells were spun down, resuspended in 5 mls of fixative, and incubated at room temperature for 30 minutes. Cells were then gently washed 3 times in fixative before resuspending in 300 μl of fixative and storing at −20°C. Cells were dropped onto slides and completely dried before pre-hybridizing in 2X SCC, 0.5% IPECAL, pH 7.0 for 30 minutes at 37°C. Cells were dehydrated using a series of ice cold ethanols (70%, 80%, 95%) for 2 minutes each. Slides were dried by placing them in an empty container at 50°C for 5 minutes. Slides were then placed in denaturation solution (28 mL formamide, 4 mL 20X SSC pH 5.3, 8 mL water) at 72°C for 2 minutes. The ethanol series was repeated again, and after drying the sections, denatured probe was added to the slides. A digoxigenin (DIG-11-dUTP, Roche)-labeled probe and a biotin (Biotin-16-dUTP, Roche)-labeled probe were mixed and hybridized to cells overnight at 37°C in a humidified chamber. To make the probe, nick translation was used to label EBV bacmid DNA (B95.8) with digoxigenin and HPV18 plasmid DNA with biotin. After washing for 30 minutes twice with 2X SSC and 50% formamide at 50°C and 30 minutes twice with 2X SSC at 50°C, signals were detected with a digoxigenin-specific antibody conjugated to fluorescein isothiocyanate (Sigma, F3523) at 2% by volume and streptavidin conjugated to Cyanine 3 (Sigma, S6402) at 1% by volume in STM solution (4X SSC, 5% non-fat dried milk, 0.05% Tween-20, 0.002% sodium azide) for 30 minutes at 37°C. Nuclei were counterstained with DAPI. The same protocol starting with pre-hybridization was used for FISH performed on formalin-fixed, paraffin-embedded tissue sections of rafts after deparaffinization. All images were taken with a Zeiss AxioImager M2 microscope using the AxioVision software version 4.8.2.

Histology

Formalin-fixed, paraffin-embedded raft sections were deparaffinized and then examined by hematoxylin and eosin (H&E), immunofluorescence (IF), and immunohistochemistry (IHC) staining. For IF staining, sections were rehydrated in a series of ethanols and washed with 1X PBS. Antigen retrieval was performed by microwaving the slides for 20 minutes in 10 mM sodium citrate (pH 6.0). After cooling, sections were blocked using 5% serum (plus 5% milk for anti-BZLF1 staining) for 1 hour at room temperature and primary antibody in 5% serum was applied overnight at 4°C in a humidified chamber. A 1:500 dilution of secondary antibody was applied at room temperature for 1 hour followed by a series of PBS washes. Sections were counterstained with Dapi. Primary antibodies used were anti-BZLF1 (BZ.1) monoclonal antibody (1:200 in 5% milk/5% donkey serum, Santa Cruz Biotechnology), anti-K10 polyclonal antibody (1:1000 in 5% donkey serum, Covance), anti-PRDM1 (BLIMP1) (1:200 in 5% goat serum, Sigma), anti-KLF4 (1:100 in 5% goat serum, Sigma), and anti-HPV18 E1^E4 (kindly provided by Dr. John Doorbar, University of Cambridge). Secondary antibodies used were Alexa 488 conjugated donkey anti-mouse, Alexa 647 conjugated donkey anti-rabbit, Alexa 594 conjugated goat anti-rabbit, and Alexa 488

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conjugated goat anti-rabbit (Life Technologies). For IHC staining, sections were rehydrated in a series of ethanols and washed with 1X PBS. Endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol. Antigen retrieval was performed by microwaving the slides for 20 minutes in 10 mM sodium citrate (pH 6.0). After cooling, sections were washed with PBS, treated for 20 minutes with 2N HCl, and washed again with PBS. Sections were blocked for 1 hour at room temperature using 2.5% horse serum from Vectastain's R.T.U. Kit. Primary antibody in 2.5% horse serum was applied overnight at 4°C in a humidified chamber. Positive staining was visualized using the R.T.U. Vectastain Kit and DAB Peroxidase Substrate Kit (Vector Laboratories) according to the manufacturer's instructions. Sections were counterstained with hematoxylin QS solution (Vector Laboratories) and dehydrated using a series of ethanols and xylenes after which Cytoseal XYL mounting medium was applied to the sections. Primary antibodies used were anti-BrdU (1:100, Calbiochem) and anti-MCM7 (1:200, NeoMarkers). All images were taken with a Zeiss AxioImager M2 microscope using the AxioVision software version 4.8.2.

