Ultrasound-assisted nonviral gene transfer of AQP1 to the irradiated minipig parotid gland restores fluid secretion

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Xerostomia is a common side effect of ionizing radiation used to treat head and neck cancer. A groundbreaking Phase I human clinical trial using Adenoviral gene transfer of Aquaporin-1 (AQP1) to a single salivary gland of individuals suffering from radiation-induced xerostomia has recently been reported. Unfortunately, the limitations of the Adenoviral vector system used in this pioneering trial preclude its advancement to a Phase II trial, and we have thus undertaken to evaluate the therapeutic potential of ultrasound-assisted nonviral gene transfer (UAGT) as an alternative means of delivering AQP1 gene therapy to the salivary gland by comparing head-to-head with the canonical Adenoviral vector in a swine model. Swine irradiated unilaterally with a 10-Gy electron beam targeted at the parotid gland suffered from significant, sustained hyposalivation that was bilateral, despite irradiation being confined to the targeted gland. Unilateral AQP1 gene therapy with UAGT resulted in bilateral restoration of stimulated salivary flow at 48 h and 1 week post treatment (1.62 ± 0.48 ml and 1.87 ± 0.45 ml) to preinjury levels (1.34 ± 0.14 ml) in a manner comparable to Adenoviral delivery (2.32 ± 0.6 ml and 1.33 ± 0.97 ml). UAGT can replace the Adenoviral vector as a means of delivering AQP1 gene therapy in the irradiated swine model, and it is a candidate for advancement to a Phase I human clinical trial.

INTRODUCTION

Cancers of the head and neck comprise roughly 3% of all cancer cases in the United States,1 with an incidence estimated at 52,000 cases in the United States in 2013.2 Incidence of these cancers varies globally, according to the prevalence of risk factors in various populations. Throughout the developed world, external beam radiation remains a mainstay of therapy for most types and stages of head and neck cancer, either alone or in combination with surgery and/or chemotherapy. Co-irradiation of the salivary glands during radiotherapy is common, and it results in severe and irreversible hyposalivation, which in turn leads to a constellation of oral morbidities including xerostomia and dental disease.3

Treatment options for radiation-induced xerostomia are extremely limited and consist of exogenous rehydration and management of progressive damage to the oral mucosa and dentition. In light of these limited treatment options and the chronic and debilitating nature of this condition, gene therapy for radiation-induced xerostomia has been developed and successfully demonstrated in a Phase I clinical trial.4,5 This gene therapy strategy is targeted to the ductal cells of the salivary gland, which, in contrast to the saliva-producing acinar cells, are resistant to ionizing radiation and survive radiotherapy largely intact. Using an Adenoviral vector to express Aquaporin 1 (AdAQP1) in parotid gland ductal cells, Baum et al.6 developed a treatment paradigm that results in the transcellular flux of interstitial fluid across the ductal cell layer and into the intraductal labyrinth of the salivary gland, where the fluid can be expelled to produce palliative oral wetness.

This AdAQP1 clinical trial has successfully established the safety of AQP1 gene therapy, as well as demonstrating objective improvements in parotid salivary flow rates and subjective improvement in xerostomia in patients receiving the treatment, in a dose-dependent manner. Despite this remarkable success, a Phase II trial of AdAQP1 is not planned, as the therapeutic effect is transient and this vector is not suitable for the re-administration required to treat this chronic condition. Adenovirus elicits a strong host immune response in humans, and this response is thought to be progressive with repeated exposure.6,7 In aggregate, successful application of AQP1 gene therapy in radiation-induced xerostomia is dependent upon the clinical implementation of a gene therapy technique that evades host immune response and allows for periodic re-administration throughout the lifetime of the individual.

Our efforts toward this goal have focused on ultrasound-assisted gene transfer (UAGT) to the salivary gland, which combines the use of a nonviral DNA vector and lipid/perfluerten microbubbles with a low-frequency acoustic field to create a ‘sonoporation’ effect, allowing gene transfer to the cells of the salivary gland without the introduction of viral antigens. This method has been shown to successfully express transgenes within the salivary gland of rodents,8,9 and it has advantages over conventional viral vectors. Most notably, this method uses three safe and clinical-grade components: (1) clinical ultrasound; (2) perfluoten lipid microbubbles, which have been approved for intravascular administration to humans;10 and (3) plasmid DNA vectors. Although intracellular host immune response can still occur with nonviral DNA vectors,9 extracellular host response, either humoral or cell-mediated, is thought to be minimized in the
absence of viral antigens, provided that the transgene itself is native to the host.

This report describes our efforts to develop and characterize a swine model of radiation-induced xerostomia, to effect UAGT to the parotid gland of swine subjects, and ultimately to compare the therapeutic efficacy and duration of AQP1 gene therapy in the swine using UAGT and Adenoviral gene transfer head-to-head. As the swine is the penultimate preclinical model of radiation-induced xerostomia, our efforts were designed to mimic those of the AdAQP1 preclinical development effort,11 and to potentially provide a rationale for the translation of UAGT/AQP1 to a Phase I clinical trial in patients suffering from radiation-induced xerostomia. If successful, these efforts could provide an alternative to viral-based gene therapy, and, by obviating host immune response, establish a gene therapy protocol that could be periodically re-administered throughout the lifetime of the patient.

RESULTS

Figure 1 illustrates, in graphical and table format, the experimental groups used in this study and the treatments, sample collections and experimental end point of each group.

