

# Recombinant AAV9-TLK1B Administration Ameliorates Fractionated Radiation-Induced Xerostomia

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## Abstract

Salivary glands are highly susceptible to radiation, and patients with head and neck cancer treated with radiotherapy invariably suffer from its distressing side effect, salivary hypofunction. The reduction in saliva disrupts oral functions, and significantly impairs oral health. Previously, we demonstrated that adenoviral-mediated expression of Tausled-like kinase 1B (TLK1B) in rat submandibular glands preserves salivary function after single-dose ionizing radiation. To achieve long-term transgene expression for protection of salivary gland function against fractionated radiation, this study examines the usefulness of recombinant adeno-associated viral vector for TLK1B delivery. Lactated Ringers or AAV2/9 with either TLK1B or GFP expression cassette were retroductally delivered to rat submandibular salivary glands ( $10^{11}$  vg/gland), and animals were exposed, or not, to 20Gy in eight fractions of 2.5Gy/day. AAV2/9 transduced predominantly the ductal cells, including the convoluted granular tubules of the submandibular glands. Transgene expression after virus delivery could be detected within 5 weeks, and stable gene expression was observed till the end of study. Pilocarpine-stimulated saliva output measured at 8 weeks after completion of radiation demonstrated >10-fold reduction in salivary flow in saline- and AAV2/9-GFP-treated animals compared with the respective nonirradiated groups (90.8% and 92.5% reduction in salivary flow, respectively). Importantly, there was no decrease in stimulated salivary output after irradiation in animals that were pretreated with AAV2/9-TLK1B (121.5% increase in salivary flow;  $p < 0.01$ ). Salivary gland histology was better preserved after irradiation in TLK1B-treated group, though not significantly, compared with control groups. Single preemptive delivery of AAV2/9-TLK1B averts salivary dysfunction resulting from fractionated radiation. Although AAV2/9 transduces mostly the ductal cells of the gland, their protection against radiation assists in preserving submandibular gland function. AAV2/9-TLK1B treatment could prove beneficial in attenuating xerostomia in patients with head and neck cancer undergoing radiotherapy.

## Introduction

A MAJORITY OF PATIENTS with head and neck cancer are treated with therapeutic radiation alone or in combination with other treatment modalities. Since radiation portals, most often, encompass a large part or whole of the major salivary glands, radiotherapy results in the unavoidable destruction of these tissues. Consequently, diminution of salivary gland function occurs in almost all patients undergoing regional radiotherapy and ~80% of patients undergoing total body radiation before bone marrow transplantation (Sciubba and Goldenberg, 2006; Chambers *et al.*, 2007a).

In clinical therapy, patients are exposed to small doses of radiation with the underlying premise of minimizing dam-

age to normal tissues. However, salivary glands are highly sensitive to radiation, and salivary gland function is compromised despite fractionated radiotherapy. Within the first week of radiotherapy, salivary function drops significantly, and the chronic decline in function continues through therapy. Although some recovery of parotid gland function has been documented in the months and years after radiotherapy (Braam *et al.*, 2005), the probability of gland dysfunction (a reduction to <25% preradiation levels) correlates well with the increase in the mean radiation dose (Roesink *et al.*, 2001; Dijkema *et al.*, 2010). Patients with head and neck cancer are generally treated with a total therapeutic dose of 65–70 Gy (Sciubba and Goldenberg, 2006), and nearly 95% patients have poor parotid function 1 year after radiation (Dijkema

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*et al.*, 2010). Therefore, it is not surprising that the most common complaint in head and neck cancer survivors is the subjective perception of oral dryness, or xerostomia. Although the underlying radiobiological mechanism of salivary dysfunction remains unclear, the early reduction in salivary flow is argued to be caused by compromised membrane functioning of acinar cells (Konings *et al.*, 2005), whereas the chronic and persistent decline in function is attributed to apoptosis of cells with latent DNA damage (Nagler, 2002).

Normal salivary flow facilitates key oral activities, and its reduction impairs functions of chewing, swallowing, speech, and taste. Further, hyposalivation accentuates radiation-induced oral mucositis, ulcerations, and odynophagia, and it increases the host's susceptibility to rampant dental decay, mucosal trauma, and infections (Sciubba and Goldenberg, 2006; Chambers *et al.*, 2007a). The comorbidity associated with xerostomia severely limits patients' well-being and their prospect of continuing cancer treatment (Epstein *et al.*, 2001; Bruce, 2004). Palliative relief of symptoms with artificial salivary substitutes and the use of prosecretory drugs, such as pilocarpine and cevimeline, have had limited success in treating xerostomia (Chambers *et al.*, 2007b; Berk, 2008). The incorporation of salivary gland-sparing radiation techniques and the use of radioprotective drug, Amifostine, have, to some measure, diminished the severity of xerostomia (Rudat *et al.*, 2008), but the chronic functional deterioration of the gland remains a major concern.

Human Tausled-like kinase 1 (TLK 1) is a 788 amino acid serine/threonine kinase that has important functions in DNA replication, chromatin assembly checkpoint arrest, and DNA repair (Sillje *et al.*, 1999; Carrera *et al.*, 2003; Groth *et al.*, 2003; Hashimoto *et al.*, 2008; Takayama *et al.*, 2010). A spliced variant of TLK1, TLK1B, is identical to full-length protein except from the loss of 237 N-terminal amino acids (Li *et al.*, 2001). TLK1 and TLK1B are known to phosphorylate the same substrates identified to date, namely, histone H3, anti-silencing factor 1, and Rad9 (Li *et al.*, 2001; Sillje and Nigg, 2001; Sunavala-Dossabhoy *et al.*, 2003; Sunavala-Dossabhoy and De Benedetti, 2009). We have shown that expression of TLK1B in mouse epithelial cells does not induce oncogenic transformation, but it does increase the cell's ability to overcome radiation injury (Li *et al.*, 2001; Sunavala-Dossabhoy *et al.*, 2003; Sunavala-Dossabhoy *et al.*, 2005). This holds true for irradiated normal rat salivary acinar and ductal cells transduced with exogenous TLK1B as well (Palaniyandi *et al.*, 2011; Sunavala-Dossabhoy *et al.*, 2012). Although TLK1B facilitates repair of double-strand breaks, its precise role in this process is not completely elucidated. It is postulated that the coordinated chaperone and kinase functions of the protein facilitate repair through checkpoint arrest and through chromatin remodeling at break sites (Canfield *et al.*, 2009; Sunavala-Dossabhoy and De Benedetti, 2009).

