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ADVANCES IN SALIVARY GLAND GENE THERAPY – ORAL AND SYSTEMIC IMPLICATIONS

Bruce J. Baum[*], **Ilias Alevizos**, **John A. Chiorini**, **Ana P. Cotrim**, and **Changyu Zheng**

Molecular Physiology and Therapeutics Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD USA 20892-1190

Abstract

Introduction—Much research demonstrates the feasibility and efficacy of gene transfer to salivary glands. Recently, the first clinical trial targeting a salivary gland was completed, yielding positive safety and efficacy results.

Areas covered—There are two major disorders affecting salivary glands; radiation damage following treatment for head and neck cancers and Sjögren's syndrome. Salivary gland gene transfer has also been employed in preclinical studies using transgenic secretory proteins for exocrine (upper gastrointestinal tract) and endocrine (systemic) applications.

Expert opinion—Salivary gland gene transfer is safe and can be beneficial in humans. Applications to treat and prevent radiation damage show considerable promise. A first-in-human clinical trial for the former was recently successfully completed. Studies on Sjögren's syndrome suffer from an inadequate understanding of its etiology. Proof of concept in animal models has been shown for exocrine and endocrine disorders. Currently, the most promising exocrine application is for the management of obesity. Endocrine applications are limited, as it is currently impossible to predict if systemically required transgenic proteins will be efficiently secreted into the bloodstream. This results from not understanding of how secretory proteins are sorted. Future studies will likely employ ultrasound assisted and pseudotyped adenoassociated viral vector-mediated gene.

Keywords

salivary glands; gene therapy; viral vectors; non-viral gene transfer; Sjögren's syndrome; radiation damage; secretory proteins

[*]For correspondence: bbaum@mail.nih.gov.

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1. Introduction

The notion of transferring genes or other oligonucleotides for therapeutic purposes is at least 50 years old [1], but has been relatively slow in reaching clinical fruition. Nonetheless, there are now several clear examples of how this biological therapy can be clinically beneficial for both general systemic illness (e.g.,[2, 3]) and local, organ-specific disease (e.g., [4, 5]). While originally gene transfer was considered primarily for use in treating cancers refractory to conventional therapy or congenital genetic disorders (e.g., [6, 7]), the basic principles have now been applied to virtually every organ, for acquired as well as inherited disorders, and for both life-threatening and quality of life concerns.

Salivary glands were first targeted for intended clinical gene transfer applications in the early 1990s [8]. Since then a considerable body of research has shown proof of concept for several applications, including frank glandular disorders [9, 10], upper gastrointestinal tract diseases, (i.e., exocrine uses, e.g., [11]) and systemic diseases, (i.e., endocrine uses, e.g., [12, 13]). Additionally, clinical utility for one specific application, salivary gland hypofunction secondary to therapeutic radiation [14], has been recently demonstrated. Each of these applications takes advantage of the localized nature of gene transfer, i.e., a gene being any form of oligonucleotide, via direct cannulation of a gland's main excretory duct and the subsequent retrograde infusion of the therapeutic agent into the gland (see Table 1 and [15, 16] for description). The procedure, which mimics a long used technique for obtaining contrast x-rays of a gland (sialography), requires no patient anesthesia and leads to little or no patient discomfort. The use of anesthesia for this procedure in experimental animals is solely for restraint. In our experience, the only true barriers to salivary gene transfer using this approach are (i) reduced access to the orifices of the main excretory ducts, e.g., if a patient has a limited jaw opening, and (ii) lack of patency in the main excretory ducts, such that a cannula cannot be inserted sufficiently.

Several reviews of salivary gland gene therapy, i.e., gene transfer for therapeutic purposes, have been published, e.g., [17, 18]. The present review will focus on very recent advances, i.e., articles published from January 2012 through December 2014, and be organized primarily by applications: glandular, exocrine, endocrine, and methodological advances. We will highlight what we consider to be the most significant developments for each application rather than providing an all-encompassing literature review.

2. Glandular disorders

There are two major disorders that affect salivary glands: iatrogenic damage to the glands subsequent to therapeutic radiation for head and neck cancers [19,20] and Sjögren's syndrome (SS), a very common autoimmune disease [21,22]. Both conditions have been widely studied regarding possible applications for salivary gland gene therapy.

