Development of a novel mouse model of spontaneous high-risk HPVE6/E7-expressing carcinoma in the cervicovaginal tract

Talia R Henkle¹, Brandon Lam¹, Yu Jui Kung¹, John Lin¹, Ssu-Hsueh Tseng¹, Louise Ferrall¹, Deyin Xing¹,²,³, Chien-Fu Hung¹,²,³, T.-C. Wu¹,²,³,⁴

Department of Pathology¹, Department of Oncology², Department of Obstetrics and Gynecology³, Department of Molecular Microbiology & Immunology⁴ The Johns Hopkins Medical Institutions, Baltimore, MD 21287 USA.

Running Title: HPV-16 E6/E7-expressing spontaneous carcinoma

Keywords: Human papillomavirus, pre-clinical model, cervical cancer, HSIL, HPV16

*Contact information for corresponding authors:

Dr. Chien-Fu Hung
Departments of Pathology and Oncology,
The Johns Hopkins Medical Institutions
CRB II Room 307
1550 Orleans St
Baltimore, MD, 21231, USA
Email: chung2@jhmi.edu
Phone: 410-502-8215
Fax: 442-287-4295

Dr. T.-C. Wu
Departments of Pathology, Oncology, Obstetrics and Gynecology and Molecular Microbiology and Immunology,
The Johns Hopkins Medical Institutions
CRB II Room 309
1550 Orleans St
Baltimore, MD, 21231, USA
Email: wutc@jhmi.edu
Phone: 410-614-3899
Fax: 442-287-4295

Conflict of Interest Disclosure: Dr. Wu is a co-founders of and has an equity ownership interest in Papivax LLC. Also, Dr. Wu owns Papivax Biotech Inc. stock options and is a member of Papivax Biotech Inc.’s Scientific Advisory Board.

Acknowledgements: This work was supported the National Institute of Health and the National Cancer Institute under award number R01CA237067 and P50CA098252.
Abstract

Current pre-clinical models for cervical cancer lack important clinical and pathological features. To improve upon these models, we aimed to develop a novel, spontaneous HPV16-expressing carcinoma model that captures major aspects of HPV-associated cancer in the female genital tract. This novel pre-clinical model features 1) expression of HPV oncogenes E6 and E7 in the tumors in female reproductive tract of mice, 2) spontaneous progression through high-grade squamous intraepithelial lesion (HSIL) to carcinoma, and 3) flexibility to model cancers from different high-risk HPV genotypes. This was accomplished by injecting plasmids expressing HPV16 E6/E7-Luciferase, AKT, c-myc, and Sleeping Beauty transposase into the cervicovaginal tract of C57BL/6 mice followed by electroporation. Cell lines derived from these tumors expressed HPV16 E6/E7 oncogenes, formed tumors in immunocompetent mice, and displayed carcinoma morphology. In all, this novel HPV-associated cervicogenital carcinoma model and HPV16E6/E7-expressing tumor cell line improves upon current HPV16-E6/E7-expressing tumor models. These tumor models may serve as important pre-clinical models for the development of therapeutic HPV vaccines or novel therapeutic interventions against HPV E6/E7-expressing tumors.

Statement of Significance: This study describes the development of a clinically relevant mouse model of cervicovaginal carcinoma that progresses from high grade lesions and recapitulates key features of human HPV+ cervical cancer.
Introduction

Human Papillomavirus (HPV) is the etiological factor for cervical cancer (1), with high-risk types such as HPV16 and HPV18 causing the majority of cases (2). Currently, cervical cancer remains the fourth most common type of cancer affecting women worldwide (1). Although the prophylactic vaccines Gardasil® and Cevarix™ have been developed and widely administered, they cannot cure previously established HPV infections. HPV is currently the most common venereal disease. Although usually self-limited, after HPV infection a small fraction of infections will progress to precancerous high-grade squamous intraepithelial lesions (HSIL), of which a subsequent fraction will progress to HPV+ cancer. The HPV oncogenes E6 and E7 play a critical role in inducing tumorigenesis (3,4), and HPV DNA encoding HPV E6 and E7 is found integrated and expressed in the vast majority of cervical cancer cells (5).

Cervical cancer progresses through high-grade squamous intraepithelial lesions (HSIL) to squamous cell carcinoma (SCC) in a stepwise process. There is currently substantial interest in developing improved treatments for both HSIL and cervical cancer. Currently, efficacy of HSIL treatments remains 75-85% (6,7), and the treatments are accompanied by various adverse effects, including increased risk for pre-term birth (8). After HSIL progression to cancer, the 5-year survival rate for cervical cancer patients is 66-79% (9). Novel immunotherapies that target HPV proteins E6 and E7 are particularly promising strategies to improve prognoses of patients with HPV-associated cancers (9). Adequate pre-clinical animal models are necessary to test such therapies, yet the existing pre-clinical models for cervical cancer have drawbacks that limit their utility in accurately evaluating novel therapies. For instance, transgenic mice that express HPV16E6E7 under the K14 epithelial cell promoter develop gynecological malignancies after 6 months when treated chronically with high dose estrogen (10). However, 1) the lengthy time for tumor outgrowth, 2) the asynchronous outgrowth of tumors and HSIL, and 3) the development of central tolerance to HPV16 E6E7 mitigate the usefulness of this model for the evaluation of novel therapies for cervical cancer (11).
Alternatively, HPV-associated malignancies can be studied through Patient Derived Xenograft (PDX) models, which involve challenging immune-compromised (SCID) mice (12) with cell lines from patient-derived cervical cancers. However, PDX models 1) are incapable of evaluating novel cancer treatments that require an intact immune system, 2) grow implanted tumors at irrelevant anatomical sites, and 3) do not model clinical progression through pre-cancerous lesions.

Furthermore, there are multiple immortalized cell lines that express HPV16 E6E7 and induce tumor outgrowth in syngeneic mice (13-17). These existing cervical cancer cell lines exhibit multiple drawbacks including that 1) they are not derived from anatomically relevant tissue, 2) they do not model clinical progression through pre-cancerous lesions, 3) and perhaps most notably, they have failed to predict clinical outcomes of novel treatments in past studies (16).