Using localized adenovirus gene delivery to rat submandibular glands, we recently demonstrated that TLK1B expression effectively attenuates salivary dysfunction after single-dose radiation (Palaniyandi *et al.*, 2011). Nonetheless, short-lived gene expression and immunogenicity of the vector on repeated administration limit the prospect of translating adenovirus-TLK1B to head and neck cancer patient care where radiotherapy is delivered in small doses, daily, for 5–7 weeks. Adeno-associated viral vectors, alternately, provide lasting gene expression, and are relatively nonpathogenic and nonimmunogenic. Naturally, they are in

greater use in clinical trials for the treatment of a number of human diseases and genetic disorders (Gaudet *et al.*, 2012; Gray, 2013; Weinberg *et al.*, 2013). In preparation for a prospective clinical transition of TLK1B for radiotherapy-induced xerostomia, this study investigates the efficacy and safety of AAV-TLK1B gene transfer to submandibular salivary glands in mitigating the disorder.

## Materials and Methods

### Vector constructs and AAV2/9 preparations

GFP or TLK1B sequence was inserted between the hybrid cytomegalovirus immediate early enhancer/chicken beta-actin promoter sequence and the bovine growth hormone polyadenylation sequence. The entire expression cassette was incorporated between AAV2 terminal repeats. Cross-packaged, recombinant AAV2/9 was generated as described previously (Klein *et al.*, 2008). In brief, human embryonic kidney cells, 293T, were cotransfected using calcium phosphate with AAV2-terminal repeat-containing plasmid and packaging plasmids, pAd-deltaF6 and pAAV9, which provided adenoviral helper genes and the AAV9 capsid genes, respectively. Cells were collected 72 hr after transfections and lysed by three freeze-thaw cycles. The crude lysates were treated with benzonase (Sigma) before applying them to iodixanol gradients (OptiPrep; Greiner Bio-One) and centrifuging at 70,000 *g* for 75 min at 20°C. The density gradient fraction containing AAV was collected, and the virus was washed and concentrated in lactated Ringer's solution (Baxter) using Amicon-Ultra 15 (100 kDa) filter units. The virus stock was titered for viral genome copies by qPCR assay (Bio-Rad) with primers targeted to GFP or TLK1B sequence. The viruses were aliquoted and stored at –80°C.

### Cell culture and fluorescence microscopy

Rat salivary gland cell lines, A5 and ParC5, were kind gifts from Dr. Bruce Baum (National Institutes of Health [NIH] Clinical Center) and Dr. David Quissell (University of Colorado Health Sciences Center [UCHSC]), respectively. A5 cells are submandibular ductal cells, whereas ParC5 are parotid acinar cells. Cells were grown at 37°C in the presence of 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM; for A5 cells) or modified DMEM/F12 (for ParC5 cells) that was supplemented with 10% bovine growth serum (BGS; Hyclone), 2 mM L-Glutamine, and 1% antibiotic-antimycotic solution (Invitrogen). Cells were plated in six-well plates, and when 40–50% confluence was reached, AAV2/9-GFP (10<sup>9</sup> vg/well) was overlaid on cells in reduced-serum medium, OPTI-MEM (Life Technologies). After 4 hr of incubation, the cells were supplemented with equal volume of the complete medium containing 20% BGS. Native GFP fluorescence was evaluated on a fluorescence microscope using an fluorescein isothiocyanate filter daily from the day after transduction to day 7.

### Salivary gland transduction in vivo

Male Sprague-Dawley rats (225–250 g) obtained from Harlan Laboratories, were anesthetized by intramuscular administration of ketamine chloride (42 mg/kg), xylazine (8 mg/kg), and acepromazine (1.4 mg/kg). Salivary secretions were suppressed by subcutaneous delivery of atropine (0.5 mg/kg). The submandibular salivary gland ducts were cannulated with thin polyethylene tubing, and virus (10<sup>11</sup> vg/gland) or

lactated Ringer's solution (vehicle) was instilled in the glands. The tubing was retained in place for 30 min before being removed. Animals were kept warm during recovery and placed in cages after they were ambulatory. All animals had access to food and water *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee and were in accordance with NIH guidelines.

#### Fractionated irradiation and collection of submandibular saliva

Five weeks after virus transduction, animals were anesthetized by intramuscular delivery of a mix of ketamine (33 mg/kg), xylazine (6 mg/kg), and acepromazine (1 mg/kg) for irradiation. They were placed in a supine position, and a 1 cm tissue-equivalent bolus was placed over the skin. Radiation collimated to the neck was delivered at 6 MV using the Elekta RTD with MLC Linear Accelerator (LSUHSC–Radiation Oncology). Animals were irradiated with 2.5 Gy/day, 4 times/week, for 2 weeks with a 2-day weekend interspersed in between. Animals received a cumulative dose of 20 Gy. After recovery from anesthesia, animals were returned to their cages and housed at the animal facility.

Stimulated submandibular salivary secretions were collected 8 weeks after completion of radiation. Pilocarpine (0.5 mg/kg) was subcutaneously administered in anesthetized animals, and collection started after saliva was observed at the ductal orifices. Submandibular/sublingual saliva that accumulated at the floor of the mouth was aspirated and collected in prechilled tubes over 20 min. To avoid collecting parotid secretions, absorbent cotton rolls were placed in the buccal pouches and at the rear of the mouth. Animals were later perfused with 4% paraformaldehyde/phosphate buffered saline (PBS), and submandibular glands and major organs were harvested for histological and immunohistochemical analysis. A schematic of the experiment schedule is shown in Fig. 1.

