Artificial Induction of Native Aquaporin-1 Expression in Human Salivary Cells

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Abstract

Gene therapy for dry mouth disorders has transitioned in recent years from theoretical to clinical proof of principle with the publication of a first-in-man phase I/II dose escalation clinical trial in patients with radiation-induced xerostomia. This trial used a prototype adenoviral vector to express aquaporin-1 (AQP1), presumably in the ductal cell layer and/or in surviving acinar cells, to drive transcellular flux of interstitial fluid into the labyrinth of the salivary duct. As the development of this promising gene therapy continues, safety considerations are a high priority, particularly those that remove nonhuman agents (i.e., viral vectors and genetic sequences of bacterial origin). In this study, we applied 2 emerging technologies, artificial transcriptional complexes and epigenetic editing, to explore whether AQP1 expression could be achieved by activating the native gene locus in a human salivary ductal cell line and primary salivary human stem/progenitor cells (hS/PCs), as opposed to the conventional approach of cytomegalovirus promoter-driven expression from an episomal vector. In our first study, we used a cotransfection strategy to express the components of the dCas9-SAM system to create an artificial transcriptional complex at the AQP1 locus in A253 and hS/PCs. We found that AQP1 expression was induced at a magnitude comparable to adenoviral infection, suggesting that AQP1 is primarily silenced through pretranscriptional mechanisms. Because earlier literature suggested that pretranscriptional silencing of AQP1 in salivary glands is mediated by methylation of the promoter, in our second study, we performed global, chemical demethylation of A253 cells and found that demethylation alone induced robust AQP1 expression. These results suggest the potential for success by inducing AQP1 expression in human salivary ductal cells through epigenetic editing of the native promoter.

Keywords: epigenetic repression, genetic therapy, genetic vectors, salivary glands, xerostomia, progenitor cells

Introduction

The application of gene therapy to the salivary gland began in 1994 with the first report of vector-mediated gene transfer to the salivary glands of a mammal (Mastrangeli et al. 1994). This novel basic science discovery provided the rationale for a translational research effort over the next 12 y, concluding in clear clinical impact with the report of safety and therapeutic efficacy in a first-in-man phase I/II clinical trial (NCT00372320) in 2012 (Baum et al. 2012). This elegant story of determined translational research has been reviewed previously (Baum et al. 2010), establishing aquaporin-1 (AQP1) as a transgene able to partially restore the function of salivary glands damaged by radiotherapy.

Hyposalivation and xerostomia are a common consequence of radiotherapy, affecting tens of thousands of survivors of head and neck cancers (Vissink et al. 2010). The persistent unmet need for palliative therapy in this condition, combined with the encouraging success of the AdAQP1 clinical trial, justify serious consideration of the remaining technological hurdles forestalling the widespread application of AQP1 gene therapy in patients with radiation-induced xerostomia. Chief among these considerations is the duration of transgene expression as adenoviral-mediated gene therapy is not permanent, and periodic readministration of viral gene therapy vectors to humans may be problematic. Interestingly, the functional

benefits of AQP1 gene therapy in the AdAQP1 clinical trial are inexplicably long-lived (Zheng et al. 2015), greatly exceeding what was predicted from animal models. Nevertheless, patients with this condition will require years of therapeutic efficacy, and refinement of the gene transfer technology to improve duration of expression and/or tolerability of the vector for repeated dosing are important clinical priorities.

In this study, we evaluated 2 alternative strategies for achieving expression of the endogenous AQP1 gene in a human salivary ductal cell line, A253, and primary multipotent salivary human stem/progenitor cells (hS/PCs) previously described by

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our group (Srinivasan et al. 2017) to have the ability to controllably differentiate into salivary acinar-like cells upon neurotransmitter stimulation. With the notable exception of integrating vectors, all conventional gene therapy approaches achieve expression of the therapeutic transgene through the persistence of an extrachromosomal, episomal vector after nuclear translocation and entry. These episomal vectors are eventually silenced and may themselves present targets for intracellular immunity due to the presence of nonhost sequences (Geguchadze et al. 2014) (e.g., the cytomegalovirus [CMV] promoter). The recent development of gene editing technologies (Naldini 2015) and even epigenetic editing (Thakore et al. 2016) raises the possibility that expression of AQP1 might be achieved in the human salivary gland by activation of the latent, native gene rather than through expression from an episomal vector, avoiding the introduction of nonhuman agents.