The limitations of the currently existing pre-clinical models for cervical cancer impede accurate evaluation of novel cancer therapies and necessitate the development of an improved pre-clinical model for cervical cancer that more faithfully mirrors the clinical scenario. We reasoned such a model would 1) locally express HPV E6E7 in the reproductive tract of mice 2) have monitorable tumor progression through HSIL to squamous cell carcinoma 3) be accompanied by a mutation in the PI3K/AKT pathway, as mutations in the PI3K/AKT pathway are the most common somatic mutations found in cervical cancers (18), 4) be amenable for the evaluation of novel treatments for HSIL and cervical cancer, including immunotherapies, 5) and display flexibility in which HPV genotype is used for tumorigenesis as there are multiple high risk HPV types beyond HPV16 (1).

In this study, we developed a novel strategy to induce HPV16-E6E7 expressing (HPV+) cancer in the murine reproductive tract. Since HPV E6E7 is insufficient to induce cervical cancer alone, we hypothesized that if we integrated clinically relevant oncogenes along with HPV E6E7 oncogenes in mouse cervicovaginal tract, we may be able to locally induce HPV+ cancer. Such
a tumor model would likely represent an improved pre-clinical model for cervical cancer. To accomplish this, we utilized the Sleeping Beauty transposase (SB100) to induce local HPV+ tumor outgrowth in the reproductive tract of mice. SB100 integrates DNA flanked by specific inverted repeat sequences into AT dinucleotides in genomic DNA (19,20), reflecting the random integration of HPV DNA seen in cervical cancer. We integrated plasmids that encoded 1) constitutively active Akt (myrAkt) as it is an oncogene in the PI3K/AKT pathway, 2) c-myc, because the upregulation of c-myc has been noted in HPV E6E7 transfected cells as well as cancer patients, where it has been found to be a negative prognostic factor for survival (21-27), and 3) HPV16-E7E6 with a luciferase reporter gene, abbreviated as AMES-16 plasmids. Using this methodology we transfected AMES-16 oncogenes into the reproductive tract of C57BL/6 mice which induced HPV+ tumor outgrowth that progressed through HSIL to invasive squamous cell carcinoma (SCC). Additionally, we isolated and characterized a HPV16+ cancer cell line, Tal3, from an intraperitoneal (IP) metastasis of a tumor induced by the AMES-16 model. The AMES-16 model and Tal3 cell line have potentially important implications in the future of pre-clinical evaluations of treatments for HSIL and cervical cancer.

Materials & Methods

Mice and Animal Care

Six- to eight-week-old female C57BL/6NTac mice were purchased from Taconic (Rensselaer, NY). All mice were maintained at the Johns Hopkins University School of Medicine Animal Facility (Baltimore, MD) under specific pathogen-free conditions. All procedures were performed according to protocols approved by the Johns Hopkins Institutional Animal Care and Use Committee and in accordance with recommendations for the proper use and care of laboratory animals.

Plasmid Vectors
The construction of the Pkt2-Luc-T2a-E7-T2a-E6 plasmid has been described previously (28).

To generate Pkt2-LucHPV18E7E6, HPV18E7E6 was synthesized by GenScript, and the sequence was cloned into the Xhol and BstXI sites of the Pkt2-LucHPV16E7E6, as described previously (28). The construction of the pCMV(CAT)T7-SB100 plasmid has been described previously (29). The plasmid was purchased from Addgene (plasmid #34879). The construction of the pT2/C-Luc//PGK-SB13 plasmid has been described previously (30). The plasmid was purchased from Addgene (plasmid #20207). The construction of the pKT2/CLP-AKT plasmid has been described previously (30). The plasmid was purchased from Addgene (plasmid #20281). To generate Pkt2-cMyc, mouse cMyc was first amplified by PCR using pCAGMKOSiE (from Addgene, # 20865) as a template and the following set of primers: 5' AAATCTAGAGCCACCATGCCCCTCAACGTGAACTT -3' and 5' TTTAGATCTTTATGCACCAGAGTTTCGAAGCT -3'. The PCR product was cloned into the XbaI and Bgl II sites of the Pkt2/clp-akt vector (from Addgene, #20281).

In Vivo Tumor Formation Experiments

To generate cervicovaginal tumors in C57BL/6NTac mice, mice (5 mice/group) received intraperitoneal (IP) injection of monoclonal anti-CD3 antibody (200μg/mouse) for three consecutive days prior to plasmid electroporation. Mice were anesthetized via intramuscular injection of 80μL of a solution of 16.7% ketaset (100mg/mL) 16.7% anased (20mg/mL) and 66.6% PBS. Mice were then injected with plasmids, dubbed AME-16 plasmids, that encoded Luciferase and HPV16-E6/E7 or Luciferase and HPV18-E6/E7, combined with plasmids encoding myrAKT, c-myc, and SB100 (10μg DNA of each plasmid in 20μL total injection volume) in the lower third of their vaginal canal, and plasmids were locally electroporated into the site of injection. Mice then received two subsequent doses of anti-CD3 antibody (200μg/mouse) weekly for two weeks after plasmid electroporation. For comparison, mice (n=5) were injected with DNA plasmids encoding only Luciferase with HPV16-E6E7 combined with
SB100 (10µg DNA of each plasmid) or with plasmids encoding AKT, c-myc, and SB13, which contains a luciferase reporter gene (10µg DNA of each plasmid). Mice were sacrificed when 1) tumor diameter > 15mm, 2) abdomen enlarged due to IP metastasis, or 3) body mass reduced 10% compared to aged-matched mice.

In vivo Electroporation

Mice were electroporated using the ECM 830 Square Wave Electroporation System (BTX). Mice were anesthetized via intramuscular injection of 80µL of a solution of 16.7% ketaset (100mg/mL) 16.7% anased (20mg/mL) and 66.6% PBS. Using 3mm electrode tweezers, mice were electroporated at the site of plasmid injection in the lower third of the vaginal tract. Settings: 72V, 200ms (interval), 20ms (duration), 8 pulses.