2.1 Radiation damage

Each year in the USA, ~45,000 people are diagnosed with head and neck cancers, with ~600,000 people diagnosed worldwide. Most of these malignancies are squamous cell carcinomas (SCCA) and treated, at least in part with ionizing radiation [20]. Salivary glands

are frequently in the radiation field and are part of the “collateral damage” to normal tissues that occurs with radiation therapy. While the salivary gland damage for some patients is transitory, or responsive to pharmacological therapy, many patients experience a life long reduction in their ability to secrete saliva, and with that multiple oral morbidities. These include having a dry mouth (termed xerostomia), difficulty swallowing (dysphagia), increased dental caries and other oral infections, local pain and discomfort and, as a result, a significant decline in their quality of life.

Because there was no useful conventional therapy to help this latter group of patients, in the early 1990s we hypothesized a potential scheme for the “repair” of radiation-damaged salivary glands [23]. This scheme involved transferring the human aquaporin-1 (hAQP1) cDNA to the radiation surviving salivary ductal epithelial cells in the damaged gland. The hAQP1 gene encodes a water channel protein. In 1997, we constructed a serotype 5, adenoviral (Ad5) vector (AdhAQP1) and delivered it via intraductal cannulation to irradiated rat submandibular glands [9]. Impressively, this maneuver resulted in an almost complete return of salivary output to pre-irradiation levels. In 2005, working with Songlin Wang and colleagues in China, we then showed that the AdhAQP1 strategy could scale from a rat to a large animal model, the miniature pig, ~1000-fold size difference [24]. Following these two pre-clinical proofs of concept studies, and an extensive biodistribution and toxicological study of AdhAQP1 [25], we submitted a clinical protocol to use the AdhAQP1 vector in a single irradiated parotid gland of research subjects previously treated for head and neck cancer with radiation therapy [14]. This was a phase I/II study. Importantly, it showed that AdhAQP1-mediated gene transfer was safe in humans. In addition, AdhAQP1 resulted in an increased saliva flow rate from the targeted parotid gland, as well as a reduction in symptoms related to the radiation-induced xerostomia, in five of the eleven subjects studied. These benefits were vector dose-dependent, as Ad5-related inflammatory/negative responses were common in subjects who did not benefit from the treatment. Although this was a single study, it clearly demonstrated that gene therapy could be used clinically to treat a disorder affecting salivary glands and an individual’s quality of life.

Currently, our research group is planning to conduct a similar study, but transferring the hAQP1 cDNA using a markedly less immunogenic viral vector, derived from AAV2 [26]. Also of particular note, very recent studies by Wang et al [27], used ultrasound-assisted gene transfer (UAGT) to deliver both the human and porcine AQP1 cDNAs to irradiated miniature pig parotid glands. They showed that this non-viral gene transfer approach can lead to increased parotid saliva flow from the radiation damaged glands, with levels approximating those achieved with Ad5-mediated hAQP1 cDNA transfer. This also occurred in the absence of any significant local inflammatory response. These results, if duplicated in clinical studies, would mean that individuals could receive multiple hAQP1 cDNA administrations to their salivary glands to maintain elevated salivary flow (Table 2). This would be quite different from the situation with viral vectors, i.e., with only a single administration possible (see below under Methodological advances).

Although results from the AdhAQP1 clinical study were quite positive, any patient would much rather not have an iatrogenically acquired disorder than need to correct one. We developed two potentially useful gene therapy approaches to prevent gland radiation

damage, using cDNAs encoding either human fibroblast growth factor-2 (hFGF2) or human keratinocyte growth factor [28,29]. Both of those studies were conducted in mice, but recently we have shown that the hFGF2 strategy can be scaled and be effective in miniature pigs, an important consideration for eventual clinical application [30]. These studies provided proof of concept that the salivary glands can be protected from radiation-induced damage.

However, the cDNAs used in these studies also raise certain safety concerns. If any of the transgene encoding vector somehow escaped from the targeted gland it could either stimulate growth of the SCCA (hKGF) or enhance the tumor's vascular supply (hFGF2). Although these concerns are reasonable, albeit theoretical, it is important to recognize that we previously have shown it is highly unlikely that vectors delivered to human salivary glands can escape from the glands and be found systemically [31]. In that study, a single subject enrolled in the above described phase I/II clinical trial was administered AdhAQP1 to one parotid gland. We were unaware that the subject had a latent Ad5 infection of the targeted gland, apparently due to the long-term use of anti-viral medications for herpes infections. Seven days after vector administration parotid saliva from the subject's targeted gland contained high levels of both the administered vector, as well as wild type Ad5; the latter as a result of trans-complementation [31]. However, at no point was this patient viremic. All AdhAQP1 and wild type Ad5 generated escaped through the luminal membrane of transduced cells and only was found in saliva. Nonetheless, at present, there are no plans to move forward clinically with either AdhFGF2 or AdhKGF.