![Graphical representation of experimental groups and treatments](image-url)

A single 10-Gy dose of radiation delivered by an electron beam to the right parotid gland results in significant, sustained and bilateral hyposalivation in the miniature swine. The digitally reconstructed radiograph image used to treatment-plan the electron beam for all study subjects is shown in Figure 2a. To confirm that the control gland received no radiation, we placed thermo-luminescent detectors on the skin overlying the right (treated) and left (control) parotid gland during the application of the electron beam. Figure 2b shows that the control gland received no detectable radiation.

A baseline stimulated, isolated parotid saliva collection was performed on all animals \( (n = 16) \), bilaterally and immediately before irradiation (~10 weeks of age) and at 4, 6, 8, 10 and 12 weeks post irradiation. Saliva volume was determined by weight, assuming a specific gravity of 1.0. Figure 2c shows stimulated parotid saliva volumes for the irradiated (right) and control (left) sides. Notably, despite the demonstrated lack of radiation damage to the control gland, saliva output of the control side began to decline from baseline by week 8, decreasing further at week 10 and week 12. Our conservative statistical strategy did not find significant differences between Baseline and Control to support the visually obvious trend, but it suggested that Control

| Group # | Treatment                  | n  | Treatment Timepoint (Weeks Post-Irradiation) | Survival Post-Treatment (Weeks) | Comments                  | Post-Treatment Saliva Measurements |
|---------|----------------------------|----|---------------------------------------------|---------------------------------|---------------------------|-----------------------------------|
| 1       | AdhAQP1 (NIH virus)        | 4  | 12                                          | 2                               | Model Validation          | Figure 4                          |
| 2       | UAGT/pAQP1                 | 4  | 14                                          | 6                               | Experimental              | Figure 6                          |
| 3       | UAGT/MetLuc                | 4  | 14                                          | 1                               | Negative Control, Histology| Figure 3B                         |
| 4       | AdpAQP1                    | 4  | 14                                          | 1                               | Histology Only            | -                                 |

Figure 1. Experimental group assignments for the 16 animal subjects participating in this study.

Figure 2. Radiation treatment plan, dosimetry study and radiation-induced hyposalivation. (a) Digital radiography reconstruction of the head of a Yucatan minipig used in this study. The right parotid gland, the target organ for irradiation, is shown in magenta. Other structures that are important to the treatment plan include left parotid (indigo), mandible (dark blue/yellow), right orbit (cyan) and left orbit (dark green). (b) Radiation dose measured on the skin overlying the left (control) and right (irradiated) parotid glands. (c) Stimulated isolated parotid saliva volumes at preirradiation baseline and various time points post irradiation in the 16 subjects participating in this study. Error bars are ± s.e.m. Statistical analysis determined that significant differences existed between irradiated and control volumes \( (P = 0.0003) \) but no interaction between the covariates, suggesting that both groups change over time at similar rates. Evaluating the treatment status versus baseline across all weeks indicated that the irradiated side was significantly different from the baseline \( (P = 0.0002) \), but the control side was not \( (P = 0.5) \).
UAGT delivering AQP1 gene therapy in the irradiated swine model
Z Wang et al

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Ultrasound-assisted gene transfer of met-Luc to the irradiated salivary gland produces bioluminescent saliva but no increase in stimulated saliva volume. Our group has previously reported ultrasound-assisted gene transfer via ‘sonoporation’ in the mouse salivary gland, by our knowledge the application of this technology to the salivary gland of the swine has never been reported. Demonstrating this phenomenon in the pig proved challenging, as the size of the animal precludes the use of the conventional IVIS imaging technique that we had previously relied upon. We therefore turned to the secreted Metridia longa luciferase (MetLuc) reporter gene, reasoning that if we were successful in accomplishing the same manipulation as animals receiving either Adenoviral or ultrasound-assisted AQP1 gene therapy, as described below. Stimulated saliva volumes collected immediately before, and 48 h after UAGT with MetLuc (Figure 3b) demonstrate that there is no effect of the UAGT methodology per se, on stimulated parotid saliva volume.

Xerostomia in our swine model is reversed by the clinically validated Adenovirus-expressing human AQP1 (hAQP1). The intent of this study was to measure AQP1 gene therapy delivered by UAGT against the clinical standard, AQP1, delivered by Adenovirus. Human AQP1, delivered by a viral vector, has previously been used as a gene therapy for xerostomia in the rat, and ultimately humans. Thus, we first sought to demonstrate that our swine model of radiation-induced xerostomia responds to Adenoviral gene therapy in a manner consistent with what was previously reported in the canonical study by Shan et al. We obtained a nonclinical-grade prep of the AdhAQP1 virus from the NIH team (a kind gift of Dr Changyu Zheng, NIDCR) to ensure that we were using the same vector that has shown therapeutic efficacy in the human clinical trial.

Consistent with earlier preclinical work using AdhAQP1 in the swine model, our animals received $1 \times 10^{10}$ viral particles of the vector to the irradiated gland, 12 weeks following irradiation ($n = 4$). AdhAQP1 reverses hyposalivation in our swine model (Figure 4) in a manner similar to that reported by Shan et al. Further, gene therapy delivered to the irradiated gland was observed to substantially enhance stimulated fluid secretion from the contralateral gland, lending additional support for functional coupling between the left and right parotid glands. The extinction of the therapeutic effect within 2 weeks is consistent with the known gene expression dynamics of the Adenovector in mammals.

