Mouse papillomavirus MmuPV1 infects oral mucosa and preferentially targets the base of the tongue

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ABSTRACT

In 2010, a new mouse papillomavirus, MmuPV1, was discovered in a colony of NMRI- Foxn1^{nu}/Foxn1^{nu} athymic mice in India. This finding was significant because it was the first papillomavirus to be found in a laboratory mouse. In this paper we report successful infections of both dorsal and ventral surfaces of the rostral tongues of outbred athymic nude mice. We also report the observation that the base of the tongue, the area of the tongue often targeted by cancer-associated high-risk papillomavirus infections in humans, is especially susceptible to infection. A suitable animal model for the study of oral papillomavirus infections, co-infections, and cancers has long been sought. The work presented here suggests that such a model is now at hand.

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Introduction

Human papillomavirus infections are ubiquitous in the general population and a subset of these viruses is associated with significant disease. Papillomavirus infection is an obligate co-factor for most cervical cancers and this cancer is a leading cause of death in women worldwide with in excess of 300,000 deaths reported each year (Arbyn et al., 2011) The viruses are also increasingly associated with anal and oral cancers (D'Souza et al., 2007; Grulich et al., 2012; Ouhoummane et al., 2013). While two vaccines are now available that target a few of the viruses most commonly associated with clinical disease including malignant neoplasia, the uptake of these vaccines has been disappointing and the price is out of reach for use in developing nations. Importantly, the vaccines do not eliminate established infections and no effective treatment or therapy is available. More research will be necessary to achieve satisfactory control of these common

infections and to assure that the cancers to which they contribute will be minimized in the years to come.

Oropharyngeal cancers associated with papillomavirus infection are increasing, especially in the young male population (Gillison et al., 2000). The reasons are unclear although it has been postulated that an increased practice of oral sex may be a factor (Gillison et al., 2008). Papillomaviruses are strictly species specific and consequently no animal can be infected with a human virus. With the demonstration that MmuPV1 can infect oral tissues of the athymic mouse, and with the observation that the virus appears to have a preference for the base of the tongue, we have demonstrated the practicality of this model for the study of oral infections and, potentially, of papillomavirus-associated oral cancers.

Results

Both the dorsal and ventral surfaces of the rostral tongues of outbred athymic nude mice are susceptible to infection with MmuPV1

Mice infected on the dorsal and ventral surfaces of the rostral tongues were sacrificed at an early time point of 3.5 months or at

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later time points of 8, 8.5 and 9 months postinfection (one mouse per time point). By 3.5 months, a section of the rostral dorsal surface of the tongue of mouse 3–3L showed significant papillomavirus infection. Fig. 1A–C shows H and E, ISH, and IHC respectively of a dorsal lesion of this tongue.

By 8 months postinfection, both the dorsal and ventral surfaces of the tongue of animal 3–3R showed very strong hybridization signal and antigen expression. See sagittal sections Fig. 2A–C. There was a sharp demarcation between positive and negative signals, the significance of which is not yet understood. Fig. 2D–F shows a higher magnification of a dorsal section of the tongue.

Animals sacrificed at 8.5 and 9 months also exhibited extensive positivity on both dorsal and ventral surfaces of the rostral tongue (Data not shown).

A Base of tongue lesion was observed in an animal that had been infected on the rostral tongue.

Since tongue infections were initiated on the dorsal and ventral surfaces of the rostral ("mobile") tongue, we did not expect to see lesions at the caudal (base) of the tongue. However, careful examination of the sections of the entire tongue revealed a base-of-tongue lesion in one of the animals. The base of the tongue is of special interest due to its involvement in papillomavirus-associated human cancers (Woods et al., 2014). Fig. 3A and B shows H and E and ISH sections of the lesion at the base of the tongue at the dorsal fornix of animal 3–3R. Surrounding tissue is negative for the virus.

Inner cheek infections were found in animals infected on the dorsal and ventral surfaces of the tongue.

At the time of sacrifice of the four animals infected on the tongue, inner cheek samples were excised and paraffin-embedded. Our earlier studies had shown that secondary infections can occur and we wished to determine whether this proximal tissue was also infected. Fig. S1A and B shows strong in situ hybridization signal in representative sections of both left and right cheek tissue from animal 3–3L.

