Journal of Visualized Experiments

In-vitro establishment of a genetically engineered murine head and neck cancer cell line using an adeno-associated virus - Cas9 system.

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60410R2
Full Title:	In-vitro establishment of a genetically engineered murine head and neck cancer cell line using an adeno-associated virus - Cas9 system.
Section/Category:	JoVE Cancer Research
Keywords:	Head and neck cancer; murine cancer models; genomic editing- CRISPR; adeno-associated virus vector; in vitro cell transformation; tumor cell line
Corresponding Author:	Moshe Elkabets Ben-Gurion University of the Negev Beer Sheva, Israel ISRAEL
Corresponding Author's Institution:	Ben-Gurion University of the Negev
Corresponding Author E-Mail:	moshee@bgu.ac.il
Order of Authors:	Manu Prasad
	Sankar Jagadeeshan
	Maurizio Scaltriti
	Irit Allon
	Moshe Elkabets
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Ben Gurion University of the Negev, Beer Sheva, Israel

EXPLORING WAYS TO FIGHT CANCER
Elkabets' lab

15 October 2019

Dear Dr Bajaj, Review Editor *JoVE*

I am pleased to submit the revised article entitled "**In-vitro establishment of a genetically engineered murine head and neck cancer cell line using an adeno-associated virus - Cas9 system**" for consideration for publication in *JoVE*.

We are grateful to your encouraging and thoughtful comments and suggestions regarding our revised submission. In response to these comments, we have made a number of modifications to our MS.We hope the reviewers will find the MS improved following these changes and more suitable for publication in JoVE.

Thank you for your consideration of this article.

Sincerely

Moshe Elkabets

Moshe Elkabets, Ph.D,
Senior Lecturer (Assistant Professor)
The Shraga Segal Dept. of Microbiology, Immunology and Genetics
Faculty of Health Sciences,
Ben-Gurion University of the Negev
Beer-Sheva, 84105
Israel.
email: moshee@bgu.ac.il | website: www.elkabetslab.com
Phone: +972-53-2323-987 | Tel: +972-8-6428846

TITLE:

In Vitro Establishment of a Genetically Engineered Murine Head and Neck Cancer Cell Line using an Adeno-Associated Virus-Cas9 System

AUTHORS AND AFFILIATIONS:

Manu Prasad[1, 2*], Sankar Jagadeeshan[1, 2*], Maurizio Scaltriti[3], Irit Allon[2, 4], Moshe Elkabets[1, 2]

[1]The Shraga Segal Department of Microbiology, Immunology and Genetics, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

[2]Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

[3]Human Oncology & Pathogenesis Program (HOPP), Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, NY, 10065, USA

[4]Institute of Pathology, Barzilai University Medical Center, 2 Hahistadrut St, Ashkelon

[*]These authors contributed equally.

Corresponding Author:

Moshe Elkabets (moshee@bgu.ac.il)

Email addresses of co-authors:

Manu Prasad (manupras@post.bgu.ac.il)

Sankar Jagadeeshan (jagadees@post.bgu.ac.il)

Maurizio Scaltriti (scaltrim@mskcc.org)

Irit Allon (allonirit1@gmail.com)

KEYWORDS:

head and neck cancer, murine cancer models, genomic editing, CRISPR, adeno-associated virus vector, in vitro cell transformation, tumor cell line

SUMMARY:

Development of murine models with specific genes mutated in head and neck cancer patients is required for understanding of neoplasia. Here, we present a protocol for in vitro transformation of primary murine tongue cells using an adeno-associated virus–Cas9 system to generate murine HNC cell lines with specific genomic alterations.

ABSTRACT:

The use of primary normal epithelial cells makes it possible to reproducibly induce genomic alterations required for cellular transformation by introducing specific mutations in oncogenes and tumor suppressor genes, using clustered regulatory interspaced short palindromic repeat (CRISPR)-based genome editing technology in mice. This technology allows us to accurately mimic the genetic changes that occur in human cancers using mice. By genetically transforming murine primary cells, we can better study cancer development, progression, treatment, and diagnosis. In this study, we used Cre-inducible Cas9 mouse tongue epithelial cells to enable genome editing using adeno-associated virus (AAV) in vitro. Specifically, by altering KRAS, p53, and APC in normal

tongue epithelial cells, we generated a murine head and neck cancer (HNC) cell line in vitro, which is tumorigenic in syngeneic mice. The method presented here describes in detail how to generate HNC cell lines with specific genomic alterations and explains their suitability for predicting tumor progression in syngeneic mice. We envision that this promising method will be informative and useful to study tumor biology and therapy of HNC.

INTRODUCTION:

HNC is a common malignancy worldwide[1]. Modeling the genesis of HNC neoplasia is currently at a scientific turning point[2]. While many genetic mutations have been identified in HNC[2–4] (e.g., TP53, PIK3CA, NOTCH1, FAT1, and RAS) the specific combinations of genomic alterations required in concert to induce HNC remain unclear.

The current use of human HNC cell lines has significantly helped to elucidate the mechanisms associated with pathogenesis and treatment[3]. However, the study of human cell lines in immunocompromised murine systems has its limitations, because these systems do not address the in vivo neoplastic process, the role of specific gene mutations, and treatment responses in an immune microenvironment. Hence, the development and establishment of murine cell lines with specific genetic alterations are of primary importance to study how different genes contribute to the transformation process and to test novel molecule-based therapies in immunocompetent mice.

Gene function studies in biomedical research have been significantly affected by advances in DNA editing technologies, by introducing double-strand breaks (DSBs), for example[5]. These technologies, including the use of zinc finger nucleases, transcription activator-like effector nucleases, and clustered regulatory interspaced short palindromic repeats (CRISPR/Cas9), allow for the manipulation of any gene of interest at its locus. The latest CRISPR/Cas9 systems is comprised of a guide RNA (gRNA) that directs the Cas9 nuclease to generate a DSB at a specific site in the genome. This system has gained extensive recognition in modifying endogenous genes in any cell or target tissue, even in the most traditionally difficult-to-treat organisms[5]. It has multiple advantages over other systems due to its simplicity, speed, and efficiency.

In oncology, CRISPR/CAS9 technology has fulfilled the need to effectively mimic cancer cells. To establish this system in HNC, we manipulated the potent KRAS oncogene and two important tumor suppressor genes, APC and p53[6]. According to the GENIE database[7], this combination is rare in HNC. RAS mutations (HRAS, NRAS, and KRAS) are present in only ~7% of all HNC populations. These tumors tend to be resistant to therapy[8,9].

Delivery of Cas9 and its gRNA is achieved through viral transduction using either AAVs or lentiviruses. Recombinant AAV is often the preferred method for delivering genes to target cells owing to its high titer, mild immune response, ability to transduce a broad range of cells, and overall safety. Using an AAV system, various tissue-specific mouse cell lines have been generated, and new cell lines are still being developed[10–12]. However, an efficient genomic editing system that can generate murine HNC cell line models cells remains to be developed. In this study, we sought to develop an in vitro AAV-Cas9-based system for transforming primary murine tongue

cells into a tumorigenic state. This unique CRISPR/Cas9 transformation protocol and the established tumor cell line can be used to better understand the biology of HNC induced by a diversity of genomic alterations.

PROTOCOL:

This study was approved by the Ben Gurion University of the Negev Animal Care and Use Committee. Animal experiments were approved by the IACUC (IL.80-12-2015 and IL.29-05-2018(E)). All aspects of animal testing, housing, and environmental conditions used in this study were in compliance with The Guide for the Care and Use of Laboratory Animals[13].

1. Adeno-associated virus production

1.1. Day 1: Cell culture

1.1.1. Seed 4 x 10^6 HEK293T cells per 14.5 cm plate in 15 mL of DMEM. Prepare 10 plates for transfection with polyethylenimine (PEI) (1 µg/µL).

1.2. Day 2: Transfection of HEK293T cells using PEI

1.2.1. Remove media and refeed with warm DMEM 1 h before the transfection.

1.2.2. Prewarm transfection reagents and DMEM.

1.2.3. Use 10 µg of the plasmid of interest containing AAV ITR (AAV pCM109 EFS Cre sg APC sg KRAS sg P53- KRAS HDR), 10 µg of the AAV 2/9n capsid plasmid (with the rep gene of AAV2 and the cap gene of AAV9, and 10 µg of the helper plasmid (pAdDelta F5 helper) per plate (See **Table of Materials**).