Figure 4. AdhAQP1 gene therapy in our swine model of radiation-induced hyposalivation. Stimulated parotid saliva volumes at Baseline, before treatment (12 weeks post irradiation) and 48 h, 1 week and 2 weeks following gene therapy with $1 \times 10^{10}$ viral particles of AdhAQP1 ($n = 4$). Error bars are ± s.e.m. Statistical analysis determined that no significant differences existed between irradiated (R) and control (L) gland saliva volumes ($P = 0.4$), suggesting that both groups change together. Comparisons between weeks noted the following significant differences: Baseline versus pretreatment ($P = 0.01$), pretreatment versus +48 h ($0.0001$), +48 h versus +1 week ($P = 0.0001$).
Porcine AQP1 (pAQP1) expressed in MDCK cells results in transcellular water permeability

The above experiment demonstrates that our swine model responds to AdhAQP1 gene therapy in a manner similar to the earlier swine model that used unilateral irradiation with photons. However, as our UAGT or ‘sonoporation’ gene transfer technology obviates the need for a viral vector, and thus avoids the introduction of foreign antigens into the damaged salivary gland, we decided to carry our experiments forward with porcine AQP1 (pAQP1). Our clinical translational goal is to use UAGT to express hAQP1 in human salivary glands. Thus, modeling that goal using pAQP1 in pigs seems a sound way of determining the duration of therapeutic effect following UAGT absent concerns of host response against a foreign transgene (that is, hAQP1), which by themselves could limit the duration of therapeutic effect.

pAQP1 was cloned as described in Material and Methods and expressed by plasmid transfection into Madin-Darby Canine Kidney epithelial (MDCK) canine kidney epithelial cells plated as a monolayer in transwell chambers (Figure 5a). Compared with green fluorescent protein (GFP)-transfected cells, cells transfected with pAQP1 showed enhanced water flux across the monolayer (Figure 5b). These experiments demonstrate the physiological functionality of our pAQP1 transgene product.

pAQP1 gene therapy delivered to the irradiated salivary gland with UAGT increases stimulated salivary flow to levels comparable to Adenoviral gene therapy

With this preparatory work completed, we next undertook to test our main hypothesis that UAGT of pAQP1 to the irradiated gland can increase stimulated parotid saliva volume in a manner similar to that previously demonstrated with hAQP1 delivered by an Adenovirus. Increases in stimulated parotid saliva volume were observed in both treated and control sides in UAGT/pAQP1-treated animals (n=4, Figure 6), with the magnitude of the increase being roughly comparable to that seen with the AdhAQP1 virus in this model. A similar bilateral effect was also observed, despite the fact that gene therapy was delivered only to the irradiated side. The increases in saliva volume declined substantially by 2 weeks post treatment, showing a trend toward persistent therapeutic effect on the control side but no longer statistically different from pretreatment levels. Notably, function improved markedly in the control gland despite the fact that gene therapy was only delivered to the irradiated gland.

UAGT does not promote the local inflammation observed with Adenoviral gene transfer to the salivary gland

The major factor limiting the clinical utility of Adenoviral gene therapy in chronic conditions such as xerostomia is the vector’s robust immunogenicity—a phenomenon that has previously been described histologically in the salivary gland, even with UV-inactivated Adenoviral particles, suggesting that it is mediated entirely by the viral capsid. Further, systemic inflammation resulting from Adenoviral gene transfer to the salivary gland, as indicated by chronic inflammatory focal lesions and induction of anti-Adenoviral antibodies, has also been reported. As our UAGT technique accomplishes gene therapy that is comparable to Adenovirus-mediated gene transfer, but obviates the introduction of viral antigens into the subject’s body, we hypothesized that UAGT would also minimize local and systemic inflammatory response.

To test this hypothesis, we constructed an Adenoviral vector expressing the pAQP1 CDNA described above. We treated a group of swine (n=4, Group 4), irradiated on the right side as described above, with this Adenoviral porcine Aquaporin 1 (AdpAQP1) and killed this group 1 week after gene transfer. The UAGT/MetLuc negative control animals (n=4, Group 3), described previously, were killed 1 week after gene transfer and served as the UAGT control. In all experiments, the unirradiated (left) parotid gland was left untouched, and it served as an internal control for each animal. The design of this experiment was such that it allowed us to parse the effects of Adenoviral gene delivery relative UAGT upon the histology of the parotid gland.

Slides were reviewed by an oral pathologist (PCE) blinded to experimental design and animal identities. Results of this experiment are summarized in Table 1 and representative sections are shown in Figure 7. Relative to control glands, two primary histological findings were noted. First, mild acinar cell pleomorphic changes and inclusions in zymogen granules were observed in all irradiated glands (Figures 7b, d, f and h). The second finding was interstitial and periductal inflammation, which was restricted to glands treated with the Adenoviral vector (Figures 7f and h). Importantly, UAGT had no detectable effect upon the structure or morphology of the salivary gland, reinforcing the safety of this technique relative to the canonical Adenoviral vector.

Saliva produced by the damaged parotid gland after gene therapy shows a profile very similar to normal saliva, but with the loss of select proteins of putative acinar origin. Aquaporin-1 gene therapy as a treatment for radiation-induced xerostomia is predicated upon the simple mechanism of

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Gene Therapy (2015) 739 – 749

Z Wang et al

UAGT delivering AQP1 gene therapy in the irradiated swine model

Figure 5. Testing of pAQP1 channel function. (a) Schematic of the transwell culture system used to assess transcellular fluid flux across a confluent MDCK monolayer. The upper layer of the well is 440mOsmol l−1 hyperosmotic sucrose/DMEM medium and the lower layer is standard DMEM medium. (b) Quantification of transcellular fluid flux in GFP-transfected and pAQP1-transfected MDCK cells over a 48-h period. *P < 0.05 indicates statistically significant difference between GFP- and pAQP1-transfected cells. Error bars are ± s.e.m.
transcellular movement of fluid via AQP1 across surviving ductal cells. This is not fundamentally different from normal physiological salivation, which uses primarily Aquaporin-5 to drive transcellular fluid movement across acinar cells. In both instances, the source of the bulk of this fluid is ultimately the interstitium, which in turn is mainly dependent upon the serum for its protein composition. Accordingly, we explored to what degree the proteome of ‘saliva’ produced as a result of our gene therapy would differ from natural saliva.