The base of the tongue was preferentially targeted by secondary infections

During the course of this work, we also conducted infectivity studies in other animals at mucosal sites such as the vagina and anus as well as cutaneous sites including tail and muzzle. Our observation that primary infection of the rostral tongue yielded a secondary infection at the base of the tongue led us to examine the tongues of some of these animals. Tongues from a total of 18 animals infected at sites other than the oral cavity had been archived as tissue blocks. Of these, ten (56%) were found to have lesions at the base of the tongue. Eight of these involved the caudal dorsal tongue and two involved the caudal ventral tongue. Three of the 18 animals (17%) had small areas of secondary infection on the rostral tongue. Table 1 summarizes the secondary base-of-tongue infections found in animals with primary infections at sites other than the oral cavity.
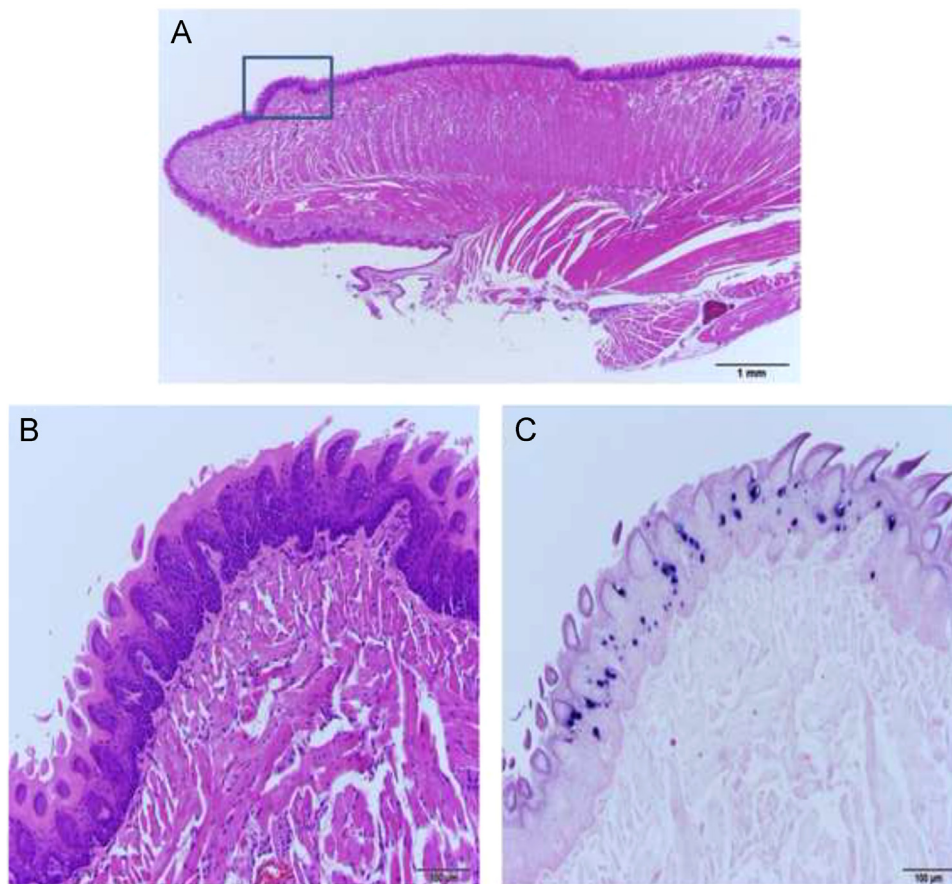

Fig. 1. (A–C) The dorsal surface of the rostral tongue is susceptible to MmuPV1 infection. (A) 10× sagittal section of the tongue of animal 3–3L sacrificed 3.5 months postinfection and stained by H and E. The dorsal lesion on the rostral tongue is boxed. (B) H and E (100×) and (C) ISH (100×) of the boxed region in A showing a discrete focus of basal cell hyperplasia and strong in situ hybridization signal.

The circumvallate papilla and adjacent Von Ebners glands represent a preferred locus for MmuPV1 infection in mice secondarily infected in the oral cavity

Careful observation of the tongue sections positive for secondary MmuPV1 infection identified the location of seven of the eight dorsal tongue infections as the single circumvallate papilla of the mouse tongue and the adjacent Von Ebners glands. More advanced infections were associated with significant hyperplasia and dysplasia (Figs. 4 A–F; 5A and B). Fig. 5C–F shows sites in two

of four additional tongues with less advanced lesions. Fig. S2A and B shows the single dorsal back of the tongue lesion not found in the area of the circumvallate papilla. This lesion was located in the dorsal pharynx/palate area of the caudal tongue.

The two secondary infections at the caudoventral tongue were found to target the same locus