Statistical analysis

For Z, FISH, and BrdU quantification, 10 images were captured and analyzed per raft section. The number of positive cells was manually counted and the total number of cells was quantified with an automated cell counting program developed by David Ornelles (Wake Forest University School of Medicine) using ImageJ software version 10.2 (NIH, Bethesda, MD). For BrdU quantification, the number of basal cells was also manually counted and subtracted from the total to obtain the total number of suprabasal cells. The percentage of positive cells per raft was calculated by dividing the total positive cells in 10 images by the total number of cells in those 10 images. For statistical analysis, positive and negative cells were counted in 10 images of each raft and the data was pooled across replicates to determine frequency of positive versus negative cells in each HPV transfection group. The Chi-square test was performed for statistical significance using MSTAT statistical software version 6.1.4 (http://www.mcardle.wisc.edu/mstat).

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Highlights

- EBV and HPV both infect oral epithelia and cause head and neck cancers
- EBV and HPV can co-infect immortalized human oral keratinocytes
- HPV increases the stability of EBV DNA genomes in latent infection
- HPV also increases the frequency of lytic reactivation of EBV in raft cultures

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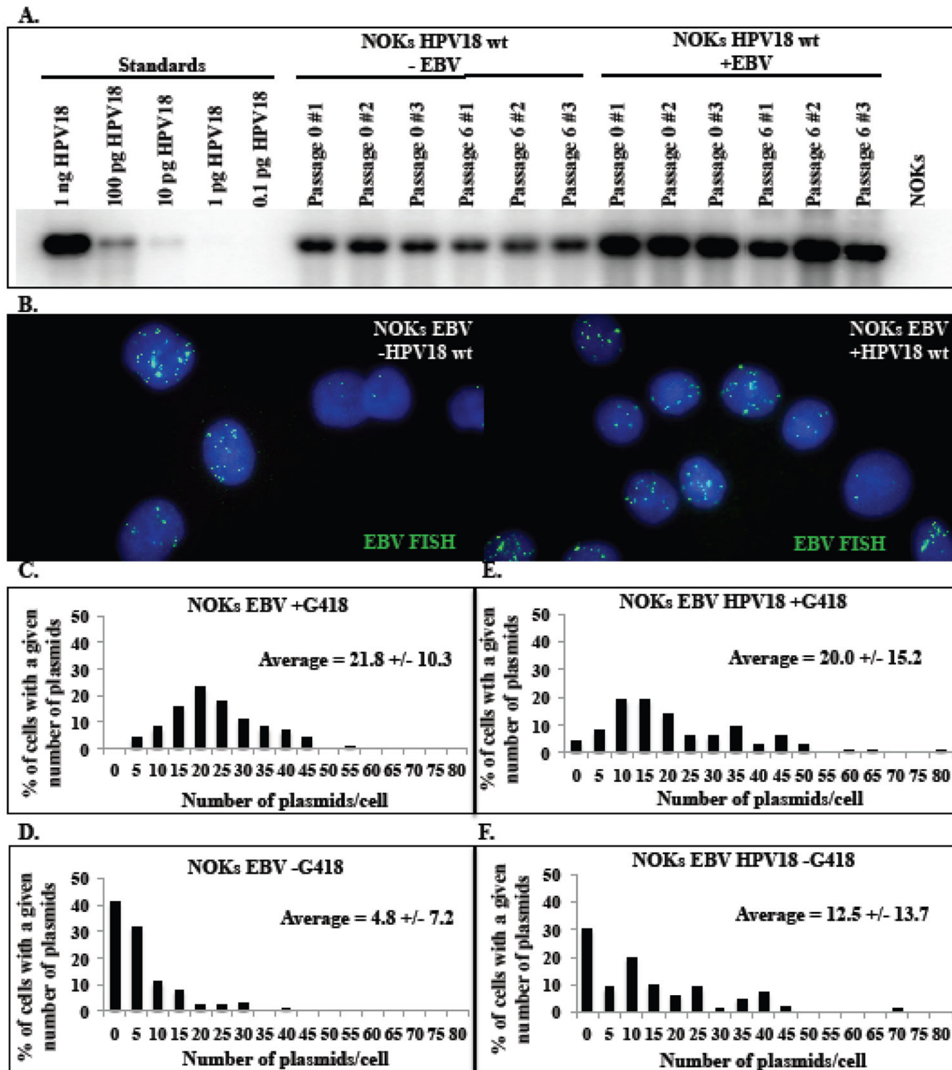

