Development of a Tongue Carcinoma Model Using Real-Time *in Vivo* Molecular Monitoring

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Abstract

Head and neck squamous cell carcinoma (HNSCC) poses a significant clinical challenge, with an incidence of 600,000 cases and a survival rate of 50%. Mouse models of cancer have provided critical insights into disease mechanisms, and there remains an urgent need for translational HNSCC models. In this proposal we describe a novel viral-mediated inducible mouse model of HNSCC based on somatic induction and tongue-specific loss of TP53/PTEN. Importantly, this model is coupled with Cre-dependent luciferase expression to facilitate longitudinal, noninvasive bioluminescence imaging of gene recombination and tumor formation. C57BL/6 mice with floxed TP53/PTEN and a lox-stop-lox luciferase allele (ROSA26 LSL-luc TP53-/- PTEN-/-) were engineered for the proposed model. Adenovirus or adeno-associated virus containing a Cre-recombinase expression vector was injected directly into the tongue mucosa to promote site-specific Cre expression and gene recombination. Mice were evaluated for luciferase expression using intraperitoneal luciferin delivery and *in vivo* molecular imaging using the Ami X bioluminescence imager and analysis software provided from Spectral Instruments Imaging. Injected mice were followed longitudinally with real-time bioluminescence imaging to evaluate gene recombination and tumor development.

A pilot cohort of eight ROSA26 LSL-luc TP53-/- PTEN-/- mice underwent a single tongue adeno-Cre injection with a range of viral titers. Mice were imaged 24 hours post-exposure and biweekly thereafter. The Ami X imaging system was sufficiently sensitive to detect gene recombination and luciferase expression in the tongue as early 24 hours post-injection, suggesting efficient viral delivery and recombination. In addition to luciferase expression in the tongue, signal was observed in draining lymph nodes, alerting us to limitations in the specificity of our viral delivery and prompting us to evaluate adeno-associated virus serotypes. Luciferase expression in the tongue as measured by *in vivo* bioluminescence imaging increased over time as cells with presumed TP53/PTEN loss continued to grow and divide. The initial pilot mouse, which was injected in the tongue and bilateral flanks with varying titers of adeno-Cre, developed a tumor at the site of greatest viral exposure (right flank) after 10 weeks. Bioluminescence at the flank as well as at the tongue, which received an intermediate viral dose, demonstrated a steady increase in bioluminescence over time, with *in vivo* signal significantly preceding tumor palpation or visualization.

This project describes our progress in developing an innovative model of tongue carcinoma based on viral-mediated TP53/PTEN knockout coupled with luciferase expression for *in vivo* longitudinal tumor imaging. Successful development of this model will help us gain a better understanding of HNSCC initiation and progression, and real-time molecular imaging of cancer cells *in vivo* will allow us to monitor tumor dynamics and metastasis. In the future, this model may serve as a valuable translational tool for monitoring preclinical responses to novel treatment algorithms.

Oral Tongue Cancer: Facts and Figures

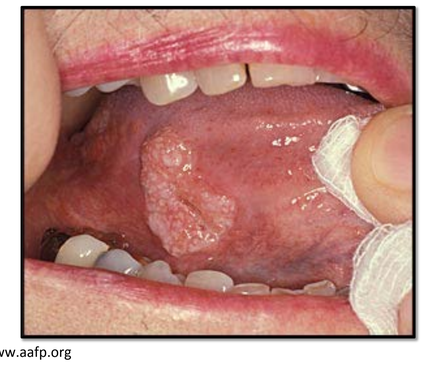


Image credit: http://www.aafp.org

- **Tongue is the most common site for oral cavity cancer**
- **ACS estimates 36,000 oral cavity diagnoses in 2013**
- **An estimated 6,850 people will die of oral cavity cancer in 2013**
- **5-year survival for Stage IV disease is 37%**
- **Mean age of diagnosis is 62 years old**
- **Risk factors: tobacco and alcohol use**
- **>90% squamous cell carcinomas**
- **Implicated tumor suppressors: TP53, NOTCH1, CDKN2A, PTEN, PI3KCA, HRAS**