NOTE: A new generation of helper plasmid -pAdDeltaF6[14–16] that can also be used for AAV production is available.

1.2.4. Mix the plasmids in 1 mL of plain DMEM per plate. Then add 90 µL of polyethylenimine (total plasmid: PEI concentration =1:3) to the plasmid mix and vortex briefly.

1.2.5. Incubate the PEI-plasmid DNA mix for 20 min at room temperature (RT).

1.2.6. Add the mix dropwise on top of the HEK293T cells and transfer the plates to the cell culture incubator at 37 °C with 5% CO_2 for 24 h.

1.3. Day 3: Medium change after transfection

1.3.1. Remove the medium completely and add 15 mL of fresh DMEM.

1.4. Day 5: Harvesting the virus

1.4.1. Prepare a dry ice/ethanol bath.

1.4.2. Collect the cells with a cell scraper and transfer them into 50 mL tubes (2 plates per 50 mL tube).

1.4.3. Spin tubes at 800 x *g* for 15 min at room temperature.

1.4.4. Discard the supernatant from all the tubes. Add 0.5 mL of the lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH = 8.5) per plate (i.e., 5 mL for 10 plates) to the first tube only. Resuspend the cells and transfer the total volume to the next tube and continue until the last tube.

1.4.5. Transfer the cell suspension to a fresh 50 mL tube. Wash the tubes with the same volume of lysis buffer (0.5 mL per plate) using the same transfer method as described in step 1.4.4.

1.4.6. Subject the cell suspension to three rounds of ~10 min freeze/thaw cycles between a dry ice/ethanol bath and a 37 °C water bath. Vortex briefly after each thawing.

1.4.7. If the purification is carried out on the same day, set the equilibration and elution buffer (see **Table 1** for the recipe) at RT.

1.4.8. Add 50 units of benzonase per plate and incubate at 37 °C for 1.5 h.

NOTE: Benzonase is used to digest residual nucleic acids from the host producer cells and the plasmid DNA present in the cell suspension.

1.4.9. Spin the tubes at 3,000 x *g* for 15 min at 4 °C.

1.4.10. Collect the supernatant in a syringe using a 18 G steel needle and push the solution through a 0.45 µm filter into a 15 mL tube to obtain the crude lysate.

1.4.11. Store the crude lysate at 4 °C for a few weeks until purification or continue purification.

2. AAV purification

2.1. Day 5 or later

2.1.1. Place the equilibration and elution buffer at RT.

2.1.2. Wash chromatography columns with 10 mL of the equilibration buffer.

2.1.3. Add 0.5 mL per plate of heparin-agarose to the column[17] and then add the equilibration buffer (4x the volume of the heparin-agarose). Mix the solutions by inverting the column and let the agarose sediment.

2.1.4. Elute the equilibration buffer from the column by gravity.

NOTE: Leave some equilibration buffer to prevent the agarose from drying out.

2.1.5. Load the crude lysate onto the column and incubate for 2 h at 4 °C with constant agitation. Bring the column into an upright position and allow the agarose to sediment.

2.1.6. Elute the crude lysate by gravity.

2.1.7. Wash the column with equilibration buffer (4x the volume of the heparin-agarose).

2.1.8. Place a 100 kDa centrifugal protein filter below the column and elute the virus using an elution buffer (3x the volume of the heparin-agarose) into the filter.

NOTE: The elution buffer should not exceed 15 mL.

2.1.9. Centrifuge the filter at 3,000 x *g* for ~30 min until less than 1 mL is left in the filter.

2.1.10. Fill up the filter with PBS and centrifuge at 3,000 x *g* for ~30 min until less than 1 mL is left in the filter. Repeat this step 2x to remove all salts.

2.1.11. Concentrate the virus solution in the filter by centrifugation at 3,000 x *g* to arrive at a volume as small as possible (less than 200 µL).

2.1.12. Aspirate the virus solution with a needle and syringe and push the solution through a 0.22 µm filter into a tube.

2.1.13. Make aliquots of concentrated viral particles for storage.

NOTE: The aliquots should be ~20 µL for short-term storage at 4 °C and ~5 µL for long-term storage at -80 °C.

2.1.14. Determine the viral titer and viral transduction efficiency as described previously[14,18–20].

3. Isolation and culture of primary cells

3.1. Day 1

3.1.1 Euthanize a 6-week-old male or female B6;129-Gt(ROSA)26Sor$^{tm1(CAG-cas9*,-EGFP)Fezh}$/J by CO_2 asphyxiation or any other IACUC approved protocol.

NOTE: These CRISPR/Cas9 knockin mice have Cre recombinase-dependent expression of *cas9* endonuclease and EGFP directed by a CAG promoter. The upstream Lox-Stop-Lox (LSL) sequence present in the genome of these mice prevent the expression of Cas9 and EGFP in the absence of Cre recombinase. When used in combination with single guide RNAs (sgRNAs) and a Cre source, they allow editing of single or multiple mouse genes in vivo or ex vivo[14].

3.1.2 Dissect the tongue from the euthanized mice using surgical scissors.

3.1.3 Manually dissociate the tissue by mincing the tongue tissue into very small fragments using a scalpel. Collect the tissue fragments in a 15 mL tube.

3.1.4 Add the triple enzyme mix (see **Table 1** for the recipe) to the tissue fragments.

3.1.5 Incubate the tissue-enzyme mix at 37 °C for 30 min and tap the tube every 10 min to enhance enzymatic dissociation of the tissue.

3.1.6 Add 5% fetal bovine serum (FBS) containing HBSS/PBS to the tissue-enzyme mix to stop the enzyme action.

3.1.7 Filter the above cell suspension through a sterile 70 µm nylon mesh to separate the dispersed cells and larger tissue fragments.

3.1.8 Wash the filtered cell suspension by centrifugation for 800 x *g* for 5 min in HBSS/PBS at RT.

3.1.9 Resuspend the pellet in culture medium (10% FBS in RPMI/DMEM) and grow in 60 mm culture dishes until distinct cell colonies are formed.

3.1.9.1. Culture cell aggregates retained on top of the filter in a 60 mm culture dish containing 3 mL of complete media (10% FBS in RPMI/DMEM) until cell colonies are formed.

3.1.10 Microscopically examine the primary cells for the presence of fibroblast contamination after 1 week of culture. Treat the primary cells developed from aggregates and cell suspensions with 0.25 trypsin 0.02% EDTA solution at 37 °C for 1 min to remove fibroblasts.

NOTE: Usually the primary culture from cell aggregates produces more colonies compared to single-cell suspensions. The cells from these colonies provide better transduction efficiency with AAV transduction.

4. AAV transduction of primary cells

4.1. Day 10

4.1.1. Seed 2 x 10^5 primary cells per well in 6 well plates in 2 mL of complete media (10% FBS in RPMI/DMEM).

4.1.2. The next day, transduce the cells with 10^{12} viral genome/mL (10^{10} transducing units/mL) and incubate the cells in viral particle-containing media for 48 h at 37 °C.

4.1.3. Remove the viral particle-containing media and feed the AAV-transduced cells with complete media.

NOTE: Only cells that underwent transduction will express GFP and start to proliferate. Cells that did not undergo transduction will eventually die within 2 weeks.

4.1.4. After 2 weeks of culture expansion, seed the cells for validation and the in vivo tumorigenic experiments.

NOTE: Genomic DNA from AAV-transduced cells and normal primary cells from Cas9 mice were extracted for validating specific genome editing using standard protocols. Sequencing of the extracted DNA and analysis of the sequenced data (**Table 2**) were performed using a hybridization capture-based next-generation sequencing assay (e.g., MSK-IMPACT platform) as described previously[21].

5. Validating the transformation of normal cells to tumorigenic cells using immunofluorescence and western blotting

5.1. Immunofluorescence

5.1.1. Seed 2 x 10^5 cells on 12 mm glass coverslips and place them in an incubator overnight.

5.1.2. Fix cells in 4% paraformaldehyde in PBS (pH = 7.4) and process for immunofluorescence labeling.

5.1.3. Incubate the fixed cells with the first primary antibody in 1% BSA or 1% serum in PBST in a humidified chamber for 1 h at RT or overnight at 4 °C. The primary antibodies used are the rabbit monoclonal anti-KRT 14, rabbit monoclonal anti-E-cadherin antibody, and Cas9 mouse mAb.

5.1.4. Remove the primary antibody solution from the coverslips by washing the cells 3x for 5 min with 1x PBS.

5.1.5. Incubate the cells with Cy3 donkey anti-rabbit IgG and/or Cy3 goat anti-mouse IgG secondary antibodies in 5% BSA in PBST for 1 h at RT in the dark.

