ORAL DISEASES

Oral Diseases (2010) 16, 269–277. doi:10.1111/j.1601-0825.2009.01631.x

http://www.blackwellmunksgaard.com

ORIGINAL ARTICLE

Evaluation of a rapamycin-regulated serotype 2 adeno-associated viral vector in macaque parotid glands

C Zheng[1], A Voutetakis[1], M Metzger[2], S Afione[1], AP Cotrim[1], MA Eckhaus[3], VM Rivera[4], T Clackson[4], JA Chiorini[1], RE Donahue[2], CE Dunbar[2], BJ Baum[1]

[1]*Molecular Physiology and Therapeutics Branch, National Institute of Dental and Craniofacial Research, Bethesda, MD;* [2]*Hematology Branch, National Heart, Lung, and Blood Institute, Bethesda, MD;* [3]*Division of Veterinary Resources, National Institutes of Health, Department of Human Health Services, Bethesda, MD;* [4]*ARIAD Pharmaceuticals, Cambridge, MA, USA*

OBJECTIVES: Salivary glands are useful target organs for local and systemic gene therapeutics. For such applications, the regulation of transgene expression is important. Previous studies by us in murine submandibular glands showed that a rapamycin transcriptional regulation system in a single serotype 2, adeno-associated viral (AAV2) vector was effective for this purpose. This study evaluated if such a vector was similarly useful in rhesus macaque parotid glands.

METHODS: A recombinant AAV2 vector (AAV-TF-RhEpo-2.3w), encoding rhesus erythropoietin (RhEpo) and a rapamycin-inducible promoter, was constructed. The vector was administered to macaques at either of two doses [1.5×10^{11} (low dose) or 1.5×10^{12} (high dose) vector genomes] via cannulation of Stensen's duct. Animals were followed up for 12–14 weeks and treated at intervals with rapamycin (0.1 or 0.5 mg kg^{-1}) to induce gene expression. Serum chemistry, hematology, and RhEpo levels were measured at interval.

RESULTS: AAV-TF-RhEpo-2.3w administration led to low levels of rapamycin-inducible RhEpo expression in the serum of most macaques. In five animals, no significant changes were seen in serum chemistry and hematology values over the study. One macaque, however, developed pneumonia, became anemic and subsequently required euthanasia. After the onset of anemia, a single administration of rapamycin led to significant RhEpo production in this animal.

CONCLUSION: Administration of AAV-TF-RhEpo-2.3w to macaque parotid glands was generally safe, but led only to low levels of serum RhEpo in healthy animals following rapamycin treatment.

Oral Diseases (2010) **16**, 269–277

Keywords: AAV2; rapamycin-regulation; gene transfer; macaque; parotid

Correspondence: Dr BJ Baum, Bldg. 10, Room 1N113, MSC-1190, MPTB/NIDCR/NIH, 10 Center Drive, Bethesda, 20892-1190 MD, USA. Tel: (301)496-1363, Fax: (301)402-1228, E-mail: bbaum@dir.nidcr.nih.gov
Received 24 July 2009; revised 9 September 2009; accepted 13 September 2009

Introduction

An important application of clinical gene transfer is gene therapeutics, use of the gene as a drug (Felgner and Rhodes, 1991; Crystal, 1995). We have made a considerable effort to apply gene therapeutics to salivary glands for both local (upper gastrointestinal tract) and systemic (serum) applications (e.g., Baum *et al*, 2004; Voutetakis *et al*, 2004, 2007a). Salivary glands are an interesting, but not often considered, target choice for gene therapeutics (Baum *et al*, 2002; Kagami *et al*, 2008). They can be easily accessed intra-orally, are capable of producing and then secreting significant amounts of protein in both exocrine and endocrine directions (Baum *et al*, 1999; Isenman *et al*, 1999; Voutetakis *et al*, 2008; Sugito *et al*, 2009), and are not critical for life in case of a severe local adverse event. Previously, in rodents, miniature pigs, and macaques, we have shown the potential utility of salivary glands for systemic gene therapeutics with such transgenes as erythropoietin (Epo; Voutetakis *et al*, 2004, 2007a,b; Hai *et al*, 2009), growth hormone (He *et al*, 1998; Wang *et al*, 2005; Zheng *et al*, 2006), and parathyroid hormone (Adriaansen *et al*, 2008).