Head and Neck Mouse Models in the Literature

Gene(s)	Model	Findings	Ref.
TP53/ Cyclin D1	EBV ED-L2 promoter drives Cyclin D1 in the presence of global TP53-/-	-L2-cyclin D1 mice develop dysplasia -L2-cyclin D1 p53-/- mice develop invasive oro-esophageal cancer and die from alternate primaries	Opitz et al. , J Clin Invest, 2002.
TP53/Rb	K14 HPV E6 and E7 transgenic mice with chemical carcinogen in drinking water	-E6/E7 expression in stratified epithelia causes spontaneous skin tumors and epithelial proliferation -E6/E7 + carcinogen mice develop oro-esophageal cancer	Strati et al., PNAS, 2006.
TGFβRII/ K-Ras	Inducer application to oral cavity causes K5-Cre TGFβRII deletion and loss of one K-Ras allele	-TGFβRII-/- only causes no spontaneous tumors -K-Ras-/+ causes benign papillomas -TGFβRII-/- and K-Ras-/+ causes aggressive oral cavity tumors	Lu et al., Gen & Dev, 2006.
TP53	TP53+/+ or -/+ with oral cavity chemical carcinogen application	-100% of mice treated with twice weekly oral cavity carcinogen developed tumors -p53 -/+ vs. +/+ mice have decreased tumor latency	Ku et al., Mol Canc Res, 2007.
TGFβRI/ PTEN	Inducer application to oral cavity causes K14-Cre TGFβRI and PTEN loss	-Single allele loss causes hyperproliferation, no tumors -TGFβRI-/- PTEN-/- mice develop spontaneous mucosa tumors	Bian et al., Oncogene, 2012.

Table: Overview of transgenic oral cavity carcinoma models described in the literature. The majority of models rely on additional chemical carcinogen application or topical application of recombination inducers to the oral cavity.

TP53/PTEN Inducible Knockout Mice and Viral Delivery Technique

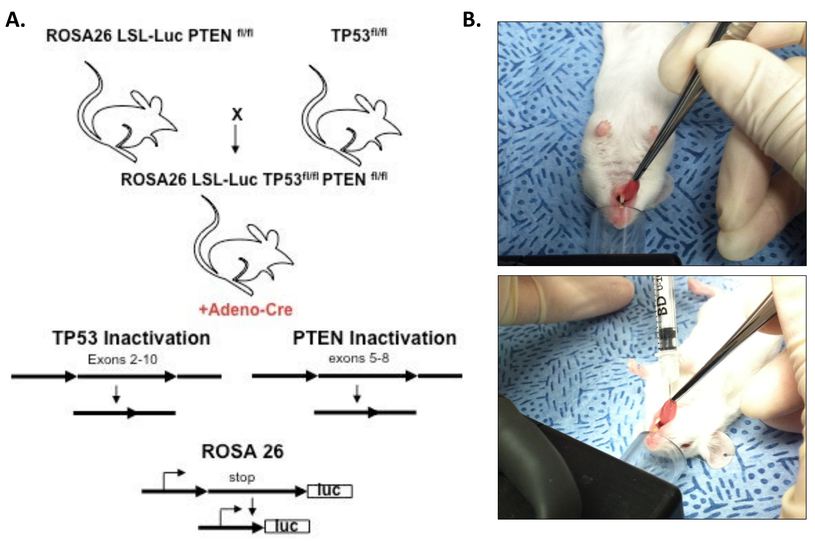
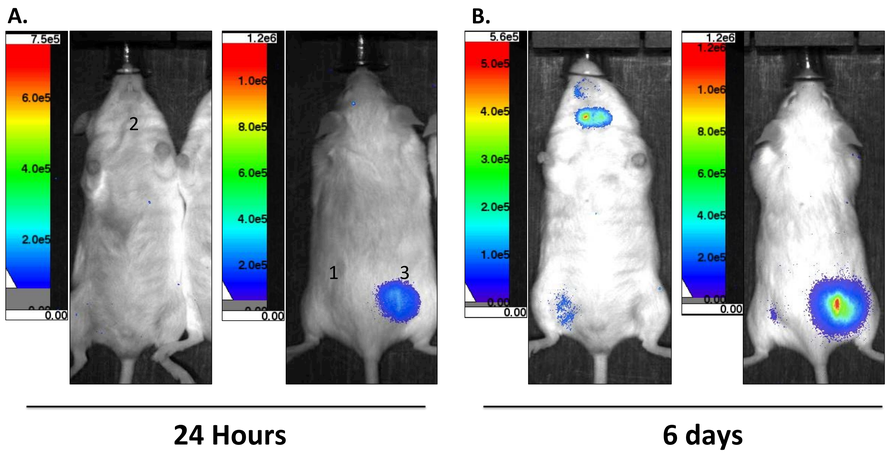