Fig 1. HPV18 and EBV genomes are maintained alone and together in NOKs cells over several passages

(A) The HPV18 genome is maintained in NOKs cells +/– EBV over six passages. Total genomic DNA (7 μg) isolated from three independent replicate populations (labeled as #1, #2, and #3) of NOKs cells +/– EBV harboring HPV18 wild-type genomes at passage 0 and 6 post-HPV transfection was double digested with a single cutter of the HPV18 genome (Nco1) and Dpn1 (an enzyme that cuts only bacterially-replicated input DNA). The digested DNA (2.5 μg) was separated using a 0.8% agarose gel and transferred to a Hybond N+ membrane followed by HPV18-specific Southern blot analysis. (B) The EBV genome is maintained over several passages (>15) in NOKs cells without HPV18 (left panel) and with HPV18 (right panel). The number of EBV genomes per cell was quantified in EBV-infected NOKs cells grown for 12 passages in the presence (C) or absence (D) of selection for EBV, highlighting the loss of EBV genomes in the absence of selection. The number of EBV genomes per cell was also quantified in EBV-infected NOKs cells harboring HPV18 grown for 12 passages in the presence (E) or absence (F) of selection for EBV.

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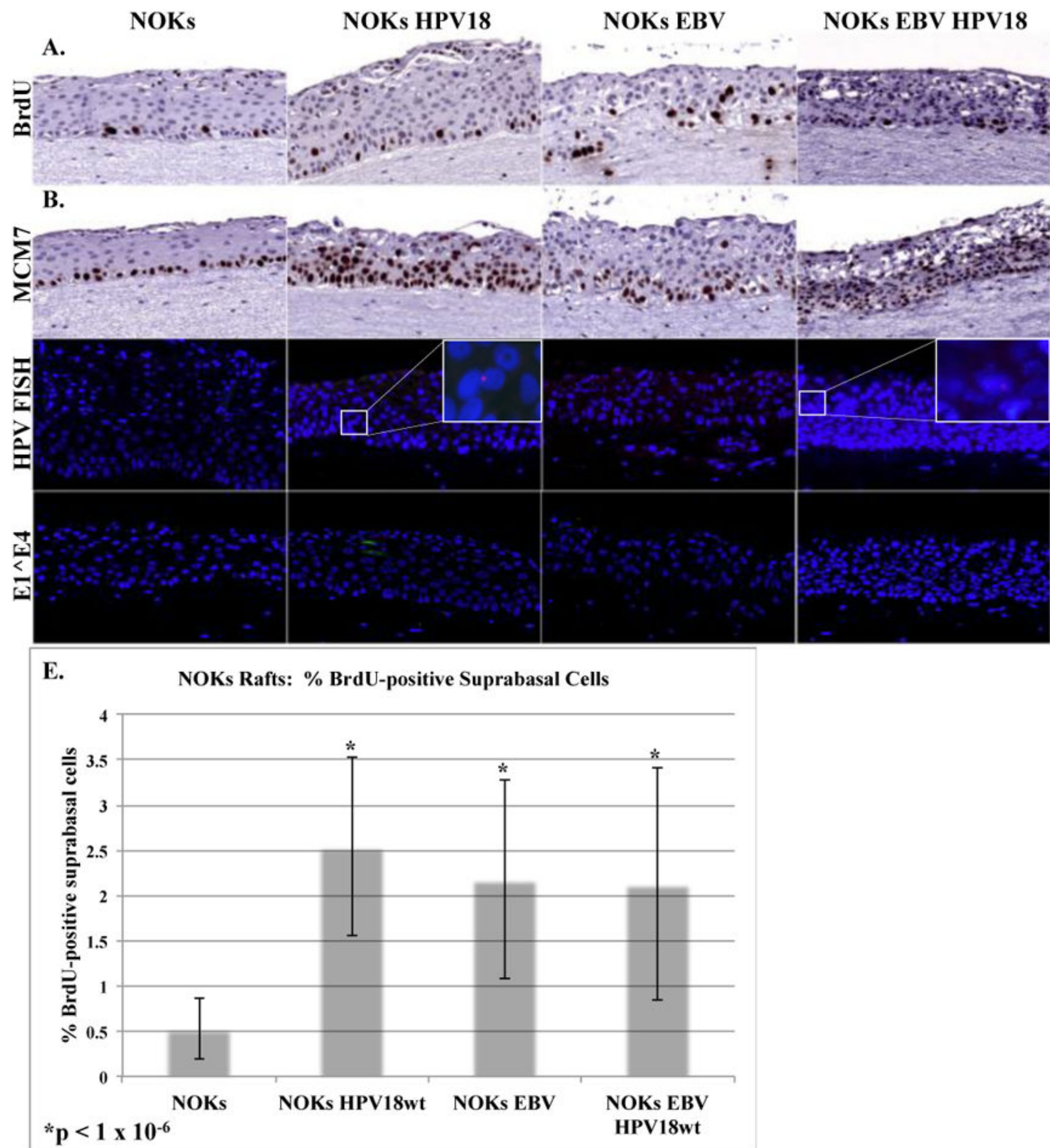