A significant issue generally for gene therapeutics, regardless of the tissue target site, is controlling the level of transgene expression. For many proteins, high and uncontrolled expression levels could be dangerous for patients, for example, with Epo. While administration of recombinant Epo is extremely valuable for patients with chronic renal failure and subsequent Epo-responsive anemia, the levels administered are very carefully monitored. The presence of high systemic Epo levels could lead to stroke and/or death. The same concern would exist for transgenic Epo produced following a gene transfer procedure. Accordingly, for successful gene therapeutics, there has been considerable research into methods to regulate transgenic protein expression

tightly and conveniently following gene transfer. An approach that has proven to be especially useful, at least in preclinical studies, is that of using small molecule regulation via activation of a chimeric transcription factor. One such system employs rapamycin or its non-immunosuppressive analogs (e.g., Rivera *et al*, 1996, 2005). We have previously shown that this system is effective in regulating transgene expression following gene transfer to rodent submandibular glands with both serotype 5 adenoviral (Ad5) and serotype 2 adeno-associated viral (AAV2) vectors (Wang *et al*, 2004, 2006).

The rapamycin-regulated transcriptional control system uses rapamycin (or an analog) to dimerize the activation (p65) and DNA-binding (ZFHD1) domains of a chimeric transcription factor through the rapamycin binding domains of the proteins FRAP and FKBP. The activated transcription factor then can bind to zinc finger motifs upstream of a minimal promoter (from human interleukin-2) and drive expression of the encoded transgene. Three major advantages of the rapamycin control system are that (i) only human components are used, reducing the potential for immunogenicity, (ii) there is little to no leakiness of transgene expression in the absence of rapamycin induction, and (iii) regulation of transgene expression is rapamycin (or analog) dose dependent (Pollock and Clackson, 2002).

For almost all published animal studies using rapamycin-based transcriptional regulation, the DNA-binding domain of the chimeric transcription factor is fused to three identical rapamycin binding domains of FKBP (Rivera *et al*, 2004). This is a concern for generating clinical grade gene transfer vectors, as these identical ~300 bp rapamycin binding domains are capable of recombination (Rivera *et al*, 2004). Accordingly, Rivera *et al* (2004) developed a non-recombinogenic AAV2 vector, which contains three non-identical FKBP domains and results in a single vector species, showing no evidence of recombination during production. In this study, we have used a baculovirus AAV2 vector production system (Urabe *et al*, 2002) to generate a non-recombinogenic vector, AAV-TF-RhEpo-2.3w, which expresses rhesus (Rh) Epo (RhEpo) in a rapamycin regulated manner. Our experimental objective was to assess the utility of this vector to direct clinically adequate, tightly controlled expression of RhEpo from transduced parotid glands of healthy rhesus macaques.

Materials and methods

Construction of AAV-TF-RhEpo-2.3w

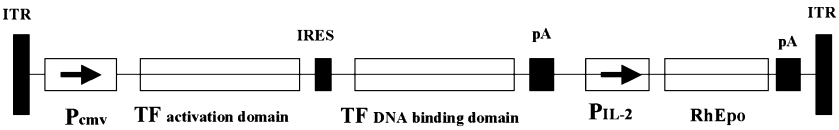
To produce sufficient amounts of the recombinant vector AAV-TF-RhEpo-2.3w for this *in vivo* macaque study, we employed a baculovirus production system (Urabe *et al*, 2002). The plasmid pFBGR is the shuttle vector for this system. To clone the rapamycin regulated RhEpo expression cassette into pFBGR, pAAV-TF-rhEpo2.3w was digested with Asc I, filled in with T4 DNA polymerase, then digested with Mlu I to obtain the ~4.7 kb cassette. Thereafter, pFBGR was digested with Bsp EI, filled in with T4 DNA polymerase, and then digested with Mlu I to delete the CMV promoter, an irrelevant transgene and the polyadenylation signal from the plasmid. Next, the digested pFBGR plasmid and the rapamycin regulated RhEpo expression cassette were ligated together. The final plasmid, pFBGR-TF.rhEpo2.3w, was then used to generate the AAV-TF-RhEpo-2.3w vector in the baculovirus production system.