5.1.6. Remove the secondary antibody solution from the coverslips and wash the cells 3x as described in step 5.1.4.

5.1.7. Mount the coverslips with a drop of mounting medium containing DAPI (DAPI Fluoromount).

5.1.8. Store in the dark at 4 °C until the slides are imaged using fluorescence microscopy.

5.2. Western blotting

5.2.1. Seed 1 x 10^6 transformed cells in a 100 mm culture dish and place them in an incubator overnight.

5.2.2. Wash and scrape the transformed cells into 200 µl ice cold PBS.

5.2.3. Centrifuge the tube containing the cell suspension for 10 min at 2,000 x *g* at 4 °C to pellet the cells.

5.2.4. Aspirate the supernatant and lyse the cells using lysis buffer (see **Table 1**) containing phosphatase inhibitor cocktails and a protease inhibitor for 10 min at 4 °C . Centrifuge the lysates for 10 min at 10,000 x *g* and 4 °C and collect the cleared lysates.

5.2.5. Use a commercially available Bradford assay kit to determine the protein concentration following the manufacturer's protocol. Use 4x sample buffer (500 mM Tris pH = 6.8, 40% glycerol, 8% SDS, 20% H_2O, 0.02% bromophenol blue) to adjust the protein samples to 0.5 or 1 µg/µL. Boil at 96 °C for 5 min.

NOTE: The samples can be stored at -80 °C or until a Western blot analysis is performed.

5.2.6. Separate equal amounts of total lysate (30 µg) by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Ensure that the dye reaches the bottom of the gel. Transfer the protein to the nylon membrane by semi-dry blotting at 25 V for 30 min.

5.2.7. Pour 5% BSA in Tris-buffered saline (TBS)-0.1% Tween (TBST) over the membrane to cover it completely. Block the membrane for 1 h at RT. Incubate the mebrane with primary antibodies (anti-Cre, anti-Cas9, anti-GFP, anti-β catenin, anti p53, anti-phospho-ERK, and anti-β actin diluted in 5% BSA TBST) at 4 °C overnight.

5.2.8. After incubation, wash the membranes for 10 min with 1x TBST making sure that the solution covers the membrane completely. Perform this wash 3x, and then add horseradish peroxidase (HRP)-conjugated secondary antibody (diluted 1:10,000 in 5% BSA TBST) to the membrane. Incubate for 1 h at RT.

5.2.9. After incubation, wash the membranes for 10 min with 1x TBST making sure that the solution covers the membrane completely. Perform the chemiluminescence imaging (see **Table of Materials**) to expose the bands, and capture images accordingly.

5.3. Validating the tumorigenic potential of transformed cells in immunocompetent mice

NOTE: Mice were maintained and treated according to the institutional guidelines of the Ben-Gurion University of the Negev. NOD.CB17-Prkdc-scid/NCr Hsd (NOD.SCID) and C57BL/6 mice were used for the in vivo studies. Mice were housed in air-filtered laminar flow cabinets with a 12 hour light/dark cycle and were fed food and water ad libitum.

5.3.1. Use 6–8 week-old female NOD.CB17-Prkdc-scid/NCr Hsd (NOD.SCID) and C57BL/6 mice for the study.

5.3.2. Trypsinize the AAV-Cas9 transformed cells. Stop the trypsinization using DMEM prewarmed at 37 °C and collect in a 50 mL tube.

5.3.3. Centrifuge the tube at 800 x *g* for 10 min at room temperature. Discard the supernatant and resuspend the cell pellet in DMEM medium without FBS. Perform the centrifugation again and resuspend the cell pellet in 1x PBS.

NOTE: Do not keep the cells in 1x PBS for too long. Always keep the cell suspension on ice to prevent cell clumping.

5.3.4. Use an automatic cell counter to count the cells and dilute the cells to the desired concentration (2.5 x 10^7 cells/mL) using 1x PBS. Generate tumors through a subcutaneous injection of the AAV-Cas9 transformed cell suspension in the right flank of each NOD.CB17-Prkdc-scid/NCr Hsd (NOD.SCID) mouse (2 x 10^6 cells/mouse). To generate an orthotropic model in syngeneic mice, inject 0.5 × 10^6 primary cells or the AAV-Cas9 transformed cells into the tongue of C57BL/6 immunocompetent mice.

5.3.5. Euthanize the animals 2 weeks postinjection by CO_2 asphyxiation, and dissect the tumors from the euthanized mice for immunohistochemistry analysis.

REPRESENTATIVE RESULTS:

Using the AAV system to transform normal Cas9 cells

Figure 1 provides a detailed vector map of the AAV transgene plasmid used in this study. **Figure 2** outlines the design and working of the AAV-Cas9 based-system. To produce viral particles, the HEK293T cells were transfected with the AAV transgene vector and other viral packaging vectors using the PEI transfection method. After transfection, the virus-containing cells were collected and lysed, and using the heparin-agarose purification process, the viral particles were purified and concentrated (**Figure 2A**). These purified viral particles were used for the in vitro transformation of primary cells isolated from Cas9 mice to check the efficacy. In our system, the AAV transgene expression cassette is introduced through homologous recombination into the Cre-dependent Cas9 Rosa26 targeting template within mouse cells that carry an ubiquitous CAG promoter (pCAG), a LoxP-Stop-LoxP cassette (LSL), a Cas9 nuclease gene, a self-cleaving P2A peptide, and the reporter gene (EGFP) flanked by two homologous arms[14]. The Cre recombinase activity of the transduced cells excises the LSL and induces Cas9 and GFP expression (**Figure 2B**).

Cas9 nuclease activity introduces a double-stranded break at the target sites of interest, directed through gRNAs, and helps in gene editing. The AAV system used in this study helps in delivering multiplexed gRNAs (sgRNAs for KRAS, p53, and APC) and also in expressing an oncogenic $KRAS^{G12D}$ allele through homology-directed repair (HDR) in the transduced cells.

[Place Figure 1 and 2 here]

Validation of the AAV-Cas9 system in vitro in isolated mouse embryonic fibroblast cells

Primary mouse embryonic fibroblast (MEF) cells have been used exclusively to study the consequences of gene ablation in cellular growth and proliferation[22]. To validate the efficiency of the AAV-Cas9 system in transforming normal cells, we transduced MEF cells isolated from Cas9 mouse embryos (E14) with AAV. The MEF cells were isolated as described previously[23,24]. MEFs from 14-day-old Cas9 mouse embryos were used because they are easy to culture and transduce in vitro. In brief, to transform MEFs via AAV transduction, primary MEFs at approximately 30% confluence were considered ready for transduction. One hour before transduction, the MEF culture media was changed. The purified AAV was thawed on ice and the MEF culture media was aspirated. Viral supernatant with a titer varying from 10^6–10^{14} viral genome/mL was added to each well and incubated overnight at 37 °C. The viral supernatant was removed after 48 h, replaced with 2 mL of MEF culture medium, and then incubated at 37 °C until GFP expression, about 5 days after transduction. GFP-expressing MEFs were subcultured and validated. Note that for this vector we found that 10^{12} viral genomes/mL (equivalent to 10^{10} transducing units/mL) could efficiently transform primary MEF cells to tumorigenic MEFs (**Figure 3A**). The transduced cells expressed Cre, Cas9, and GFP (**Figure 3B** and **3C**). To access the tumorigenic potential of the AAV transformed MEF cells, 0.5 x 10^6 cells (primary/AAV-transduced MEF) were injected into the tongue, lip, and subcutaneously in NOD-SCID mice. Transformed cells formed tumors in these mice, compared with the primary MEF, which did not form tumors (**Figure 3D**–**F**).