Figure 1: Engineering TP53/PTEN inducible knockout mice and method of viral delivery to the oral tongue. A) C57BL/6 ROSA26 LSL-luciferase PTEN$^{fl/fl}$ mice were crossed with TP53$^{fl/fl}$ mice to create offspring with floxed TP53 and PTEN alleles susceptible to inducible somatic knockout. These mice are designed to lose TP53 and PTEN expression and gain luciferase expression in the presence of Cre-recombinase. B) Cre-recombinase is delivered by direct adenoviral injection into the anterior dorsal tongue. Once anesthetized, the mouse is placed supine and the oral tongue is gently retracted using blunt forceps (top panel). With the anterior oral tongue exposed, a 30 gauge insulin syringe is advanced superficially into the mucosa of the tongue and the viral suspension is injected (bottom panel).

Increase in Luciferase Signal Intensity Post-Injection: Pilot Mouse



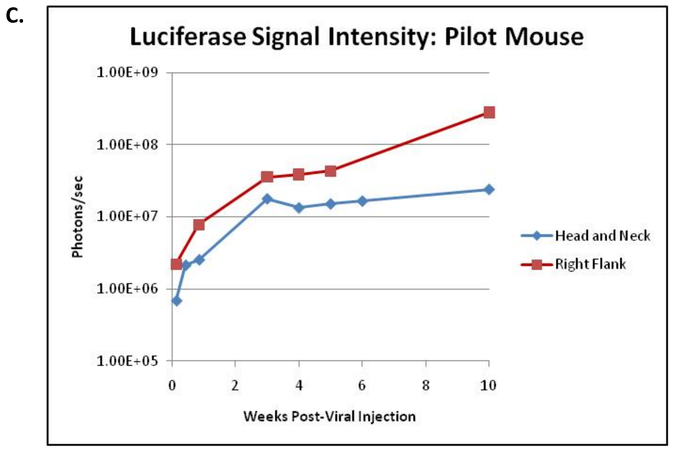


Figure 2: C57BL/6 LSL-luc TP53$^{fl/fl}$ PTEN$^{fl/fl}$ pilot injections and luciferase signal intensity. A) Luciferase expression is visualized in the pilot mouse as early as 24 hours post-viral exposure. Ad5 CMV Cre-eGFP was obtained from the Gene Transfer Vector Core and was resuspended in PBS at various titers. Injections were performed in the oral tongue and bilateral flanks. Site 1 = 1 x 10^7 PFU, Site 2 = 1 x 10^8 PFU, Site 3 = 1 x 10^9 PFU. The pilot mouse was injected intraperitoneally with D-luciferin at 24 hours and 6 days post-viral exposure, and real-time *in vivo* bioluminescence was measured using the Ami X imaging system. B) Luciferase signal intensity increases over time. Luciferase signal intensity was measured in the head and neck region or the right flank using AMIView software to specify the region of interest. Signal intensity is depicted in photons per second on a logarithmic scale over the course of 10 weeks.

Tumor Growth: Pilot Mouse

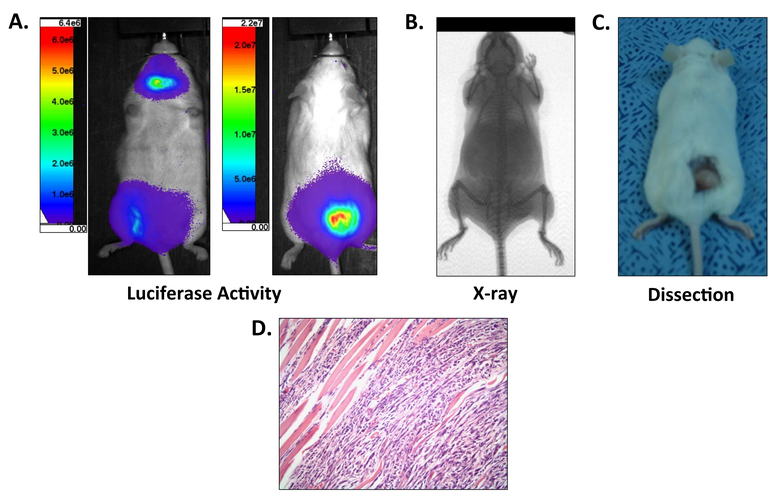


Figure 3: Tumor growth in the TP53$^{fl/fl}$ PTEN$^{fl/fl}$ pilot mouse at the site of greatest viral injection. A) Luciferase expression in the pilot mouse 10 weeks post-viral injection demonstrating high luciferase signals in the right flank and head and neck regions. B) X-ray image of the flank mass demonstrating a soft tissue tumor with no apparent bony involvement. C) Photograph of the mouse during dissection. Exposed is a solid tumor mass, approximately 1.1cm in diameter, overlying and slightly adherent to the underlying muscle. D) Hematoxylin and eosin image from the flank tumor depicting a sarcoma with invasion into the adjacent skeletal muscle.