To investigate this issue, we performed proteomic profiling using difference gel electrophoresis on matched samples of saliva obtained 48 h after gene therapy from the right (irradiated, treated) and left (control, untreated) parotid glands within the same animal relative to baseline saliva collected and banked from the same animal before irradiation. Figure 8 shows composite proteomic profiles of saliva at
baseline, from the control gland, and from the gene therapy-treated gland. The global similarity of Baseline and Control saliva relative to UAGT/pAQP1 saliva is visibly evident (Figures 8a and b versus Figure 8c).

Biological variation analysis was performed using the DeCyder platform, and we found the following differences: Baseline versus Control, 152 of 2253 matched spots significantly changed ($P < 0.05$); and Baseline versus Treated, 186 of 2362 matched proteins significantly changed ($P < 0.05$). To evaluate the potential clinical significance of salivary proteins lost as a result of radiation injury to the parotid gland, we selected and extracted spots determined to be significantly altered in UAGT/pAQP1 saliva versus Baseline saliva, but not significantly altered in Control versus Baseline saliva (a manual step confirmed this as a check on the software). Of the 108 spots extracted, 71 were positively identified using mass spectrometry, and results are shown in Table 2.

**DISCUSSION**

The development of salivary gland gene therapy from proof of concept in 1994 through to successful Phase I human clinical trial in 2012 is one of the finest examples of translational gene therapeutics yet reported. Although xerostomia is not life-threatening or life-limiting, the impact of this condition on the quality of life, nutrition, oral and digestive health is severe and can be devastating.

When it is considered that patients suffering from radiation-induced xerostomia, as well as other causes of xerostomia such as Sjogren’s syndrome and drug-related xerostomia, can live for decades with this condition, the development of a clinically practicable salivary gland gene therapy has the potential to mitigate the suffering of millions of people.

The present study is the first to show that UAGT can provide effective gene therapy for radiation-induced xerostomia in the penultimate preclinical model, the irradiated miniature swine. We have developed and now report a novel swine model wherein irradiation of the salivary gland is accomplished with an electron beam, obviating the possibility of radiation damage to the contralateral gland. On the basis of this model, we report a novel observation that unilateral damage to one parotid gland impairs the function of the contralateral gland. This mechanism should be considered in interpreting the results of the AdhAQP1 Phase I clinical trial.

The observation that the functions of the left and right parotid glands are functionally coupled is interesting and likely of great clinical importance. Two previous studies in a similar irradiated pig model noted possible reduction in salivary flow from the uninjured gland, but as these studies used photons it was assumed that the contralateral effects were owing to some spillover and/or scatter of radiation from the targeted side. In our study, that possibility has been excluded, demonstrating that the reduction in flow on the contralateral side is related in some way to the damage to the targeted gland. There is evidence of a neurological interplay between different salivary glands in rats, although this is not well characterized. Of interest, one study showed that unilateral parotid irradiation was sufficient to ameliorate sialorrhoea in patients suffering from amyotrophic lateral sclerosis.

To our knowledge, this study is the first to demonstrate nonviral UAGT to the parotid glands of swine. Our technique delivers therapeutic benefit equivalent to the clinically validated Adenoviral gene therapy approach, but it obviates the need to expose the patient to the systemic toxicity and local inflammation associated with the Adenoviral vector. This host immune response to viral vectors has historically presented the greatest challenge to the mainstreaming of gene therapy, and in the case of gene therapy for radiation-induced xerostomia this limitation makes Adenovirus unsuitable for advancement to a Phase II trial. Histometrics of damage to the salivary gland resulting from Adenoviral gene transfer have not been evaluated in humans, but significant morphological and functional damage to the salivary glands following Adenoviral gene transfer has been noted in animal studies, and we now report data confirming these safety considerations. Alternative strategies, such as Adeno-associated virus delivery of AQP1, are being evaluated, but would still necessarily involve re-exposing the patient to a viral vector every few years, risking progressive antivector host response.

The proteome of the fluid produced as a result of our gene therapy intervention is qualitatively very similar to natural saliva but lacks certain proteins normally supplied by the parotid acinar cells (see for a global overview of this topic). The clinical significance of this observation is complex and not yet entirely clear. On one hand, there is no doubt that the fluid produced is likely to be palliative, and it can be expected to relieve subjective xerostomia in patients, as it is reported to have done in the AdAQP1 clinical trial. On the other hand, the absence of, or the presence of alternative isoforms of, major salivary proteins such as Amylase, Lactoferrin and Lactoperoxidase (Table 2) makes it likely that the fluid produced may lack the efficacy of natural saliva in preventing caries and preserving the overall health of the oral mucosa. Finally, perhaps the most clinically relevant observation made in this study is the ability of AQP1 gene therapy delivered to the irradiated parotid gland to restore and even augment the function of the contralateral, undamaged gland. Integrating area under the curve in Figure 6, it appears that the majority of the saliva produced as a result of our UAGT/AQP1 gene therapy intervention actually came from the undamaged parotid gland, and if this principle holds true in human patients the benefit to the maintenance of oral and dental health is likely to be very significant.