Alternatively, studies by Sunavala-Dossabhoy and colleagues [32,33] and by Ovitt and colleagues [34,35] have employed quite different strategies to prevent radiation-induced salivary gland damage. Both of these strategies show considerable promise. Sunavala-Dossabhoy et al [32] initially used an Ad5 vector, and later employed an AAV vector [33], to deliver the cDNA for a protein termed tousled kinase 1B (TK1B). TK1B is critically important in chromatin remodeling. In their most relevant pre-clinical study to date [33], an AAV vector encoding T1KB was administered to rats before fractionated radiation (2.5 Gy/day; 8 days). Animals treated with this vector experienced no reduction in their salivary output, while animals administered a control AAV vector experienced a >90% reduction in salivary flow.

Using a completely different and a particularly exciting strategy, Ovitt and colleagues [35] administered a small-interfering RNA (siRNA) to mouse submandibular glands employing pH-dependent nanoparticles. This non-viral delivery method was used as a way to inhibit the expression of two genes encoding the pro-apoptotic proteins PKCδ and Bax. Both of these genes are normally activated following cellular radiation. Since the effect of irradiation on cells is transitory, use of siRNA delivery to the glands would not be a concern despite the short duration of a siRNA's action. Specifically, for this very important clinical indication, the transient inhibition of apoptosis using siRNA allowed the salivary epithelial cells to recover, apparently without significant permanent genetic alterations. Indeed, this strategy led to markedly higher levels of saliva secretion 3-months following radiation in treated mice versus mice receiving the control nanoparticles [35]. Thus, the retroductal administration allowed the nanoparticles to reach sufficient numbers of salivary gland cells

as well as get internalized and efficiently exert their effects on their target mRNA. The nanoparticles used were comprised of cationic micelles that interact with the siRNA electrostatically, preventing nucleases from cleaving them [34]. Additionally, they included a pH sensitive endosome-processing system that facilitated siRNA release within the cytoplasm [34]. Of note, the immune response raised by the nanoparticles, although not severe, was not insignificant and it will be interesting to observe the immune responses to this approach in larger animal models.

Each of these novel approaches, thus, appears useful in pre-clinical, proof of concept, rodent experiments. However, both must now be tested in a large animal model, e.g., as noted above [24,30], as well as shown to be safe in toxicological and biodistribution studies, e.g., [25,26] before any clinical trial can be initiated.

2.2 Sjögren's syndrome

SS comes in two general varieties: primary SS (pSS), which exists in the absence of another autoimmune disease, and secondary SS, which occurs concurrently with another autoimmune disease, e.g., rheumatoid arthritis or systemic lupus erythematosus. The introduction of recombinant proteins for the treatment of autoimmune rheumatic diseases has led to major beneficial effects on patient outcomes. However, many of the biological agents useful in the management of rheumatoid arthritis are either ineffective or have a negative impact on SS patients, e.g., [36,37]. Thus, in the absence of effective conventional therapy for SS, gene transfer has been viewed as offering both a potential therapy for patients as well as providing an experimental approach to explore the different arms of the immune system in animal models. The applications of gene therapy to SS have been restricted to mouse models. Unfortunately, at present, there is no simple and coherent understanding of the clinical pathogenesis of SS. While it is considered to affect principally two exocrine glands, the salivary and lacrimal glands, with the most common symptoms being a dry mouth and/or dry eyes, respectively, SS is in fact a multi-system disease. SS can lead, for example, to pulmonary, renal and neurological manifestations. All recent gene transfer studies related to SS have been an extension of the earlier reports, using NOD mice, e.g., by Kok et al [10] and Lodde et al [38]. The major drawbacks of these SS-related salivary gland gene transfer studies, both past and recent, are (i) the relevance of the mouse models employed to the actual human disease and (ii) the targeting of only a single diseased tissue in a systemic condition. Despite these caveats, a good deal has been learned recently, at the very least about the models available to study SS.