#### Statistical analysis

Salivary flow rate data are expressed as mean  $\pm$  SEM. Statistical computing software, SAS, version 9.2, was used to analyze the data. A two-way analysis of variance was used to determine significant effects of treatment (lactated Ringers, AAV2/9-GFP, and AAV2/9-TLK1B), irradiation (non-exposed, exposed), and treatment–irradiation interaction on

the salivary flow rate. The Bonferroni multiple comparison test was used to determine which pair-wise comparisons were significant.

#### In Vivo Imaging Systems imaging for GFP epifluorescence

To ascertain AAV9 transduction of submandibular salivary glands and associated vector spread, GFP gene expression was analyzed by *In Vivo* Imaging Systems (IVIS) imaging at 5 weeks after virus delivery. Animals were euthanized under anesthesia, and submandibular glands and major organs, including the heart, lung, liver, spleen, kidneys, and intestines, were evaluated by biophotonic GFP imaging using Xenon IVIS 100/XFO-12 apparatus. Images were acquired at the same settings: emission and excitation filters (GFP), exposure time (5 s), binning (8), and *f*/stop (1). Pseudocolored images showing a spatial distribution of photon counts were overlaid on photographs. Rats instilled with lactated Ringer's solution were negative controls. Images were analyzed using Living Image 4.0 (Caliper Life Sciences).

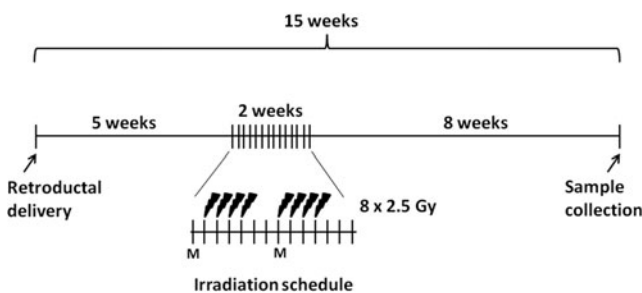
#### Histology and GFP immunohistochemistry

Submandibular glands and major organ tissues were collected and fixed in 4% paraformaldehyde overnight at 4°C before being processed for paraffin-embedding and sectioning. Sections of tissue samples were stained with hematoxylin and eosin, or processed for immunohistochemistry with rabbit anti-GFP (Invitrogen), or mouse anti–glutamate-glutamic acid-rich proteins (GRP) (an antibody to acinar cell-specific GRPs; a kind gift from Dr. Lawrence Tabak, NIDCR). Endogenous peroxidase activity was quenched in deparaffinized, dehydrated tissue sections with 0.3% H<sub>2</sub>O<sub>2</sub>/PBS for 30 min. Sections were permeabilized with 0.3% Triton X-100/PBS for 10 min, and blocked in 10% goat serum/5% bovine serum albumin with the avidin blocking solution (Vector Labs) to suppress nonspecific antibody binding. Primary antibody incubation (1:300) with the biotin blocking solution (Vector Labs) was done for 16 hr at 4°C followed by biotinylated anti-rabbit (1:200; Dako Cytomation) or biotinylated anti-mouse (1:50; BD Pharmigen) for 30 min. Sections were labeled with horseradish peroxidase (HRP)-conjugated Extravidin (1:2000; Sigma) for 30 min before proceeding to detect avidin–biotin peroxidase complexes with diaminobenzidine (DAB). Lactated Ringers-treated glands were negative controls for immunohistochemical staining.

Histopathological analysis of submandibular glands was performed using a focus score. Nested mononuclear cells that were  $>5$  per 4 mm<sup>2</sup> were scored. A minimum of four sections per animal were scored, and the total number of foci per animal were plotted. Scoring of GFP immunostaining was performed on the subjective assessment of stain intensity and percent area staining. Quantification of surface acinar cell area was done using ImageJ software (NIH). A minimum of three random microscope fields (200 $\times$ ) per gland were analyzed, and GRP-stained areas were summed.

#### Immunoblotting for TLK1B

Submandibular glands treated with lactated Ringers or AAV2/9-TLK1B were removed at 5 or 15 weeks after retroductal instillation. The tissues were minced and sonicated in the radio immunoprecipitation assay (RIPA) buffer



**FIG. 1.** A schematic representation of an experiment schedule. Radiation commenced 5 weeks after delivery of virus ( $10^{11}$  vg/gland) or lactated Ringers to both submandibular glands. A radiation dose of 20 Gy was fractionated in eight doses of 2.5 Gy. Sample collection was done 8 weeks after irradiation. M, Monday.



with protease inhibitor cocktail (Roche), 5 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenates were centrifuged, and protein concentrations were determined using BCA Protein Assay (Pierce/Thermo Scientific). Equal amount of total protein (100  $\mu$ g) from each sample was loaded on 10% sodium dodecyl sulfate polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked in 7% milk/PBS before incubating with rabbit anti-TLK1 (1:1000) followed by HRP-conjugated anti-rabbit (1:1000; Vector Laboratories), or HRP-conjugated anti- $\beta$  actin (1:10,000; Sigma). The membrane was reacted with DAB for visualization of protein bands.

## Results

### AAV2/9 transduction in rat salivary gland cells in culture

Before investigating AAV2/9 transduction in salivary glands *in vivo*, virus transduction was assessed in rat salivary cells in culture. A5, ductal cells of the submandibular gland, and ParC5, acinar cells of the parotid, were transduced with AAV2/9-GFP. As shown in Fig. 2, native GFP fluorescence was detected in 60–70% of each cell type, although the intensity of fluorescence was higher in A5, ductal cells, than in Par C5, acinar cells. GFP expression was observed starting at day 3 post-transduction (Fig. 2A and B), and fluorescence intensity increased with time. Peak fluorescence intensity was recorded at day 7 (Fig. 2C and D).