Fig 2. Biomarker analysis of the HPV18 productive phase in NOKs +/− EBV organotypic raft cultures

Raft cultures of NOKs cells support the productive HPV18 life cycle and the presence of EBV blocks HPV18 genome amplification and late gene expression. Shown is immunohistochemical analysis for BrdU (A) and the E2F-responsive protein MCM7 (B), HPV18-specific FISH analysis (C), and immunofluorescence staining for the HPV productive phase protein E1^E4 in NOKs rafts harboring HPV18, EBV, neither or both viruses. (E) Quantitation of BrdU-positive suprabasal cells from part A. Asterisks indicate statistical significance compared to NOKs rafts with neither virus ($p < 1 \times 10^{-6}$, Chi-square test). Error bars indicate standard deviation.

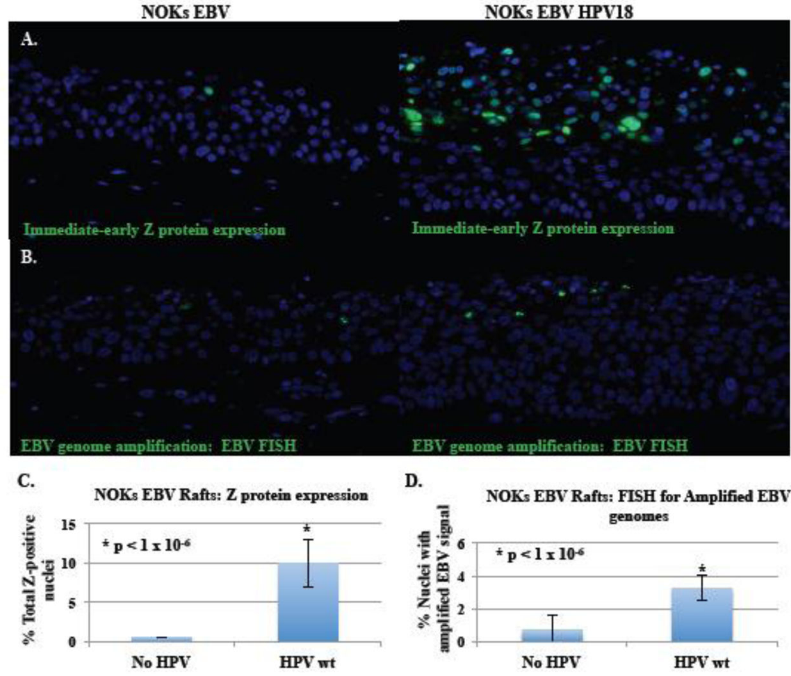

Fig 3. HPV18 induces EBV lytic reactivation in differentiated oral epithelial cells

(A) Immunofluorescence staining for the EBV immediate-early Z protein (BZLF1) in NOKs rafts with EBV only (left panel) or with EBV and HPV18 (right panel). (B) EBV-specific FISH in NOKs rafts with EBV only (left panel) or with EBV and HPV18 (right panel). (C) Quantitation of Z-positive nuclei from part A. (D) Quantitation of nuclei with amplified EBV FISH signal from part B. Asterisks indicate statistical significance compared to NOKs rafts with EBV only ($p < 1 \times 10^{-6}$, Chi-square test). Error bars indicate standard deviation.