For example, Roescher et al [39] showed that expression of a soluble form of the CD40 ligand in the salivary glands of NOD mice did not lead to a reduction in overall inflammation and/or improved salivary gland function. Conversely, impedance of cytotoxic T-lymphocyte antigen 4 (CTLA-4), which is a key negative co-stimulatory molecule that displays a wide range of anti-inflammatory properties, seems to offer beneficial effects [40]. The recombinant fusion protein CTLA4IgG, which consists of the extracellular portion of murine CTLA-4 and the Fc portion of human IgG1, is currently approved to treat rheumatoid arthritis and is now in clinical trials for the treatment of SS. Theoretically, transferring the CTLA4IgG cDNA offers the possibility of local expression of this

immunomodulatory protein in the salivary glands to gain continuous benefit. That hypothesis has been evaluated in mouse models and its efficacy in blocking B7 expression on macrophages in vitro also has been assessed [40]. In this study CTLA4IgG expressed locally in the salivary glands of C57BL/6.NOD-Aec1Aec2 mice (another, more specific model of SS) inhibited of the loss of salivary gland activity and decreased T and B cell infiltration, as well as dendritic cells and macrophages, in the glands, compared with findings in control mice.

Although elevated levels of certain proinflammatory cytokines can be observed in the serum of SS patients, several studies point to the salivary gland epithelium itself as being a source of their expression. For example, BAFF (B cell–activating factor of the tumor necrosis factor family) is a critical cytokine for B cell activation. BAFF is over-expressed by salivary gland epithelial cells in pSS. ΔBAFF, a minor alternative splice variant of BAFF, is a physiological inhibitor of BAFF. As with CTLA4IgG, Δ BAFF offers the possibility to express this inhibitor within the targeted salivary glands. Roescher et al [41] engineered U7 RNA antisense sequences targeting BAFF splice regions and achieved a functional shift in message utilization that favored the production of Δ BAFF. This maneuver increased ΔBAFF mRNA and resulted in a marked decrease of BAFF mRNA production and BAFF protein secretion in vitro. In vivo, the salivary glands of NOD mice were administered, by retrograde infusion, a serotype 2 adeno-associated viral (AAV2) vector encoding the modified U7 RNA that targeted the BAFF transcript. The transduced salivary glands showed significantly decreased BAFF protein expression and lymphocytic infiltrates, as well as enhanced saliva production [41]. In particular, this study offers a rationale for the localized inhibition of BAFF production in pSS patients and represents a proof of concept for the use of exon skipping as a therapeutic strategy in autoimmune diseases. Interestingly, AAV (AAV2-TACI-Fc) mediated expression of a soluble Fc fusion protein of one receptor for BAFF, TACI, significantly reduced the number of inflammatory foci in the targeted salivary glands, as well as global IgG and IgM levels, but did not decrease IgA levels [42]. Furthermore, while overall expression of pro-inflammatory cytokines also tended to be lower in AAV2-TACI-Fc treated mice, salivary flow was unaffected.

Gene transfer vectors are also useful tools for functional genomics studies that have been beneficial to understanding SS pathogenesis. For example, the membrane protease matriptase is a critical factor in maintaining the epithelial barrier function [43]. Embryonic ablation of matriptase expression in mice results in the loss of secretory epithelial cell function and the induction of autoimmunity similar to that observed in pSS [44]. Acute ablation of matriptase expression, via use of the CRE recombinase locally in salivary glands after gene transfer, also results in significant salivary gland dysfunction in the absence of overt immune activation [44]. Functional analysis of these salivary glands shows a loss of electrical potential across the epithelial layer, as well as the altered distribution of a key tight junction protein, claudin 3. Additionally, an apparently significant decrease in matriptase gene expression was detected in the minor salivary glands of pSS patients compared with healthy volunteers [44], an interesting observation that requires further exploration. This study is consistent with past reports suggesting the importance of epithelial barrier function in SS pathogenesis, e.g., [45–47].

The application of genome wide transcriptome analysis has opened the possibility of broadly studying gene expression changes and the context in which they are occurring. Not surprisingly, such studies also have been useful in SS. Yin et al [48] examined the transcriptome in biopsies of minor salivary glands from SS patients and identified an increase in expression of the cytokine bone morphogenetic protein 6 (BMP-6). Subsequent expression of this protein in the salivary or lacrimal glands of healthy mice, following local BMP-6 cDNA transfer, suggested its possible etiologic role in the loss of salivary and lacrimal gland function [48].