[Place Figure 3 here]

In vitro generation of the tumorigenic murine tongue epithelial cell line from primary tongue epithelial cells using the AAV-Cas9 system

After validating the transforming property of the AAV system using the MEF cells, we isolated primary tongue epithelial cells from Cas9 mice and cultured them in vitro. The primary tongue cells were transduced with AAV viral particles (10^{10} cfu/mL), and after one week cells started to express GFP. The tumorigenic transformation of primary tongue epithelial cells (**Figure 4A**) was confirmed using immunofluorescence and Western blotting (**Figure 4B**, **4C**). The transformed cells exhibit enhanced KRT 14, E-cadherin, and GFP expression (**Figure 4B**). The tumorigenic transformation of primary tongue epithelial cells was confirmed by the expression of GFP (i.e., the Cre-dependent expression of Cas9 and GFP) (**Figure 4C**). The expression of KRT14 and E-cadherin confirms their epithelial cell characteristics. Protein validation confirmed enhanced β-catenin, phospho-ERK, and reduced p53 expression (**Figure 4C**), indicating the effective downregulation of APC and p53 and the incorporation of mutant KRAS into the tongue cells, thus making them tumorigenic. Genomic analysis by deep sequencing of the DNA isolated from the transformed cells confirmed the effective gene editing and frame-shift of the TP53 and APC genes

by the AAV-Cas9 system (**Table 2**). The tumorigenic property of the AAV-transformed tongue cell line was further assessed by injecting it into the tongue of immunocompetent wild type C57BL/6 mice. The transformed tongue cells, designated as KRAS mutant epithelial cells of the tongue (KRAS Mut EpT), form tumors efficiently in C57BL/6 mice (**Figure 4D**). KRAS Mut EpT tumors were evaluated by an oral and maxillofacial pathologist by routine hematoxylin and eosin (H&E) staining and by immunohistochemistry. The tumors exhibited a spindle cell morphology with prominent nuclear atypia: pleomorphism, hyperchromatism, giant nuclei, several nucleoli, and a high mitotic rate with atypical mitotic figures. Perivascular invasion and angioinvasion were also noted. These morphological features were accompanied by very limited to nonexistent tumor-associated inflammation. Immunohistochemically, the neoplastic cells were cytoplasmically positive for E-cadherin and KRT 14, both markers of epithelial tissue. KRT 14 is typical of squamous epithelium, supporting the squamous origin of the tumor cells and thus the diagnosis of squamous cell carcinoma (**Figure 4E**). Hence, this approach provides a versatile methodology for transforming primary cells in vitro with a desired gene alteration. In addition, this methodology facilitates the use of these developed cell lines in vivo in syngeneic mice for better understanding of neoplasia in a real tumor microenvironment milieu.

[Place Figure 4 here]

FIGURE AND TABLE LEGENDS:

Figure 1: Vector map of the AAV transgene plasmid used in this study. This AAV system has an AAV backbone that expresses Cre recombinase and three U6-driven sgRNAs targeting KRAS, p53, and APC. It also contains a KRAS G12D homology-directed repair (HDR) donor.

Figure 2: Design and working of a AAV-Cas9 system in transforming normal cells. (**A**) Outline of the production and purification of AAV particles from HEK293T after transfecting them with the AAV transgene and packaging plasmids. (**B**) Viral transduction of primary cells with AAV particles and the mechanism by which Cre-dependent Cas9 expression enables CRISPR editing and the transformation of primary cells to tumorigenic cells. A single AAV vector integrating gRNAs for three different genes with a knockin of the KRAS G12D allele through HDR with a Cre recombinase expression cassette was delivered into Cre-dependent Cas9 mouse primary cells. Cre recombinase activity in the transduced cells leads to excision of the LoxP-Stop-LoxP cassette and induces Cas9 and GFP expression.

Figure 3: Validation and in vitro transformation of primary MEF cells using the AAV-Cas9 system. (**A**) Outline of the in vitro transformation of fibroblasts from embryos collected from Cas9 mice using AAV particles. (**B**) Representative immunofluorescence image of primary MEF and AAV-transduced MEF cells showing the expression of GFP, Cre, and Cas9. DAPI was used to stain the nucleus. (**C**) Western blotting showing Cre, Cas9, GFP, and β-actin in embryonic normal fibroblasts and genetically edited fibroblasts. (**D**) Representative image of a tongue tumor that developed in an NOD-SCID mouse after injecting it with transformed fibroblast cells. (**E**) Representative image of a lip tumor that formed in an NOD-SCID mouse after injecting it with transformed fibroblast cells. (**F**) Representative image of a flank tumor that developed in an NOD-SCID mouse after injecting it with transformed fibroblast cells. The arrows indicate a tumor.

Figure 4: In vitro transformation of primary tongue epithelial cells using the AAV-Cas9 system. (**A**) Schematic representation of the in vitro transformation of normal epithelial cells obtained from the tongue of Cas9 mice. (**B**) Representative immunofluorescence image of AAV-transduced tongue epithelial cells showing the expression of KRT14, E-cadherin, and GFP. (**C**) Western blot showing Cas9, β-catenin, and phospho-ERK upregulation with p53 protein level downregulation, showing the genetically edited epithelial cells. (**D**) Representative image of a tumor that developed in the tongue of the immunocompetent mice after injecting them with transformed tongue epithelial cells (KRAS Mut EpT). (**E**) Representative images of tumor tissue sections for H&E, E-cadherin, and KRT 14 staining at 20x and 40x magnification.

Table 1: Recipes of buffers used in this study.

Table 2: Genome analysis data of Kras Mut EpT cells.

DISCUSSION:

Several methods have previously been used to transform primary cells in culture with variable degrees of success[25–28]. Most of these methods use mouse fibroblast cells for transformation[14,17–19] or use carcinogens such as 4-nitroquinoline-1-oxide (4-NQO)[21,22] for developing murine cell line models. Use of knockout and transgenic mouse models has also greatly enhanced our understanding of the various molecular pathways that govern the development and differentiation of HNC. Use of murine oral epithelial cell lines with a unique genetic pattern would certainly complement these studies, and their subsequent biochemical assays would contribute towards the treatment and diagnosis of HNC. However, only a few reports have described the establishment of mouse oral epithelial cells[28–33] and these cell lines are not in widespread use.

The major limitation of these murine oral epithelial cell lines is that they are not characterized extensively at the molecular level and were found to express uncommon gene patterns. Thus, the limited molecular understanding of these murine cell line models and the lack of an efficient methodology prompted us to harness the unique potential of the AAV-CRISPR genome editing technology in Cas9 knock-in mice for establishing murine cell lines with specific gene alterations. In this study, we introduced and developed an in vitro AAV-Cas9 system for transforming primary murine cells into a tumorigenic state to confirm the genetic alterations that give rise to carcinoma cells. We successfully established murine tongue epithelial cells with specific gene mutations with high efficiency using AAV-mediated CRISPR-induced somatic gene editing by several sgRNA combinations.

This methodology offers a new experimental approach for better understanding the cell-autonomous etiology of HNC. Starting with murine primary tongue epithelial cells, we were able to transform such cells to tumorigenicity by deleting a limited set of genes and introducing a single KRAS mutation. Using this methodology, it is now possible to investigate whether other genes known to be implicated in other carcinomas can produce malignancies that more closely resemble HNC. The morphological characteristics of the tumors from KRAS Mut EpT confirmed squamous cell carcinoma. The histopathological analysis of KRAS Mut EpT tumors in syngeneic

mice is associated with and supportive of the aggressive biological behavior of head and neck carcinoma. Thus, it may be possible to link the genotypes of cancer cells to specific clinical and histopathological features of the tumors.

The main advantages of this methodology are the ability to develop cell lines from primary cells from any organ using specific gene mutations and the use of AAV-Cas9, which makes the system more robust and stable. The endogenous Cas9 system makes it possible to utilize other gene knockouts. The major disadvantage of the current methodology is the time required to isolate and establish the primary culture as well as the high titer of viral particles needed to transduce the primary cells. However, this could be overcome by standardizing the isolation process for each cell type. The problem of requiring a high AAV viral titer for efficient transduction could be alleviated by use of the latest generation of capsid and helper plasmids for viral packaging and a better purification process.

In the future, a better and more efficient AAV system could be designed and extrapolated in vivo to make organ-specific genetically modified tumor models. In conclusion, establishing the murine cell lines by this approach will serve as a valuable in vitro cell culture-based tool to test several hypotheses in the context of oncology.

ACKNOWLEDGMENTS:

We wish to thank Dr. Daniel Gitler for providing us with the pAd Delta 5 Helper plasmid. This work was funded by the Israel Science Foundation (ISF, 700/16) (to ME), the United States-Israel Binational Science Foundation (BSF, 2017323) (to ME and MS), the Israel Cancer Association (ICA, 20170024) (to ME), the Israel Cancer Research Foundation (ICRF, -1693-17RCDA) (to ME), and the Concern Foundation (#7895) (to ME). Fellowship: the Alon fellowship to ME and BGU Kreitman fellowships to SJ and MP.