Current TP53/PTEN Cohort

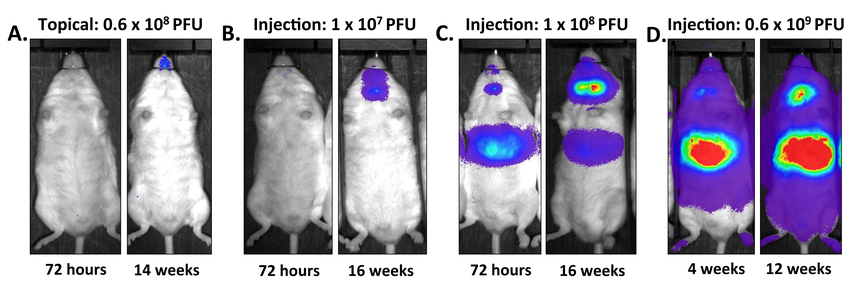


Figure 4: C57BL/6 LSL-luc TP53$^{fl/fl}$ PTEN$^{fl/fl}$ mice exposed to various titers of Ad5 CMV Cre-eGFP. Luciferase signal is shown at an early timepoint and at a later timepoint for each titer tested. Signal intensity increases over time in all experimental groups. A) Luciferase activity in a mouse exposed to topical virus on the dorsal tongue for 45 minutes. B) Mouse injected with low-dose virus. C) Mouse injected with intermediate-dose virus. D) Mouse injected with high-dose virus.

Testing Adeno-Associated Virus Serotypes

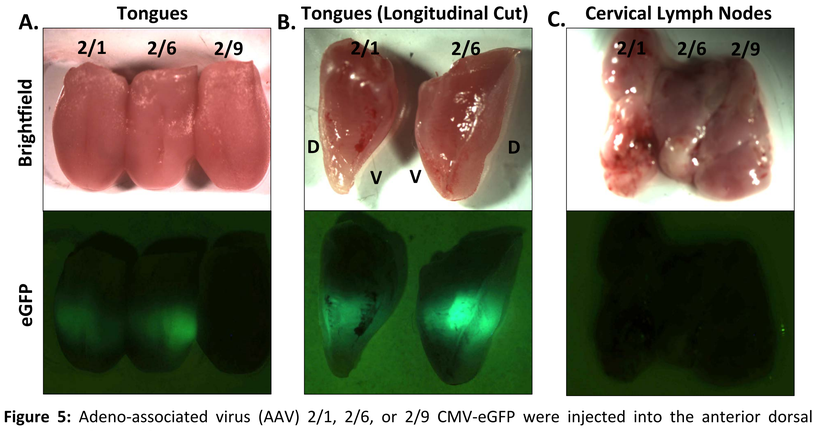


Figure 5: Adeno-associated virus (AAV) 2/1, 2/6, or 2/9 CMV-eGFP were injected into the anterior dorsal tongue of C57BL/6 mice to test for tongue-specific vector delivery. Mice were euthanized 14 days post-injection. Tongues and cervical lymph nodes were dissected and examined for eGFP expression. A) Brightfield images of tongues injected with AAV 2/1, 2/6, or 2/9 are shown demonstrating no gross signs of inflammation. Corresponding eGFP native fluorescence shows eGFP expression by AAV2/1 and 2/6. B) Tongues injected with AAV2/1 or 2/6 were sectioned longitudinally and imaged cut-side up. (D) = dorsal, (V) = ventral. C) Cervical lymph node chains corresponding to tongues injected with AAV 2/1, 2/6, or 2/9 were examined for eGFP expression. Brightfield images demonstrate no gross asymmetry or lymph node enlargement. Fluorescent images show no eGFP expression in the draining lymph nodes.

Next Steps

- **Monitor C57BL/6 LSL-luc TP53$^{fl/fl}$ PTEN$^{fl/fl}$ Ad5 CMV Cre-eGFP mice for progression of luciferase signal and tumor growth**
- **Inject new cohort with AAV 2/1 or 2/6 CMV-Cre to better characterize specificity and recombination efficiency**
- **Troubleshoot viral topical delivery technique**

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