Activation of latent AQP1 expression in these salivary gland-derived cells was attempted using 2 novel approaches. The first method used was the dCas9-SAM system (Konermann et al. 2015), which uses a mutant Cas9 protein to recruit an artificial transcriptional complex to a specific site targeted by a guide RNA. The second method employed a global demethylation technique (Shao et al. 2011) intended to simulate the effect of epigenetic editing of the heavily methylated AQP1 promoter (Tan et al. 2014). To provide a measure of comparison to conventional gene therapy strategies, we compared the magnitude of AQP1 expression *in vitro* achieved with both of these approaches to infection of the same cellular targets with the clinically validated AdAQP1 viral vector.

Materials and Methods

Human Subjects Protection

Human salivary gland tissues were procured from consented patients undergoing surgery for head and neck cancer following protocols approved by institutional review boards (IRBs) at both Christiana Care Health Systems and the University of Delaware.

Cell Cultures

The A253 cell line was purchased from ATCC and grown in McCoy's 5a media (also sourced from ATCC), per supplier's guidance. Primary salivary hS/PCs were obtained as previously described (Pradhan-Bhatt et al. 2013, 2014; Srinivasan et al. 2017) and grown in Hepatocyte Defined media (Discovery Labware) containing epidermal growth factor at a concentration of 5 μ g/500 mL. Cells in passages 5 to 7 were used for all experiments.

Vectors and Cell Transfection

Figure 1A shows maps of the 3-plasmid system used to achieve dCas9-SAM activation of AQP1 transcription. The plasmids were obtained from AddGene after being deposited by

Dr. Feng Zhang (Massachusetts Institute of Technology). Six guide RNAs were designed to target various loci (Fig. 1B) in the human AQP1 promoter using the "CRISPR Design" software (available free at <http://crispr.mit.edu>).

A253 cells and hS/PCs were grown in McCoy's 5a Medium Modified medium (ATCC) and HepatoSTIM growth medium (Corning) supplemented with 1 μ g/100 mL epidermal growth factor (EGF). The 1×10^6 cells were seeded in 6-well plates the day before transfection. Twenty-four hours after plating, cells were transfected in triplicate with total 6 μ g of the 3 plasmids: 1) single-guide RNA (sgRNA)-expressing plasmid, 2) MS2-effector plasmid, and 3) dCas9-effector plasmid at a 1:1:1 mass ratio. All transfections were performed in triplicate. For A253 cell transfection, polyethylenimine (PEI; Polyscience) polymer was used because the cells were permissive. For hS/PCs, Lipofectamine 2000 (Thermo Fisher Scientific) was used to enhance transfection efficiency. For A253 cell transfection, 20 μ L PEI solution was mixed with 6 μ g total plasmids in 200 μ L McCoy's 5a Medium Modified medium (without fetal bovine serum [FBS] or antibiotics) for 10 min. For hS/PC transfection, 6 μ g total plasmids and 15 μ L Lipofectamine 2000 transfection reagent were incubated with 200 μ L opti-MEM reduced serum medium (Thermo Fisher Scientific) separately for 5 min and then incubated together for another 5 min. After replacement of growth medium with fresh medium in the wells, the transfection mix complexes were evenly deposited in A253 and hS/PC cultures. Forty-eight hours posttransfection, cells were harvested and total RNA were isolated for quantitative polymerase chain reaction (PCR) to measure relative AQP1 transcripts.