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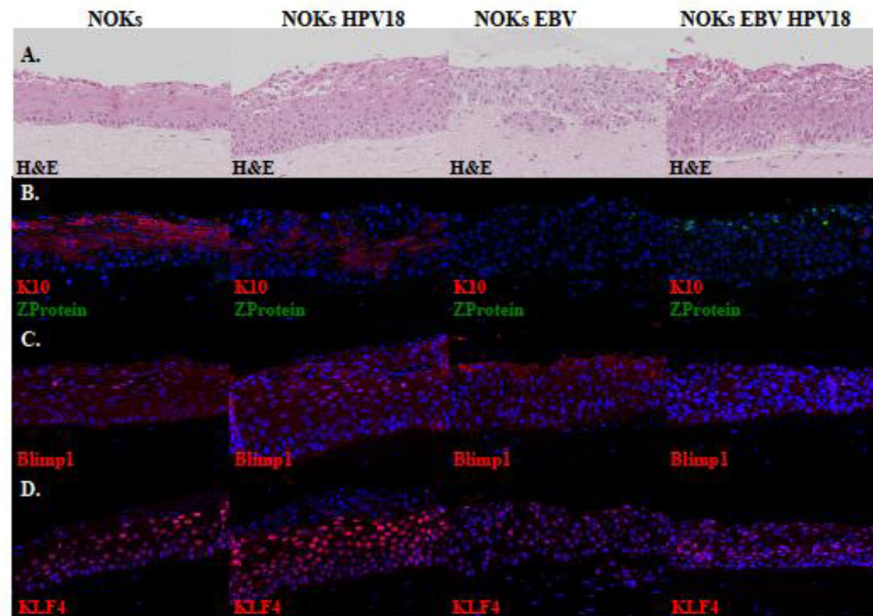

Fig 4. Histological and differentiation marker analysis of NOKs organotypic rafts harboring HPV, EBV or both

H&E staining (A), immunofluorescence co-staining for differentiation marker (K10) expression and EBV immediate-early Z protein expression (B), and immunofluorescence staining for two cellular factors that promote terminal differentiation, Blimp1 (C) and KLF4 (D) in NOKs organotypic rafts harboring HPV18, EBV, both or neither.

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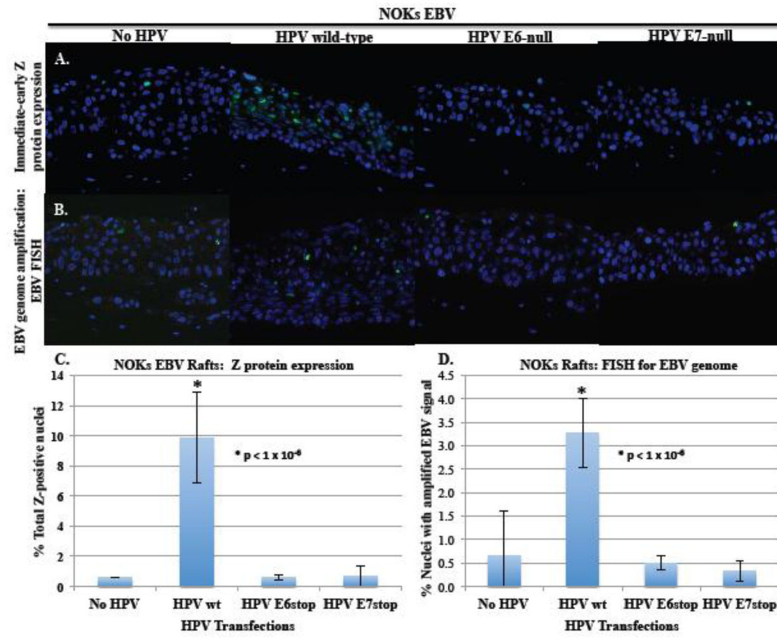