In Vivo Bioluminescence Imaging

In vivo bioluminescence imaging was performed using the IVIS Spectrum in vivo imaging system series 2000 (PerkinElmer). Weekly after electroporation, mice were injected IP with 200µL of D-luciferin (Goldbio) substrate. Mice were anesthetized in an isoflurane chamber and bioluminescence imaging for luciferase expression was conducted on a cryogenically cooled IVIS system using Living Image acquisition and analysis software (Xenogen). Images were acquired 10mins after D-luciferin injection. The levels of light from the bioluminescent cells were detected by the IVIS imager, integrated, and digitized. The region of interest from displayed images was quantified as total flux using Living Image 2.50 software (Xenogen).

Histology and Immunohistochemistry Staining

After euthanizing the tumor-bearing mice, the cervicovaginal tumors were surgically removed, isolated, and placed into 10% neutral buffered formalin solution for adequate fixation with a minimum of 48hrs at room temperature. The tumor samples were then...
sent to the Johns Hopkins University Oncology Tissue Services for subsequent procedures including paraffin embedding, tissue sectioning, hematoxylin and eosin (H&E) staining, and immunohistochemical (IHC) staining. The histology slides were reviewed by two board-certified gynecologic pathologists (Dr. T.C. Wu and Dr. Deyin Xing) of the Pathology Department in the Johns Hopkins University School of Medicine.

In Situ hybridization to Detect the Expression of HPV16 Oncogenes

In situ hybridization was performed using the RNAscope® 2.0 HD Brown Chromogenic Reagent Kit (Advanced Cell Diagnostics, Hayward, CA) using the supplied protocol and a target probe against HPV16-E6 (Advanced Cell Diagnostics #450591). In short, fresh-cut, formalin-fixed, paraffin-embedded slides were baked at 60°C for 1 hour. After deparaffinization, slides were air-dried, circled with a hydrophobic barrier pen and then exposed to pretreatment solutions 1, 2, and 3. Target probes were hybridized on slides for 2hrs at 40°C in oven, followed by a series of signal amplification and washing steps. The signals were detected by chromogenic reactions using DAB chromogen followed by counterstaining of hematoxylin (mixed 50/50 with distilled water Sigma-Aldrich, St. Louis, MO).

RT-PCR to Verify HPV16 Oncogene Expression

Cell lines [Tal3, TC-1 (positive control) and HEK293 (negative control)] were cultured in vitro and lysed in TRIzol®. Cellular RNA was isolated using Direct-zol MicroPrep kit (Zymogen) and cDNA was made using iScript Reverse Transcription Supermix for RT-qPCR (Bio Rad) according to the supplied protocols. Gene transcript was amplified using the isolated RNA as a template and HPV16 E6 primers (Forward Primer: ACAAACCGTTGTGTGATTTGTT; Reverse Primer: CAGTGGCTTTTGACAGTTAATACA) with a Touchdown qPCR assay, as described previously (31). Subsequently, 1μL of qPCR product was transferred to a 1% agarose gel with
0.01% ethidium bromide and run at 130V for 45min. Resultant bands were visualized using the ChemiDoc™ Touch Imaging system (Bio Rad).

**T cell Recovery Flow Cytometry**

To evaluate the kinetics of T cell recovery post T cell depletion, peripheral blood was collected via facial vein bleeding into 1.5mL Eppendorf tubes with 100μL of .05M EDTA. RBC lysis buffer (ChemCruz, Dallas, TX) was used to lyse red blood cells and then peripheral blood mononuclear cells (PBMCs) were subsequently washed in MACs buffer. PBMCs were then stained with the antibodies as described in Supplementary Table 1 and characterized by flow cytometry analysis.

**Isolation of Tumor-Infiltrating Lymphocytes**

Tumor-bearing mice were euthanized, and the tumor tissues were surgically removed and placed into C tubes (Miltenyi) containing 3mL of MACS buffer (Miltenyi) supplemented with magnesium and calcium per manufacturer’s recommendation. Tumors were then cut into 1-2mm pieces and digestive enzymes were added (0.05mg/mL collagenase I, 0.05mg/mL collagenase IV, 0.25mg/mL DNase I). The tumor/enzyme mixture was incubated at 37°C with periodic agitation for 20-30min. The tumors were homogenized on the gentleMACS™ dissociator (C tube protocol m_impTumor_1.01) and then filtered through a 70μm nylon filter to remove undigested tissue fragments. Tumor lysate was then pelleted at 500xg for 5min and supernatant was aspirated off. The tumor pellet was resuspended in 10mL DMEM (10% FBS) media and slowly layered on top of 5mL of Ficoll-Paque™ PLUS. The tubes were then centrifuged at 400xg for 30min at room temperature with no acceleration and no brake. Leukocytes localized in cloudy layer at the interface of the Ficoll and media interface were pipetted off. Leukocytes were then washed in PBS and then counted for flow-cytometry analysis. Cells were then split in half and then stained as indicated in Table 1.
Isolation of Lymphocytes from Tumor Draining Lymph Nodes

When tumor-bearing mice were sacrificed to analyze tumor immune infiltrate, the inguinal lymph nodes were also harvested for further analysis. Lymph nodes were suspended in 2mL of MACs buffer and then ground through a 70μm nylon filter to achieve a single cell suspension. Cells were then washed and stained with the tumor infiltrate and tumor exhaustion panels as indicated in Table 1 and 2.