AdAQP1 was obtained as a kind gift from Dr. Changyu Zheng (NIDCR) and upscaled and purified using methods we have previously described (Wang et al. 2015). Viral infections were carried out at the specific multiplicity of infection (MOI) (defined as viral particles/cell) in serum-free media for 2 h at 37°C. Thereafter, media were discarded and growth media added and the cells were harvested 48 h later.

Global Demethylation

A253 cells and hS/PCs were plated in 24-well plates and treated in triplicate with 5-aza-20-deoxycytidine (5-AZA; Sigma-Aldrich) for 72 h. A 5- μ M final concentration was used for A253 cells, and a 0.5- μ M final concentration was used for hS/PCs to reduce cytotoxicity. Subsequently, 3 wells from both cell lines were treated with trichostatin A (TSA; Sigma-Aldrich) at a 300- μ M final concentration for an additional 24 h. Total cellular RNA or total cellular DNA of all different treatments was isolated using the RNeasy Kit (Qiagen) and QIAamp DNA mini kit (Qiagen), respectively, according to the manufacturer's instructions.

Quantitative PCR for AQP1 Expression

Cells were harvested at the time points specified and total RNA was isolated using the RNeasy mini kit (Qiagen). Using oligo DT primers, 1 μ g total RNA was used to generate first-strand

Figure 1. Targeting of the aquaporin-1 (AQP1) promoter with Cas9-SAM. **(A)** Guide RNAs (gRNAs) were designed to target various loci in the AQP1 promoter. The precise loci are indicated by their position on chromosome 7. TSS indicates the transcription start site for the AQP1 messenger RNA. The PAM sequence of each gRNA is indicated in red. **(B)** Plasmid maps of the 3 vectors cotransfected to target cells to drive expression of the Cas9-SAM system. The open reading frames for each component of the Cas9-SAM system are indicated, as well as major features of the plasmid backbone. WRPE, Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element.

complementary DNA (cDNA) (First Strand cDNA Synthesis kit; Roche Life Science). Quantitative PCR (qPCR) was carried out in a LightCycler 480 system (Roche Life Sciences). The AQP1 primer sequences used were as follows: forward, 5'-CTC TCAGGCATCACCTCCTC-3'; reverse, 5'-GGAGGGTCCCCG ATGATCT-3'. Quantitative gene expression data of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as a reference for AQP1 expression. The GAPDH primer sequences were as follows: forward, 5'-CAT GGG TGT GAA CCA TGA GAA-3'; reverse, 5'-GGT CAT GAG TCC TTC CACGAT-3'. Amplification conditions were as follows: 95°C for 5 min followed by 45 cycles at 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. The relative level of AQP1 in each sample was normalized to GAPDH expression, and relative mRNA levels were determined by setting the control samples as 1.

Bisulfite Treatment and Sequencing

The EpiTect Bisulfite Kit (Qiagen) was used to convert unmethylated cytosines in genomic DNA to uracil according to the manufacturer's instructions. Bisulfite primers, which contain

no CG dinucleotides, were designed by MethPrimer (Li and Dahiya 2002) to span areas of CpG island(s) in the promoter. We designed the following primers to amplify a portion of the AQP1, which contains 8 potential CpG sites: forward, 5'-TTTTGT AGTTGGTTGATGG TGTG-3'; reverse, 5'-AAAAAACTAAACCACCAAAAT CA C-3'. Hotstart Tag@ DNA polymerase (Qiagen) was used and amplification conditions were as follows: 95°C for 15 min followed by 35 cycles of 95°C for 45 s, 60°C 1 min, and 72°C for 1 min. The final PCR products were purified using the QIAquick PCR Purification Kit (Qiagen). The sequencing primer 5'-AGTGGGTG TGGATCCGGCTT-3' was used to amplify a region of the AQP1 promoter between 30911726 and 30911954 on chromosome 7, and the amplicon was subjected to Sanger sequencing. Next-generation sequencing of the amplicon is described in the Supplementary Material.

Statistical Analysis

The data shown in Figures 2, 3, and 4 were analyzed using a 1-way analysis of variance (ANOVA) and post hoc Scheffe test. A *P* value of <0.05 was considered significant.