2.3 Other applications of gene therapy for glandular disorders

Very recently Li et al [49] used gene transfer for a completely unique purpose, for a salivary gland disorder not previously studied. They used Ad5-mediated transfer of the N-myc downstream-regulated gene 2 (NDRG2) to correct the salivary hypofunction, altered ion fluxes and increased water intake that occur in an estrogen-deficient rat model of post-menopausal xerostomia. Rats were ovariectomized to mimic the estrogen deficiency that occurs during human menopause, as well as the salivary hypofunction that can accompany this condition in some women. Three days following retroductal delivery of the Ad5-NDRG2 vector into the submandibular glands, Li et al [49] found that the ovariectomized rats exhibited a normalized level of salivary secretion, increased salivary duct NaCl reabsorption, and a reduction in their water intake. The authors suggest that use of NDRG2 gene transfer may be useful clinically for postmenopausal women who suffer from salivary hypofunction and do not want to risk the side effects of estrogen supplementation therapy. As impressive as the study of Li et al [49] is, it is particularly important to see if their therapeutic strategy can extend and scale to examine salivary hypofunction during estrogen deficiency in a large animal model with a menstrual cycle, i.e., non-human primate, versus their rat model, i.e., a species that has an estrus cycle. In addition, as noted above, appropriate toxicological and biodistribution studies are needed to examine vector safety.

3. Exocrine applications

That gene transfer to salivary glands might be employed for exocrine purposes, to address clinical problems in the mouth and upper gastrointestinal tract, is hardly surprising. These are exocrine glands and the overwhelming majority of protein secretion from these glands is into the forming saliva. Thus, one assumes gene transfer for exocrine applications in salivary glands should be relatively easy. In fact, Voutetakis et al [50] showed that transfer of the cDNA for human erythropoietin (hEpo), a protein normally secreted from the kidney directly into the bloodstream, is predominantly secreted into saliva (ratio; total hEpo in saliva/serum) in rats (1.74), miniature pigs (13,900) and macaques (12.9), but not in mice (0.1). Despite this, little clinically relevant research has occurred on exocrine applications of salivary gland gene transfer since the original report of O'Connell et al [11].

Indeed, in the last 2–3 years we think that there has only been one development, carried into preclinical studies, that is important to mention; the research of Zolotukhin and colleagues [51–53] involving the AAV-mediated transfer of the small peptide hormone PYY(3–36), a member of the neuropeptide Y family. PYY is normally secreted from gut epithelia into the bloodstream and mediates the sensation of satiety. However, these investigators discovered

the presence of PYY in both murine and human whole saliva. Although, using a mouse model, they showed the salivary PYY was in fact derived from the bloodstream, they hypothesized that it may be involved in an oral element of satiety control [51]. Importantly, several subtypes of PYY receptors are specifically and abundantly expressed in murine lingual epithelium [51]. As Hurtado et al [52] note, it is likely that these receptors are involved in the mediation of a wide variety of functions, including proliferation, differentiation, motility, taste perception and satiation.

In their 2011 report, Acosta et al [51] conducted a long-term study with diet-induced obese mice. The mice experienced a sustained increase in salivary PYY(3–36) using AAV vector-mediated gene delivery to the salivary glands. Compared to findings from control animals, the extended increase in salivary PYY(3–36) resulted in a significant long-term reduction in food intake and body weight in the obese mice. This study demonstrated that there are new oral functions of PYY(3–36) and suggested a potential alternative therapy for the treatment of obesity. In a related study, La Sala et al [53], used a similar gene transfer approach to identify a role for PYY signaling in taste. Specifically, they showed that (i) PYY is expressed in a specific subset of murine taste cells and (ii) PYY signaling modulates responsiveness to bitter-tasting stimuli, as well as to lipid emulsions [53]. Again, using AAV vector-mediated PYY gene delivery to augment salivary PYY in PYY knockout mice, they could rescue murine behavioral responsiveness to a lipid emulsion but not to bitter stimuli, and in so doing, suggested that this was likely a result of the activation of Y2 receptors found in taste cells. Thus, this study implies that PYY produced locally in taste cells has a different, yet still important, function from the PYY produced in the gut that circulates in the bloodstream.

Furthermore, this same group recently addressed a key problem with previous clinical applications of systemically administered PYY for weight reduction [54], albeit not using gene transfer. As they noted [54], when PYY is given systemically to humans the weight loss benefits are compromised because of accompanying visceral illness and nausea. They used a novel approach to administer PYY, increasing its salivary concentration in mice with an intraoral puff spray [54]. Earlier they showed this approach is able briefly to increase salivary concentrations of PYY and lead to strong anorexic responses [51]. In the Hurtado et al [54] study they showed this delivery method does not cause aversive reactions in mice. The increased salivary PYY activated forebrain areas known to mediate feeding, hunger, and satiation while minimally affecting brainstem chemoreceptor zones triggering nausea [54]. They discovered an alternative neuronal circuit for ingestive behavior that does not induce taste aversion, as well as supported their earlier suggestion that this may lead to therapeutic uses of PYY for the treatment of obesity via direct oral applications [54]. Of course, this proposal must now be tested in a large animal model (as noted above), as well as shown to be safe in toxicological and biodistribution studies.

