APOBEC3A Functions as a Restriction Factor of Human Papillomavirus

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ABSTRACT

Human papillomaviruses (HPVs) are small DNA viruses causally associated with benign warts and multiple cancers, including cervical and head-and-neck cancers. While the vast majority of people are exposed to HPV, most instances of infection are cleared naturally. However, the intrinsic host defense mechanisms that block the early establishment of HPV infections remain mysterious. Several antiviral cytidine deaminases of the human APOBEC3 (hA3) family have been identified as potent viral DNA mutators. While editing of HPV genomes in benign and premalignant cervical lesions has been demonstrated, it remains unclear whether hA3 proteins can directly inhibit HPV infection. Interestingly, recent studies revealed that HPV-positive cervical and head-and-neck cancers exhibited higher rates of hA3 mutation signatures than most HPV-negative cancers. Here, we report that hA3A and hA3B expression levels are highly upregulated in HPV-positive keratinocytes and cervical tissues in early stages of cancer progression, potentially through a mechanism involving the HPV E7 oncoprotein. HPV16 virions assembled in the presence of hA3A, but not in the presence of hA3B or hA3C, have significantly decreased infectivity compared to HPV virions assembled without hA3A or with a catalytically inactive mutant, hA3A/E72Q. Importantly, hA3A knockdown in human keratinocytes results in a significant increase in HPV infectivity. Collectively, our findings suggest that hA3A acts as a restriction factor against HPV infection, but the induction of this restriction mechanism by HPV may come at a cost to the host by promoting cancer mutagenesis.

IMPORTANCE

Human papillomaviruses (HPVs) are highly prevalent and potent human pathogens that cause >5% of all human cancers, including cervical and head-and-neck cancers. While the majority of people become infected with HPV, only 10 to 20% of infections are established as persistent infections. This suggests the existence of intrinsic host defense mechanisms that inhibit viral persistence. Using a robust method to produce infectious HPV virions, we demonstrate that hA3A, but not hA3B or hA3C, can significantly inhibit HPV infectivity. Moreover, hA3A and hA3B were coordinately induced in HPV-positive clinical specimens during cancer progression, likely through an HPV E7 oncoprotein-dependent mechanism. Interestingly, HPV-positive cervical and head-and-neck cancer specimens were recently shown to harbor significant amounts of hA3 mutation signatures. Our findings raise the intriguing possibility that the induction of this host restriction mechanism by HPV may also trigger hA3A- and hA3B-induced cancer mutagenesis.

Human papillomaviruses (HPVs) are small, nonenveloped DNA viruses known as one of the most prevalent sexually transmitted pathogens. Among almost 200 different genotypes, ~24 high-risk HPV genotypes are causally associated with multiple human cancers, including nearly all cervical cancers and a portion of head-and-neck squamous cell carcinomas (1). From 1988 to 2004, the incidence of HPV-associated oropharyngeal squamous cell carcinoma (OPSCC) has increased by 225% and surpassed the incidence of HPV-negative, smoking-related OPSCC (2). Recently developed HPV vaccines will provide some protection against new HPV infections. However, these vaccines cover only two high-risk genotypes (HPV16 and HPV18) and do not affect established HPV infections. Interestingly, continued expression of HPV oncogenes is required for cancer maintenance, suggesting that these oncogenes could be specific and effective targets for curing HPV-associated cancers (3). Thus, there is an urgent need to develop novel approaches to prevent and treat HPV infection.

While the majority of people become infected with HPV during their lifetime, only 10 to 20% of infections are established as

persistent infections, while the rest clear the infection within 1 to 2 years (4–6). This suggests that host immune responses may efficiently eliminate most HPV infections. Cell-mediated immune responses may confer protection, as a fraction of patients with HPV16-associated lesions show T cell responses specific to HPV16 E2 and E6 (7–10). Because HPV infection rarely triggers viremia and strong adaptive immune responses (11), it is believed

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that innate immune responses also play a critical role in viral clearance. However, very little is known about the innate mechanisms that can restrict HPV infection in human keratinocytes.

Members of the apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3 (APOBEC3) family of cytidine deaminases were first discovered as important host restriction factors that block the replication of HIV-1 (12–14). Human APOBEC3 (hA3) consists of seven members: hA3A, hA3B, hA3C, hA3D, hA3F, hA3G, and hA3H (15, 16). A subset of these enzymes deaminate cytidines (C) to uridines (U) in retroviral, single-stranded (minus-strand) DNA, ultimately resulting in G-to-A mutations during plus-strand DNA synthesis (17, 18). Of these seven hA3 family members, hA3A, hA3B, and hA3H genes are expressed in skin keratinocytes, the primary host cell for HPV infection (19). Recent studies have shown that hA3A could restrict certain DNA viruses, including adeno-associated viruses (AAVs) and other parvoviruses (20–22). In addition, other groups found evidence for editing of herpes simplex virus 1 and Epstein-Barr herpesvirus (23) and, notably, HPV DNA in precancerous lesions and cell lines (24). The latter study (24) raised the possibility that certain hA3 proteins may restrict HPV. However, there is currently no evidence that hA3 proteins could directly interfere with HPV infection of critical target cells.

Recently, several high-profile studies identified significant hypermutation signatures in genomic DNA from many different cancer cells, including breast, bladder, cervix, lung, and head-and-neck cancer cells (25–27). Since these mutations fit known hA3 mutational hot spots, the “APOBEC mutator hypothesis” was formulated. This hypothesis states that hA3 proteins introduce critical mutations in host genomes that eventually promote cancer progression (19, 26, 28). Other studies suggested that genomic mutations following double-strand DNA breaks could also be induced by hA3 (29, 30). Notably, hA3-associated mutational signatures are highly enriched in HPV-positive cervical and head-and-neck cancers (26, 31, 32). However, the molecular triggers for hA3-dependent mutagenesis in HPV-positive cancers remain unknown.

Here, we report that hA3A and hA3B are significantly upregulated in HPV-positive keratinocytes and cervical lesions from early disease stages to cancer. Using a robust method that we pioneered to encapsidate full-length HPV into infectious virions (33), we provide the first evidence for direct inhibition of HPV infectivity by hA3A. Our results suggest that the proposed editing of HPV and host genomes in precancerous lesions and cervical cancer (24, 26, 27) may be linked to HPV-mediated induction of an hA3A-dependent intrinsic host defense mechanism.