Fig 5. HPV oncogenes E6 and E7 are necessary to promote EBV lytic reactivation in NOKs rafts

(A) Immunofluorescence staining for EBV immediate-early Z protein (BZLF1) in EBV-positive NOKs rafts harboring HPV18 wild-type, HPV18 E6-null, or HPV18 E7-null genomes. (B) EBV-specific FISH in EBV-positive NOKs rafts harboring HPV18 wild-type, HPV18 E6-null, or HPV18 E7-null genomes. (C) Quantitation of Z-positive nuclei from part A. (D) Quantitation of nuclei with amplified EBV FISH signal from part B. Asterisks indicate statistical significance compared to NOKs rafts with no HPV18, HPV18 E6-null genomes or HPV18 E7-null genomes ($p < 1 \times 10^{-6}$, Chi-square test). Error bars indicate standard deviation.

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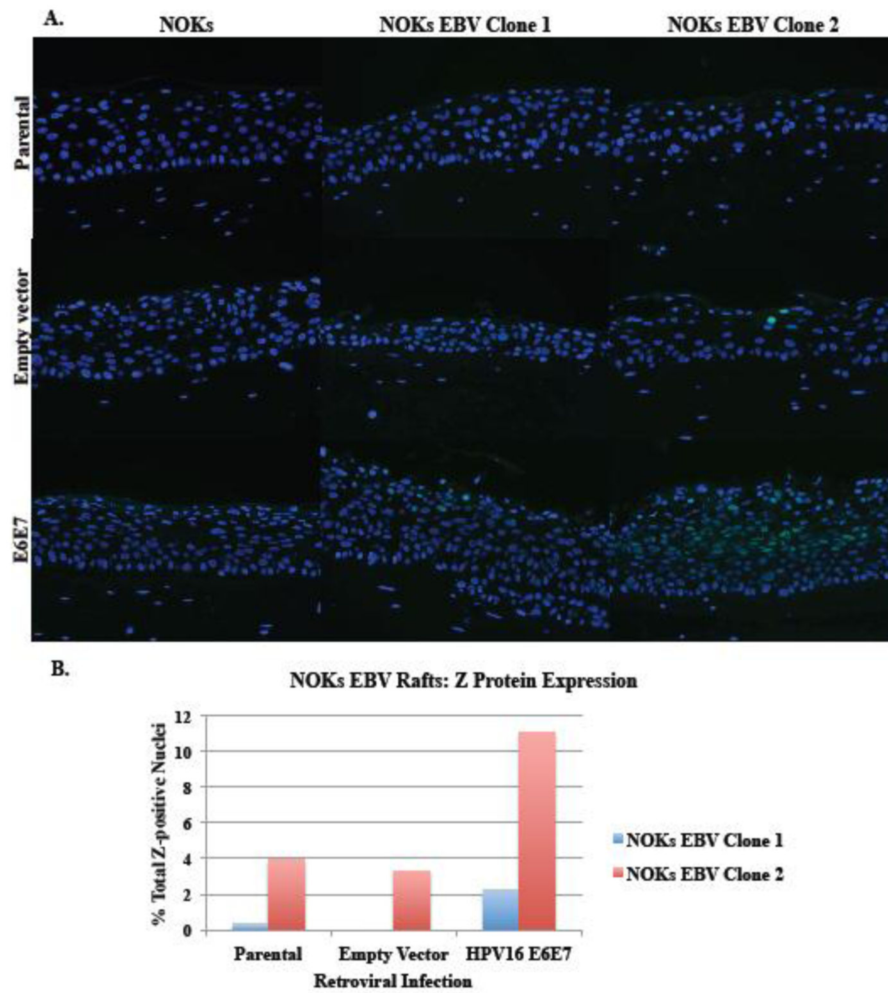

Fig 6. HPV oncogenes E6 and E7 are sufficient to promote EBV lytic reactivation in NOKs rafts

(A) Immunofluorescence staining for the EBV immediate-early Z protein (BZLF1) in rafts containing either parental NOKs cells or two populations of EBV-infected NOKs cells (clone 1 and clone 2) transduced with a pBabe recombinant retrovirus to stably express HPV16 E6 and E7. (B) Quantitation of Z-positive nuclei from part A.