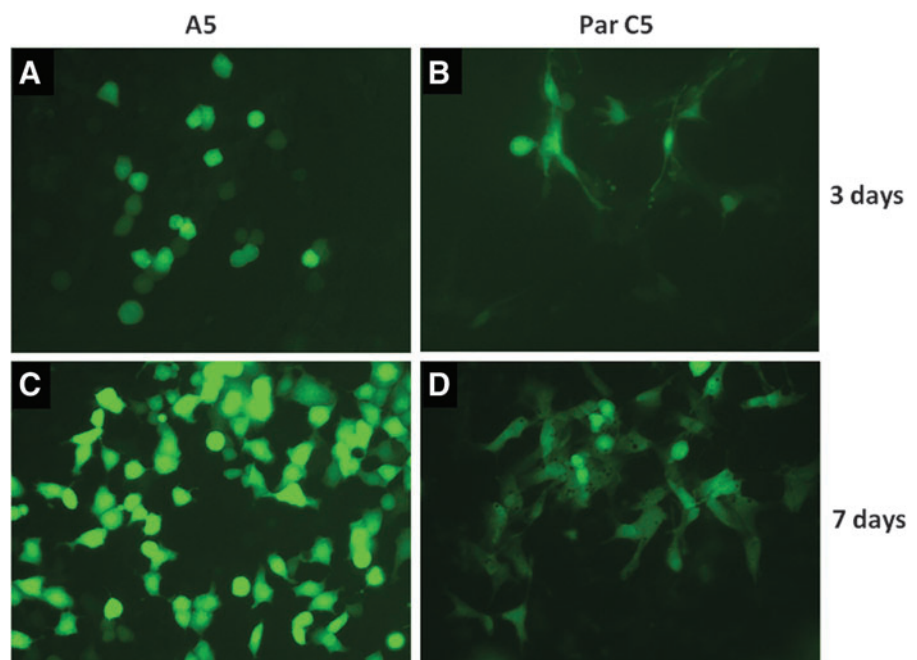
### AAV2/9-associated transgene expression in submandibular salivary glands in vivo

GFP gene expression ascertained after retroductal delivery of AAV2/9-GFP to submandibular glands revealed transgene expression in the tissue within 5 weeks. GFP epifluorescence measured at 5 and 15 weeks after virus transduction indicates that AAV2/9-mediated transgene expression in submandibular glands is long-term and stable (Fig. 3B and C). Immunogenicity of the vector was determined by evaluation of

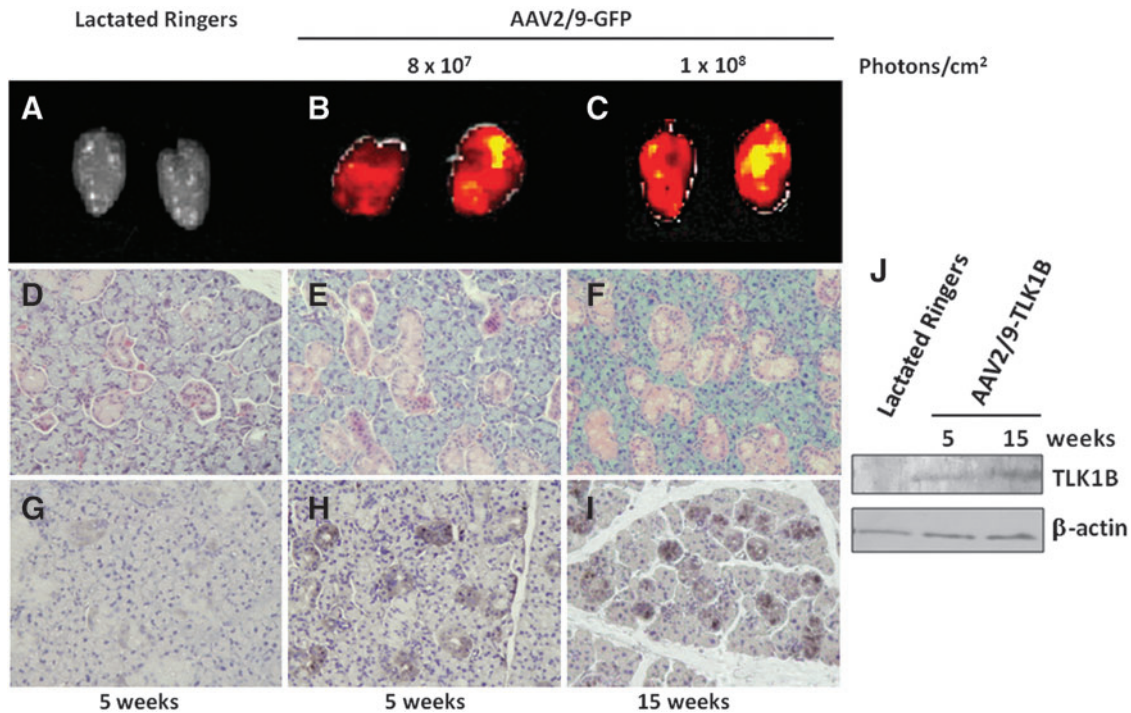
tissue sections, microscopically. No histological differences in gland architecture in virus-treated or lactated Ringers-treated animals were noted (Fig. 3D–F). To ascertain virus tropism for salivary cell type, immunohistochemical localization of GFP was performed. There was no GFP staining in lactated Ringers-treated glands, but in glands treated with AAV2/9-GFP, GFP expression was predominantly in the ductal epithelial cells with marginal number of acinar cells staining positive for it (Fig. 3H and I). Similar to results of virus transduction in salivary cells in culture, *in vivo* results confirm superior AAV2/9 transduction in the ductal cells. Immunohistochemical staining was observed in the intercalated and striated ducts and in convoluted granular tubules 5 weeks after virus delivery (Fig. 3H), and transgene expression was sustained up to the end of the study at 15 weeks (Fig. 3I). Submandibular glands treated with AAV2/9-TLK1B confirm longevity of transgene expression after virus delivery (Fig. 3J).