MATERIALS AND METHODS

Cell lines and reagents. The 293FT cell line, a clonal isolate derived from 293T cells that provides high-yield HPV stocks, was purchased from Life Technologies and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) fetal bovine serum (FBS). HaCaT cells (34) were cultured in E-medium (3 parts DMEM, 1 part Ham's F-12 medium) supplemented with 5% FBS. Normal human immortalized keratinocyte (NIKS) cells (obtained from Lynn Allen-Hoffman) (35), NIKS-16 derivatives stably transfected with HPV16 genomes (36), W12E clone 20850 cells (37), and W12I cells (38) were cocultured with mitomycin C-treated NIH 3T3 cells in E-medium supplemented with 2.5% FBS, 0.4 µg/ml hydrocortisone, 8.4 ng/ml cholera toxin, 5 µg/ml insulin, 24 µg/ml adenine, and 10 ng/ml epidermal growth factor, as previously described (35). Cell pellets of parental human tonsillar epithelial (HTE) cell

TABLE 1 Target DNAs for HPV quasivirion and pseudovirion production

Construct	Backbone (reference or source)	HPV genome	HPV early gene expression	Reporter gene expression
HPV16-5.0kb	HPV16-5.0kb (33)	Yes	Yes	None
HPV16RL	HPV16-5.9kb (33)	Yes	Yes	RL
HPV16GFP	HPV16-5.9kb (33)	Yes	Yes	GFP
hpv16-phRL	phRL-null (Promega)	No	No	RL

lines and stable HTE cell lines expressing HPV16 E6 or E7 were obtained (39). Human recombinant beta interferon (IFN-β) (catalog number NR-3085) was obtained from BEI Resources (Manassas, VA).

Plasmid constructs. Packaging construct p16SheLL (40) was provided by John Schiller. pEGFP-N3 (Clontech) and phRL-null (Promega) were purchased. hA3A, hA3A/E72Q, hA3B, and hA3C expression plasmids were obtained from Warner Greene. pEGFP-SV40p was prepared from pEGFP-N3 by replacing the cytomegalovirus (CMV) promoter with the simian virus 40 (SV40) early promoter. phRL-SV40p was prepared from phRL-null by inserting the SV40 early promoter. To generate chimeric HPV16 genome constructs with reporters (pHPV16RL and pHPV16GFP), the enhanced green fluorescent protein (EGFP) or human renilla luciferase (hRL) gene along with the SV40 early promoter were removed from pEGFP-SV40p and phRL-SV40p, respectively, and inserted inversely into the 5.9-kb pHPV16 W12 genome construct in the region of the L2 and 5' L1 genes (positions 4295 to 6302) (33). pHPV16-5.0kb contains a truncated HPV16 W12 genome without late genes (33). These target DNAs are summarized in Table 1. None of the target DNAs for virus assembly contains the SV40 origin for self-replication in the packaging cells.

Production of HPV16 virions and pseudovirions. For transfection into 293FT cells, the full-length HPV or chimeric HPV DNAs were released from the bacterial vector by restriction enzyme digestion (BamHI for full-length HPV16 and XhoI for chimeric HPV16 clones) and recircularized by ligation, as previously described (41). HPV virions and pseudovirions were prepared as described previously (33, 42). Briefly, we cotransfected 293FT cells with the HPV16 capsid protein expression plasmid p16SheLL (gift from John Schiller) as well as one of the target DNAs for encapsidation. After incubation for 60 h at 37°C, cells were harvested, and virions were purified by using Optiprep gradient centrifugation.

Lentiviral transduction of shRNA. Lentiviruses were produced by cotransfecting packaging plasmids pVSV-g (obtained from Jerome Schaeck) and pCMV-HIVdeltaR8.2 (catalog number 12263; Addgene), as previously described (43, 44). Three clones of hA3A short hairpin RNA (shRNA) expression plasmids (TRCN0000049958, TRCN0000049959, and TRCN0000049960) were obtained from Sigma-Aldrich (RNAi Consortium, Cambridge, MA) through the Functional Genomics Facility at the University of Colorado—Boulder. Keratinocytes were inoculated with each lentivirus. Cells with stable shRNA expression were selected with 2 µg of puromycin and maintained with 1 µg of puromycin.

Infectivity assays. 293FT and keratinocyte cell lines were infected with HPV16 virions containing full-length or chimeric genomes or HPV16 pseudovirions containing a nonviral reporter gene, as previously described (33, 45). At 48 h postinfection, infectivity and cell viability assays were performed by using a renilla luciferase (RL) assay system and a Cell-Titer-Glo luminescent cell viability assay according to the supplier's instructions (Promega). Luminescence was measured with a GloMax-Multi+ detection system with Instinct software (Promega). At 48 h postinfection, green fluorescent protein (GFP) expression was analyzed by fluorescence microscopy. Infectivity of HPV16 virions containing truncated wild-type genomes (HPV16-5kb) was examined by reverse transcriptase quantitative PCR (RT-qPCR) by amplifying viral mRNA, as described below.

TABLE 2 PCR, RT-qPCR, and MiSeq primers and probes used in this study

Gene	Primer use	Sequence ^a
GAPDH	RT-qPCR forward	5'-CCCATGTTTCGTCATGGGTGT
GAPDH	RT-qPCR reverse	5'-TGGTCATGAGTCCTTCCACGAT
GAPDH	Probe	5'-(6-FAM)CTGCACCACCACTGCTTAGCACCC(TAMRA-6-FAM)
hA3A	RT-qPCR forward	5'-TTGGAAGGCATAAGACCTACCTG
hA3A	RT-qPCR reverse	5'-CAGAGAAGATTCTTAGCCTGGTTGTG
hA3A	Probe	5'-(6-FAM)TCTTGACCGAGGTGCCATTGTCC(TAMRA-6-FAM)
hA3B	RT-qPCR forward	5'-GACCCCTTGGTCCTTCGAC
hA3B	RT-qPCR reverse	5'-GCACAGCCCCAGGAGAAG
hA3B	Probe	5'-(6-FAM)CCAGCACATGGGCTTTCTAT(TAMRA-6-FAM)
HPV16 E1/E4	RT-qPCR forward	5'-AAATGACAGCTCAGAGGAGGAG
HPV16 E1/E4	RT-qPCR reverse	5'-GAGTCACACTTGCAACAAAAGG
β-Actin	RT-qPCR forward	5'-TCACCACACTGTGCCCATCTA
β-Actin	RT-qPCR reverse	5'-TGAGGTAGTCAGTCAGGTCCCCG
LCR-out	Nested-PCR	5'-TGTTTTCTGACCTGCACTG
LCR-out	Nested-PCR	5'-CATCCTCCTCCTCTGAGCTG
LCR-in	MiSeq forward	5'-AATGATACGGCGACACCGAGATCTACACTCTTTCCCTACACGACGCTCTCC GATCTNNNNINDEX1ACTTCTAAGGCCAACTAAATGT
LCR-in	MiSeq reverse	5'-CAAGCAGAAGACGGCATAACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCG ATCTNNNNINDEX2TATCACATACAGCATATGGATT
mA3	RT-qPCR forward	5'-CTGCCATGGACCTATACGAA
mA3	RT-qPCR reverse	5'-TCCTGAAGCTTAGAATCCTGGT
MmuPV1 E2	PCR forward	5'-CATTGCAATTCTGTGCTCGT
MmuPV1 E2	PCR reverse	5'-AGGTCCTGCTGGAGTCTCTG
MmuPV1 E6	PCR forward	5'-GAAATCGGCAAAGGCTACAC
MmuPV1 E6	PCR reverse	5'-GTTTGCGAAGCCCGCAGTAG
MmuPV1 E7	PCR forward	5'-ACCAACAATTGCTGACATCG
MmuPV1 E7	PCR reverse	5'-TTTCCATTCTGAGGTTACAG
MmuPV1 early transcript	RT-qPCR forward	5'-TTTGCTGCGACACTGTTCTC
MmuPV1 early transcript	RT-qPCR reverse	5'-CGGGCAGACAAAGCTAAGAT