Establishment of Tal3 Cell Line

The Tal3 cell line was derived from an IP metastasis of an AMES-16 plasmid induced tumor. The IP tumor was excised, sliced into small pieces, then filtered through a 70μm Falcon cell strainer (Fischer Scientific, Waltham, MA). Tal3 cells were then cultured in complete DMEM media supplemented with 10% FBS, Falcon, NY Sodium Pyruvate, MEM Non-Essential amino acids solution, L-Glutamine, Penicillin-Streptomycin, and 2-mercaptoethanol (all purchased from ThermoFisher Scientific, Waltham, MA). The cells were then harvested using 0.05% Trypsin-EDTA (ThermoFisher Scientific, Waltham, MA). The polyclonal cell population was then expanded across three cell passages. The tumor cells (1 million cells/mouse) were injected subcutaneously into C57BL/6 mice to induce tumor outgrowth (first in vivo passage). A resultant tumor from the first Tal3 in vivo passage was excised, processed and expanded as before and then challenged once again in new C57BL/6 mice (second in vivo passage). A resultant tumor from the second in vivo passage was excised, processed and expanded as before. The expanded Tal3 tumor cells were diluted into a single cell dilution and expanded to develop a clonal population of Tal3 cells. Tal3 cells were then used to induce tumor via submucosal injection into the reproductive tract or subcutaneous injection into naïve C57BL/6 mice.

Statistical Analysis
The statistical analyses were performed with GraphPad Prism V.8 software (La Jolla, CA, USA). Data were expressed as means ± standard deviations (SD). Kaplan-Meier survival plots were constructed to estimate either tumor-free rate or survival percentage. The log-rank test was used to compare survival times between treatment groups. Comparisons between individual data point was analyzed by two-tailed Student’s t test. A p-value < 0.05 was considered statistically significant.

Results

Transfection of plasmids encoding high-risk HPV E6E7 oncogenes with AKT and c-myc oncogenes in the reproductive tract of C57BL/6 mice in the presence of transient T cell depletion induces tumor growth.

To induce tumor outgrowth in C57BL/6 mice, we mixed together 10μg of each AMES-16 plasmid (Fig 1B) (20μL total injection volume), injected them submucosally in the lower third of the vaginal tract of C57BL/6 mice and subsequently electroporated the mice at the site of injection (Fig 1A). We found that administration of AMES-16 plasmids was not able to induce tumor growth in naïve immunocompetent C57BL/6 mice. However, we previously reported that HPV+ oral tumor outgrowth via the integration of oncogenes was achievable in conjunction with transient T cell depletion via administration of anti-CD3 antibody (28). Similarly, when mice were treated with subsequent doses of anti-CD3 antibody (200μg/mouse/injection) for three consecutive days prior and two weekly doses after plasmid electroporation (Fig 1A), cervicovaginal electroporation of AMES-16 plasmids induced HPV16+ tumorigenesis 3-5 weeks post-electroporation (Fig 1C), which is monitorable via in vivo bioluminescence imaging (Fig 1D). In comparison, mice were injected with either HPV16-E7E6 Luciferase and SB100 plasmids alone, or injected with AKT, c-myc, and a SB13-luciferase plasmids alone, which failed to induce tumorigenesis, demonstrating the importance of including all four AMES-16 plasmids to achieve tumor outgrowth (Fig 1D and 1E). Tumor penetrance among AMES-16 challenged
mice was 80% (4 out of 5 mice) (Fig 1E). Interestingly, resultant AMES-16 tumors metastasize to the IP cavity in approximately 40% of mice, and manifest as poorly differentiated SCCs (Supplementary Figure 1A-D).

Our methodology offers unique flexibility in which oncogenes are utilized for tumorigenesis. As such, we constructed a plasmid that encodes HPV18E7E6 (Fig 1B) and found that cervicovaginal electroporation of myrAKT, c-myc, SB100, and HPV18E7E6Luc (AMES-18) plasmids in transiently CD3 depleted C57BL/6 mice also induced tumorigenesis (Fig 1D) in 80% of mice. Ultimate survival at 12 weeks post-plasmid injection was similar for AMES-18 tumors compared to AMES-16 tumors (p = 0.3039), and tumor penetrance upon injection of AMES-18 followed by electroporation was likewise 80% (4 out of 5 mice) (Fig 1E). Such findings demonstrate that our methodology can assess tumorigenic properties across different high-risk HPV strains and may be appropriate for assessing HPV-targeted therapies for high-risk strains beyond HPV16. Additionally, transfection of AMES-16 DNA caused tumor growth, leading to shorter survival compared with mice treated with transfection with E7E6Luc and SB plasmids alone (p < 0.0001), and likewise AMES-18 caused tumor growth, resulting in shorter survival compared with mice treated with E6E7 plasmids alone (p = 0.0023), suggesting the importance of including AKT and c-myc plasmids to achieve appreciable oncogenicity (Fig 1E).

AMES-16 induced cervicovaginal tumors present as squamous cell carcinomas, express relevant tumor markers and are infiltrated by diverse immune cell populations.

Tumors induced by cervicovaginal electroporation of AMES-16 plasmids present as squamous cell carcinomas (SCC) with morphological features showing either well-differentiated (Fig 2A) or poorly differentiated (Fig 2B) carcinomas, reflective of what is seen clinically. AMES-16 plasmids induced cervicovaginal tumors that display expression of HPV16 E6 as measured by RNA in situ hybridization (Fig 2C-D), integrated AKT and c-myc oncogenes, and typical carcinoma marker, CK14 (Fig 2 E-H).
After transient CD3 depletion, mouse T cell populations gradually recover over the course of tumor outgrowth and neared the CD3 T cell levels of naïve mouse by week 6 (Fig 3A).

Lymphocytes were identified via flow cytometry using a gating strategy as shown in Supplementary Figure 2. AMES-16-induced tumors are infiltrated with a variety of immune cells (Fig 3B), and the tumors display strong expression of immune exhaustion markers (Fig 3C), suggesting these tumors may be amenable for study of novel immunotherapies.

AMES-16 induced cervicovaginal tumors progress from high-grade lesions to invasive carcinoma

Since cervical cancer progresses through HSIL before forming SCC, we sought to investigate whether our AMES-16 induced tumors similarly progressed through HSIL. Weekly after AMES-16 plasmid electroporation and correlating with specific luminescence values, mice were sacrificed (n=3) and their reproductive tracts were harvested for histological analysis.