Only two secondary infections targeted the caudoventral tongue and both of these were located in an area near the reflection to
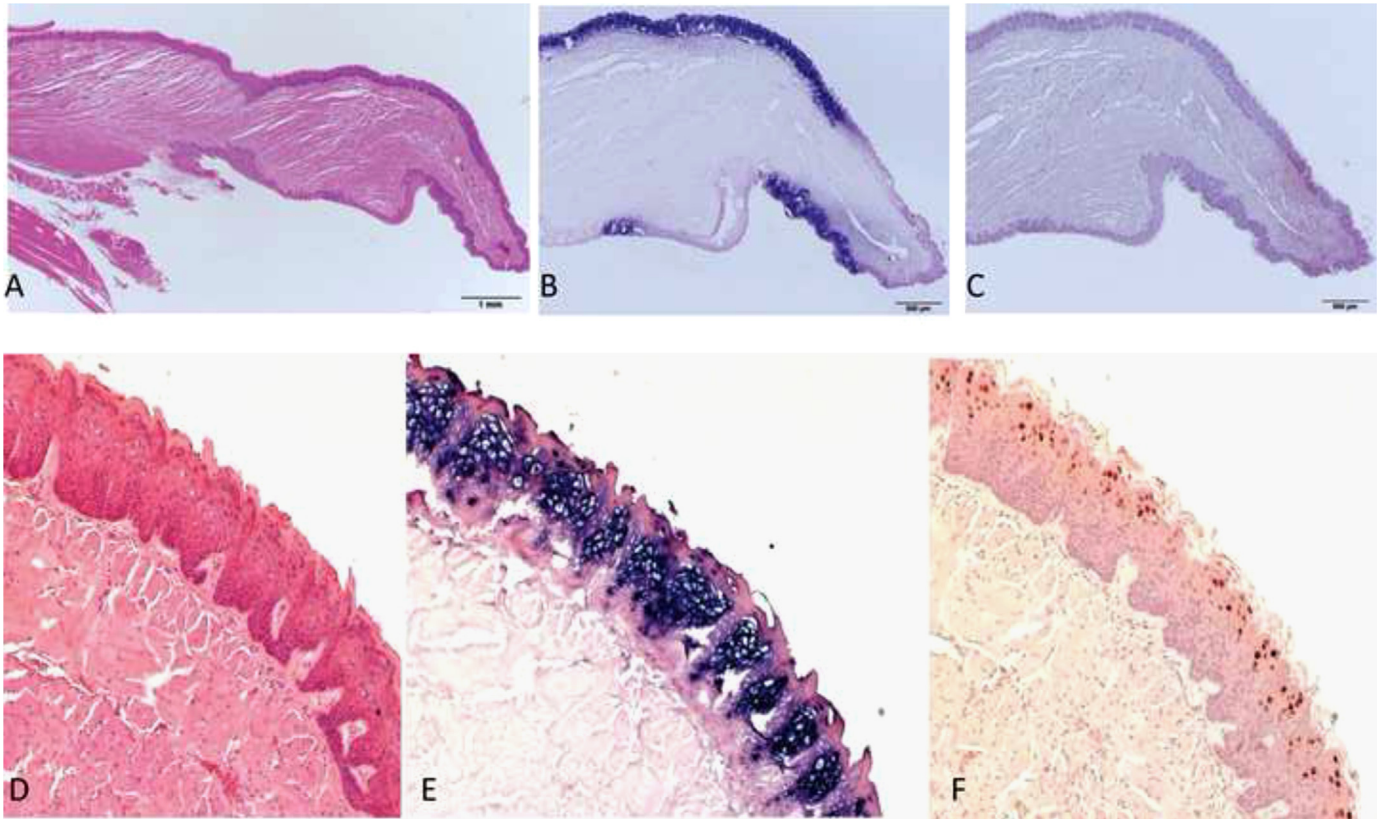

Fig. 2. (A–F) By 8 months postinfection both dorsal and ventral surfaces exhibit strong positivity for MmuPV1. (A) Sagittal section of the tongue of mouse 3–3R sacrificed at 8 months postinfection. H and E at 10 × . (B) ISH (20 ×) of the sagittal section showing strong ISH signal with clear demarcation of positive and negative sites. (C) IHC (20 ×) of same section showing signal in the DNA-positive sites. (D) H and E (100 ×), (E) ISH (10 ×) and (F) IHC (100 ×) illustrating the magnitude of viral infection.
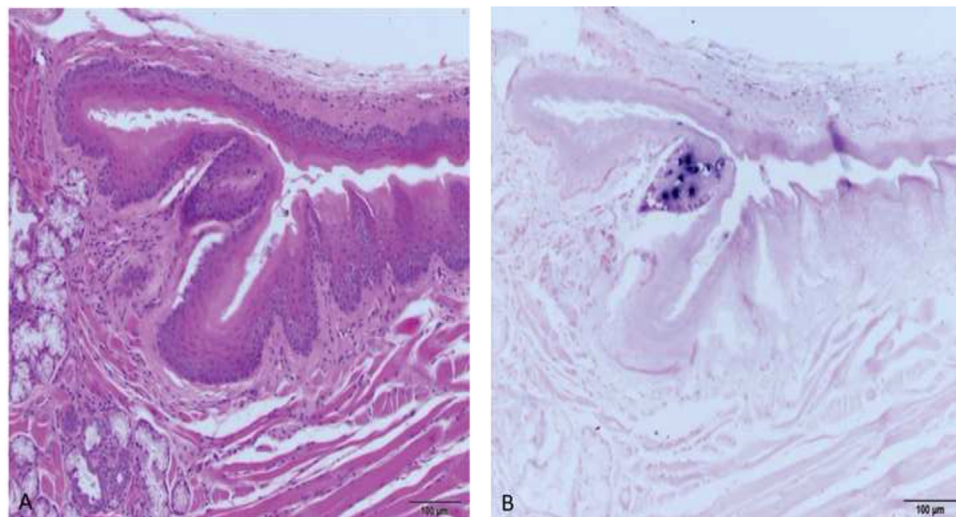

Fig. 3. (A and B) A secondary back of the tongue lesion was found. (A) H and E (100 ×) of a sagittal section of the caudal tongue encompassing the dorsal fornix of animal 3–3R. (B) ISH (100 ×) of an adjacent section showing a focus of positivity near the dorsal fornix.

the sublingual oral mucosa. The limited number of samples does not allow the conclusion that this is a preferred site on the ventral tongue but the data are suggestive Fig. 6A–E).