AAV-TF-RhEpo-2.3w vector stocks were produced in *Spodoptera frugiperda* Sf9 cells, as previously described (Urabe *et al*, 2002). Briefly, these insect cells require both cis and trans factors from AAV2 for replication, packaging and particle formation. Accordingly, Sf9 cells were transfected with a set of three baculovirus plasmid constructs: pBac-VP (expresses the three AAV capsid proteins), pBac-Rep (expresses AAV2 Rep 52 and Rep 78, required for vector DNA replication), and pFBGR-TF.rhEpo2.3w, which is 'rescued' in the presence of Rep proteins and is then able to replicate to very high copy numbers in the Sf9 cells.

The three different baculovirus constructs were used to transfect 200 ml of Sf9 cells (2×10^6 cells ml^{-1}). The suspension was cultured on a shaker at 135 rpm and 28°C for 72 h. Thereafter, the culture was subjected to three freezing/thawing cycles and then the supernatant was clarified by centrifugation at 2000 *g* for 10 min at room temperature. Polyethylene glycol (PEG-8000; 2% final concentration; Sigma-Aldrich, St. Louis, MO, USA) was added to the supernatant and shaken gently at 4°C for 48 h, followed by centrifugation at 5000 ***g*** for 30 min. The pellet was then resuspended in 12 ml of CsCl solution with a refractive index of 1.372 and centrifuged in a swinging bucket rotor (SW40, Beckman Coulter, Fullerton, CA, USA) at 178 300 ***g*** for 72 h. The gradients were then fractionated, and the titer of the resulting AAV-TF-RhEpo-2.3w vector (Figure 1) was determined by quantitative (Q) PCR using plasmid DNA standards. DNase-resistant vector genomes (vg) in the vector stock were determined by QPCR using the SYBR green system (PE Applied Biosystems, Foster City, CA, USA; see below) with primers specific to the cytomegalovirus promoter (274F:CATCTACGTATTAGTCATCGCTATTACCAT and 367R:TGGAAATCCCCGTGAGTCA).

In vitro *demonstration of rapamycin-regulated RhEpo expression*

Expression of RhEpo initially was evaluated in 293 cells. The cells were grown in improved Eagle's minimal essential medium, supplemented with 10% bovine serum, 100 U ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin (all from Biosource, Camarillo, CA, USA) at 37°C in a humidified, 5% CO_2 atmosphere, incubator. AAV-TF-RhEpo-2.3w was used to transduce the 293 cells at a multiplicity of infection (MOI) of either $\sim 6 \times 10^3$ or $\sim 6 \times 10^4$ vg per cell. After 24 h, the medium was removed, the cell layer was washed with phosphate-buffered saline, and fresh medium without serum, but containing rapamycin (1.25 or 12.5 μg ml^{-1}), was then added. Thereafter, the cells were allowed to incubate for an additional 24 h. This medium was then collected,



AAV-TF-RhEpo-2.3w

Figure 1 Schematic representation of the AAV-TF-RhEpo-2.3w vector. ITR, inverted terminal repeat; Pcmv, cytomegalovirus promoter; TF, transcription factor; IRES, internal ribosome entry site; pA, polyadenylation signal; PIL-2, interleukin-2 promoter; RhEpo, rhesus erythropoietin cDNA. See Materials and Methods for additional details

centrifuged at 2500 ***g*** for 3 min and the resulting supernatants assayed for RhEpo expression with a human Epo enzyme-linked immunosorbent assay (ELISA), which cross-reacts, ~40%, with RhEpo (Rivera *et al*, 2005; Voutetakis *et al*, 2009; StemCell Technologies, Vancouver, BC, Canada; detection limit 0.6 mU ml^{-1} based on human Epo standards).