Remarkably, we saw that transfection of AMES-16 DNA produced HSIL in the cervicovaginal tract by week 3 (Fig 4A-D), and we discovered that AMES-16 DNA induced tumors progress through HSIL to invasive SCC in a stepwise fashion (Fig 4E). HSIL, illustrated by near-full-thickness cytological atypia and increased nuclear-to-cytoplasmic (N:C) ratio, present as early as 2 weeks post-electroporation and had progressed to invasive SCC by week 4 (Fig 4E).

Similar to squamous intraepithelial lesions in some immunocompromised patients in a clinical setting, multiple HSIL lesions could be found in the mouse reproductive tract as early as week 2 post AMES-16 electroporation, appearing both on the murine cervix and along the vaginal wall.

In mice electroporated with only HPV16-E6E7 and SB100 DNA constructs, HSIL were identified at week 3 post electroporation (Supplementary Figure 3A-C). Furthermore, HSIL appearance reliably correlates with luminescence values of around $1 \times 10^6$ in mice electroporated with AMES-
16 plasmids (Fig 4F). These results suggest that this model may be appropriate for assessing novel treatments for HSIL prior to SCC progression.

**Tal3 cell line derived from the AMES-16 induced tumor cells forms SCC in challenged immunocompetent mice**

Transplantable tumor cell lines are still widely used and can be useful for evaluating certain cancer treatments. As such, we sought to create an HPV+ cell line that displayed carcinoma morphology and was accompanied by a PI3K/AKT pathway mutation, characteristics not displayed by current HPV-E6E7 expressing cell lines. To this end, a single cell clone derived from an IP metastasis from a cervicovaginal AMES-16 tumor, designated Tal3, was selected and expanded for further analysis. To test tumorigenicity, Tal3 was subcutaneously injected into C57BL/6 mice at different doses. We observed that Tal3 cells achieved 100% tumor penetrance at a dose of $1 \times 10^5$. (Fig 5B-C, Supplementary Table 2). Upon sacrifice, Tal3 tumors were analyzed histologically and found to express relevant tumor markers including c-myc, AKT, HPV16 oncogenes (Fig 5A, Fig 6B-C) and proliferation marker ki-67 (Fig 6D) and found to display SCC morphology (Fig 6A). Additionally, we measured the tumor infiltrating leukocytes frequencies by flow cytometry. As shown in Figure 6E, Tal3 tumors were infiltrated with a variety of immune cells, and the tumor infiltrating lymphocytes were shown to display exhaustion markers (Fig 6F). Additionally, we challenged C57BL/6 mice with Tal3 cells submucosally in the cervicovaginal tract of C57BL/6 mice. Mice achieved 100% tumor growth and demonstrated squamous cell carcinoma morphology resembling that of the Tal3 tumor generated by orthotopic tumor challenge (Supplementary Figure 4A-D). These findings suggest that Tal3 may serve as a potentially important transplantable pre-clinical tumor model for the studies of novel treatments for HPV16+ cancer.

**Discussion**
Pre-clinical models of cervical cancer should recapitulate critical aspects of the human malignancy. Here we present the development of a novel pre-clinical model of cervical cancer that utilizes SB100 to integrate clinically relevant AMES-16 oncogenes to induce tumor outgrowth in the reproductive tract of mice. Remarkably, our model recapitulates key features of 1) gynecologic tumor outgrowth 2) progression through pre-cancerous lesions to SCC 3) and local expression of HPV-oncogenes. Such innovation has been sought after in the field and is particularly important upon documentation of the inadequacy of existing pre-clinical models to predict clinical outcomes (15-17).

Although transient T cell depletion by anti-CD3 monoclonal antibody is necessary to induce tumor formation via integration of AMES-16 plasmids, the T cell population gradually recovers over the course of tumor growth (Fig 3A). In addition, the resultant tumors are infiltrated with a variety of immune cells and express various exhaustion markers resembling cervical cancer (Fig 3B-C). Thus, this model may be appropriate for the evaluation of novel cancer therapies that require a functioning immune system, including therapeutic HPV vaccines and/or checkpoint blockade therapies. This pre-clinical tumor model may be potentially useful due to high clinical and translational interest in using immunotherapy for HPV+ associated cancers (32,33). To better determine whether such a pre-clinical tumor model has clinical relevance, further studies need to be conducted to evaluate if outcomes of the therapeutic interventions tested in this model correlate with responses of the therapeutic interventions in clinical trials.

It was an interesting and unexpected finding that our AMES-16 model metastasizes to the IP cavity in 40% of mice (Supplementary Figure 1A). All IP tumors present as poorly differentiated squamous carcinomas. The squamous morphology of the IP tumors suggest that these tumors likely represent true metastases derived from the reproductive tract. Furthermore, since there is no route from uterine lumen to IP cavity via fallopian tube because the bursa covers ovary and fallopian tube, the tumor in the peritoneal cavity likely represent a metastasis.
from uterine serosa to IP cavity. It is noteworthy that, although most advanced human cervical cancers directly extend to vagina and/or uterine corpus, parametrium, bladder and rectum, they only occasionally spread to the peritoneal cavity. This aberrant metastasis site may represent a limitation of murine models to anatomically mimic metastatic human cancers.

Our pre-clinical model’s ability to demonstrate tumor progression from HSIL to SCC is an unprecedented strength in modeling cervical cancer. HSIL lesions are detected by pap smears and are treated by surgical or ablative therapies with the success of such therapies reaching 75-85% (6,7). However, such surgical therapeutic approaches may potentially result in cervical incompetence, leading to preterm birth (34). Thus, the availability of such a model will allow the development of medical interventions for control of HSIL, such as vaccines, in pre-clinical models. Novel therapeutic approaches that minimize potential detrimental reproductive effects are particularly important for women who wish to bear children. Here we show that AMES-16 plasmid-induced cervicovaginal tumors progress through HSIL, and these HSIL are potentially monitorable via bioluminescence imaging (Fig 3A), opening an unprecedented opportunity for pre-clinical evaluation of novel treatments for HSIL. One potential limitation of our model for the assessment of novel HSIL therapies is that the model HSIL progresses to SCC within about a week (Fig 4A). Thus, this model may not be suitable for assessing treatments for HSIL that require a lengthy mechanism of action.