This study has several limitations that should be noted, and they are imposed either owing to logistical challenges with this large animal model or economy of animal use for experimental design. First, the 10-Gy radiation is a mild insult, and it is delivered as a single dose, in contrast to the fractionated dosing used clinically. As noted in Methods, a 20-Gy electron beam causes severe radiation burns, and our ongoing experiments suggest that 15 Gy may be suitable for future studies. Second, we have not shown immunohistochemical localization of pAQP1 expression in our treated minipigs, although we have previously shown immunohistochemical distribution of a transgene in the salivary glands of mice following UAGT and expect a similar pattern in minipigs.

As now it is possible that UAGT can replace Adenovirus as the gene transfer technology to carry forward as a gene therapy for radiation-induced xerostomia, it is important to consider the state of the art vis-à-vis the plasmid vector. Conventional plasmid vectors contain extensive backbone regions of bacterial origin, and these regions are thought to stimulate host immunity and transgene silencing through various mechanisms that are as yet incompletely characterized. Minimalist plasmid constructs, termed ‘minicircles’ or ‘mini-intronic plasmids’ delete all or most of the foreign backbone sequences, but they are more difficult and costly to manufacture than conventional plasmids, particularly at the microgram quantities required for our application. Nevertheless, work from our own group has shown minicircles to be superior vectors for UAGT to the salivary gland. Our gene therapy strategy, as well as others in the preclinical pipeline, would likely benefit from technological advancement, allowing for GMP production of minicircles in the microgram range, at reasonable cost. Finally, UAGT is theoretically suitable for the delivery of gene editing components (for example, CRISPR/Cas) to the salivary gland, and the potential for this emerging technology as a means of permanent insertion of AQP1 into surviving ductal cells should be considered in future studies.
MATERIALS AND METHODS

Animals and husbandry
The Institutional Animal Care and Use Committee of Allegheny Singer Research Institute approved all animal experimentation described herein. Yucatan miniature swine aged ~10 weeks were purchased from Sinclair BioResources (Columbia, MO, USA). This well-characterized colony is certified free of common swine diseases and is vaccinated for Haemophilus, erysipelas, PCV and mycoplasma. Animals were group housed in large pens, with temperature maintained at 21–26 °C and 30–70% humidity under positive pressure. Enrichment with toys and human contact was provided. Subjects were fed twice daily, according to the breeder’s recommendations, and allowed free access to water via an automatic system. Subjects were acclimated to our colony for 1 week before being introduced into the study. Experimental group assignments are outlined in Figure 1. Group sizes were based upon pilot studies indicating that n = 4 gave a significant effect in gene therapy-treated animals. Animals received a name designation and were randomly assigned to their experimental group upon receipt in our facility.

Electron beam treatment planning and irradiation
Earlier studies (data not shown) using a 20-Gy electron beam revealed extremely severe damage to the parotid gland, well in excess of that experienced by most patients suffering from radiation-induced xerostomia. We calculated a single 10-Gy dose of electrons to be approximately bioequivalent to the threshold dose for parotid gland dysfunction. The functional deficits reported in Figure 2c validate this mild dose of radiation as being sufficient to elicit profound functional deficits in the parotid glands of our swine subjects.

A treatment plan was devised by computerized tomographic scanning of a single minipig of similar size and weight to all others in the study. This animal subject was used as a model for a vacuum formed bag to ensure targeting accuracy and immobilization of all subjects. This vacuum formed bag was subsequently used for immobilization of all animals participating in the study. CT scanning of this animal was performed using a Siemens Somatom Sensation scanner (Concord, CA, USA), and radiation treatment planning was performed on a Computerized Medical Systems XIO (Maryland Heights, MO, USA) planning system. A digitally reconstructed radiograph image of the treatment plan is presented in Figure 2a, showing the anatomical location of the left and right parotid glands in relation to the mandible and to one another.

On the basis of this treatment plan, irradiation of the right parotid gland was performed in all animal subjects using a Siemens Primus (Concord, CA, USA) linear accelerator. Animals were sedated and placed in the vacuum form bag in the linear accelerator. A 12 MeV electron beam calculated at the 90% isodose line was used to treat the gland, exempting a small portion of the anteromedial region to avoid unintended irradiation of the contralateral gland owing to the anatomic proximity of the two structures in this area. Cerrobend (Bolton Metal Products, Bellefonte, PA, USA), an eutectic alloy comprising 90% bismuth, 26.7% lead, 13.3% tin and 10% cadmium by weight, was used to block the electron beam from surrounding structures following custom anatomic fitting to match the right swine parotid gland. A 1.0-cm margin around the target gland was added to the aperture of the block to allow for respiratory motion and to allow build-up of electron dose. After irradiation and recovery from sedation, all animals were returned to the husbandry unit without event.