^a 6-FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

Western blotting. Whole-cell lysates from 293FT cells transfected with plasmids pcDNA-hA3A-HA, pcDNA-hA3A/E72Q, pCDNA3.1 (Life Technologies), p3XFLAG-hA3B, p3XFLAG-hA3C, and p3XFLAG (Sigma) or from NIKS, NIKS-16, and NIKS-16ΔE7 cells were prepared by using a 1% SDS-phosphate-buffered saline (PBS) lysis buffer containing a Complete EDTA-Free Mini protease inhibitor cocktail (Roche Applied Science) and boiling for 10 min. QIAshredder homogenizer columns (Qiagen) were used to reduce viscosity. The protein concentration was determined by using the Pierce BCA protein assay kit (Thermo Scientific/Pierce Biotechnology), and 20 to 40 μg total cell lysate or virion lysate was electrophoresed in a 12% SDS-PAGE gel and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore). The blots were blocked with 5% nonfat dried milk and incubated with an hA3G antibody cross-reactive with hA3A (46), HPV16 E7 antibody (catalog number ED17; Santa Cruz Biotechnology), hemagglutinin (HA) antibody (catalog number ab9110; Abcam), FLAG-alkaline phosphatase antibody (M2; Sigma), or β-actin (8H10D10) antibody (Cell Signaling), followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Protein bands were visualized by using SuperSignal West Pico (Thermo Scientific/Pierce Biotechnology) or Clarity Western ECL (Bio-Rad) chemiluminescent substrates and the ChemiDoc XRS+ system (Bio-Rad).

PCR and RT-qPCR. Genomic DNA was extracted by using a DNeasy blood and tissue kit (Qiagen). The HPV16 genome was detected by PCR with GoTaq Green master mix (Promega) and specific primers, as previously described (45). Total RNA was extracted by using an RNeasy minikit (Qiagen) and an RNase-free DNase set (Qiagen) according to the supplier's instructions. First-strand cDNA was reverse transcribed with SuperScript II reverse transcriptase (Life Technologies) and oligo(dT)₁₆ (Integrated DNA Technologies) from total RNA. Real-time PCR was performed by using the Bio-Rad CFT Connect real-time system and Fast-

Start Universal SYBR green master (Rox) (Roche Applied Science) or TaqMan gene expression master mix (Life Technologies) in a reaction mixture of 20 μl containing 0.5 μM each PCR primer with or without 0.2 μM probe, 10 μl of SYBR green PCR master mix, 4 μl of cDNA template, and nuclease-free water to complete the 20-μl volume. The primers and probes used in this study are listed in Table 2.

Next-generation sequencing. The HPV16 long control region (LCR) (positions 7758 to 296) and E2 region (positions 3098 to 3536) were amplified from purified virions by using a nested-PCR approach. Briefly, encapsidated DNA was released by heating the samples to 98°C for 5 min. Each sample was amplified independently, three times, for 35 cycles with 0.5 μM LCR-out primers. First-round PCR products were cleaned up by using a PCR purification kit (Promega). Second-round PCR followed with gene-specific primers containing an Illumina MiSeq adapter extension and barcoded indexes (Table 2). To increase the diversity in the sample, a four-nucleotide random sequence (NNNN) was added 5' of the 6-bp index (or barcode) sequence. The primers were purchased from Eurofins MWG Operon (Huntsville, AL). FASTQ output files from MiSeq were sorted into samples by using 6-bp barcodes. The quality score (Q score) for each base was used to filter out low-scoring segments of sequence, as previously described (47). After contigs were built with paired reads by using the PANDASEQ program (48), the mutation rate of each read was evaluated by comparing the sequence to the W12 genome reference sequences of HPV LCR and E2. All contigs were aligned to the reference sequence by using USEARCH (49). Custom PERL scripts were used to scan and compare every possible deamination site in the reference sequence to the aligned contig sequences in order to identify A3A-type mutations based on their consensus dinucleotide or trinucleotide context. To quantify the percentage of A3A-type mutations, we utilized mutations in C or G bases as a denominator, since these are the mutations that could be directly mapped as an A3A-type deamination.

FIG 1 hA3A and hA3B expressions are significantly induced in low- and high-grade cervical lesions. Gene expression data for human APOBECs from a global gene expression study of 128 cervical tissue samples in different disease stages were analyzed: normal tissue ($n = 24$), low-grade lesion ($n = 36$), high-grade lesion ($n = 40$), and cancer ($n = 28$) (D. Pyeon, J. A. den Boona, M. Horswill, Z. Wang, S. Hewitt, M. Schiffman, M. Sherman, R. Zuna, J. Walker, S. S. Wang, M. A. Newton, P. F. Lambert, N. Wentzensen, and P. Ahlquist, unpublished data). (A and B) Normalized fluorescence intensities ($\log_2$) of hA3A and hA3B expression from each group are shown in box-and-whisker plot. P values were calculated by the Student t test. (C) Gene expression data for hA3A, hA3B, hA3C, hA3D, hA3F, and hA3G determined as described above for panel A. Averages of normalized fluorescence intensities ($\log_2$) from each group are shown. (D) Expression levels of both hA3A (x axis) and hA3B (y axis) from each patient. The correlation coefficient (R^2) was determined by linear regression. The significance of a correlation coefficient (P value) was calculated by using VassarStats software (<http://www.vassarstats.net/>).

In vivo analysis of mouse papillomavirus infection. C57BL/6J (B6) wild-type mice were purchased from the Jackson Laboratory. Athymic nude (Ncr-nu/nu) mice were obtained from the National Cancer Institute (NCI). C57BL/6J *Apobec3* knockout (B6 *Apobec3*^{-/-}) mice were generated previously (50). This animal study has been approved by the Institutional Animal Care and Use Committee at the University of Colorado [approval number 93913(12)1E]. *Mus musculus* papillomavirus 1 (MmuPV1) infections were carried out as follows. The tails of wild-type B6, B6 *Apobec3*^{-/-} (12 months old), and nude (2 months old) mice were gently scarified and infected with 9.8×10^{10} viral genome equivalents (vge) of MmuPV1. Mice were sacrificed at 12 weeks postinfection, and ~25-mg tail tissue sections were cut and stored at -80°C until analyses were performed. DNA extractions were performed by using a DNeasy blood and tissue kit (Qiagen) according to the manufacturer's protocol. For RNA isolation, tail sections were resuspended in 800 μl of lysis solution (Zymogen) with 0.5-mm silica beads (BioSpec), and tissues were homogenized via a FastPrep FP120 instrument (Thermo Savant). RNA isolation was continued by using an RNA isolation kit (Zymogen) according to the manufacturer's protocol. PCR and RT-qPCR were performed as described above, using primers detailed in Table 2.