To gain insight on the role of T cells in HSIL development, we electroporated fully immunocompetent mice with AMES-16 DNA plasmid without CD3 depletion and sacrificed them at different time points to evaluate their reproductive tracts for HSIL. We found that HSIL lesions could be found in fully immunocompetent AMES-16 DNA electroporated C57BL/6 mice by week 3 post plasmid-injection, suggesting that the presence of T cells does not prohibit normal epithelium from developing into HSIL (Supplementary Figure 5A-D). However, no lesions were observed on week 4 post AMES-16-injection, suggesting that the presence of T cells eventually eliminate the HSIL lesions and preclude cancer formation (data not shown).
In our studies, HSIL and SCC were found to express HPV16 oncogenes by RNA in situ hybridization (ISH). The RNA ISH stain for HSIL (Fig 4F) demonstrated mainly nuclear speckle staining pattern, probably due to weak cross-hybridization with integrated HPV DNA and relatively lower levels of HPV E6 RNA in the cytoplasm. In comparison, the RNA ISH stain for SCC (Fig 2 C&D) demonstrated both nuclear and cytoplasmic staining pattern, probably due to relatively higher levels of HPV E6 RNA in the nuclear and cytoplasm in cancer. It has been demonstrated that HPV-associated malignancies tend to upregulate E6/E7 RNA due to multiple mechanisms related to HPV genome integration (for review see (35)).

Furthermore, we evaluated if in vivo electroporation of HPV16-E6E7Luc and SB100 DNA constructs alone could form HSIL in the absence of the AKT and Myc oncogenes. We found that this treatment could induce HSIL 2-3 weeks post-electroporation but that no lesions were observed by week 4 post-electroporation (Supplementary Figure 3 A-C), suggesting the importance of AKT and Myc oncogenes for cancer formation. P16INK4A is frequently overexpressed in HPV-associated malignancies. To test whether our model additionally overexpresses p16, we stained AMES-16 DNA electroporated tumors with mouse p16 -specific antibody using Abcam antibody Ab51243. While we observed up-regulation of p16 protein in HSIL as well as squamous cell carcinoma in mice, the p16 staining pattern does not show the “block” staining pattern (diffuse nuclear and cytoplasmic staining) that is typically encountered in human samples infected with HR-HPV (Supplementary Figure 6A-D). Our findings are consistent with those reported by other investigators (13).

In our AMES-16 model, multiple HSILs occur in different regions of the lower reproductive tract of AMES-16 DNA electroporated mice, including on the cervix and on the vaginal wall (Fig 4). Due to the small size of the murine reproductive tract, our electroporation methodology cannot induce HSIL on only the cervix. This may represent a limitation of our model in capturing clinical features of typical cervical cancers. However, in humans although high-risk HPV most often affects squamous epithelial cells in the cervix, it can also cause
squamous intraepithelial lesion and carcinoma in the vagina, vulva, and other locations, suggesting a common HPV-related oncogenic mechanism in the reproductive tract was recapitulated in our model system.

As cancer therapies become more personalized, it is important to have pre-clinical models that represent diverse clinical scenarios. To that end, the methodology presented in this study offers unique flexibility in which oncogenes are used to induce tumorigenesis. Here, we showed evidence that this methodology can be used to induce tumor outgrowth using both HPV16 E6E7 and HPV18 E6E7 (Fig 1). Although HPV16 and HPV18 are responsible for causing the majority of cervical cancers, there are at least 13 other high risk HPV types that cause cervical cancer (1). This study suggests that this methodology may be amenable to evaluating targeted therapies for diverse high-risk HPV genotypes. Of additional interest, this methodology may also be able to lend insight to mechanistic differences in tumorigenesis between the high-risk HPV genotypes.

Additionally, we utilized myrAKT and over-expression of c-myc to help drive tumorigenesis in our model. However, the AMES-16 plasmid combination can be easily altered to reflect the oncogene profile of interest by adding or replacing relevant plasmids encoding different oncogenes to investigate their roles. Furthermore, this methodology could be expanded in mouse strains beyond C57BL/6. A previous study has shown that FVB/n mice have increased susceptibility to HPV16 E6E7-driven malignancy compared to C57BL/6 (36). Thus, it would be of interest to investigate if FVB/n mice would require the same degree of immune suppression to induce tumorigenesis compared to C57BL/6 mouse. It would be also of particular interest in evaluate this methodology in different human MHC class I transgenic mice or genetically outbred mice and subsequently evaluate their immune responses to novel treatments such as E6E7-targeted vaccines. Such information may be important for future clinical translation.

Although our methodology of HPV+ tumor induction via the integration AMES-16 oncogenes in the reproductive tract of C57BL/6 mice may be the pre-clinical model most closely
related to human cervical cancer to date, we also found merit in developing the Tal3 cell line, which we derived from an IP metastasis of an AMES-16 induced cervicovaginal tumor. Despite the previously discussed limitations of transplantable cell lines for the study of cervical cancer, which remain for Tal3, transplantable tumor-forming cell lines are still useful in rapid screening of novel drugs or vaccines. Prior to the development of Tal3 cell line, there were no HPV16 E6E7-expressing cell lines that demonstrated squamous cell carcinoma phenotype, a clinically important parameter in modeling cervical cancer. As Tal3 displays these important characteristics, it may serve as an appropriate pre-clinical tumor model for the studies of HPV+ cancer. Together, our AMES-16 plasmid-induced cervicovaginal tumors and Tal3 cell line represent marked advancements in the pre-clinical modeling of HSIL and/or cervical cancer and hold promise to more reliably predict clinical outcomes of novel therapeutic interventions.