DISCLOSURES:

M.S. is on the Advisory Board of the Bioscience Institute, and Menarini Ricerche has received research funds from Puma Biotechnology, Daiichi-Sankio, Targimmune, Immunomedics, and Menarini Ricerche. M.S. is also a co-founder of Medendi Medical Travel, and in the past 2 years, has received honoraria from Menarini Ricerche and ADC Pharma. All other authors have nothing to disclose.

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Figure 1

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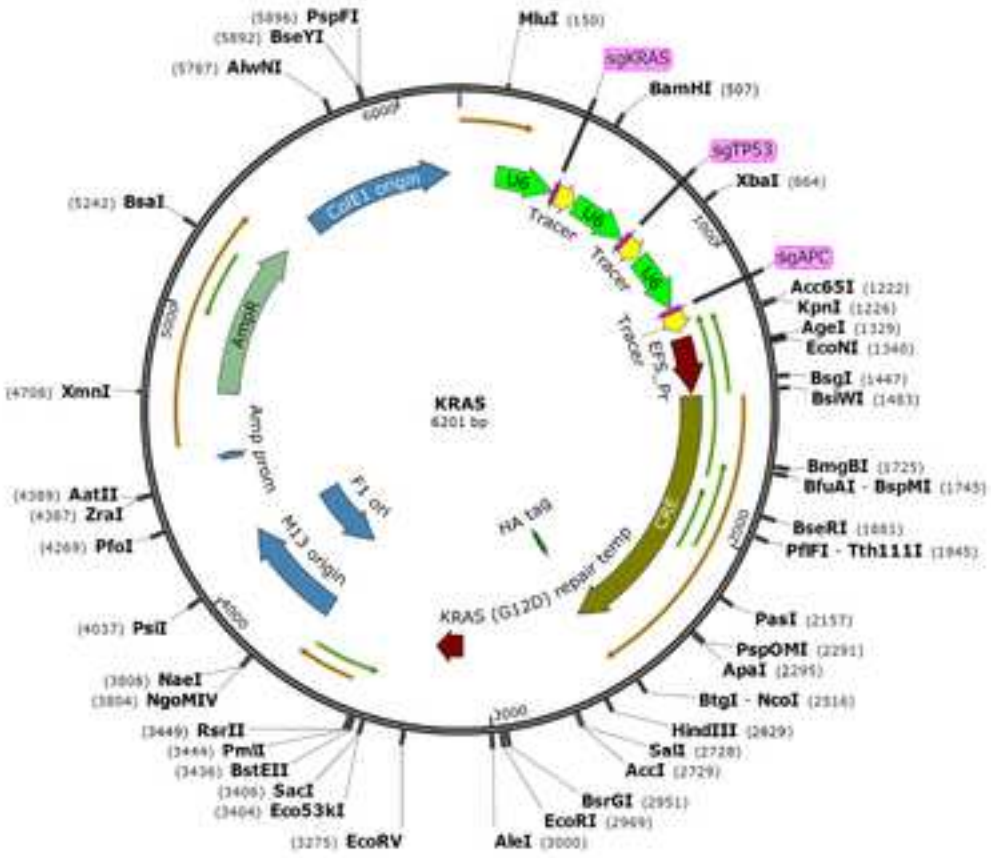