### Vector spread after submandibular gland delivery in vivo

To evaluate the spread of vector after localized delivery of AAV2/9-GFP, submandibular glands and major organ tissues were excised and studied using IVIS biphotonic GFP imaging at 5 weeks after virus delivery. Apart from the submandibular salivary glands, the heart, lung, liver, kidneys, spleen, and intestine were evaluated for GFP expression by IVIS imaging 5 weeks after virus delivery. All major organs, except the intestine of virus-treated animals, demonstrated signal at background levels similar to no vector-treated controls (Supplementary Fig. S1A–E and G–K; Supplementary Data are available online at [www.liebertonline.com/hum](http://www.liebertonline.com/hum)). GFP signal could be detected in intestines of treated animals (Supplementary Fig. S1L), but not in those of lactated Ringers-treated controls (Supplementary Fig. S1F). Similar to submandibular salivary glands, transgene expression in the intestines was long-lived, and it could be detected immunohistochemically within the simple columnar epithelial



**FIG. 2.** AAV2/9 transduction in rat salivary gland cells. AAV2/9-GFP ( $10^9$  vg) transduction in rat submandibular ductal cells, A5 (A and C), and rat parotid acinar cells, ParC5 (B and D). Native GFP fluorescence recorded on a fluorescence microscope with fluorescein isothiocyanate filters at 3 (A and B) and 7 days (C and D) after virus transduction. Magnification, 400 $\times$ .



**FIG. 3.** AAV2/9-associated stable transgene expression in rat salivary glands *in vivo*. (A–C) Biphotonic imaging of GFP in salivary glands at an expression interval of 5 weeks ( $n=3$  each) or 15 weeks ( $n=4$  each) after instillation of lactated Ringer's solution or AAV2/9-GFP ( $10^{11}$  vg/gland). (D–F) Histological evaluation of hematoxylin and eosin-stained salivary gland sections, and (G–I) immunohistochemical localization of GFP at 5 and 15 weeks after delivery of lactated Ringers or AAV2/9-GFP ( $10^{11}$  vg/gland). Nearly 25–50% of ductal cells were immunostained at moderate (score 2) intensity. Magnification,  $200\times$ . (J) Western blotting of submandibular gland tissue lysates with antibodies to TLK1 and  $\beta$ -actin at 5 and 15 weeks after retroductal delivery of lactated Ringers or AAV2/9-TLK1B ( $10^{11}$  vg/gland).

cells that line the crypts and the lamina propria at 15 weeks post-virus delivery (Supplementary Fig. S2).

#### *TLK1B ameliorates fractionated radiation-induced hyposalivation*

Previously, we had shown that a single-dose of 16Gy compromises rat submandibular gland function, and pretreatment with adenovirus-TLK1B effectively salvaged salivary flow (Palaniyandi *et al.*, 2011). To assess the effect of TLK1B on clinically relevant radiation dosing, animals were irradiated in fractions of 2.5Gy daily, up to a total of 20Gy (Fig. 1). Nonirradiated animals pretreated with lactated Ringers, AAV2/9-GFP, or AAV2/9-TLK1B showed average salivary flow rates of  $40.5 \pm 7.9 \mu\text{l}/\text{min}$ ,  $30.6 \pm 4.5 \mu\text{l}/\text{min}$ , and  $33.4 \pm 3.2 \mu\text{l}/\text{min}$ , respectively. Radiation treatment resulted in a significant reduction in salivary flow in animals that were pretreated with lactated Ringers or AAV2/9-GFP ( $3.7 \pm 0.7 \mu\text{l}/\text{min}$  and  $2.3 \pm 0.6 \mu\text{l}/\text{min}$ , respectively). Unlike control animals, animals treated with AAV2/9-TLK1B demonstrated no drop in salivary flow after radiation ( $40.54 \pm 5.4 \mu\text{l}/\text{min}$ ; Fig. 4). When compared with the corresponding nonirradiated groups, the lactated Ringers- and AAV2/9-GFP-treated animals exhibited a >90% reduction in salivary flow after radiation, but more importantly, AAV2/9-TLK1B groups showed no decline in salivary flow after radiation (121% increase); salivary flow was not significantly different in irradiated and nonirradiated animals treated similarly with TLK1B (Fig. 4).

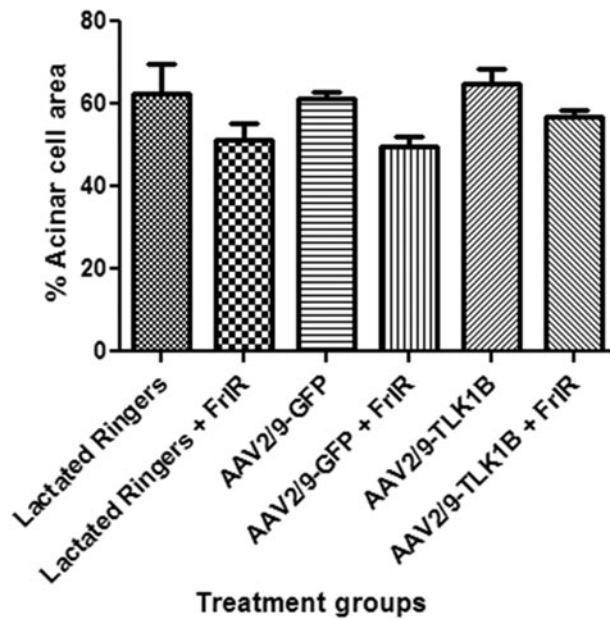
Statistical analyses of salivary flow rates showed that treatment, irradiation, and interaction effects were highly significant (each with  $p < 0.01$ ). Pair-wise comparisons between AAV2/9-TLK1B and lactated Ringers groups, and between AAV2/9-TLK1B and AAV2/9-GFP groups were significant ( $p < 0.01$ ), but there was no significant difference between lactated Ringers and AAV2/9-GFP groups. A significant effect of radiation–treatment interaction ( $p < 0.01$ ) suggested that the effect of irradiation on salivary flow rate was different among the three treatment groups. Radiation had a significant effect on the AAV2/9-GFP-treated and lactated Ringers-treated animals (with significantly lower mean salivary flow rates than the corresponding nonirradiated animals), but radiation had no effect on the AAV2/9-TLK1B-treated animals.