RESULTS

hA3A and hA3B are upregulated during HPV-associated cancer progression. Previous studies showed that in HPV-positive cervical lesion and cancers, HPV DNA and host genomes contain hA3A- and hA3B-associated mutation signatures (24, 26). However, no study has shown if expression levels of hA3A and hA3B in HPV-positive cervical lesions and cancers are upregulated in comparison with normal tissues. In addition, it was

unclear if high-level expression of hA3B appears during the early or late stages of cervical cancer progression (51). To determine the molecular signatures and key pathways involved in HPV-associated cancer progression, we previously performed a global gene expression analysis of cervical tissue samples in various disease stages, including normal tissue, low-grade and high-grade intraepithelial lesions, and invasive cancer (D. Pyeon, J. A. den Boona, M. Horswill, Z. Wang, S. Hewitt, M. Schiffman, M. Sherman, R. Zuna, J. Walker, S. S. Wang, M. A. Newton, P. F. Lambert, N. Wentzensen, and P. Ahlquist, unpublished data). This study was performed by using 128 fresh-frozen cervical tissue samples under the Study To Understand Cervical Cancer Early Endpoints and Determinants (SUCCEED), as previously described (52). To determine gene expression patterns of APOBEC family members during cervical cancer progression, we analyzed this gene expression data set and found significantly higher hA3A and hA3B mRNA expression levels in low- and high-grade lesions than in normal tissue (Fig. 1A and B). In contrast, there was little or no change in expression levels of hA3C, hA3D, hA3F, and hA3G (Fig. 1C) as well as APOBEC1, -2, and -4 (data not shown). These data indicate that hA3A and hA3B expression levels are increased from the early stages of HPV-associated cervical neoplasia, a necessary prerequisite to cervical cancer. Furthermore, hA3A expression levels were significantly correlated with hA3B expression levels in the entire patient cohort, suggesting that hA3A and hA3B are coordinately regulated during HPV-associated cancer progression (Fig. 1D).

FIG 2 Increased hA3A and hA3B expression levels in HPV16-positive cells are dependent on the E7 oncoprotein. Total RNA was extracted from NIKS, NIKS-16 (HPV16-positive), and NIKS-16ΔE7 cells (A and B) and from parental human tonsillar epithelial (HTE) cells, HTE cells with the vector only, and HTE cells expressing HPV16 E6 or E7 (D and E). Relative expression levels of hA3A and hA3B were measured by RT-qPCR and normalized by using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. (C) HPV16 E7 protein was detected by Western blotting using mouse anti-HPV16 E7 (ED17; Santa Cruz), as previously described (79). Data are shown as means $\pm$ standard deviations. *P* values were calculated by the Student *t* test. *, *P* < 0.05; **, *P* < 0.01. (F) Total RNA was extracted from NIKS and HPV31-positive NIKS-31 and CIN612 9E cells. Expression levels of hA3A were measured by RT-qPCR and normalized to the β -actin expression level as an internal control. Data are shown as fold changes $\pm$ standard deviations relative to HPV-negative NIKS cells. *P* values were calculated by the Student *t* test. *, *P* < 0.05; **, *P* < 0.001.

hA3A and hA3B upregulation in HPV-positive cells is dependent on HPV16 E7. To determine whether HPV infection specifically affects hA3A and hA3B expression, we analyzed mRNA levels of hA3A and hA3B in HPV-positive and HPV-negative cells by RT-qPCR. We utilized *in vitro* cell culture models of normal immortalized skin keratinocytes (NIKS cells) (35) and immortalized human tonsillar epithelial (HTE) cells (39). Our experiments showed a significant upregulation of hA3A and hA3B expression in NIKS cells containing established HPV16 genomes (NIKS-16 cells) compared to HPV-negative NIKS cells (Fig. 2A and B). Interestingly, an upregulation of hA3A and hA3B was not observed in NIKS-16ΔE7 cells, which contain an E7-deficient HPV16 genome created by inserting a translational termination linker (TTL) in the E7 gene in the context of the full HPV16 genome (36) (Fig. 2A to C). In addition, the expression levels of both hA3A and hA3B were increased dramatically in HTE cells expressing the HPV16 oncoprotein E7, while the hA3B expression level was also increased in HTE cells expressing the HPV16 oncoprotein E6 (Fig. 2D and E). Further analysis of the high-risk HPV31-positive cell lines NIKS-31 and CIN612 9E, a cell line derived from an HPV31-positive cervical intraepithelial neoplasia lesion, also revealed a significant upregulation of A3A mRNA (Fig. 2F). Collectively, these results indicate that HPV16 and at least one other high-risk HPV genotype, HPV31, induce the expression of hA3A and hA3B in keratinocytes through a mechanism involving HPV oncoproteins.

hA3A, but not hA3B or hA3C, restricts HPV16 infection. To investigate interactions between host factors and HPV genomes, we generated and encapsidated HPV16 genomic DNA containing either renilla luciferase (HPV16RL) or GFP (HPV16GFP) reporter genes driven by the SV40 early promoter. To minimize the effect of the viral enhancers, the reporter genes were inserted into the region of the L2 and 5' L1 genes of the HPV16 W12 genome (bp 4296 to 6300) (33) in the inverse orientation of the direction of viral gene expression. Insertion of the reporter gene did not alter the rest of the viral genome, including the early genes and viral regulatory regions. The sizes of the pHPV16RL and pHPV16GFP recombinant viral DNAs, 7,349 bp and 7,157 bp, respectively, were within the packaging limit of the viral capsids (~8 kb) (Fig. 3A). These modified HPV DNAs were encapsidated into virions by using the 293FT transfection method as described previously, yielding HPV16RL and HPV16GFP virions (33). 293FT cells infected with the HPV16GFP virions expressed amounts of HPV16 E1/E4 mRNA transcripts, a readout for initial steps in HPV infection, comparable to those observed with virions containing intact HPV16 DNA genomes (Fig. 3B). Thus, these modified viruses provide a useful tool in the investigation of interactions between viral DNA and host factors during early steps in infection, as they contain functional viral genomes as well as useful reporter genes.