Saliva collection
All saliva collections were performed between 0630 hours and 1000 hours, and the time points of saliva collection are shown in the respective figures. No blinding was used in this study. Animals were initially sedated by
| Accession # | Description | Fold-change (AQP1-treated/baseline) |
|-------------|-------------|-----------------------------------|
| **Pig database** |  |  |
| CP881_PIG | 5-beta-cholestan-3-alpha,7-alpha-diol 12-alpha-hydroxylase | −2.2 |
| SNT7D_PIG | 5-hydroxtryptaminereceptor1D (Fragment) | −2.5 |
| ACT5_PIG | Actin, alpha skeletal muscle | 2.2 |
| ACTB_PIG | Actin, cytoplasmic | 2.2 |
| ADM1L_PIG | ADMS = Sus scrofa GN = ADMD PE = 1 SV = 1 | |
| ATPD_PIG | ATP synthase subunit delta, mitochondrial (Fragment) | 3.4 |
| CAN1_PIG | Calpain-1 catalytic subunit OS | 2.2 |
| CHLE_PIG | Cholinesterase (Fragment) | −6.1 |
| CO3_PIG | Complement C3 OS | 2.9 |
| CP2E1_PIG | Cytochrome P450 2E1 OS | 2.1 |
| HPT_PIG | Haptoglobin OS = Sus scrofa GN = HP PE = 1 SV = 1 | 2.5 |
| LAC_PIG | Ig lambda chain C region OS | 3.3 |
| MOES_PIG | Mesoins OS = Sus scrofa GN = MN PN PE = 2 SV = 3 | 2.7 |
| MYH1_PIG | Myosin-1 OS = Sus scrofa GN = MYH1 PE = 2 SV = 1 | 2.4 |
| OPTN_PIG | Optineurin OS = Sus scrofa GN = OPTN PE = 1 SV = 1 | 2.5 |
| AMYP_PIG | Pancreatic alpha-amylase OS | 2.9 |
| PECA1_PIG | Platelet endothelial cell adhesion molecule OS | 2.2 |
| COL1P1 | Pro-Opio-melanocortin OS = Sus scrofa GN = POMC PE = 1 SV = 1 | 2.0 |
| S10A1C_PIG | Protein S100-A12 OS = Sus scrofa GN = S100A12 PE = 1 SV = 2 | 3.5 |
| SAL_PIG | Salivary lipocalin OS = Sus scrofa GN = SAL1 PE = 1 SV = 1 | 6.7 |
| TREFE_PIG | Serotransferrin OS = Sus scrofa GN = TF PE = 1 SV = 1 | 3.3 |
| ALBU_PIG | Serum albumin OS = Sus scrofa GN = ALB PE = 1 SV = 2 | 2.1 |
| SDHB_PIG | Succinate dehydrogenase (ubiquinone) iron-sulfur subunit, mitochondrial OS = Sus scrofa GN = SDHB PE = 1 SV = 1 | 2.0 |
| TPM4_PIG | Tropomyosin alpha-4 chain OS = Sus scrofa GN = TPM4 PE = 2 SV = 3 | 4.5 |
| TRPY_PIG | Trypsin OS = Sus scrofa PE = 1 SV = 1 | 2.3 |
| MYO7A_PIG | Unconventional myosin-VIIa (Fragment) OS = Sus scrofa GN = MYO7A PE = 2 SV = 1 | 3.4 |
| UPK2_PIG | Uroplakin-2 OS = Sus scrofa GN = UPK2 PE = 2 SV = 3 | −6.0 |
| **Other mammalian database (excluding primates and rodents)** |  |  |
| 1433B_BOVIN | 14-3-3 protein beta/alpha OS = Bos taurus GN = YWHAB PE = 1 SV = 2 | 5.5 |
| 1433E_BOVIN | 14-3-3 protein epsilon OS = Bos taurus GN = YWHAE PE = 2 SV = 1 | 5.5 |
| 1433F_BOVIN | 14-3-3 protein eta OS = Bos taurus GN = YWHAE PE = 1 SV = 2 | 5.5 |
| 1433G_BOVIN | 14-3-3 protein gamma OS = Bos taurus GN = YWHAG PE = 1 SV = 2 | 5.5 |
| 1433S_BOVIN | 14-3-3 protein sigma OS = Bos taurus GN = SFN PE = 2 SV = 1 | 5.5 |
| 1433T_PIG | 14-3-3 protein theta OS = Bos taurus GN = YWHAQ PE = 2 SV = 1 | 5.5 |
| 1433Z_BOVIN | 14-3-3 protein zeta/delta OS = Bos taurus GN = YWAHZ PE = 1 SV = 1 | 5.5 |
| ACTC_BOVIN | Actin, alpha cardiac muscle 1 OS = Bos taurus GN = ACTC1 PE = 2 SV = 1 | 2.2 |
| ACTB_PIG | Actin, aortic smooth muscle OS = Bos taurus GN = ACTB2 PE = 1 SV = 1 | 2.2 |
| ACTH_BOVIN | Actin, gamma-entric smooth muscle OS = Bos taurus GN = ACTG2 PE = 2 SV = 1 | 2.2 |
| ADA_BOVIN | Adenosine deaminase OS = Bos taurus GN = ADA PE = 1 SV = 3 | 2.3 |
| SNTA1_BOVIN | Alpha-1-syntrophin OS = Bos taurus GN = SNTA1 PE = 2 SV = 1 | 5.5 |
| AP2M1_BOVIN | AP-2 complex subunit mu OS = Bos taurus GN = AP2M1 PE = 1 SV = 1 | 3.5 |
| CALM_BOVIN | Calmodulin OS = Bos taurus GN = CALM PE = 1 SV = 2 | 2.6 |
| COMT_PIG | Catechol O-methyltransferase OS = Bos taurus GN = COMT PE = 2 SV = 1 | 3.5 |
| CHLE_HORSE | Cholinesterase OS = Equus caballus GN = CHLE PE = 1 SV = 1 | −6.1 |
| CFTFR_LOXAF | Cystic fibrosis transmembrane conductance regulator OS = Loxodonta africana GN = CFTF PE = 3 SV = 1 | −2.3 |
| DPP6_BOVIN | Dipeptidyl aminopeptidase-like protein 6 OS = Bos taurus GN = DPP6 PE = 1 SV = 1 | 3.6 |
| RN220_BOVIN | E3 ubiquitin-protein ligase RNF220 OS = Bos taurus GN = RNF220 PE = 2 SV = 1 | 3.6 |
| FACR2_BOVIN | Fatty acyl-CoA reductase 2 OS = Bos taurus GN = FAR2 PE = 2 SV = 1 | 6.7 |
| GBR2R2_BOVIN | Gamma-amino- butyric acid receptor subunit rho-2 OS = Bos taurus GN = GABRR2 PE = 2 SV = 4 | 5.5 |
| IL15_BUBBU | Interleukin-15 OS = Bubalus bubalis GN = IL15 PE = 2 SV = 1 | 5.6 |
| IL2_CEREL | Interleukin-2 OS = Cervus elaphus GN = IL2 PE = 2 SV = 1 | 5.6 |
| IL4_BUBBU | Interleukin-4 OS = Bubalus bubalis GN = IL4 PE = 2 SV = 1 | 5.6 |
| K1C10_BOVIN | Keratin, type I cytoskeletal 10 OS = Bos taurus GN = KRT10 PE = 3 SV = 1 | 5.6 |
| K1C14_BOVIN | Keratin, type I cytoskeletal 14 (Fragment) OS = Bos taurus GN = KRT14 PE = 2 SV = 1 | 5.6 |
| K1C15_SHEEP | Keratin, type I cytoskeletal 15 OS = Ovis aries GN = KRT15 PE = 2 SV = 1 | 5.6 |
| K1C17_BOVIN | Keratin, type I cytoskeletal 17 OS = Bos taurus GN = KRT17 PE = 2 SV = 1 | 5.5 |
| K1T22_BOVIN | Keratin-like protein KRT222 OS = Bos taurus GN = KRT222 PE = 2 SV = 1 | 5.5 |
| KIF22_BOVIN | Kinesin-like protein KIF22 OS = Bos taurus GN = KIF22 PE = 2 SV = 1 | 3.5 |
| PERL_BOVIN | Lactoperoxidase OS = Bos taurus GN = LPO PE = 1 SV = 1 | 3.3 |
| TRF1_BUBBU | Lactotransferrin OS = Bubalus bubalis GN = LTF PE = 1 SV = 1 | 3.3 |
| MDC2_BUBBU | Mammalian aminopeptidase 2 OS = Bos taurus GN = MAPP2 PE = 2 SV = 1 | 2.2 |
| MYLK_SHEEP | Myosin light chain kinase, smooth muscle (Fragment) OS = Ovis aries GN = MYLK PE = 2 SV = 2 | 2.1 |
| PARP1_BOVIN | Poly [ADP-ribose] polymerase 1 OS = Bos taurus GN = PARP1 PE = 2 SV = 2 | 5.4 |
| RSRC1_BOVIN | Serine/Arginine-related protein 53 OS = Bos taurus GN = RSRC1 PE = 2 SV = 1 | 2.9 |
| NAC1_FELCA | Sodium/calcium exchanger 1 OS = Felis catus GN = SLCB1A PE = 2 SV = 1 | 5.4 |