Figure 2

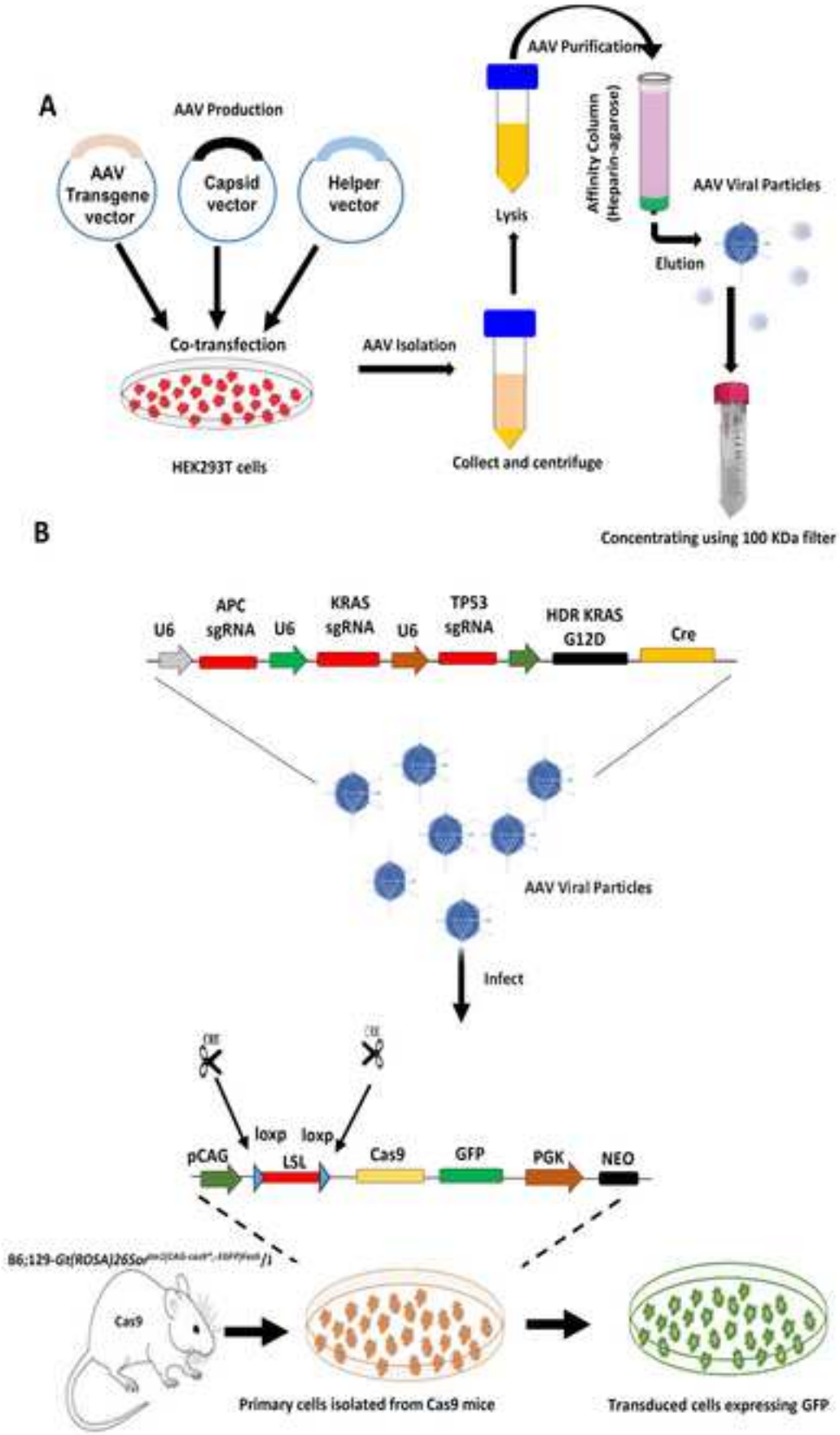

Figure 3

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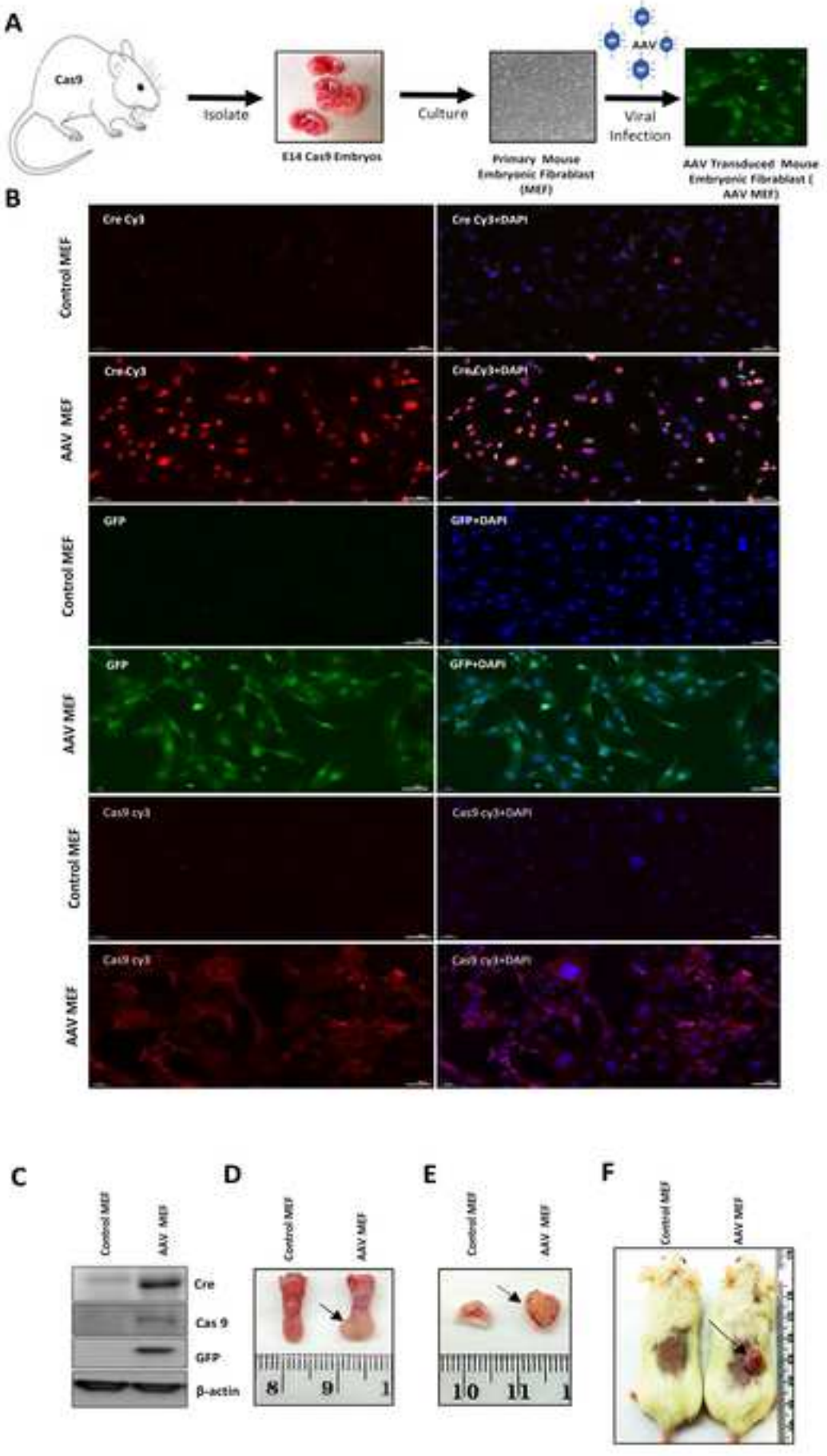

Figure 4

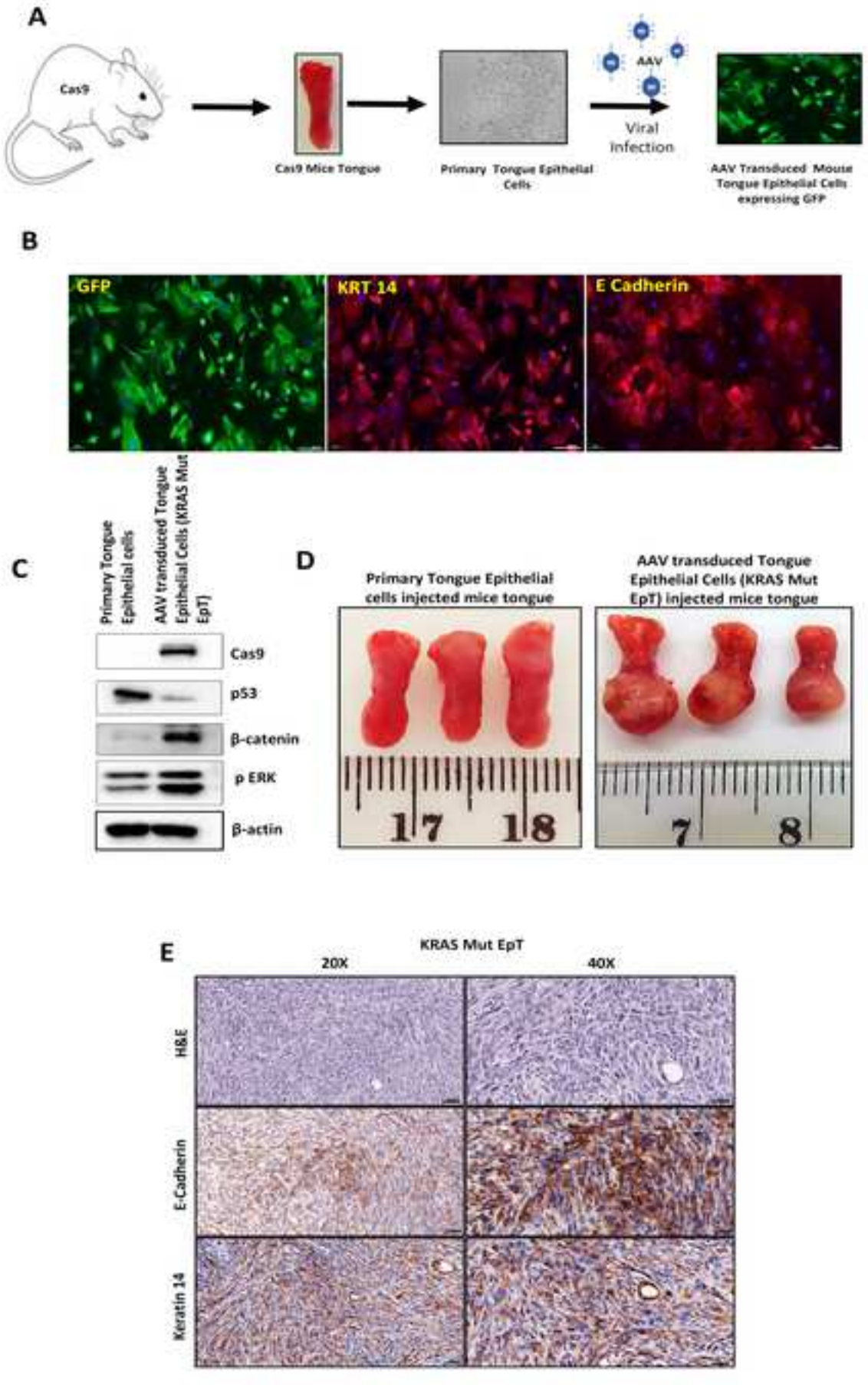

Table 1: Recipe of buffers used in this study

Cell lysis buffer for viral harvesting	Volume
150 mM NaCl	876.6mg
50 mM Tris-HCl	605.7mg
Use HCl to adjust pH 8.5	
Molecular grade water	Make upto 100 mL
Equilibration buffer	
1 mM $MgCl_2$	9.52 mg
2.5 mM KCl	18.64mg
Mix all in PBS 1X and adjust pH to 7.2	Make upto 100 mL
Elution buffer	
0.5 M NaCl	2.92g
Mix all in PBS 1X and adjust pH to 7.2	Make upto 100 mL
10X Triple Enzyme Stock Solution:	
Collagenase	1 g final conc. [10 mg/ml]
Hyaluronidase	100 mg [1 mg/ml]
DNase	20,000 Units final conc. [200 mg/ml]
PBS 1X	Make upto 100 mL
Plasmid mix (for one 14.5cm Plate)	
AAV pCM109 EFS Cre sg APC sg KRAS sg P53- KRAS HDR	10µg
AAV 2/9 capsid vector	10µg
pAD Delta F5 helper	10µg
PEI(1µg/µl Stock)	90µl
DMEM	1 mL

Table 2: Genome analysis data of the Kras Mut EpT cells

Hugo_Syml	Chromosome	Start_Positi	End_Positi	Variant_Classificati	Variant_Ty	Refer	Tumor	Tumor_Seq
Trp53	11	69589194	69589195	Frame_Shift_Ins	INS	-	-	A
Apc	18	34296075	34296076	Frame_Shift_Ins	INS	-	-	TC

dbSNP_R	Tumor_Sample_Barco	HGVSc	HGVSp	HGVSp_Sh	Transcript_	Exon_Num	t_depth
novel	s_KrasMutEpT	c.717_718i	p.Met240A	p.M240Nfs	ENSMUST0	7/11	814
novel	s_KrasMutEpT	c.873_874i	p.Ser293Le	p.S293Lfs*	ENSMUST0	9/16	188

t_ref_coun	t_alt_coun	n_depth	n_ref_cour	n_alt_coun	all_effects	Allele	Gene	Feature
129	685	768	768	0	Trp53,fram	A	ENSMUSG(	ENSMUST0
76	112	150	150	0	Apc,frames	TC	ENSMUSG(	ENSMUST0