We hypothesized that hA3 proteins may restrict HPV infection by editing viral genomic DNA, thereby inhibiting virus infection. To determine whether hA3A, hA3B, and/or hA3C affects HPV

FIG 3 hA3A, but not hA3B or hA3C, significantly restricts HPV16 infection. (A) Map of the HPV16 genome containing the renilla luciferase (RL) reporter or GFP driven by the SV40 promoter. The cloning sites are indicated on the HPV16 genome locations. (B) 293FT cells were infected with chimeric HPV16GFP virions. At 48 h postinfection, HPV16 early gene expression was determined by amplification of HPV16 E1/E4 mRNA by RT-PCR (33). (C) Expression of HA-hA3A (~23 kDa) and FLAG-tagged hA3B (~46 kDa) and hA3C (~23 kDa) in transfected 293FT cells was detected by specific antibodies, as described in Materials and Methods. 293FT cells were cotransfected with each target HPV16 genome containing GFP (HPV16GFP) or RL (HPV16RL), the HPV16 L1 and L2 expression plasmid (p16ShE1L), and either the hA3A, hA3B, hA3C, or empty expression plasmid. 293FT or HaCaT cells were infected with the purified HPV16GFP (D) or HPV16RL (E and F) virions. (D) At 48 h postinfection, GFP expression was analyzed by fluorescence microscopy. Representative of green fluorescence (top) and bright-field (bottom) images are shown. (E and F) To determine infectivity of HPV16RL, RL activity in the lysates of infected HaCaT keratinocytes (E) and 293FT cells (F) was measured by using a renilla luciferase assay (Promega). Data are shown as means $\pm$ standard deviations. *P* values were calculated by the Student *t* test. *, *P* < 0.01; **, *P* < 0.001. (G) NIKS cells were treated with 100 U/ml of recombinant IFN- β for 24 h. Expression levels of hA3A were measured by RT-qPCR and normalized to the GAPDH expression level as an internal control. Data are shown as fold changes $\pm$ standard deviations relative to PBS-treated cells. *P* values were calculated by the Student *t* test. *, *P* < 0.001.

infection, we prepared HPV16RL and HPV16GFP virions, as described above, in the presence or absence of cotransfected hA3A, hA3B, or hA3C expression plasmids (20). Expression of tagged hA3 proteins was measured by Western blotting using anti-HA (hA3A) and anti-FLAG (hA3B and hA3C) antibodies, respectively. Given the extreme sensitivity of the HA antibody, the blots were imaged separately (short exposure for HA) and are presented in separate panels (Fig. 3C). The infectivity of HPV16GFP virions assembled during hA3A, hA3B, and hA3C overexpression were assayed in 293FT cells by inoculation with ~500 viral genome equivalents (vge)/cell and monitoring GFP expression by fluorescence microscopy at 48 h postinfection. Our data demonstrated that hA3A, but not hA3B or hA3C, inhibited the infection of HPV16GFP virions relative to the empty-vector control (Fig. 3D). Furthermore, hA3A restricted the infectivity of HPV16RL virions in HaCaT human keratinocytes as well as in 293FT cells, as determined by renilla luciferase activity (Fig. 3E and F).

We previously reported that HPV infection in keratinocytes is inhibited by pretreatment with IFN- β (53). To test whether hA3A expression is induced by IFN- β stimulation in keratinocytes, we treated NIKS cells with 100 U/ml of IFN- β for 24 h and harvested total RNA for RT-qPCR analysis. We observed a significant up-regulation of hA3A in HPV-negative keratinocytes (Fig. 3G), consistent with data from a previous study utilizing HPV16-positive cell lines (54). These results indicate that the interferon-inducible protein hA3A, but not hA3B or hA3C, inhibits the production of infectious HPV16 virions.

hA3A restricts infection of HPV16 virions containing viral genomes more efficiently than pseudovirions containing nonviral DNA. To determine whether the inhibition of HPV infection is caused directly by hA3A, we titrated the hA3A expression plasmid transfected during HPV16RL virion assembly in 293FT cells. There was a clear correlation between the amount of the hA3A expression plasmid transfected and the efficiency of infectivity of

FIG 4 hA3A restricts infection of HPV16 virions containing viral genomes more efficiently than pseudovirions containing nonviral DNA. (A) HPV16RL virions were produced by cotransfecting 293FT cells with HPV16RL DNA, p16SheLL, and different concentrations of the hA3A expression plasmid. RLU, relative light units. (B) hvp16-phRL pseudovirions were also prepared by encapsidating a nonviral reporter plasmid, phRL-SV40p. 293FT cells were infected with purified virions, and HPV infectivity was determined at 48 h postinfection. Data are shown as means $\pm$ standard deviations. *P* values were calculated by the Student *t* test. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.0001.

the produced HPV16RL virions (Fig. 4A). A previous study showed that hA3A eliminated transfected plasmid DNA from human cells in a deaminase-dependent manner (55). Thus, we tested whether hA3A restriction of HPV infection is specific to HPV genomes or rather a consequence of hA3A-mediated restriction on any foreign DNA delivered by HPV pseudovirions. We generated HPV16 pseudovirions (hvp16-phRL) containing a nonviral reporter plasmid, phRL-SV40p, the parental plasmid used to generate HPV16RL. hvp16-phRL pseudovirions were produced with various amounts of the hA3A expression plasmid, as described above, and 293FT cells were infected with the HPV pseudovirions to assess virus infectivity. Our data showed that while hA3A significantly restricted infection of hvp16-phRL pseudovirions, the degree of hA3A restriction was far less dramatic than that for HPV16 virions containing viral genomes (Fig. 4). While HPV16RL infection was almost completely blocked, showing a >1,000-fold reduction at the largest amount of hA3A expression plasmid transfected (Fig. 4A), hvp16-phRL infection was only modestly inhibited (<10-fold) under the same conditions (Fig. 4B). These results suggest that restriction of HPV16 infection by hA3A is not due solely to its foreign plasmid DNA restriction properties.

Cytidine deaminase activity of hA3A is required for restriction of HPV16 infection. hA3A cytidine deaminase has a canonical deaminase domain (H-x-E-x24-28-P-C-x-x-C) in which the glutamate has a role in proton transfer and the histidine and two cysteines coordinate a zinc cofactor (20, 21). To investigate the mechanism by which hA3A restricts HPV infection, we tested the role of cytidine deaminase activity using a mutant, hA3A/E72Q, in which the catalytic glutamate is replaced with a glutamine (20). HPV16RL and hvp16-phRL virions were produced in 293FT cells cotransfected with the hA3A/E72Q expression plasmid, as described above. hA3A/E72Q expression did not affect the infectivity of either HPV16RL or hvp16-phRL pseudovirions, whereas wild-type hA3A significantly reduced infectivity (Fig. 5A and B). Protein expression of wild-type and mutant hA3A in 293FT packaging cells was confirmed by Western blotting with an anti-hA3G antibody that is cross-reactive to hA3A (46) (Fig. 5C). These results suggest that cytidine deaminase activity is critical for hA3A-mediated restriction of HPV infection.

hA3A does not degrade or affect the encapsidation of reporter or wild-type HPV16 genomes. Previous studies have shown that transfected DNA is efficiently edited by hA3A and eventually degraded (55). Degradation of transfected viral DNA in packaging cells may lead to inefficient DNA encapsidation into virions and a reduction in infectivity. To determine whether hA3A expression in 293FT packaging cells interferes with the encapsidation of these DNAs, we extracted encapsidated DNAs from HPV16RL and phRL virions purified by using Optiprep gradient centrifugation, as previously described (33). We found no observable difference in the levels of target DNA encapsidated in the presence of hA3A or the vector control in both HPV16RL and hvp16-phRL (Fig. 6A). In sharp contrast, HPV16RL infectivity was dramatically inhibited in a parallel infection assay (Fig. 6B). To verify that the extracted DNA from HPV16RL virions corresponded to the target DNA containing HPV early genes, the E7 region of the HPV16 genome was amplified by using conventional PCR, as previously reported (45). DNAs extracted from normal human keratinocytes, NIKS (HPV negative) and NIKS-16 (HPV16 positive), were used as negative and positive controls, respectively. HPV16 E7 was detected from DNA extracted from HPV16RL virions assembled with both hA3A and the vector control, while no E7 amplicon was produced from DNA extracted from hvp16-phRL pseudovirions, as expected (Fig. 6C).