4. Endocrine applications

Although salivary glands are exocrine glands, there is an extensive and old literature suggesting that they can secrete products into the bloodstream, i.e., in an endocrine manner e.g., [55, 56]. While most such reports have been described in rodent models, there are some

studies in larger animals, including pigs e.g., [55]. Furthermore, as is well recognized, human salivary epithelial cells certainly secrete their own basement membrane and salivary amylase is found in the bloodstream e.g., [56].

Based on this extensive, but still inconclusive literature, our research group has long been fascinated with the possibility that salivary glands could be used as an endogenous bioreactor, secreting transgenic proteins systemically to alleviate clinical disease. The report by Kagami et al [58] unequivocally demonstrated that both the rat parotid and submandibular glands could secrete a transgenic protein, in this case human α1-antitrypsin, into the bloodstream, while the report by He et al [12] showed that a transgenic protein, human growth hormone (hGH), secreted into the bloodstream could be biologically active. Subsequently, many additional studies, e.g., [59] with human insulin and hGH; [60] with human IL-10; [13, 61], both with hEpo; [62] with human α-galactosidase 1 in a model of Fabry disease; [63] with human exendin-4 in a model of Type 2 diabetes, have consistently shown that transgenic proteins and peptides can be secreted systemically from salivary glands and lead to biological function and/or the correction of disease in animal models.

Despite the abundance of proof on the feasibility and potential utility of salivary gland gene transfer for endocrine applications, little progress has recently occurred. There are only a few reports of small incremental steps being published since 2013, e.g., [64–67]. More importantly, there are no applications of which we are aware that are close to being considered for clinical testing. Why is this so? As noted in a 2010 review [68], the cell biology community does not yet understand fully how protein sorting is regulated under normal circumstances [69–71], something absolutely necessary for selecting transgenic molecules whose ultimate secretory destination can be precisely predicted. An excellent example of this problem was noted above, i.e., the enormous variability (close to six orders of magnitude) in the saliva/serum ratio of secreted transgenic hEpo in mice, rats, miniature pigs and macaques [50]. With such unpredictability occurring in animal models, the lack of movement towards clinical application is not surprising.

Perhaps the best example of the inadequate understanding of the complexities underlying protein sorting, when applied for the efficient use of salivary gland gene transfer for systemic disease, is research we have conducted to reroute the secretion of hGH. In the He et al [12] study mentioned earlier, we observed fairly high levels of hGH in the serum of normal rats (~16 ng/ml); hGH that was biologically active. What we did not then appreciate was that almost all of the hGH produced by the transduced glands actually was being secreted into the saliva [72]. As a regulated secretory protein, normally secreted from the anterior pituitary gland in granules by the regulated secretory pathway (RSP), transgenic hGH efficiently found its way into the RSP and was secreted into saliva, i.e., the RSP endpoint in salivary glands.

As one strategy to alter the sorting of transgenic hGH in order to increase its delivery from transduced salivary glands into the bloodstream, we focused on a report showing the presence of a C-terminal motif, originally observed in pro-opiomelanocortin (POMC) and in several neuroendocrine hormones, including hGH and insulin [73]. Simply put, at the time it was believed that proteins secreted via the RSP either contained an internal sorting signal for

their entry into or retention within secretory granules [69],[73],[74]. Thus, it seemed that the C-terminal POMC-like motif represented a probable sorting signal for hGH entry or retention in the forming secretory granules of the RSP. Over an approximately 10-year period, we focused on mutating the comparable motif in hGH with minimal success, with success being defined as significantly increasing the proportion of synthesized transgenic hGH secreted into the bloodstream [67, 75]. Thus, despite multiple studies from several laboratories showing that potentially therapeutic transgenic proteins can be secreted from salivary glands in an endocrine manner, it is currently impossible to predict how such proteins will be sorted following gene transfer to human salivary glands. In the absence of an understanding of the mechanism(s) through which both regulated and constitutive pathway secretory proteins are sorted, further clinical application for this strategy is severely hindered.

5. Methodological advances

Recent studies by Passineau and colleagues have carefully examined the utility of two different methods of gene transfer to salivary glands: use of pseudotyped AAV vectors and use of UAGT [76–78]. Their results provide important methodological information that should be valuable to all investigators planning to use salivary gland gene transfer for intended clinical applications.