Feature_ty	Consequen	cDNA_posi	CDS_positi	Protein_po	Amino_aci	Codons	Existing_va	DISTANCE
Transcript	frameshift_	874-875/17	717-718/1:	239-240/3	-/X	-/A		
Transcript	frameshift_	1184-1185,	873-874/8	291-292/2	-/X	-/TC		

STRAND_V	SYMBOL	SYMBOL_S	BIOTYPE	CANONICA	CCDS	ENSP	SWISSPRO	TREMBL
1	Trp53	MGI	protein_co	YES	CCDS3619	ENSMUSP0		Q549C9
1	Apc	MGI	protein_co	YES	CCDS2912	ENSMUSP0		B2RUG9

UNIPARC	RefSeq	SIFT	EXON	INTRON	DOMAINS	PUBMED	IMPACT	PICK
UPI000000	NM_01164		7/11		hmmpanther:PTHR114		HIGH	1
UPI000002	NM_00746		9/16		hmmpanther:PTHR126		HIGH	1

VARIANT_C	TSL	MINIMISED	GENE_PHE	FILTER	flanking_bp	variant_id	variant_qu	UpDownstr
insertion	1	1	1	.	GCA	.	51820.3	IHYKYMCN
insertion	1	1	1	.	GTT	.	6872.58	RVDHETAS

Caller	POS	TAG	LABEL	TriNuc	t_var_freq	n_var_freq	MARK_CODING_CHAN
HaplotypeC	chr11:6958	chr11:6958	chr11:6958		0.841523	0	Y
HaplotypeC	chr18:3429	chr18:3429	chr18:3429		0.595745	0	Y

GE

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Antibodies			
Anti mouse HRP	Jackson ImmunoResearch	115-035-146	
Anti rabbit HRP	Jackson	711-035-152	
Cas9 Mouse mAb	Cell Signaling Technology	14697	
Cre	BioLegend	900901	
Cy3-AffiniPure Goat Anti-Mouse IgG	Jackson ImmunoResearch	115-165-062	
Cy-AffiniPure Goat Anti-Rabbit IgG	Jackson ImmunoResearch	111-165-144	
GFP	Santa Cruz Biotechnology	sc-9996	
Phospho-p44/42 MAPK (Erk1/2)	Cell Signaling Technology	4370	
Rabbit monoclonal anti E cadherin	Cell Signaling Technology	3195S	
Rabbit monoclonal anti-KRT 14	Abcam	AB-ab181595	
β actin	MP Biomedicals	691001	
β catenin	Cell Signaling Technology	9582S	
Cell lines			
HEK93T	ATCC	CRL-3216	
Culture Media, Chemicals and Reagents			
Bradford Reagent	Bio-Rad	30015484	
BSA	Amresco	0332-TAM-50G	
DAPI fluoromount	Southern Biotech	0100-20	

DMEM	Biological Industries Israel Beit-Haemek Ltd.	01-055-1A
ECL (Westar Supernova and Westar Nova 2.0)	Cyanagen	XLS3.0100 and XLS071.0250
FBS	Biological Industries Israel Beit-Haemek Ltd.	04-127-1A
HBSS	Sigma	H6648
Heparin - Agarose	Sigma	H6508
Isolate II Genomic DNA Kit	Bioline	BIO-52066
$MgCl_2$	Panreac AppliChem	300283
NaCl	Bio Lab Ltd	1903059100
PBS	Biological Industries	02-023-1A
PEI	Polysciences	23966-1
Pen Strep Solution	Biological Industries Israel Beit-Haemek Ltd.	03-031-1B
PFA	Santa Cruz Biotechnology	30525-89-4
Phosphatase inhibitor cocktail	Biotool	B15001A/B
Protease inhibitor cocktail	MilliporeSigma	P2714-1BTL
Tris buffer	MERCK Millipore	648311-1KG

Enzymes

Benzonase	Sigma	E1014
Collagenase IV	Thermo Fisher Scientific	17104019
DNAse	Thermo Fisher Scientific	18047019
Hyaluronidase	MilliporeSigma,	H3506
Trypsin	Biological Industries	03-050-1B

Glass wares

Cover slips	BarNaor	BNCB00130RA1

Slides	Bar naor	BN9308C	
Mouse strains			
C57BL/6	Envigo		
B6;129-Gt(ROSA)26Sor$^{tm1(CAG-cas9*,-EGFP)Fe}$	Jackson labs	24857	
NOD.CB17-Prkdc-scid/NCr Hsd (Nod.Sci	Envigo		
Plasmids			
AAV pCM109 EFS Cre sg APC sg Kras sg I	Broad Institute of MIT		Kind gift from Dr Randall J Platt and Dr. Joseph Rosenbluh, Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA
AAV 2/9 capsid vector	Addgene	112865	
pAD Delta F5 helper	Ben Gurion University of the Negev		Provided by Dr Daniel Gitler, Department of Physiology and Cell Biology, Faculty of Health
Plastic wares			
Amicon-ULTRA filter 100 KDa	Millipore	UFC910024	
0.22 µm sterile filters ,4 mm	Millex	SLGV004SL	
0.45 µm sterile filters ,13 mm	Millex	SLHV013SL	
Culture plates	Greiner Bio-One		
Falcon tubes	Greiner Bio-One		

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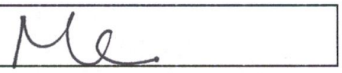
CORRESPONDING AUTHOR

Name:	MOSHE ELKABETS
Department:	THE SHRAGA SEGAL DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND GENETICS
Institution:	BEN GURION UNIVERSITY OF THE NEGEV
Title:	ASSISTANT PROFESSOR
Signature:	
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EXPLORING WAYS TO FIGHT CANCER
Elkabets' lab

14/10/2019

Reviewers' comments point by point:

We are grateful for your valuable comments and suggestions regarding our revised submission. In response to these comments, we have made considerable modifications to our MS. Below we detailed these specific modifications with the specific comments from the reviewer, point by point, in blue. We hope the reviewers will find the MS improved following these changes and more suitable for publication in JoVE.

Reviewers' comments:
Reviewer #4:
The revisions made to the manuscript are sufficient for publication.

The authors thank the reviewer for accepting the revised manuscript submitted to JoVE.

Reviewer #5:
Manuscript Summary:
The authors describe in detail how to generate HNC cell lines with specific genomic alterations and they explain their suitability to be studied for predicting tumor progression in syngeneic mice Cre-inducible Cas9. The paper is in general well written and quite relevant in the context of HNC research.

Major Concerns:
Nothing to report

Minor Concerns:
To maximize the reliability of the results is necessary to use at least two gRNA for each gene examined.

The authors thank the reviewer for the valuable suggestions, however as the vector we used in this study was already validated see Reference no. 14, this request is beyond the scope of this methodology paper. To ensure the on-target effect of the vector in our model, for the revised (1st round) we already performed genomic sequencing and western blotting analysis. Moreover, as we use one-in-all vector that contains three guides and homology-directed repair gene, generating a new vector and validating it in cell will take six to eight months, which is beyond the timeline of the revision.

Reviewer #6:
Manuscript Summary:
This manuscript describes a protocol using AAV/gRNA vectors to introduce selected mutations in Cas9-expressing mouse fibroblasts and tongue epithelial

cells as a means of transformation, which may help to study factors and pathways involved in tumorigenesis.

Major Concerns:
I have initially struggled to understand the concept underlying this work, since no details are explained at the beginning. It would help to show Fig. 2B much earlier, so that readers immediately understand what exactly is expressed from the AAV vector and how it interacts with the stable mouse cells.

We appreciate the reviewer's comment. However, this manuscipt had six other reviewers, and adjustment have been done already in the 1st revision, hence at this point this major comment can not been addressed.

The authors produce and use AAV9 vectors but purify them using heparin columns? To my knowledge, AAV9 does not bind heparin, in contrast to e.g. AAV2 or AAV3. How can this be reconciled?