The SV40 promoter, driving RL expression in HPV16RL and hvp16-phRL, is highly TC rich and thus may serve as a substrate for hA3A deamination. Although there is no difference in the levels of DNA encapsidation (Fig. 6A), it is still possible that mutation of the SV40 promoter region by hA3A may diminish RL and GFP reporter activities. To test whether reporter activity is diminished by hA3A in packaging cells, we cotransfected 293FT cells with hA3A and plasmid pHPV16RL or phRL-SV40p and measured RL activity at the indicated time points (Fig. 6D and E). Both pHPV16RL and phRL-SV40p showed a reduction in RL activity when cotransfected with hA3A; however, as with the viral infections (Fig. 4), the magnitude of the reduced RL expression was much greater when HPV16 DNA was present (Fig. 6D). These results suggest that restriction by hA3A is specific to HPV16 genomic DNA recognition. To determine whether hA3A sup-

FIG 5 The catalytic site of hA3A is required for HPV restriction. HPV16RL virions and hpv16-phRL pseudovirions were produced in the presence of wild-type (WT) hA3A and the hA3A/E72Q mutant. (A and B) 293FT cells were infected with HPV16 virions, and a renilla luciferase assay was performed at 48 h postinfection to determine the infectivity of HPV16RL virions (A) and hpv16-phRL pseudovirions (B), as described above. (C) Wild-type and mutant hA3A protein expression in transfected packaging cells was analyzed by immunoblotting using an anti-hA3G antibody that is cross-reactive with hA3A (46). Data are shown as means $\pm$ standard deviations. *P* values were calculated by the Student *t* test. *, *P* < 0.0001.

presses viral gene expression controlled by the HPV16 native promoter, we measured levels of the HPV16 early transcripts in cotransfected cells by RT-qPCR. Our results showed a considerable decrease in viral gene expression ($\sim$ 18-fold at 96 h) mediated by hA3A (Fig. 6F).

To test whether infection of HPV16 containing only viral genomes was also similarly inhibited by hA3A, we assembled HPV16 virions containing a 5.0-kb pseudogenome (HPV16 genome without late genes) in cells cotransfected with the hA3A expression plasmid or the cognate, parent expression vector. First, we measured encapsidated DNA content by qPCR. We did not detect any significant changes in the levels of encapsidated viral DNA due to hA3A expression in three independent virus preparations (Fig. 6G). To determine the infectivity of these HPV16-5.0kb virions, we assessed HPV16 early gene expression from infected 293FT cells ($\sim$ 250 vge/cell) by RT-qPCR. Strikingly, we observed a >20 -fold decrease in viral early gene expression levels when the viral genome was encapsidated in the presence of hA3A expression (Fig. 6H). Taken together, these results indicate that the expression of hA3A during HPV16 assembly does not cause reporter inhibition, degradation of viral genomes, or interference with viral DNA encapsidation.

hA3A overexpression does not change A3A-type mutation signatures in HPV16 genomes. The catalytic hA3A/E72Q mutant did not affect RL expression (Fig. 6D and E) and elicited only moderate effects on early gene expression (Fig. 6F). Consistent with the results shown in Fig. 5, these results demonstrate that hA3A restriction of HPV16 requires a functional deaminase domain. hA3 family proteins are potent cytidine deaminases that cause C-to-T mutations. This function of hA3 is a known effective antiviral host defense mechanism (17, 18). Thus, we further explored sequence modifications of the HPV genome using a recently established, sensitive, and unbiased next-generation sequencing approach (47). Based on a previous report of HPV DNA mutations by hA3s (24, 54), the HPV16 LCR (positions 7758 to 296) and E2 region (position 3098 to 3536) were amplified from

purified virions produced with the expression of hA3A or the vector by using barcoded MiSeq primers. The percentage of hA3A-type mutation signatures was calculated by using a novel hA3A mutational signature detection algorithm (47). Despite the sensitivity of this approach, we were unable to detect any evidence of mutational signatures above the background in both the HPV16 LCR and E2 region (Fig. 7).

Knockdown of hA3A enhances HPV16 infection in cervical keratinocytes. To determine the function of endogenous hA3A in HPV infection and replication, we knocked down hA3A using lentiviral delivery of shRNAs against hA3A (shRNA-hA3A). We utilized two cervical keratinocyte cell lines that express low or high levels of hA3A (Fig. 8A), compared to normal keratinocytes: W12E cells contain the episomal HPV16 genome, whereas W12I cells contain integrated HPV16 genomes. Because W12I cells express higher levels of HPV16 E7 protein (56), this result suggests that HPV16 E7 might induce hA3A expression, consistent with data shown in Fig. 2. Following lentiviral transduction and puromycin selection for 2 to 3 weeks, total RNA was extracted, and RT-qPCR was performed to quantify hA3A mRNA expression levels in W12E and W12I keratinocytes. The results showed that the hA3A mRNA expression levels in cervical keratinocytes were reduced by $>80\%$ with shRNA-hA3A compared to the scrambled control shRNA (Fig. 8B).

We first analyzed copy numbers of HPV genomes and found that there was no statistically significant change in HPV genome copy numbers by knockdown of hA3A expression in W12E and W12I cells (data not shown). To determine whether knockdown of endogenous hA3A affects HPV infectivity, we infected W12E and W12I cells with HPV16RL or hpv16-phRL virions, and infectivity was measured by using a renilla luciferase assay. Interestingly, hA3A knockdown significantly enhanced HPV infectivity by 30 to 50% (Fig. 8C and D). While the effect was modest, the increased HPV infectivity due to hA3A knockdown was consistent and statistically significant in at least three independent experiments. These results suggest that