Vector administration to macaques

The protocol for these animal experiments was approved by and conducted under the guidelines of the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute and the National Institutes of Health Institutional Biosafety Committee. The general way in which these studies were conducted has been previously reported (Voutetakis *et al*, 2007a), and will be described here only briefly. Six male rhesus macaques (~5–7 kg) were housed either singly or in compatible pairs. They were provided free access to water and received ~10–15 biscuits of Purina monkey chow 5038 twice daily. The vector was administered to macaques at either of two doses [1.5×10^{11} vg (~2.5×10^{10} vg kg^{-1}, low dose); 1.5×10^{12} vg (~2.5×10^{11} vg kg^{-1}, high dose)] via cannulation of Stensen's duct of the right parotid gland (see also O'Connell *et al*, 1999; Voutetakis *et al*, 2008). For all procedures, macaques were anesthetized with a dose of 3 mg kg^{-1} telazol and ketamine intramuscularly. Blood (femoral vein) was obtained, and rapamycin administered, at the times indicated in Results. Whole saliva was also obtained at these time points, as described (Voutetakis *et al*, 2007a, 2008). Rapamycin was dissolved in N,N dimethylacetamide to a concentration of 50 mg ml^{-1} and then diluted in polyethylene glycol (PEG-400; Sigma-Aldrich) to a stock concentration of 2 mg ml^{-1}. Just prior to the administration to macaques, the stock solution was diluted in a diluent (1.2% Tween 80, 27% PEG-400) and given intravenously at a final dose of either 0.1 or 0.5 mg kg^{-1}. In addition, all macaques also received an injection of rapamycin (0.1 mg kg^{-1}) on day 14, which had been diluted inappropriately with saline. None of the resulting blood samples from the day 14 administration was included in the analyses herein. For purposes of this report, the results obtained with five animals [#s 3034, 6847, 6852 (all low dose), 3563 and 4356 (both high dose)] generally will be distinguished from those of the remaining animal (#6851), as the latter experienced a severe adverse event (see below).

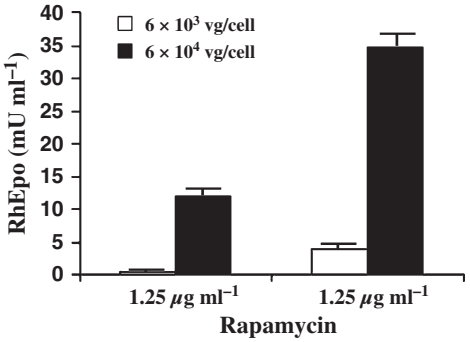


Figure 2 Demonstration of rapamycin responsiveness of the AAV-TF-RhEpo-2.3w vector *in vitro*. Vector was added to 293 cells at the indicated multiplicities of infection. After 24 h, medium was replaced with serum-free medium, and rapamycin added at the indicated concentrations. Following incubation for an additional 24 h, medium was collected and assayed for rhesus erythropoietin (RhEpo). Data shown are the average of duplicate determinations. See Materials and Methods for additional details

Serum chemistry and hematology analyses

At each designated time point (see experimental timeline in Figure 3a), a complete blood count (CBC) was performed on all animals. The CBC included the following: hemoglobin, hematocrit, platelet count, white blood cell count, red blood cell count, lymphocyte count, neutrophil count, and counts of basophils, eosinophils, monocytes, and reticulocytes. In addition, multiple serum chemistry analyses were also performed at these times: total protein, albumin, alkaline phosphatase, serum glutamic pyruvic aminotransferase, serum glutamic oxaloacetic aminotransferase, γ-glutamyl transferase, lactate dehydrogenase, creatine phosphokinase, creatinine, blood urea nitrogen, calcium, chloride, iron, magnesium, phosphorus, potassium, sodium, and total bilirubin.

Severe adverse event, necropsy, and pathology assessment

On day 37 postvector delivery, macaque #6851 was reported as not eating well. By day 43, the animal exhibited an elevation in white cells, as well as a reduced hematocrit. On day 55, the animal was reported to have a bad cough, and a chest x-ray indicated a solid mass in the left lung. No other animals showed similar signs, and chest x-rays performed on all other animals were negative. Oral voriconazole (for ~1 week) and oral cephalexin (for 3 weeks), both twice daily, were then begun for the affected macaque. Subsequently, the animal's appetite improved, the animal gained weight, and the animal's coughing ceased. However, the lung