#### *TLK1B better preserves submandibular gland structure after fractionated ionizing radiation*

Histological examination of submandibular gland tissue revealed the presence of mucinous, signet-ring cells irrespective of treatment type or radiation. Although we do not understand the nature of these vacuoles, our results suggest that their presence does not affect salivary gland function. Submandibular glands extirpated 8 weeks after fractionated radiation (15 weeks after virus infusion; Fig. 1) demonstrated focal structural abnormalities such as inflammatory infiltrate and fibrosis, which were more pronounced in irradiated animals pretreated with lactated Ringers or GFP than in TLK1B-treated animals (Supplementary Fig. S3). Histopathological







**FIG. 6.** Quantification of acinar cell surface area in rat submandibular gland tissues. Rat submandibular gland tissue sections reacted with glutamate–glutamic acid-rich proteins antibody were used for analysis. Three random microscopic fields per gland, at 200 $\times$  magnification, were evaluated using ImageJ software (NIH), and the surface area occupied by acinar cells was measured. The data shown are mean  $\pm$  SD.

glands. Likewise, expression of the transgene after AAV9 delivery to rat submandibular glands was detected within 5 weeks, and it persisted for a long period—through to 15 weeks (Voutetakis *et al.*, 2004; Katano *et al.*, 2006; Wang *et al.*, 2006). Recombinant AAV vectors are gutless vectors that only possess the inverted terminal repeats of the viral genome. They depend on the cellular machinery for replication, and therefore the time lag between virus delivery and transgene expression could be associated with the delayed conversion of single-stranded rAAV DNA to a double-stranded functional molecule in slow-replicating cells of the salivary gland. The narrow interval between virus transduction and gene expression in rapidly proliferating salivary cells in culture lends credence to this supposition. Although wild-type AAV2 integrates into the human genome at a site-specific location on chromosome 19, the integration of rAAV is relatively infrequent and inefficient (McCarty *et al.*, 2004). Therefore, long-term transgene expression in salivary glands suggests a preponderance of rAAV9 duplex DNA as episomal genomes or concatamers in submandibular salivary glands (Voutetakis *et al.*, 2010). Stable expression without chromosomal integration averts the risk of deleterious mutagenesis in the host. rAAV has not been associated with any malignancies in humans and is, therefore, regarded as a relatively nonpathogenic gene delivery vehicle.

Vector biodistribution evaluated at the macroscopic and microscopic levels confirmed GFP expression in the target organ, submandibular salivary glands, but unexpectedly, in the intestines too. Systemic administration of AAV9 is known to effectively transduce the heart, liver, and skeletal muscles (Inagaki *et al.*, 2006; Pacak *et al.*, 2006). However, the

lack of transgene expression in the heart and liver in our study suggests that vector spread to the intestines was likely a result of ingestion of residual virus that was expelled in oral cavity after the wear-down of atropine effects rather than virus spread through systemic circulation. The prospect of intestinal expression after virus ingestion is supported by a previous study that demonstrated gene transfer to gut epithelium and lamina propria after oral administration of AAV (During *et al.*, 1998). Prolonged expression of the transgene in the rapidly proliferating cells of the gut suggests the potential transduction of progenitor cells within the intestinal crypts (During *et al.*, 1998).

Salivary glands are composed of three main epithelial cell types—the fluid-producing acinar cells, the myoepithelial cells, and the ductal cells that form conduits for saliva to flow to the oral cavity. The ductal tree plays an important role in modulating the composition of saliva, and in rodents, it is comprised of the intercalated ducts, the convoluted granular tubules, and the secretory and excretory ducts. Ductal cells have gained importance in salivary gland regeneration, since they were found to harbor putative progenitor stem cells (Takahashi *et al.*, 1998; Man *et al.*, 2001). Stem cells within the intercalated ducts can differentiate into amylase-producing acinar cells to aid recovery of gland function after radiation injury and after ductal ligation-induced gland atrophy (Lombaert *et al.*, 2008; Cotroneo *et al.*, 2010). Additionally, ductal cells of rodent salivary glands also serve as reservoirs of growth hormones, known to play a physiological role during development (Barka, 1980; Mori *et al.*, 1983; Kato *et al.*, 1991). Growth hormones stimulate cells to proliferate and may play a role in tissue regeneration. Therefore, apart from fluid-producing acinar elements, the protection of ductal elements can also favor the restoration of salivary function after injury in rodents.

Multiple plausible reasons have emerged to explain the loss of salivary gland function after radiation. The drop in salivary flow within a few days after radiation does not correlate with loss of acinar cell viability, and the loss in secretory function has been attributed to molecular damage-induced cellular dysfunction and to brief inanition caused by oropharyngeal mucositis (Nagler, 2002). Delayed death in cells with irreparable damage can occur over weeks or months, and progenitor cells of the gland divide to repopulate cells lost to radiation (Konings *et al.*, 2005). Previous studies have shown an increase in proliferative activity in salivary glands within days after single-dose radiation (Ballagh *et al.*, 1994; Peter *et al.*, 1994). Increase in proliferative activity after radiation results in a more devastating effect of fractionated radiation on salivary gland function, since proliferating cells will be more susceptible to damage during subsequent doses of radiation. Therefore, our observations of a >90% decrease in salivary function after repeated small doses of 2.5 Gy is not surprising. However, with no widespread structural damage to the glands after radiation, histological manifestations alone appear to fall short in explaining the severity of gland malfunction in irradiated animals. A likely explanation for this observation is that the small doses of radiation were not sufficient to cause cell death, but they were severe enough to cause irreparable molecular changes and/or interfere with the proliferative activity of progenitors to incapacitate the function of the glands. Additionally, radiation damage to the gland

vasculature could also play a role in compromising the secretory capacity of the glands (Cotrim *et al.*, 2007).