Results

Cas9-SAM Targeted to the AQP1 Gene Locus Activates AQP1 Gene Expression in the Human Salivary Ductal Cell Line A253

In our first experiments, we sought to use the Cas9-SAM system (Konermann et al. 2015) to determine whether an artificial transcriptional start site could activate AQP1 expression in the human salivary ductal cell line A253. A series of 6 guide RNAs were designed using the CRISPR Design tool to target regions of the AQP1 promoter (see Fig. 1), and vectors (sgRNA; Fig. 1B) expressing these gRNAs were cotransfected into A253 cells (in triplicate) with the components of the Cas9-SAM system dCas9-VP64 and MS2-p65-HSF1 ("Cas9-SAM"; Fig. 1B). Transfection of A253 cells was well tolerated, and mock-transfected cells served as negative controls. Figure 2 shows relative AQP1 mRNA levels, detected by qPCR 48 h after cotransfection of plasmids.

Because the overall aim of our study was to explore the feasibility of alternative methods for activating AQP1 expression,

Figure 2. Cas9-SAM activation of aquaporin-1 (AQP1) in A253 cells. Forty-eight hours after either infection with AdAQP1 (at the multiplicity of infection [MOI] indicated) or cotransfection of pCAS9-VP64-GFP/MS2-P65-HSF1-GFP with the indicated guide RNA (gRNA), AQP1 messenger RNA (mRNA) was quantified using quantitative polymerase chain reaction (qPCR) as described in the text. Each bar represents the average of measurements from 3 wells. *Indicates a significant difference ($P < 0.05$) between gRNA5 and all other treatments tested or between AdAQP1 MOI 100 and all other AdAQP1 treatments tested. **Indicates a significant difference ($P < 0.05$) between gRNA4 and gRNA5.

we used the conventional AdAQP1 vector, which drives episomal expression of AQP1 and has been successfully used in human salivary glands, as a positive control. We observed increasing levels of AQP1 mRNA as the gRNA position approached the ATG site but a marked decrease when the gRNA (i.e., gRNA6) crossed the ATG site. Furthermore, the most efficient gRNA (gRNA5) actually drove expression of AQP1 at levels comparable to AdAQP1 at a moderate/high MOI of 100 viral particles/cell.

Guide RNA Efficiency Using the Cas9-SAM System Varies Slightly between A253 and Primary Salivary hS/PCs

We repeated the same experiment in primary hS/PCs, but in this experiment, we used only one well/gRNA due to the scarcity of the primary human cells. We found that the efficiency of the gRNAs showed a very similar trend, but slight variation in that gRNA4 was more efficient at activating AQP1 expression in these cells (Fig. 3). Again, transfection was well tolerated in the primary cells. In interpreting these data, it is clear that there is a region of the AQP1 promoter that is ideal for targeting with gRNA to induce Cas9-SAM-mediated transcription of AQP1, but slight differences (e.g., gRNA5 superior in A253, gRNA4 superior in hS/PCs) between cellular substrates are not surprising and are probably of little significance vis-à-vis clinical translation. We suspect these differences reflect slight variation in gRNA affinity for the targeted region due to polymorphisms in the AQP1 promoter sequence in the A253 cell line as opposed to the primary cells. It is important to remember that little more will be learned about this particular issue as this technology is advanced through preclinical animal models, as

Figure 3. Cas9-SAM activation of aquaporin-1 (AQP1) in primary human salivary stem/progenitor cells (hS/PCs). Forty-eight hours after cotransfection of pCAS9-VP64-GFP/MS2-P65-HSF1-GFP with the indicated guide RNA (gRNA), AQP1 messenger RNA (mRNA) was quantified using quantitative polymerase chain reaction (qPCR) as described in the text. Each bar represents the average of 3 measurements from the same well. *Indicates a significant difference ($P < 0.05$) between gRNA5 and all other treatments tested. **Indicates a significant difference ($P < 0.05$) between gRNA4 and gRNA5.

gRNAs targeted to AQP1 in human salivary glands can only be optimized through clinical trials.