FIG 6 hA3A does not degrade or affect the encapsidation of reporter or wild-type HPV16 genomes. (A) Encapsidated DNA was extracted from HPV16RL and hpv16-phRL virions assembled in the presence of the hA3A expression plasmid or vector by using a PCR purification kit (Qiagen) and analyzed on an agarose gel with ethidium bromide (33). (B) In parallel, infectivity assays were performed with the same sets of virions described above. Data are shown as means $\pm$ standard deviations. *P* values were calculated by the Student *t* test. *, *P* < 0.0001. (C) The encapsidated DNA was verified by amplification of the HPV16 E7 region using conventional PCR with specific primers (44). NIKS (HPV-negative) and NIKS-16 (HPV16-positive) keratinocytes were used as negative and positive controls, respectively. (D and E) pHPV16RL (D) and phRL-SV40p (E) were transfected into 293FT cells with the vector or hA3A or hA3A/E72Q expression plasmids. RL activity in the transfected 293FT cell lysates was measured at the indicated time points by using an RL assay (Promega). Data are shown as means $\pm$ standard deviations of results from quadruplicate samples. (F) pHPV16RL was cotransfected with the vector or hA3A or hA3A/E72Q expression plasmids as described above for panel D. The HPV16 early gene transcript was measured in transfected-cell lysates at the indicated time points by RT-qPCR and normalized to β -actin expression levels. Data are shown as relative expression levels of the HPV16 early gene transcript $\pm$ standard deviations. (G) A 1:10,000 dilution of clarified virus prep was used as the input for encapsidated DNA detection by qPCR, as described in Materials and Methods. The copy number was determined by using serially diluted plasmid standards. Data are shown as the mean genome copy numbers/ μ l, with error bars representing the standard errors of the means from three independent virus production schemes. (H) The infectivity of viruses described above for panel G was measured by quantifying the expression of the HPV16 early gene transcript in infected 293FT cell lysates ($\sim$ 250 vge/cell) at 48 h postinfection by using RT-qPCR, as described above. Data are shown as fold changes relative to the vector-only control from three independent experiments. *P* values were calculated by the Student *t* test. *, *P* < 0.01.

endogenous hA3A could interfere with HPV infection in human keratinocytes.

Mouse Apobec3 knockout is not sufficient for tumor formation by mouse papillomavirus infection in immunocompetent mice. As described above, hA3A expression during the assembly

of HPV particles significantly abrogates virus infectivity *in vitro*. In an initial attempt to extend these findings *in vivo*, we used the recently described *Mus musculus* papillomavirus 1 (MmuPV1) (57–59) infection model. As with HPV-positive human keratinocytes, mouse tonsillar epithelial (MTE) cells expressing HPV16 E6

FIG 7 hA3A overexpression does not change A3A-type mutation signatures in HPV16 genomes. The HPV16 LCR (positions 7758 to 296) and the E2 region (positions 3098 to 3536) were amplified from purified virions produced with the overexpression of A3A or the vector by using barcoded MiSeq primers. The PCR products were gel purified and used as the input for PCR amplicon sequencing (Illumina MiSeq). The percentage of A3A-type mutation signatures was calculated by dividing the total number of A3A-specific dinucleotide (dark gray) or trinucleotide (light gray) motif transitions (Y represents any pyrimidine) by the total number of C>T and G>A nucleotide changes. The HPV16 W12 genome sequence was used as a reference for sequence alignment. Data are shown as percent means $\pm$ standard deviations.

and E7 (60) have significantly increased mouse Apobec3 (mA3) expression levels compared to HPV-negative MTE cells (Fig. 9A and B). To test whether endogenous mA3 expression affects MmuPV1 infection, the tails of three wild-type C57BL/6J (B6) and three B6 Apobec3 knockout (B6 *Apobec3*^{-/-}) mice were inoculated with MmuPV1, and tumor formation was monitored for 12 weeks. Three athymic Ncr-nu/nu (nude) mice, which are susceptible to MmuPV1 infection, were used as positive controls (59). While all of the nude mice infected with MmuPV1 showed clearly visible papillomas by 6 weeks, none of wild-type B6 and B6 *Apobec3*^{-/-} mice developed papillomas (data not shown). Next, we harvested tissue samples at 12 weeks postinfection to analyze persistent viral DNA replication and RNA expression. As expected, nude mice infected with MmuPV1 showed high levels of MmuPV1 DNA and mRNA in infected tail tissue (Fig. 9C and D). However, one of three B6 and none of the B6 *Apobec3*^{-/-} mice showed detectable levels of MmuPV1 DNA (Fig. 9C). Additionally, MmuPV1 early gene expression in B6 and B6 *Apobec3*^{-/-} mice was below the detection limit by RT-qPCR (Fig. 9D). While MmuPV1 DNA was detected in one B6 mouse, these viral DNAs were likely in the process of clearance. These results suggest that mA3 deficiency alone is not sufficient to overcome host innate and adaptive immune responses against MmuPV1 infection in immunocompetent mice.

FIG 8 HPV infectivity is increased in keratinocytes with knockdown of hA3A expression. (A) Total RNA was extracted from NIKS, W12E (episomal HPV16), and W12I (integrated HPV16) cells. Expression levels of hA3A were measured by RT-qPCR and normalized to the GAPDH expression level as an internal control. Data are shown as fold changes $\pm$ standard deviations relative to values for HPV-negative NIKS cells. W12E (episomal HPV16-positive), and W12I (integrated HPV16-positive) cervical keratinocytes were transduced with lentiviruses containing shRNA-hA3A (TRCN0000049960; Sigma) or a scrambled shRNA control. Transduced cells were selected by puromycin for 2 to 3 weeks. (B) Relative expression levels of hA3A were measured by RT-qPCR using TaqMan probes and normalized to the GAPDH level as an internal control. (C and D) Established W12E and W12I cell lines expressing hA3A-shRNA were infected with HPV16RL or hvp16-phRL virions. At 48 h postinfection, infectivity was determined by using a renilla luciferase assay and normalized by cell viability. Shown are the percent infectivities of HPV16 in W12E (C) and W12I (D) keratinocytes relative to cells with the scrambled shRNA control. Data are shown as means $\pm$ standard deviations. *P* values were calculated by the Student *t* test. *, *P* < 0.05; **, *P* < 0.001.

FIG 9 Apobec3 knockout is not sufficient for tumor formation by mouse papillomavirus infection in immunocompetent mice. (A and B) Total RNA was extracted from HPV16 E6/E7-positive mouse tonsillar epithelial cells (MTE E6/E7) and HPV-negative MTE cells. (A) mA3 expression was measured in cell lysates by RT-qPCR and normalized to β -actin expression levels. Data are shown as fold changes $\pm$ standard deviations for mA3 expression relative to the expression level in HPV-negative MTE cells. *P* values were calculated by the Student *t* test. (B) HPV16 E1/E4 mRNA expression in MTE cells was measured as described above. Data are shown as mean E1/E4 mRNA expression levels $\pm$ standard deviations. (C) Total DNA was harvested from $\sim$ 25-mg tissue sections by using a DNeasy blood and tissue kit (Qiagen). MmuPV1 E2, E6, and E7 were amplified by PCR from B6 wild-type, B6 *Apobec3*^{-/-} (triplicates), and nude (single representative) mice. The MmuPV1 plasmid was used as a positive control. Data are representative of results from two PCR amplifications of two tail tissue sections. (D) Total RNA was extracted from MmuPV1 tail tissue as described in Materials and Methods. The copy number of MmuPV1 E7 early gene mRNA was determined as described in the legend of Fig. 6.