Discussion

Papillomavirus-associated diseases pose a major problem throughout the world. To date, more than 150 species of human PVs have been fully sequenced. Most of these are relatively benign but a number are implicated in cancers, especially anogenital cancers, as well as some skin cancers and head and neck malignancies. Others do not cause cancer under normal conditions but do present significant medical challenges (reviewed in Doorbar et al. (2015)). Because human papillomaviruses do not infect animals, finding a suitable in vivo model to study the disease has been challenging. Our laboratory (Hu et al., 2006; Christensen et al., 1996; Han et al., 2000, 2005; Cladel et al., 2013; Hu et al.,

2007, 1998) and those of others (Brandsma et al., 1992; Du et al., 2005; Peh et al., 2004; Han et al., 1992; Salmon et al., 2000) have used the cottontail rabbit papillomavirus (CRPV) model to good advantage. We have also pioneered the rabbit oral papillomavirus (ROPV) model (Christensen et al., 2000; Wilgenburg et al., 2005). Due to limited reagents for the rabbit and technical challenges with ROPV, we sought another small laboratory animal model to complement our studies. We were especially interested in a mouse model because of the abundance of reagents available for this common laboratory animal. When the new mouse virus, MmuPV1, was reported (Ingle et al., 2011) we initiated studies to see if this virus would aid our studies on preclinical papillomavirus infections.

MmuPV1 was reported to be a strictly cutaneous pathogen (Ingle et al., 2011) but our early studies revealed mucosal secondary infections (Cladel et al., 2013, 2015). This observation was later corroborated by Sundberg et al (Sundberg et al., 2014). We have shown that MmuPV1 has a broad tropism in athymic $Foxn1^{nu}$

Table 1
Secondary oral lesions found in animals infected with MmuPV1 at sites other than the oral cavity.