Global Epigenetic Demethylation in A253 Activates AQP1 Gene Expression

We interpreted the robust expression of AQP1 in both human salivary cell types with the Cas9-SAM system to indicate that suppression of AQP1 in human salivary cells is likely to be primarily pretranscriptional. When considered in light of the evidence from earlier studies that transcriptional suppression of AQP1 in the salivary gland is mediated by methylation of CpG sequences in the native promoter, these observations support a hypothesis that AQP1 silencing can be overcome by demethylation of the promoter. Targeted demethylation, referred to as “epigenetic editing,” has been proposed, and some early studies suggest it may be feasible with conventional gene therapy techniques (Vojta et al. 2016).

We undertook a proof-of-principle experiment to determine if demethylation of the AQP1 promoter alone could activate AQP1 expression in the A253 human salivary ductal cell line and primary hS/PCs. Cells were plated as described above (in triplicate) and treated with 5-AZA for 72 h $\pm$ trichostatin for an additional 24 h. Afterward, genomic DNA was recovered for bisulfite treatment and sequencing, and RNA was collected for qPCR for AQP1 expression levels. Figure 4 shows results of this experiment. 5-AZA is a nonselective demethylating agent that has been previously shown to activate AQP1 in cells derived from primary adenoid cystic carcinoma (ACC) (Shao et al. 2011). For reasons that are unclear to us, 5-AZA alone was sufficient to accomplish this phenomenon, in the absence of trichostatin (TS), despite earlier reports showing synergy

Figure 4. Global demethylation with 5-AZA in A253 cells. Ninety-six hours after treatment with either 5-AZA alone or 48 h after treatment with 5-AZA and trichostatin A (TSA) (24 h 5-AZA + 24 h TSA), aquaporin-1 (AQP1) messenger RNA (mRNA) was quantified using quantitative polymerase chain reaction (qPCR). *Indicates a significant difference ($P < 0.05$) between 5-AZA treatment and both untreated and 5-AZA/TSA-treated cells ($n = 3$ per treatment).

Figure 5. Bisulfite sequencing of the aquaporin-1 (AQP1) promoter after global demethylation. Blue peaks represent C; red, T; black, G; and green, A. Putative demethylated cytosines in CpG regions of the AQP1 promoter region are indicated by rectangles.

between these agents (Shao et al. 2011). It is also notable that global demethylation in A253 cells led to AQP1 expression that was similar in magnitude to 100 vp/cell treatment with AdAQP1.

AQP1 Activation after Global Demethylation Is Associated with Demethylation of the AQP1 Promoter

Bisulfite genomic sequencing of a portion of the AQP1 promoter is shown as a chromatograph in Figure 5. In this chromatograph, blue peaks represent C; red, T; black, G; and green, A. Cytosines in CpG regions of the AQP1 promoter region are indicated by rectangles. Note that in the 5-aza-dC-treated cells, the sequencing returned an approximately 50/50 mixture of T and C, whereas in the mock-treated cells, the cytosines were preserved after bisulfite treatment. This inferred demethylation was also shown in the 5-AZA/TSA-treated cells but was less pronounced, in precise agreement with the qPCR results for AQP1 transcripts in Figure 4A. This demonstrates that global demethylation with 5-AZA treatment accomplishes demethylation of the AQP1 promoter and strongly suggests but

does not prove that AQP1 upregulation in 5-AZA-treated A253 cells is due to demethylation of the promoter region. Next-generation sequencing results confirm these findings and are included in Supplemental Material.

Discussion

Our results demonstrate that the native AQP1 gene, normally latent in human salivary gland cells, can be activated by both promoter-independent artificial transcription and epigenetic editing of the promoter. These approaches are fundamentally different from conventional gene therapy strategies that require exogenous delivery and episomal vector-mediated transcription of the transgene of interest. These findings align with a pair of earlier reports (Shao et al. 2011; Tan et al. 2014) suggesting that promoter methylation is the principal mechanism by which AQP1 expression is suppressed in human salivary gland cells. Overcoming this suppression presents an intriguing potential alternative to AQP1 gene transfer in accomplishing gene therapy for radiation-induced xerostomia in humans.