DISCUSSION

HPV is one of the most prevalent sexually transmitted pathogens worldwide. Current studies have shown that the overall prevalence of HPV among sexually active men and women is $\sim$ 50% (61, 62). However, only a small subset of these infections result in persistent infections, and even a smaller percentage of persistently infected individuals develop cancer. A mechanistic understanding of how most infections fail to progress to disease while others do could have major implications in developing novel anti-HPV treatment strategies. As with many viral pathogens, innate host defense mechanisms likely play an important role in counteracting HPV infection, but only a few examples have been documented (40, 43). In this study, we focused on the role of the APOBEC3 cytidine deaminases in restricting HPV infection.

In 2008, Vartanian and colleagues revealed a potential role for hA3 in restricting HPV infection when they showed that HPV1a and HPV16 were hyperedited in skin warts and precancerous cervical lesions, respectively (24). To link this observation to the hA3 proteins, those authors showed that cotransfection of HPV DNA with hA3A, hA3C, and hA3H expression plasmids resulted in substantial hypermutation (24). However, a direct impact of the hA3 genes on HPV infection has not been assessed. The ratio of infectious to noninfectious HPV virions is relatively low (43), and the

procedures that those authors used to quantify editing frequencies were biased to yield AT-rich/low-denaturation amplicons through a method known as differential DNA denaturation (3D)-PCR (63). Thus, it is quite possible that the true editing frequencies in HPV are not significantly impacting HPV infection. In addition, the hA3-dependent editing observed may just be the consequence of foreign DNA restriction, as various hA3A proteins were reported to deaminate transfected plasmid DNA (55).

We previously developed a method to produce infectious HPV virions that encapsidate full-length HPV DNA (33). Using this method, and under careful consideration of potential effects of hA3s on plasmid DNA, we provide the first evidence that hA3A can directly inhibit HPV infection. Similar to the impact of various hA3 proteins on retroviruses, the impact of hA3A on HPV was observed primarily in the next round of infection. Mutation of hA3A residue E72Q, which is essential for catalytic activity, led to a loss of antiviral activity against HPV. However, even using a highly sensitive next-generation sequencing approach (47), we were unable to detect any significant levels of hA3A mutational signatures within sites previously identified to be edited by A3s (24, 54) (Fig. 7). Further analysis of whole-genome sequence modifications may identify potential modified sites that may disrupt infection.

The ability of hA3A to restrict HPV is likely deaminase dependent, since catalytically inactive hA3A could not restrict HPV. These results mirror data from previous studies suggesting a deaminase-dependent mechanism for hA3A inhibition of retrotransposons, AAV-2, and parvoviruses (20–22, 64, 65). Importantly, using an shRNA knockdown approach, we document that endogenous hA3A can significantly inhibit HPV in keratinocytes. While the impact of endogenous hA3A on HPV infection was relatively modest, our knockdown approach was only partially efficient. Nevertheless, a 30 to 50% effect on HPV infectivity could still have a substantial impact after multiple rounds of HPV infections. While mA3 deficiency was not sufficient to overcome MmuPV1 restriction in immunocompetent mice (Fig. 9C and D), it is possible that inhibition of papillomaviruses is an APOBEC3 function that developed late during vertebrate evolution. In fact, mA3 was also found to have no impact on murine gammaherpesvirus 68, another DNA virus (66). We speculate that other type I interferon-responsive proteins may further augment the antiviral effect in early MmuPV1 infection in mice (53, 67, 68).

In contrast to hA3A, both hA3B and hA3C did not exert a substantial effect on HPV infection. These results could be due to differences in nucleic acid substrate specificity (69, 70) and cellular localization (71, 72) between the different hA3 family members. hA3A is unique among the primate APOBEC3A gene family since it appears to have lost its restriction property against lentiviruses but gained activity against DNA viruses (22). In fact, it appears that this property is shared among hominid A3As due to the 3-amino-acid deletion in active center loop 1 of the enzyme (73). Further studies are warranted to investigate the specific stage(s) of the HPV life cycle that hA3A restricts and which domains of hA3A are critical for restricting HPV. Additionally, despite the lack of transcriptional upregulation of other hA3 family members during virus persistence (Fig. 1C), our data do not fully rule out their contribution to HPV restriction through alterations in their levels/activities at posttranscriptional levels.

Showers of hypermutation, or “kataegis,” in multiple cancers have raised the intriguing possibility that hA3A and hA3B may be involved in cancer development (25–27, 29). In part due to mutational profiles, the Harris laboratory suggested that hA3B was primarily involved, and in fact, those researchers suggested that hA3B might be responsible for up to half of all mutations in breast cancer (25). In addition, frequent mutations that can be ascribed to hA3B and hA3A (5'-TC context) were found in host DNA sequences of other cancers, including bladder, cervix, lung, and head-and-neck cancers (25). Kataegis were frequently found near chromosome rearrangement breakpoints (27), and hA3A can induce DNA breaks and activate DNA damage responses (30, 74, 75). Thus, hA3A may also contribute to cancer development due to its potential genotoxicity. However, the notion that hA3A is genotoxic is challenged by a recent report that endogenous hA3A is primarily cytoplasmic (71). Interestingly, large deletions of the hA3 gene cluster that include the hA3A and hA3B regions were associated with increased breast cancer risk (76, 77). These findings appear to counter the “APOBEC mutator hypothesis,” but on closer inspection, these genomic alterations did not remove the coding region of hA3A and in fact led to higher genome APOBEC3-type mutation rates (78). It would be of interest to determine how hA3-hA3B genomic alterations in human populations affected endogenous hA3A expression and enzymatic activity.

Our global gene expression analyses revealed that hA3A and

FIG 10 Potential link between virus restriction and cancer mutagenesis during HPV-associated cancer progression. Our working model suggests that hA3A and hA3B are induced to restrict persistent HPV infection. However, during periods of virus persistence and enhanced E7 expression, likely upon integration, hA3A and hA3B expression is further promoted. This specific increase in hA3A and hA3B expression levels may predispose genomic DNA to A3-mediated hypermutations. The accumulation of A3-dependent somatic mutations during decade-long disease may be a likely contributor to cellular transformation.

hA3B transcription levels were significantly upregulated in early-stage, low-grade cervical lesions and further increased in high-grade lesions. Interestingly, hA3A and hA3B expressions were coordinately regulated during cancer progression, suggesting that similar signaling mechanisms may be inducing these factors. In fact, we provide evidence that the coordinate induction of hA3A and hA3B appears to be dependent on the HPV E7 oncoprotein. Notably, hA3-related signatures are the only significant mutation signatures in cervical cancer, while most other cancers show variable arrays of mutation signatures (19). Taken together, these findings suggest that pathways that result in the induction of an intrinsic host defense mechanism against HPV (hA3A) may also be responsible for the long-term accumulation of genomic mutations (hA3B and possibly hA3A) that contribute to HPV-associated cancers (Fig. 10). Further studies would be required to test this intriguing link between hA3A-mediated intrinsic immunity and the APOBEC mutator hypothesis in the context of HPV infection.

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