| Animal	Primary infection	Location of secondary infection	Fig.
5–2L	Tail	Caudodorsal tongue;	ND
7–2L	Back and tail	Caudodorsal tongue extending into the duct of the serous lingual accessory salivary gland	5E and F
8–2R	Anus	Caudodorsal tongue extending into the duct of the serous lingual accessory salivary gland	4A–C
10–2L	Vagina	Caudodorsal tongue extending into the duct of the serous lingual accessory salivary gland	4D–F
1–3R	Anus	Dorsal/pharynx in area of palate	S2 A and B
4–3L	Vagina	Caudal margin of dorsal tongue near reflection to palatine mucosa; lingual salivary gland duct involvement	5A and B
5–3R	Vagina	Caudal margin of dorsal tongue near reflection to palatine mucosa; lingual salivary gland duct involvement	ND
6–3R	Muzzle, tail, anus, vagina	Caudoventral tongue near reflection to sublingual oral mucosa	6A and B
7–3L	Muzzle, tail, anus, vagina	Caudoventral tongue near reflection to sublingual oral mucosa	6 C–E
10–3	Muzzle, tail, anus, vagina	Caudodorsal tongue extending into the duct of the serous lingual accessory salivary gland	5C and D
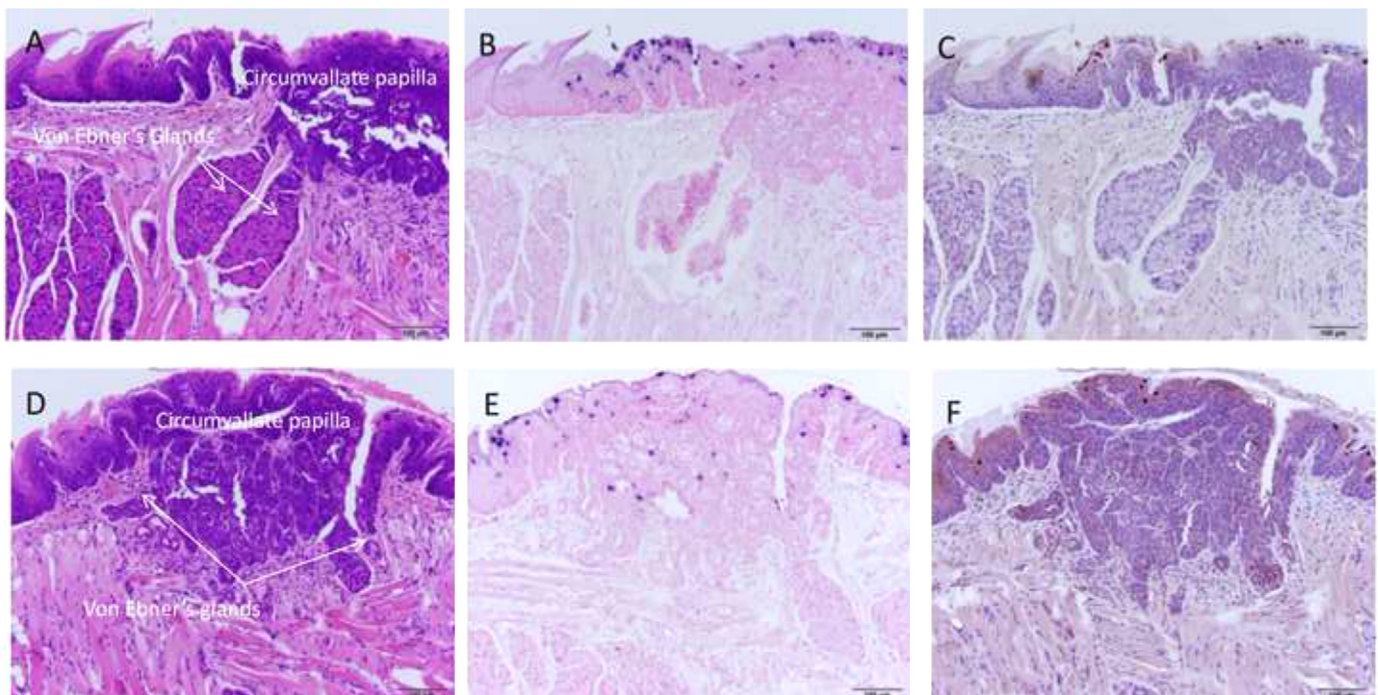
Fig. 4. (A–F) Back of the tongue secondary lesions involved the circumvallate papilla and adjacent Von Ebner's glands and exhibited marked dysplasia. (A) H and E (100 ×) of a sagittal section of the caudodorsal tongue of animal 8–2R demonstrating florid pseudocarcinomatous hyperplasia of the epithelium of the circumvallate papilla of the dorsal tongue adjacent to and extending into the duct of the serous lingual accessory salivary (Von Ebner's) glands. Adjacent duct of the mucinous salivary gland is unaffected. (B) ISH (100 ×) of an adjacent section demonstrating strong staining in the superficial epithelial cells, correlating