Gene activation with Cas9-SAM is elegant, but the resultant gene expression remains active only as long as the Cas9-SAM complex remains bound to the genetic target locus, a phenomenon whose durability is measured in hours. In this sense, there is no apparent advantage over conventional gene therapy techniques, in that the Cas9-SAM proteins, as well as the guide RNA, must be continuously produced by an episomal vector. In other words, even though expression of AQP1 in salivary gland cells using this system does not require an episomal vector, the mechanism by which the gene is activated does. Furthermore, arranging for convergence of all of the Cas9-SAM complex elements and the gRNA in the target cell requires either a polycistronic vector or transfection of multiple vectors to the same target cell, as we have performed in this study. This is presumably more troublesome than simply expressing the transgene through an episomal vector in the conventional way. However, these results tell us that suppression of AQP1 gene expression in A253 and hS/PCs is pretranscriptional, meaning that if suppression of transcription can be removed in a way that is long-lasting, this may be a viable new method for extending the duration of expression of AQP1 in a gene therapy application for radiation-induced xerostomia. Extending the duration of action of the Cas9-SAM complex is an intriguing possibility, and we presume this is an active area of research.

AQP1 hypomethylation has been observed in salivary gland ACC, resulting in significant overexpression of AQP1 in tumor

samples from 2 independent patient cohorts (Shao et al. 2011). The mechanisms by which this hypomethylation occurs, as well as the specific cell types in which it occurs, are unknown. While some questions remain as to the role of AQP1 in ACC, the possibility that AQP1 may itself be a novel oncogene, rather than simply associated with ACC, is not well supported by cell biology experimentation. Furthermore, the apparent safety of clinical gene transfer of AQP1 to the human salivary gland in the AdAQP1 clinical trial further belies the idea that AQP1 upregulation in the salivary gland is a causative mechanism of oncogenesis.

Based on these earlier studies of AQP1 promoter methylation in the salivary gland, we hypothesized that experimental demethylation of the CpG-rich AQP1 promoter might upregulate AQP1 in A253, and hS/PCs particularly, since our experiments with the Cas9-SAM system suggest that pretranscriptional regulation seems to be the major mechanism by which inactivation occurs. Our data support this hypothesis, and epigenetic editing through demethylation of the AQP1 promoter presents an attractive approach to durable AQP1 expression in the salivary gland, as reversal (i.e., remethylation) of this process may be very gradual, if it occurs at all, in slowly dividing cells of the salivary gland. Further experimentation will be needed to address this issue, as the existing literature does not provide a conclusive answer.

Various approaches to targeted epigenetic editing have been proposed, and future studies should explore whether it would be possible to accomplish specific demethylation of the AQP1 promoter, possibly with a fusion of dCas9 and Tet2 (Chen et al. 2014; Huisman et al. 2016). Epigenetic editing is a very promising, if still embryonic, new paradigm in gene therapy. For the past 3 decades, the gene therapy field has striven for clinical proof of principle, with ever-increasing success. At this juncture, with the field in general and with salivary gland gene therapy in particular, practical considerations such as duration of expression now merit full attention. Epigenetic editing has the potential to deliver what episomal vectors cannot—namely, months to years of expression with a single treatment that can restore salivary fluid flow. Future studies will be needed to further refine our epigenetic editing approach to achieving long-lived expression of AQP1 as a treatment for radiation-induced xerostomia.

Author Contributions

Z. Wang, contributed to data acquisition, analysis, and interpretation, critically revised the manuscript; S. Pradhan-Bhatt, M.C. Farach-Carson, contributed to data analysis and interpretation, critically revised the manuscript; M.J. Passineau, contributed to design and data analysis, drafted the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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