In 2010, this group first reported using ultrasound induced microbubble destruction for plasmid gene transfer to mouse salivary glands [76]. This general technique, termed sonoporation, causes transient pores that greatly facilitate the uptake of nucleic acids in the glands. They employed a luciferase reporter gene plasmid with a cytomegalovirus promoter, and retroductal administration to a single submandibular salivary gland. The plasmid was delivered in a solution containing microbubbles composed of lipid-encased perfluoropropane gas [76]. UAGT with a 15% microbubble solution led to robust and fairly stable transgene expression for up to 28 days [76]. Very recently [78], they compared UAGT efficiency and host response in murine submandibular glands using a conventional plasmid versus a minicircle, both encoding luciferase. Minicircles are miniaturized, episomal covalently closed, circular gene expression vectors, generally biosynthesized in recombinant bacteria, with long-lasting gene expression and a safe clinical profile [79]. The minicircle vector led to more efficient gene transfer [78]. Next, they characterized physiological responses of the targeted glands to UAGT using global proteomic profiling. Sonoporation alone, in the absence of a gene transfer vector, was without significant effect on the salivary gland proteome. While a plasmid vector profoundly perturbed the proteome, to a level comparable to that seen with high doses of AAV, UAGT with a minicircle vector resulted in minor proteomic changes, similar to sonoporation alone [78]. Thus, using the same expression cassette and equimolar amounts of gene, the authors found that UAGT with minicircles eliminated >95% of the protein changes associated with plasmid vectors. As noted above, Passineau and colleagues have extended these studies to examine delivery of the AQP1 cDNA to irradiated porcine parotid glands with considerable success [27], essentially replicating the results of Shan et al [37] without requiring the need to employ an Ad5 vector. These results offer considerable promise for future uses for sonoporation, microbubbles and minicircles in all clinical salivary gene transfer applications.

The Geguchadze et al [77] study examined the efficacy of AAV vector-mediated gene transfer to mouse submandibular glands. They used the key genetic elements of AAV2, i.e., the inverted terminal repeats, and made pseudotyped vectors, that were packaged using the capsids of either AAV serotypes 5, 8, or 9. Initially they determined that pseudotyping, in particular with the AAV5 capsid, increased expression of the transgene used, luciferase, in salivary glands by several orders of magnitude compared with AAV2 [77]. They achieved a level of gene transfer similar to that seen with an AAV2 vector using an AAV2/5 pseudotyped vector at a 100-fold lower dose [77]. This is consistent with the previous report of increased gene transfer to salivary glands using AAV5 vectors versus AAV2 and AAV4 vectors [80]. Additionally, they examined the use of the AAV2/5 pseudotyped vector on pre-clinical safety. They found that using the AAV2/5 pseudotyped vector at the 100-fold lower dose decreased vector levels recovered in the liver by 300-fold at one week after delivery. Using whole salivary gland-proteome profiling they found minimal alterations with this pseudotyped vector [77].

Overall, these studies indicate that should gene transfer to salivary glands require use of a viral vector, an AAV2/5 pseudotyped vector offers the potential of much greater efficacy, and much lower toxicity, than a traditional AAV2 vector. However, it must be recalled that there are many long-term studies that attest to the safety of AAV2 vectors in multiple tissues. In particular, in two toxicology and biodistribution studies with mice, conducted under Good Laboratory Practice conditions, AAV2 vectors targeted to salivary glands clearly have been shown to be safe and without significant adverse effects ([81], submandibular gland; [26] parotid gland).

6. Conclusion

While salivary glands are not considered a major target for clinical gene transfer applications, there is a growing body of research from many laboratories that attests to its feasibility and utility for certain clinical conditions. These applications will likely grow considerably with increased understanding of key mechanistic processes in the biology and pathology of salivary glands.

7. Expert Opinion

Salivary gland gene transfer has been shown to be safe and beneficial in a recent human clinical trial to repair radiation damaged salivary glands. The results of that study should provide substantial impetus for increased research on similar, as well as additional, clinical applications. Of particular note, three recent, quite different approaches to the prevention of salivary gland radiation damage have shown considerable potential in preclinical studies. Two of these (with the TK1B cDNA and a novel siRNA) are particularly promising, as their use is focused on events within cells and, therefore, pose a reduced safety risk, unlike hFGF2, which apparently is secreted in an autocrine manner.

Studies focused on SS suffer from an inadequate understanding of the etiology of this disease, and from primary attention to local exocrine gland manifestations (dry mouth and dry eyes) when it is clearly a multisystem disease. Nonetheless, recent studies have reinforced earlier reports that the transfer of oligonucleotides, both RNA and cDNAs

encoding or enhancing specific immunomodulatory molecules, can correct SS-like disease in animal models, as well as increase our understanding of its etiopathogenesis.