Acknowledgments.

Funding
This work was supported the National Institute of Health and the National Cancer Institute under award number R01CA237067 and P50CA098252.

Contributions
TS contributed to the conduction of the experiment. S-HT contributed to the performance of the experiments. LF contributed to the original draft and preparation of the manuscript. DX contributed to interpretation of the data. C-FH contributed to the design of the study. T-CW contributed to the design of the study and supervised the study.

Competing Interests
Dr. Wu is a co-founder of and has an equity ownership interest in Papivax LLC. Also, Dr. Wu owns Papivax Biotech Inc. stock options and is a member of Papivax Biotech Inc.’s Scientific Advisory Board.

Ethics Approval
All procedures were performed under prior-approved protocols of the Johns Hopkins Animal Care and Use committee in accordance with recommendations for the proper use and care of laboratory animals.

References

8. Lamont RF, Sarhanis P. Precancerous changes in the cervix and risk of subsequent preterm birth. BJOG 2007;114:775-6; author reply 6-7


18. Litwin TR, Clarke MA, Dean M, Wentzensen N. Somatic Host Cell Alterations in HPV Carcinogenesis. Viruses 2017;9


Table 1

**Tumor Infiltrate Panel**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Fluorophore</th>
<th>Company</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live/Dead</td>
<td>Zombie Aqua™ (BV510)</td>
<td>Biolegend</td>
<td>423101</td>
</tr>
<tr>
<td>CD19</td>
<td>Alexa488</td>
<td>Biolegend</td>
<td>115524</td>
</tr>
<tr>
<td>CD11b</td>
<td>PE</td>
<td>Biolegend</td>
<td>101207</td>
</tr>
<tr>
<td>DEC205</td>
<td>PE-Dazzle594</td>
<td>Biolegend</td>
<td>138217</td>
</tr>
<tr>
<td>NK1.1</td>
<td>PE-Cy5</td>
<td>Biolegend</td>
<td>108715</td>
</tr>
<tr>
<td>I-A/I-E</td>
<td>PE-Cy7</td>
<td>Biolegend</td>
<td>107629</td>
</tr>
<tr>
<td>GR1</td>
<td>APC</td>
<td>Biolegend</td>
<td>108411</td>
</tr>
<tr>
<td>CD45</td>
<td>APC-R700</td>
<td>BD Horizon</td>
<td>565478</td>
</tr>
<tr>
<td>CD25</td>
<td>APC-Fire750</td>
<td>Biolegend</td>
<td>102053</td>
</tr>
<tr>
<td>CD3</td>
<td>BV421</td>
<td>Biolegend</td>
<td>100228</td>
</tr>
<tr>
<td>CD8</td>
<td>BV650</td>
<td>Biolegend</td>
<td>100741</td>
</tr>
<tr>
<td>CD4</td>
<td>BV785</td>
<td>Biolegend</td>
<td>100551</td>
</tr>
<tr>
<td>TruStain FcX™</td>
<td>N/A</td>
<td>Biolegend</td>
<td>101319</td>
</tr>
</tbody>
</table>

Table 2

**Tumor Exhaustion Panel**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Fluorophore</th>
<th>Company</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live/Dead</td>
<td>Zombie Aqua™ (BV510)</td>
<td>Biolegend</td>
<td>423101</td>
</tr>
<tr>
<td>PD1</td>
<td>Alexa488</td>
<td>Biolegend</td>
<td>143719</td>
</tr>
<tr>
<td>TIGIT</td>
<td>PE</td>
<td>Biolegend</td>
<td>142103</td>
</tr>
<tr>
<td>TIM3</td>
<td>PE-Dazzle594</td>
<td>Biolegend</td>
<td>134013</td>
</tr>
<tr>
<td>NK1.1</td>
<td>PE-Cy5</td>
<td>Biolegend</td>
<td>108715</td>
</tr>
<tr>
<td>LAG3</td>
<td>PE-Cy7</td>
<td>Biolegend</td>
<td>125226</td>
</tr>
<tr>
<td>CTLA4</td>
<td>APC</td>
<td>Biolegend</td>
<td>106309</td>
</tr>
<tr>
<td>CD45</td>
<td>APC-R700</td>
<td>BD Horizon</td>
<td>565478</td>
</tr>
<tr>
<td>CD25</td>
<td>APC-Fire750</td>
<td>Biolegend</td>
<td>102053</td>
</tr>
<tr>
<td>CD3</td>
<td>BV421</td>
<td>Biolegend</td>
<td>100228</td>
</tr>
<tr>
<td>CD8</td>
<td>BV650</td>
<td>Biolegend</td>
<td>100741</td>
</tr>
<tr>
<td>CD4</td>
<td>BV785</td>
<td>Biolegend</td>
<td>100551</td>
</tr>
<tr>
<td>TruStain FcX™</td>
<td>N/A</td>
<td>Biolegend</td>
<td>101319</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1. Generation of spontaneous HPV+ cervicovaginal carcinoma model in C57BL/6 mice. Mice received transient CD3 cell depletion at day -3, -2, -1, 7, and 14. On day 0, C57BL/6 mice received 10µg of each DNA plasmid (total injection volume 20µg) as a submucosal injection in the vaginal tract, followed by electroporation. (A) Schematic of experimental design. (B) Schematic of plasmids used to induce HPV+ cervicovaginal tumors via oncogene integration. Arrows indicate direction of plasmid integration. (C) Representative images of the development of AMES-16 induced cervicovaginal tumor post-electroporation as measured by IVIS Spectrum imaging. Bioluminescence was recorded by IVIS Spectrum after intraperitoneal injection of luciferin solution. (D) Tumor growth as monitored by bioluminescence imaging. (E) Survival rate shown by percentage. Mice were considered dead due to tumor when tumor diameter >15mm, and the mice were subsequently sacrificed. ** p = 0.0023. *** p = <0.0001.