High-dose radiation used to treat patients with head and neck cancer is associated with irreversible xerostomia, and clinical measures to protect salivary glands and preserve its function have shown benefit (Scrimger, 2011). Nonetheless, radiation-resultant compromised salivary gland function continues to be a challenge. Tousled-like kinase 1B and its larger variant TLK1 are cellular proteins that play a role in cell cycle arrest, chromatin remodeling, and DNA repair (Groth *et al.*, 2003; Sunavala-Dossabhoy *et al.*, 2005; Canfield *et al.*, 2009; Sunavala-Dossabhoy and De Benedetti, 2009; De Benedetti, 2010). Their role in facilitating DNA repair is emphasized by observations that overexpression of the protein protects cells against death by radiation (Li *et al.*, 2001; Sunavala-Dossabhoy *et al.*, 2005), whereas suppression of its kinase activity or knockdown of the protein enhances the cell's susceptibility to DNA damage-induced cell death (Sunavala-Dossabhoy *et al.*, 2003; Takayama *et al.*, 2010). We have shown before that exogenous TLK1B in rat submandibular salivary glands effectively attenuates xerostomia associated with a single large-dose radiation (Palaniyandi *et al.*, 2011; Sunavala-Dossabhoy *et al.*, 2012). Now, we demonstrate that sustained expression of TLK1B associated with a single instillation of rAAV9 to rat submandibular glands is sufficient to completely ameliorate salivary dysfunction caused by fractionated radiation. Our results on TLK1B efficacy in ameliorating fractionated radiation-inflicted xerostomia generate enthusiasm for analyzing, in depth, the safety of the therapeutic with a prospect of furthering AAV2/9-TLK1B to cancer patient care.

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### Author Disclosure Statement

No competing financial interests exist.

### References

- Ballagh, R.H., Kudryk, K.G., Lampe, H.B., *et al.* (1994). The pathobiology of salivary gland. III. PCNA-localization of cycling cells induced in rat salivary gland by low-dose x-radiation. *Oral Surg. Oral Med. Oral Pathol.* 77, 27–35.
- Barka, T. (1980). Biologically active polypeptided in submandibular glands. *J. Histochem. Cytochem.* 28, 836–859.
- Bell, C.L., Gurda, B.L., Van Vliet, K., *et al.* (2012). Identification of the galactose binding domain of the AAV9 capsid. *J. Virol.* 86, 7326–7333.
- Berk, L. (2008). Systemic pilocarpine for treatment of xerostomia. *Expert Opin. Drug Metab. Toxicol.* 4, 1333–1340.
- Braam, P.M., Roesink, J.M., Moerland, M.A., *et al.* (2005). Long-term parotid gland function after radiotherapy. *Int. J. Radiat. Oncol. Biol. Phys.* 62, 659–664.
- Bruce, S.D. (2004). Radiation-induced xerostomia: how dry is your patient? *Clin. J. Oncol. Nurs.* 8, 61–67.
- Canfield, C., Rains, J., and De Benedetti, A. (2009). TLK1B promotes repair of DSBs via its interaction with Rad9 and Asf1. *BMC Mol. Biol.* 10, 110.
- Carrera, P., Moshkin, Y.M., Gronke, S., *et al.* (2003). Tousled-like kinase functions with the chromatin assembly pathway regulating nuclear divisions. *Genes Dev.* 17, 2578–2590.
- Chambers, M.S., Rosenthal, D.I., and Weber, R.S. (2007a). Radiation-induced xerostomia. *Head Neck* 29, 58–63.
- Chambers, M.S., Jones, C.U., Biel, M.A., *et al.* (2007b). Open-label, long-term safety study of cevimeline in the treatment of postirradiation xerostomia. *Int. J. Radiat. Oncol. Biol. Phys.* 69, 1369–1376.
- Cotrim, A.P., Sowers, A., Mitchell, J.B., *et al.* (2007). Prevention of irradiation-induced salivary hypofunction by microvessel protection in mouse salivary glands. *Mol. Ther.* 15, 2101–2106.
- Cotroneo, E., Proctor, G.B., and Carpenter, G.H. (2010). Regeneration of acinar cells following ligation of rat submandibular gland retraces the embryonic-perinatal pathway of cytodifferentiation. *Differentiation* 79, 120–130.
- De Benedetti, A. (2010). Tousled kinase TLK1B mediates chromatin assembly in conjunction with Asf1 regardless of its kinase activity. *BMC Res. Notes* 3, 68.
- Dijkema, T., Raaijmakers, C.P., Ten Haken, R.K., *et al.* (2010). Parotid gland function after radiotherapy: the combined Michigan and Utrecht experience. *Int. J. Radiat. Oncol. Biol. Phys.* 78, 449–453.
- During, M.J., Xu, R., Young, D., *et al.* (1998). Peroral gene therapy of lactose intolerance using an adeno-associated virus vector. *Nat. Med.* 4, 1131–1135.
- Epstein, J.B., Robertson, M., Emerton, S., *et al.* (2001). Quality of life and oral function in patients treated with radiation therapy for head and neck cancer. *Head Neck* 23, 389–398.
- Gaudet, D., Méthot, J., and Kastelein, J. (2012). Gene therapy for lipoprotein lipase deficiency. *Curr. Opin. Lipidol.* 23, 310–320.
- Gray, S.J. (2013). Gene therapy and neurodevelopmental disorders. *Neuropharmacology.* 68, 136–142.
- Groth, A., Lukas, J., Nigg, E.A., *et al.* (2003). Human Tousled like kinases are targeted by an ATM- and Chk1-dependent DNA damage checkpoint. *EMBO J.* 22, 1676–1687.
- Hashimoto, M., Matsui, T., Iwabuchi, K., *et al.* (2008). PKU-B/TLK1 regulates myosin II activities, and is required for accurate equaled chromosome segregation. *Mutat. Res.* 657, 63–67.
- Inagaki, K., Fuess, S., Storm, T.A., *et al.* (2006). Robust systemic transduction with AAV9 vectors in mice: efficient global cardiac gene transfer superior to that of AAV8. *Mol. Ther.* 14, 45–53.
- Kaludov, N., Brown, K.E., Walters, R.W., *et al.* (2001). Adeno-associated virus serotype 4 (AAV4) and AAV5 both require sialic acid binding for hemagglutination and efficient transduction but differ in sialic acid linkage specificity. *J. Virol.* 75, 6884–6893.
- Katano, H., Kok, M.R., Cotrim, A.P., *et al.* (2006). Enhanced transduction of mouse salivary glands with AAV5-based vectors. *Gene Ther.* 13, 594–601.
- Kato, K., Yokose, S., and Tajima, Y. (1991). Use of silver enhancement technique for immunohistochemical detection of EGF in rat submandibular gland. *Biotech. Histochem.* 1, 87–88.
- Klein, R.L., Dayton, R.D., Tatom, J.B., *et al.* (2008). AAV8, 9, Rh10, Rh43 vector gene transfer in the rat brain: effects of serotype, promoter and purification method. *Mol. Ther.* 16, 89–96.
- Konings, A.W.T., Coppes, R.P., and Vissink, A. (2005). On the mechanism of salivary gland radiosensitivity. *Int. J. Rad. Oncol. Biol. Phys.* 62, 1187–1194.
- Li, Y., DeFatta, R., Anthony, C., *et al.* (2001). A translationally regulated Tousled kinase phosphorylates histone H3 and