Perhaps the most surprising finding of this review, because salivary glands are active secretory tissues, is that there has been so little accomplished recently towards advancing clinical applications employing cDNAs encoding secretory proteins. This is true for both local upper gastrointestinal tract as well as systemic uses. Proofs of concept for such exocrine and endocrine applications have long been known via studies in preclinical animal models. With the exception of the promising exocrine application for an orally centered management of obesity, only small incremental advances otherwise have been made. While apparently all secretory proteins will, to a variable degree, find their way into saliva (exocrine), protein secretion into the bloodstream for endocrine applications are significantly hindered. This is because at present it is impossible to predict if systemically required transgenic proteins will be efficiently secreted in an endocrine manner in humans. This impediment stems from the absence of any major recent progress in understanding the sorting behavior of all secretory proteins.

Methodologically, there seems to be enough evidence to suggest that most future salivary gland studies involving oligonucleotide transfer likely should benefit from employing non-viral approaches, e.g., either UAGT of minicircles or nanoparticles. Given the possibility of attaining equal levels of functional gene transfer, we would argue that non-viral methods of gene transfer are preferable to those using a viral vector. The reason for this bias is that ideally we would prefer to limit the oligonucleotides administered to only those absolutely necessary for clinical efficacy. However, should use of a viral vector be deemed preferable, for whatever reason, employing pseudotyped AAV vectors currently appears to offer the greatest benefits for future gene transfer endeavors.

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Highlights

- Salivary gland gene transfer is easily accomplished in an essentially non-invasive manner.
- Salivary gland gene transfer is safe, efficacious and shown useful for treating radiation-damaged glands in humans.
- Several gene transfer strategies appear potentially useful for preventing salivary gland radiation damage.
- Salivary gland gene transfer, in preclinical models, can be used to help understand the immunopathogenesis of Sjögren's syndrome and possibly for treating local glandular dysfunction.
- At present, the most promising exocrine secretory application appears to be for obesity via transfer of the PYY cDNA.
- There are many promising endocrine secretory applications. However, the sorting behavior in salivary glands of transgenic secretory proteins needed systemically (saliva: serum distribution ratio) is currently unpredictable.
- Salivary gland gene transfer with ultrasound assisted gene transfer and pseudotyped adeno-associated viral vectors shows considerable promise.

Table 1

Advantages and disadvantages of salivary glands for gene therapy

Advantages

- There is easy access of a gene transfer vector to almost all epithelial cells in a gland via intraoral cannulation of the main excretory duct and retrograde infusion
- There is a readily defined fluid volume that can be predetermined and infused into each gland
- The concentration of vector in the infusate fluid is not further diluted following vector delivery
- The luminal membrane of almost all epithelial cells in the gland is a potential target for infused vectors
- Salivary epithelial cells divide slowly making them a relatively stable target population with non-integrating gene transfer vectors
- It is easy to measure neurotransmitter coupled secretory responses in human salivary glands via the gustatory stimulation of salivation
- Human salivary glands are well encapsulated minimizing the potential for vector spread beyond the targeted gland
- If a severe and life-threatening adverse event were to occur, a single salivary gland is not essential for life and could be removed

Disadvantages

- There is a relatively small number of investigators studying salivary gland gene transfer, hindering rates of progress
- There is a critical lack of understanding of the basic biology regulating the sorting of secretory proteins within salivary glands into either saliva or the bloodstream

Modified from Samuni and Baum [18]

Table 2

Major gene transfer methods used for salivary gland gene transfer

Clinical studies

- Replication deficient, serotype 5 adenoviral vector (Ad5) [14,82] [1]
- Serotype 2 adeno-associated viral (AAV2) vector; protocol submitted for review

Pre-clinical animal model studies

- Ad5 vectors in mice, rats, miniature pigs and macaques, e.g., [8,24,28,50]
- AAV2, and other AAV serotypes including many pseudotyped, vectors in mice, rats, miniature pigs and macaques, e.g., [10,33,61,83] [2]
- Moloney murine leukemia viral vector in rats [84] and lentiviral vector in mice [85]
- Multiple non-viral methods including cationic liposomes [86], ultrasound assisted microbubble destruction [27], pH-dependent nanoparticles [35]

[1] Note that the length of expression from the AdhAQP1vector in human salivary glands seems much longer than that seen in pre-clinical rat and miniature pig studies with this vector, apparently related to the methylation status of the CMV promoter used [82].

[2] Note that traditional AAV2 vectors are ineffective in rat salivary glands [87]