Table 2. Identities of proteins determined to be significantly reduced in saliva obtained from irradiated, UAGT/pAQP1-treated parotid glands relative to saliva obtained at the same time from contralateral, control glands.
intraductal injection of a mixture of ketamine (20 mg kg\(^{-1}\)) and xylazine (2 mg kg\(^{-1}\)), and then placed on an operating table; they were then intubated and placed in the prone position, and maintained with isoflurane anesthesia (1–1.5%) via artificial ventilation. An intramuscular injection of 1 mg kg\(^{-1}\) Pilocarpine was given and 10–15 min were allowed to elapse for the sialogogue to take effect (determined by pooling of saliva in the anterior floor of the mouth). An oral swab with a 2-ml capacity (Salimetrics, Carlsbad, CA, USA) was weighed and then placed over the opened parotid duct on the right side, in the buccal corridor, and mechanically secured. Saliva was collected by capillary action for 10 min, and the oral swab was replaced if it appeared to be approaching capacity. After 10 min, the oral swabs(s) were weighed and saliva volume was calculated by subtracting initial weight from final weight, assuming a specific gravity of 1 for the saliva. Saliva was removed from the oral swab by centrifugation at 3000 r.p.m. for 15 min at 4 °C and analyzed as described.

pAQP1 construction and testing
Porcine (Sus scrofa) AQP1 (NCBI, NM_414454) was synthesized by Integrated DNA Technologies (Coralville, IA, USA). The cDNA was amplified with the primers—Forward: 5′-ATAGATGCTTCCAGGACGATGATCGAACGAAG-3′; Reverse 5′-TATTCGGAATTATGTCGACCTCCAC-3′—and cloned into pCMV-MCS (Agilent Technologies, Santa Clara, CA, USA) using BamHI and XhoI restriction sites, resulting in pCMV-pAQP1.
pAQP1 channel function was tested by expressing the pCMV-pAQP1 plasmid in Madin-Darby Canine Kidney epithelial cells (Source: ATCC). Transfection was performed using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA), using a GFP-expressing plasmid in parallel cultures as a control for transfection efficiency. At 48 h after transfection, cells were seeded on collagen-coated polycarbonate filter inserts in six-well plates (Corning, Corning, NY, USA) and placed in a transwell system, as shown in Figure 5a. After cells formed a confluent monolayer, the apical chamber was replaced with 1.5 ml of hypotonic sucrose (DMEM medium (440 mOsmol l\(^{-1}\)), and the medium in the basal chamber was replaced with fresh 2.6 ml of DMEM medium. After 48 h, the fluid volume in the individual apical chambers was measured by pipette and the transepithelial net fluid movement was calculated relative to GFP controls.