with viral cytopathic effect seen on H&E section. (C) IHC (100 ×) of an adjacent section showing capsid antigen presence in the same areas positive for MmuPV1 DNA. (D) H and E (100 ×) of a sagittal section of the caudodorsal tongue of animal 10–2L demonstrating florid pseudocarcinomatous hyperplasia of the epithelium of the circumvallate papilla of the dorsal tongue adjacent to and extending into the duct of the serous lingual accessory salivary (Von Ebner's) glands. Adjacent duct of the mucinous salivary gland is unaffected. (E) ISH (100 ×) of an adjacent section demonstrating strong staining in the superficial epithelial cells, correlating with viral cytopathic effect seen on H&E section. (F) IHC (100 ×) of an adjacent section showing capsid antigen presence in the same areas positive for MmuPV1 DNA.
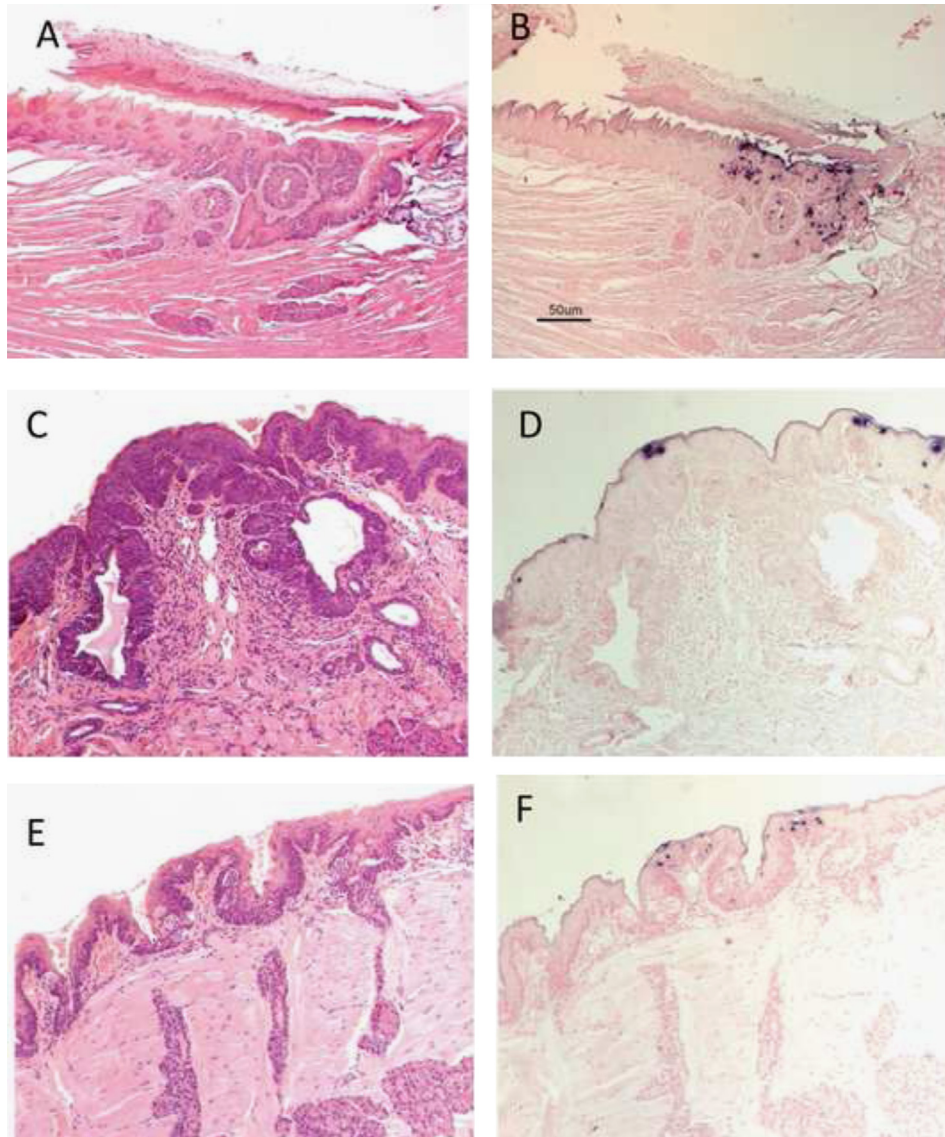
Fig. 5. (A–F) Additional back of the tongue lesions targeted the circumvallate papilla. (A) H and E (40 ×) of a longitudinal parasagittal section of the tongue of animal 4–3L experimentally infected vaginally. At the caudal margin of the dorsal tongue near the reflection to the palatine mucosa and involving the ducts of the lingual salivary (Von Ebner's) glands and associated circumvallate papilla, there is focally extensive epithelial hyperplasia with dysplasia and viral cytopathic effect (CPE). (B) ISH (40 ×) of an adjacent section showing strong DNA positivity in the same dysplastic area. (C) H and E of lesion targeting the circumvallate papilla of animal 10–3 (100 ×). (D) ISH of the same lesion showing DNA positivity. (E) H and E of lesion targeting the circumvallate papilla of animal 7–2L(100 ×). (F) ISH of the same lesion showing DNA positivity (100 ×).