- confers radioresistance when overexpressed. *Oncogene* 20, 726–738.
- Lombaert, I.M.A., Brunsting, J.F., Wierenga, P.K., *et al.* (2008). Rescue of salivary gland function after stem cells transplantation in irradiated glands. *PLoS One*. 3, e2063.
- Man, Y.G., Ball, W.D., Marchetti, L., *et al.* (2001). Contributions of intercalated duct cells to the normal parenchyma of submandibular glands of adult rats. *Anat. Rec.* 263, 202–214.
- McCarty, D.M., Young, S.M., Jr., and Samulski, R.J. (2004). Integration of adeno-associated virus (AAV) and recombinant AAV vectors. *Annu. Rev. Genet.* 38, 819–845.
- Mori, M., Hamada, K., Naito, R., *et al.* (1983). Immunohistochemical localization of epidermal growth factor in rodent submandibular glands. *Acta Histochem. Cytochem.* 16, 536–547.
- Nagler, R.M. (2002). The enigmatic mechanism of irradiation-induced damage to the major salivary glands. *Oral Dis.* 8, 141–146.
- Pacak, C.A., Mah, C.S., Thattaliyath, B.D., *et al.* (2006). Recombinant adeno-associated virus serotype 9 leads to preferential cardiac transduction *in vivo*. *Circ. Res.* 99, e3–e9.
- Palaniyandi, S., Odaka, Y., Green, W., *et al.* (2011). Adenoviral delivery of Tousled kinase for the protection of salivary glands against ionizing radiation damage. *Gene Ther.* 18, 275–282.
- Peter, B., Van Waarde, M.A., Vissink, A., *et al.* (1994). Radiation-induced cell proliferation in the parotid and submandibular glands of the rat. *Radiat. Res.* 140, 257–265.
- Roesink, J.M., Moerland, M.A., Battermann, J.J., *et al.* (2001). Quantitative dose-volume response analysis of changes in parotid gland function after radiotherapy in the head-and-neck region. *Int. J. Radiat. Oncol. Biol. Phys.* 51, 938–946.
- Rudat, V., Münter, M., Rades, D., *et al.* (2008). The effect of amifostine or IMRT to preserve the parotid function after radiotherapy of the head and neck region measured by quantitative salivary gland scintigraphy. *Radiother. Oncol.* 89, 71–80.
- Sciubba, J.J., and Goldenberg, D. (2006). Oral complications of radiotherapy. *Lancet Oncol.* 7, 175–183.
- Scrimger, R. (2011). Salivary gland sparing in the treatment of head and neck cancer. *Expert Rev. Anticancer Ther.* 11, 1437–1448.
- Sillje, H.H., and Nigg, E.A. (2001). Identification of human Asf1 chromatin assembly factors as substrates of Tousled-like kinases. *Curr. Biol.* 11, 1068–1073.
- Sillje, H.H., Takahashi, K., Tanaka, K., *et al.* (1999). Mammalian homologues of the plant Tousled gene code for cell-cycle-regulated kinases with maximal activities linked to ongoing DNA replication. *EMBO J.* 18, 5691–5702.
- Sunavala-Dossabhoy, G., and De Benedetti, A. (2009). Tousled homolog, TLK1, binds and phosphorylates Rad9; TLK1 acts as a molecular chaperone in DNA repair. *DNA Repair (Amst.)* 8, 87–102.
- Sunavala-Dossabhoy, G., and Klein, R. (2009). Adeno-associated virus-mediated gene transfer in rat salivary glands. *Gene Therapy Symposium, New Orleans, LA.*
- Sunavala-Dossabhoy, G., Li, Y., Williams, B., *et al.* (2003). A dominant negative mutant of TLK1 causes chromosome mis-segregation and aneuploidy in normal breast epithelial cells. *BMC Cell. Biol.* 4, 16.
- Sunavala-Dossabhoy, G., Balakrishnan, S.K., Sen, S., *et al.* (2005). The radioresistance kinase TLK1B protects the cells by promoting repair of double strand breaks. *BMC Mol. Biol.* 6, 19.
- Sunavala-Dossabhoy, G., Palaniyandi, S., Richardson, C., *et al.* (2012). TAT-mediated delivery of Tousled protein to salivary glands protects against radiation-induced hypofunction. *Int. J. Radiat. Oncol. Biol. Phys.* 84, 257–265.
- Takahashi, S., Schoch, E., and Walker, N.I. (1998). Origin of acinar cell regeneration after atrophy of rat parotid induced by duct obstruction. *Int. J. Exp. Pathol.* 79, 293–301.
- Takayama, Y., Kokuryo, T., Yokoyama, Y., *et al.* (2010). Silencing of Tousled-like kinase 1 sensitizes cholangiocarcinoma cells to cisplatin-induced apoptosis. *Cancer Lett.* 296, 27–34.
- Voutetakis, A., Kok, M.R., Zheng, C., *et al.* (2004). Reengineered salivary glands are stable endogenous bioreactors for systemic gene therapeutics. *Proc. Natl. Acad. Sci. U. S. A.* 101, 3053–3058.
- Voutetakis, A., Zheng, C., Cotrim, A.P., *et al.* (2010). AAV5-mediated gene transfer to the parotid glands of non-human primates. *Gene Ther.* 17, 50–60.
- Wang, J., Voutetakis, A., Papa, M., *et al.* (2006). Rapamycin control of transgene expression from a single AAV vector in mouse salivary glands. *Gene Ther.* 13, 187–190.
- Weinberg, M.S., Samulski, R.J., and McCown, T.J. (2013). Adeno-associated virus (AAV) gene therapy for neurological disease. *Neuropharmacology.* 69, 82–88.

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