Figure 2. Representative images of HPV16+ spontaneous cervicovaginal tumor. Mice received transient CD3 cell depletion at day -3, -2, -1, 7, and 14. On day 0, C57BL/6 mice received 10µg of each AMES-16 DNA plasmid (total injection volume 20µg) as a submucosal injection in the vaginal tract, followed by electroporation. Representative H&E of tumor displaying (A) well-differentiated or (B) poorly differentiated SCC. (C-D) RNA in situ hybridization of HPV16 E6 for corresponding tumors shown in A and B. (E) IHC of tumor proliferation marker ki-67, (F) IHC of c-myc, (G) IHC of carcinoma marker CK14, (H) IHC of AKT.

Figure 3. T cell characterization in HPV16+ spontaneous cervicovaginal tumor. (A) Time course of T cell recovery post-depletion. Mice received transient T cell depletion via administration of intraperitoneal injection of 200µg anti-CD3 antibody daily three days prior to AMES-16 electroporation and weekly for two weeks after electroporation. (B) Tumor-bearing
mice were sacrificed 6 weeks post AMES-16 electroporation and tumor immune infiltrate was evaluated by flow cytometry. Bar graph summary of AMES-16 induced cervicovaginal tumor infiltrating lymphocytes (TIL). (C) Bar graph summary of exhaustion markers expressed by TIL 6 weeks post AMES-16 electroporation.

Figure 4. Anatomical orientation and lesion progression from HSIL to SCC in spontaneous HPV+ cervicovaginal carcinoma model. At each time point following the AMES-16-plasmid electroporation, 3 mice were sacrificed and their reproductive tracts were harvested and fixed in formalin for sectioning and histological analysis. Representative images of the histological examination were selected. (A) 2X magnification of mouse reproductive tract 3 weeks post AMES-16 DNA electroporation anatomically labeled. (B) 40X magnification showing cervical HSIL in black box with orientation as shown by cervix in (A). (C) 40X magnification showing vaginal HSIL in dotted black box as shown in lower left corner of (A). (D) 20X magnification showing vaginal HSIL in dashed black box and carcinoma in black circle with orientation as shown by (A). (E) Development of SCC from HSIL. (Left) Emergence of HSIL [see box] corresponding with luminescence values ~1x10^6. (Center) Appearance of SCC [see box] and corresponding with luminescence values ~1x10^7. (Right) Appearance of HSIL and invasive carcinoma corresponding with luminescence values ~1x10^8. (F) (Left) Representative H&E of HSIL lesion 2 weeks post AMES-16 DNA electroporation. (Right) HPV16 RNA (black arrows) co-localizes with HSIL lesion at week 2 post AMES-16 DNA electroporation with no evidence of HPV RNA expression in dermal compartment. HPV16 RNA was performed by RNAscope in situ hybridization.

Figure 5: Characterization of Tal3 cell line induced tumor size and penetrance in immunocompetent C57BL/6NTac mice. (A). E6 expression in the Tal3 cell line was measured by RT-PCR and gel electrophoresis. C57BL/6NTac mice were subcutaneously injected with
8x10^3, 8x10^4, 8x10^5 or 8x10^6 Tal3 cells in the vaginal tract. (B) Tumor growth curve of submucosal injection of indicated doses of Tal3 cells/mouse. N= 5. (C) Representative image of Tal3 tumor as measured by IVIS Spectrum imaging. Bioluminescence was recorded by IVIS Spectrum after intraperitoneal injection of luciferin solution.

**Figure 6: Characterization of SCC, tumor markers, and tumor infiltrate by Tal3 cell line.**

C57BL/6NTac mice were submucosally injected with 8x10^5 Tal3 cells in the vaginal tract. (A) Representative H&E of Tal3 cell line displaying SCC morphology, (B) IHC of c-myc, (C) IHC of AKT (D) IHC of tumor proliferation marker ki-67. (E) Mice were sacrificed 3 weeks post submucosal injection of Tal3 and TILs were evaluated by flow cytometry. A bar graph result summary of TILs. (F). Bar graph summary of exhaustion markers expressed by Tal3 TILs.
Fig 3

A. T Cell Recovery Post-Depletion
- Naive C57Bl/6 mice
- C57Bl/6 mice with transient T cell depletion

% CD3+ T cells (gated on lymphocytes)

Week post plasmid electroporation

B. Leukocyte Frequencies in Tumor Infiltrate

% of Live CD45+ Cells

CD4+ T Cells, Tregs, CD8+ T Cells, B Cells, NK Cells, MDSCs

C. Exhaustion markers in Tumor Infiltrate

Exhaustion marker

- CD8+ T cell
- CD4+ TIL
- NK cell TIL

MFI
Fig 6

H&E

IHC

A

B

c-myc

C

AKT

D

ki-67

Leukocyte Frequencies in Tumor Infiltrate

E

Exhaustion markers in Tumor Infiltrate

F

CD4+ T Cells

CD8+ T Cells

Tregs

B Cells

NK Cells

MDSCs

% of Live CD4+ Cells

CD4+ TIL

CD8+ TIL

NK cell TIL

Exhaustion marker

Downloaded from cancerres.aacrjournals.org on July 5, 2021. © 2021 American Association for Cancer Research.
Development of a novel mouse model of spontaneous high-risk HPVE6/E7-expressing carcinoma in the cervicovaginal tract

Talia R Henkle, Brandon Lam, Yu Jui Kung, et al.

*Cancer Res* Published OnlineFirst July 2, 2021.

<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-21-0399</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Access the most recent supplemental material at: <a href="http://cancerres.aacrjournals.org/content/suppl/2021/07/01/0008-5472.CAN-21-0399.DC1">http://cancerres.aacrjournals.org/content/suppl/2021/07/01/0008-5472.CAN-21-0399.DC1</a></td>
</tr>
<tr>
<td>Author Manuscript</td>
<td>Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.</td>
</tr>
</tbody>
</table>

**E-mail alerts** Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions** To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/early/2021/07/02/0008-5472.CAN-21-0399. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.