mice and that it infects anal, vaginal and cervical tissues (Cladel et al., 2015). In this paper we extend our observations to demonstrate that the virus can also infect oral mucosal sites, specifically both the dorsal and ventral surfaces of the rostral tongue, the inner cheek, and the oropharyngeal cavity. We show that the oropharyngeal cavity, including base of tongue and surrounding tissues, is especially susceptible to infection and we have localized most of the infections to the circumvallate papilla and associated Von Ebner's glands. The more advanced lesions (Fig. 4A–F; 5A and B) exhibit florid pseudocarcinomatosis and cytopathic effect (CPE) associated with papillomavirus infections.

Oropharyngeal squamous cell carcinomas have been on the rise in much of the world in the past few decades (Woods et al., 2014). Two different populations of individuals have been identified as having this disease and it appears to present differently in these two populations (Gillison et al., 2008). One of the groups is older on average and has risk factors such as heavy drinking and

smoking. The other group tends to be younger and healthier. In the latter case, disease is frequently associated with papillomavirus infection, commonly HPV16 but also other types. These infections tend to be located at the base of the tongue and in the tonsillar region (Ramqvist et al., 2015). We have demonstrated in this study that the base of the tongue of the nude mouse appears to be a preferred target for oral cavity MmuPV1 infection. We postulate that the MmuPV1/mouse model could be very useful for the study of papillomavirus infection at this anatomical site. We also anticipate that the model will be useful for studies of cancer initiation and progression, studies that will have significant relevance to the human condition. Extensive hyperplasia and dysplasia as well as viral CPE were noted in some of the lesions reported here. Cancers develop over time and we hypothesize that given more time these lesions might have progressed to cancer. Addition of a cofactor such as tobacco smoke or ozone could accelerate malignant conversion. Our research with the rabbit model has
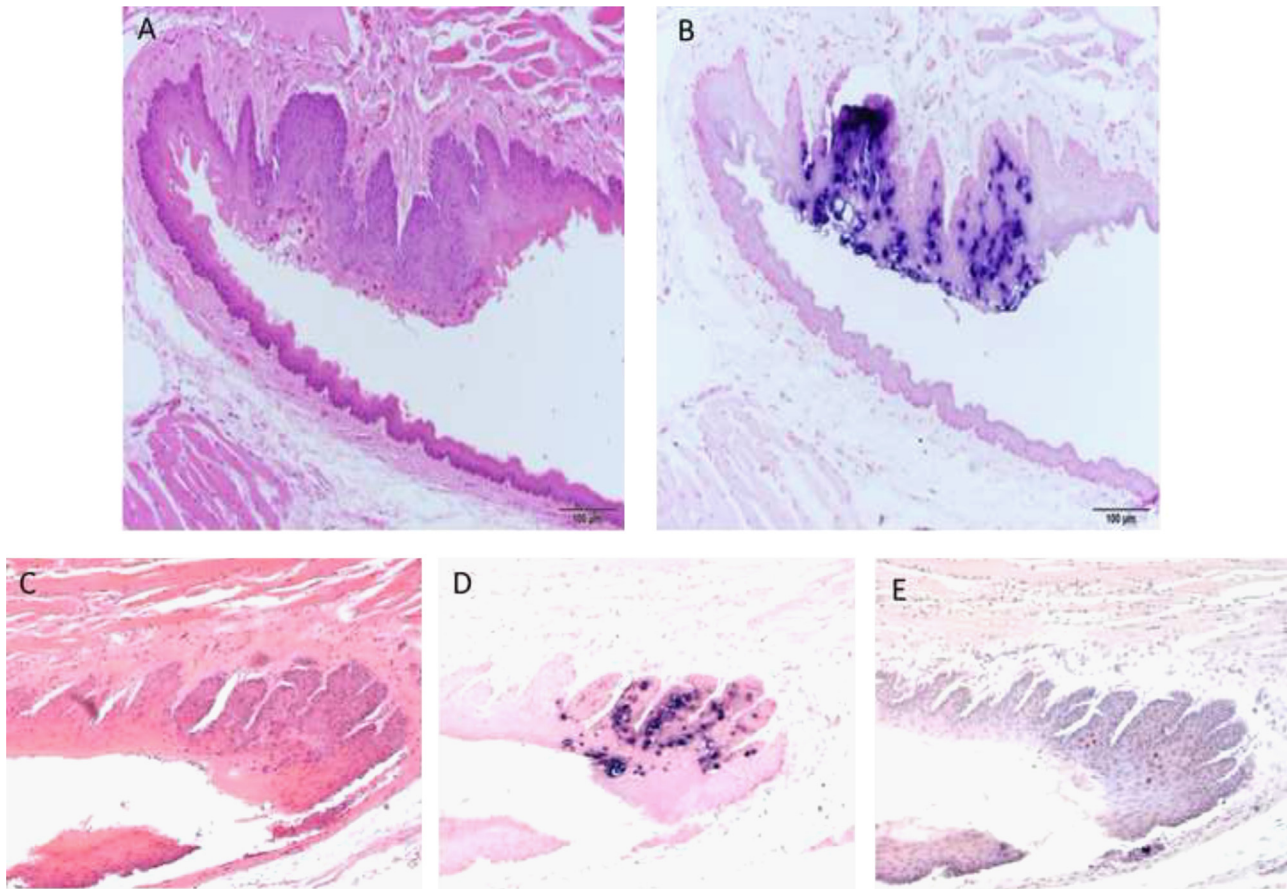
Fig. 6. (A–E) Two lesions were found at the caudal ventral tongue. (A) H and E (100 ×) of a sagittal/oblique section of the caudoventral tongue of animal 6–3R showing the area near the reflection to the sublingual oral mucosa. The animal was experimentally infected at several non-oral mucosal and cutaneous sites. (B) ISH (100 ×) of an adjacent section showing strong staining near the reflection to the sublingual oral mucosa. (C) H and E (100 ×) of a longitudinal parasagittal section of the tongue of animal 7–3L showing the reflection to the sublingual oral mucosa. The animal was originally infected at several non-oral mucosal and cutaneous sites. (D) ISH (100 ×) of an adjacent section showing strong positivity at the reflection to the sublingual oral mucosa. (E) IHC (100 ×) of an adjacent section showing capsid antigen presence in the same areas positive for MmuPV1 DNA.

shown that synonymous codon modifications in the oncogenes E6 and E7 of CRPV result in genomes that yield infections that rapidly progress to cancer (Cladel et al., 2013). MmuPV1 genomes with codon modifications may similarly enhance the malignant potential of this virus.

Another use for this model will be to study papillomavirus infection under conditions of immune suppression, conditions that are naturally present in the Hsd: NU-*Foxn1^(nu)* mouse. As the population of individuals infected with the Human Immunodeficiency Virus (HIV) increases and as more patients receive organ transplants, papillomavirus infections are becoming more prevalent as are associated oral cancers (Syrjanen, 2011; Gaester et al., 2014). Until now, there has been no in vivo model to study the disease under these unique conditions. The MmuPV1/athymic mouse model is ideally suited for these studies.