We thank reviewer for the raising this question, but we think the reviewer did not pay attention that we have used a hybrid plasmid AAV2/9n (was a gift from James M. Wilson (Addgene plasmid # 112865 ; http://n2t.net/addgene:112865 ; RRID:Addgene_112865) which have *rep* gene of AAV2 and *cap* gene of AAV9. Recent studies have shown that cross packaging of AAV2 vectors on to different serotypes have enhanced efficiency of transduction (Chao H, Liu Y, Rabinowitz J, Li C, Samulski RJ, et al. Several log increase in therapeutic transgene delivery by distinct adeno-associated viral serotype vectors. Mol Ther. 2000;2:619–623.). Cross-packaged AAV2 genomes in AAV1, AAV3, and AAV4 capsids showed 900, 30 and 3-fold enhanced gene expression, respectively, in skeletal muscle compared to AAV2. AAV9 exhibits a similar profile with widely disseminated transduction as AAV2, albeit with much higher efficiency than AAV2. It is also proved the use of an AAV2 vector with AAV9 capsid causes stable transduction in cells (J Surg Res. 2012 Jun 1; 175(1): 169–175.). Heparin columns have been widely used for the purification of AAV2 related viral purification, hence we used heparin-agarose column for virus purification in this study.

Why do the authors use AAV9 to begin with? Have they tested multiple capsid variants and was this the best? Considering that AAV9 typically transduces cultured cells very poorly, I am surprised by the choice of capsid.

The reviewer raised a major question, that worth an investigation in a separate work, which is beyond the scope of this work.
In our laboratory and in our collaborating labs we have been working with AAV2/9 hybrid plasmid and standardized protocols with the same. The current protocol outlined in the JoVE manuscript works well for us and has been using in the establishment of various other murine models both in vitro and in vivo. We have not tested any other capsid variants and we will be doing other variants in future studies in the process of identifying better purification processes with high viral titer.

To my understanding, the authors are trying to make the point that their protocol is versatile and can also be used to knock out and study other genes. If so, it would be good to spend more time on explaining how the gRNA vectors can be customized for other targets, rather than describing a generic AAV production and purification protocol.

We appreciate the reviewer's suggestion but the major theme of this manuscript is to establish a murine cell line that harbors specific mutations that are found in human cancers. Moreover, similar studies has been already published in various journals that address the reviewer's suggestion (Cell. 2014 Oct 9;159(2):440-55., Science. 2012 Nov 23;338(6110):1080-4., Mol Ther. 2018 Jul 5;26(7):1818-1827.). We would like to highlight that the scope of the current work is to provide the reader's protocol and methodology with the potentiality of the AAV-Cas9 system in making murine head and neck cancer lines with specific human gene mutations which are currently lacking in HNC studies.

There is no description of a protocol for AAV vector titration (following their purification). Without this, readers will not be able to quantify their vectors and adjust the MOIs.

The protocol for AAV vector titration has been incorporated in the form of reference 18-20 in the revised manuscript. Since we do not want to repeat the previously published protocol, in consultation with the JoVE editor we have provided the citation for the same.

Lock, M. *et al.* Characterization of a Recombinant Adeno-Associated Virus Type 2 Reference Standard Material. *Human Gene Therapy*. **21** (10), 1273, doi: 10.1089/HUM.2009.223 (2010).
Challis, R.C. *et al.* Systemic AAV vectors for widespread and targeted gene delivery in rodents. *Nature Protocols*. **14** (2), 379–414, doi: 10.1038/s41596-018-0097-3 (2019).
Rabinowitz, J.E. *et al.* Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. *Journal of virology*. **76** (2), 791–801, doi: 10.1128/jvi.76.2.791-801.2002 (2002).

Why are chapters 5.2 to 5.4 formatted differently (not in protocol style)?

As per the suggestion of the reviewer, chapters 5.2 and 5.4 has been formatted in the form of step by step protocol for better understanding for the JoVE readers.

As it is, chapter 5.3 is not helpful since it is very superficial, to an extent that no-one will be able to reproduce this workflow.

As per the reviewer's suggestion chapter 5.3 has been removed from the protocol as the specific workflow of the MSK-IMPACT platform is under the regulation of Memorial Sloan Kettering Cancer Center Genomics and

computational Core facility and it works just like any other next-generation sequencing.

Similarly, chapter 5.4 is is not a protocol but rather reads like a specific methods section from a regular paper; thus, I am not sure this is useful for JoVE readers?

Chapter 5.4 has been modified in the revised manuscript for better clarity to the readers.

How would the challenge of having to use high viral titers be solved "by using a better purification process", as stated in lines 480-1? Likewise, how would "using the latest generations of capsid and helper plasmids" help? This sounds very generic; what are these latest generations?

We are thankful for the concerned raised by the reviewer but recent study from Challis et al., 2019 has shown more efficient purification methods using various capsid and helper plasmids, which support our claim.
Challis, R.C. *et al.* Systemic AAV vectors for widespread and targeted gene delivery in rodents. *Nature Protocols*. **14** (2), 379–414, doi: 10.1038/s41596-018-0097-3 (2019).

Please use correct terminology throughout:
1) In the context of viral vectors, the correct term is "transduction", not "infection" (the latter is only used for wild-type viruses)
2) "vectors" is not the same as "plasmids"; thus, one uses e.g. "packaging plasmids" for AAV production, but not "vectors"
3) "cfu/ml" is used extremely rarely for AAV vectors, since they do not form colonies. The correct measure is MOI, i.e., multiplicity of infection (which is unfortunately misleading, since infection is the wrong term, as noted above), which is (physical) particle number per cell.

We apologize for the error in terminology and the authors are very thankful to the reviewer for pointing this mistake. The authors have taken extra care in re-editing the manuscript with the correct terminology.

Minor Concerns:

On page 2, the authors mention that a "new generation of helper plasmid - pAdDeltaF6 is available", but there is no reference and it remains unclear what the difference to F5 is or why it would be preferred.

Reference for pAd Delta F6 helper plasmid has been incorporated. The difference between pAd Delta 5 and pAd Delta 6 is in their sequence. Deletions of a 2.3 kb NruI fragment and, subsequently, a 0.5 kb RsrII/NruI fragment from pAd Delta F1 helper generated helper plasmids pAd.DELTA.F5 and pAd.DELTA.F6, respectively. The helper plasmid, termed p.DELTA.F6, provides the essential helper functions of E2a and E4 ORF6 not provided by the E1-expressing helper cell, but is deleted of adenoviral capsid proteins and functional E1 regions.

Line 156: "steel needle" is not helpful; which size was used?

The sentence has been modified as "Collect the supernatant in a syringe using a 18 gauge steel needle and push the solution through a 0.45 µm filter into a 15 mL tube"

Line 159: Where should the particles stored for longer periods? -20 or -80°C?

From our experience the storage of crude lysate for longer periods in -20 or -80℃ has been shown to reduce the viral titre and transduction efficiency of the virus.

Line 205: Why are different volumes stored at 4 or -80°C?

In order to reduce repeated freeze-thawing and to increase the transduction efficiency, we used to store less volume for longer storage at -80°C.

Line 213: What is a "scalpel method"?

Scalpel method is the mechanical dissection of tissue using the scalpel blade and the sentence in the revised manuscript has been modified as "Use manual dissociation of tissue by mincing the tongue tissue into very small fragments using the scalpel".

Line 250-1: As noted, cfu/mL should not be used. The commentary in brackets is very confusing. MOI refers to the number of AAV particles, not the cells. And MOI is typically expressed as e.g. 1e5 (particles per cell), but not in cfu/mL.

The sentence has been modified as "transduce the cells with 10^{12} viral genome/mL (10^{10} transducing units/mL) and incubate the cells in viral particle-containing media for 48 h at 37 ˚C."

Reviewer #7:

The authors established a new method to generate murine head and neck cancer (HNC cell lines with specific genetic mutations by infecting normal cells with the AAV-Cas9 system. They have provided detailed protocol for conducting their methods. This protocol will open a new era in research into understanding the molecular mechanisms of head and neck cancers as currently there are very few mouse models for this disease. This protocol will be useful for studying how certain genes regulate the tumourigenesis and development of HNC. The revised version addressed all comments that was was raised by the initial reviewers and now is suitable for publication in JoVe.

The authors thank the reviewer for accepting the revised manuscript submitted to JoVE.

EXPLORING WAYS TO FIGHT CANCER

Elkabets' lab

Moshe Elkabets, Ph.D,
Senior Lecturer (Assistant Professor)

The Shraga Segal Dept. of Microbiology, Immunology and Genetics
Faculty of Health Sciences,
Ben-Gurion University of the Negev
Beer-Sheva, 84105
Israel.
email: moshee@bgu.ac.il | website: www.elkabetslab.com
Phone: +972-53-2323-987 | Tel: +972-8-6428846