Adenoviral vector construction, purification and testing
The AdhAQP1, obtained as an aliquot of the clinically validated vector, was upscaled in HEK293 cell cultures, and purified by protein G chromatography. The protein concentration was determined using a Bradford assay and applied to the skin and moved over the outline of the parotid gland using a gliding motion. A total ultrasound exposure of 4 treatments comprising 30 sec each, 2 W cm\(^{-2}\) at a 50% duty cycle with 10 sec between treatments was delivered. Following gene transfer, the animal was allowed to awaken and returned to its housing.

Tissue processing and histological analysis
Parotid glands were dissected from the specimen, partially sectioned across the transverse plane (that is, ‘bread loafed’) to facilitate fixation and immediately fixed in 20 volumes of formalin per weight. The glands were examined and at least 5 representative sections were harvested per gland. Tissue sections were processed for conventional H&E staining.

Statistical analysis
For statistical comparisons of measurements at a single time point, including Figures 2b, 3a and 5b, an unpaired, single-tailed t-test was used. For statistical comparisons of multiple saliva measurements made over time, including Figures 2c, 3b, 4 and 6, a repeated Measures ANOVA with mixed effects using a compound symmetry variance and post-hoc Tukey adjusted differences of least squares mean was used. In all analyses, \(P\) values of <0.05 were considered significant.

Proteomic profiling of swine saliva
Proteomic profiling was performed on pig saliva using methods similar to those we have previously described. Briefly, a clean-up step was performed using a 2-D Quant kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and dissolved using 2D gel rehydration buffer with 50 mM Tris-HCl, pH 8.5. Protein concentrations were evaluated using the Bradford method, and samples were appropriately diluted to a final protein concentration of 1 μg ml\(^{-1}\). A volume of 1 μl of Cy3 or Cy5 NHS-conjugated dyes (Lumiprobe, Inc., Hallandale Beach, FL, USA) were added to 50 μg of protein sample and incubated on ice for 30 min in the dark. A volume of 1 μl of 10 mM lysine was used to quench the reaction by incubating on ice for 10 min in the dark.

| Accession # | Description | Fold-change (AQP1-treated/baseline) |
|-------------|-------------|-----------------------------------|
| SPICE_BOVIN | Spindle and centriole-associated protein 1 OS = Bos taurus GN = SPICE1 PE = 2 SV = 1 | 5.6 |
| SMC3_BOVIN | Structural maintenance of chromosomes protein 3 OS = Bos taurus GN = SMC3 PE = 1 SV = 1 | 5.3 |
| STX17_BOVIN | Syntaxin-17 OS = Bos taurus GN = STX17 PE = 2 SV = 1 | 2.2 |
| TFR1_HORSE | Transferrin receptor protein 1 OS = Equus caballus GN = TFRC PE = 2 SV = 1 | 3.6 |
| TAGL_BOVIN | Transgelin OS = Bos taurus GN = TAGLN PE = 1 SV = 4 | 3.5 |
| ZNH11_BOVIN | Zinc-finger HIT domain-containing protein 1 OS = Bos taurus GN = ZNH11 PE = 2 SV = 1 | 3.5 |
| ZN184_BOVIN | Zinc-finger protein 184 OS = Bos taurus GN = ZN184 PE = 2 SV = 1 | 3.5 |

Data are separated according to their database identification, either from the swine database or the other mammals database.
2-D sodium dodecyl sulfate gels were run by mixing 10 μg of each paired sample and diluting further in rehydration buffer to 450 μL and placing it on a 24-cm IEF strip (pH3-10NL). These strips were previously rehydrated using 2% DTT, 0.5% iodoacetic acid and 0.002% 2-mercaptoethanol. The strips were then equilibrated with 1% DTT followed by 2.5% iodoacetamide for 15 min each. The second dimension was carried out in a homogeneous 13.5% sodium dodecyl sulfate gel.

Completed gels were scanned using a Typhoon 9400 scanner (GE Healthcare). The pictures were edited using the ImageQuant TL 7.0 software and Differential In-gel Analysis, and Biological Variation Analysis of the 2D difference gel electrophoresis results were performed using the DeCyder 2D 7.0 Software (GE Healthcare). All protein spots of interest, identified by Variation Analysis and Differential In-gel Analysis, were manually checked to ensure that they were not changed in the Baseline versus Control gels, and thus the decrease or loss of these proteins can be definitively attributed to the effects of irradiation and/or gene therapy. Spots identified as being significantly decreased in the Baseline versus UAGT/pAQ1 gels but unchanged in the Baseline versus Control gels were extracted using an Ettan Spot Picker (GE Healthcare).

Protein identification was performed as previously described. Briefly, protein spots of interest from 2D gels were excised, reduced with DTT and alkylated with iodoacetamide, digested with trypsin and desalted with C18 ZipTips (MilliPore, Billerica, MA, USA). Both MS and MS/MS analyses of the digested peptides were performed on a MALDI-TOF-TOF tandem MS (Bruker UltrafleXtreme, Bruker Daltonics InC, Billerica, MA, USA). The database search and analysis were performed using FlexAnalysis and BioTools software (Bruker Daltonics Inc) against ‘Other Mammalia’ (excluding primate and rodents) SwissProt protein database using a local Mascot server.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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