Conclusions

We have shown in this study that mouse papillomavirus 1 (MmuPV1) infects the oral cavity of outbred nude mice and has a preference for the base of the tongue. Of the 18 tongues of animals infected at sites other than the oral cavity, 10 (56%) had secondary infections at the base of the tongue. The circumvallate papilla and associated Von Ebner's glands on the caudal dorsal tongue was the preferred site of infection with 7 (70%) of infected animals

demonstrating secondary infections only at this locus and not at other oral sites. Two animals (20%) had lesions on the caudal ventral tongue and these lesions targeted the same location suggesting that this might also be a susceptible site. A single lesion was found on the caudal dorsal tongue at the pharynx/palate area. To date, there has been no suitable in vivo model to study oral papillomavirus-associated lesions. Human lesions associated with papillomavirus-induced cancers also tend to occur at the back of the tongue. The MmuPV1 model for papillomavirus infection should provide a viable option for gaining insights into human lesions and associated cancers.

Materials and methods

Viral stock

MmuPV1 virus was isolated from lesions on the tails of mice from our previous study (Cladel et al., 2013). The lesions were scraped from the tail with a scalpel blade and homogenized in phosphate buffered saline (PBS) using a Polytron homogenizer (Brinkman PT10-35) at highest speed for three minutes while chilling in an ice bath. The homogenate was centrifuged at 10,000 rpm and the supernatant was decanted into Eppendorf tubes for storage at $-20\ °C$. For these experiments, the virus was diluted 1:5 in PBS and 200 µl was passed through a 0.2 µm

cellulose acetate sterile syringe filter. This was chased by the addition of 200 µl PBS. The PBS filtrate was added to the filtered virus to give a total of 250 µl sterile virus solution when taking into account loss in the filter. Viral DNA was quantitated following extraction of the DNA from 5 µl of this stock. The DNA was isolated in 50 µl water and SYBR green Q PCR assay was conducted on a series of 1:2 dilutions. Dilutions of plasmid DNA of known concentration were used to develop a standard curve. 1 µl of DNA was determined to contain 0.012ng viral DNA. 1 ng contains 1.2×10^8 copies of viral DNA (http://cels.uri.edu/gsc/cndna.html). Thus, 1 µl of the DNA extract contains 1.4×10^6 copies of viral DNA meaning such that 1 µl of the viral extract contains 1.4×10^7 viral genome equivalents.

MmuPV1 tongue infections

All mouse work was approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University College of Medicine. Outbred athymic nude mice (Hsd:NU-*Foxn1*^nu^) were obtained from Harlan Laboratories and were housed in sterile microisolator cages and were fed sterilized food and water. Four mice, ages 8–12 weeks, were sedated i.p. with ketamine/xylazine mixture (100 mg/10 mg per kg body weight in PBS). Tongues were withdrawn using a sterile forceps and microneedles (Microneedle Systems, LLC, GA) were used to superficially wound both the dorsal and ventral surfaces of the rostral tongues. Some bleeding occurred but care was taken to minimize this bleeding. Animals were allowed to recover overnight. The following day, each animal was again anaesthetized. Tongues were again gently abraded with the microneedles and 10 µl of sterile virus (1.4×10^8 viral genome equivalents) was applied to the freshly abraded surfaces. Animals were monitored weekly and photodocumentation was performed. All work was conducted in biosafety cabinets.

Harvest and analysis of tissues

Animals were euthanized by ketamine/xylazine anesthesia followed by cervical dislocation and the entire tongue, with submandibular and palatine mucosae attached, was excised. Specimens were routinely processed to paraffin blocks. Sequential sections were cut for H and E analysis, in situ hybridization and immunohistochemistry. Standard conditions were used for H and E staining. A subgenomic fragment of MmuPV1 (3913bp EcoRV/BamH1 fragment) was used as an in situ hybridization probe for the detection of MmuPV1 DNA in tissues. The probe was biotinylated using the random priming method and diluted in a hybridization cocktail described in previous work (Cladel et al., 2013). Access to target DNA was obtained with 0.2 mg/ml pepsin in 0.1 N HCl incubation at 37 °C for 8 min. After thorough washing, the biotinylated probe was applied and heated to 95 °C for 5 min to achieve dissociation of target and probe DNA. Reannealing was allowed to occur for 2 h at 37 °C. Target-bound biotin was detected using a streptavidin AP conjugate followed by colorimetric development in BCIP/NBT. For immunohistochemical (IHC) L1 capsid detection, a goat Group Specific Antibody (GSA) to a conserved region of L1 (ViroStat #5001) was used on FFPE sections. Detection was achieved using the ImmPRESS anti-goat IgG polymer system (Vector #MP-7405). Tissues were examined by an ACVP diplomate pathologist blinded to treatment. All images were obtained with an Olympus BX51 microscope and DP71 digital camera using cellSens Standard 1.12 imaging software (Olympus America, Center Valley, PA).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.10.030.

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