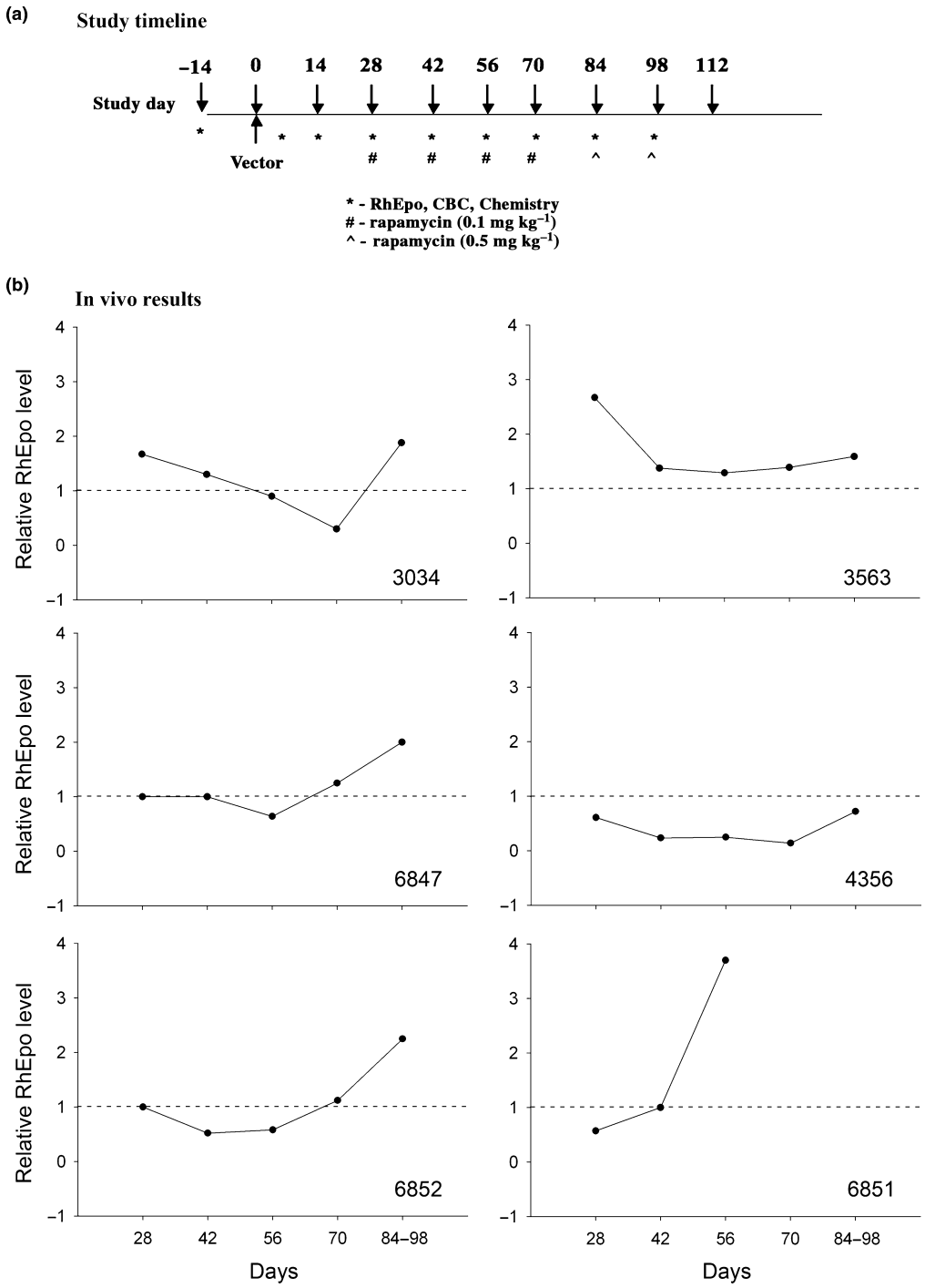


Figure 3 Effect of rapamycin administration on serum RhEpo (rhesus erythropoietin) levels in all six macaques across the entire time course of this study. (**a**) Experimental timeline for this study. Upward facing arrow indicates time when vector was administered (day 0), while downward facing arrows indicate times (* symbol) when blood samples were collected for determination of RhEpo (rhesus erythropoietin) levels, CBC (complete blood counts) and serum chemistry values. The # and ^ symbols indicate times when rapamycin was administered. Note that only one animal was administered rapamycin on day 98 (# 6852), because of difficulty in getting venous access on day 84. See text for additional details. (**b**) Each panel represents data from an individual monkey. The '*y*-axis' indicates the relative level of RhEpo detected 24 h after rapamycin administration with 1 (dashed line in each panel) equal to no difference in serum RhEpo levels from before rapamycin administration, i.e., Relative RhEpo level = serum RhEpo after rapamycin/serum RhEpo before rapamycin. Thus, values <1 indicate a reduction in serum RhEpo levels compared with before rapamycin administration, and values >1 indicate an increase in serum RhEpo levels compared with before rapamycin administration. The three left panels indicate low dose animals and the three right panels indicate high dose animals. Specific animal study numbers are shown in the bottom right corner of each panel. Animal #6851 is the macaque that experienced the severe adverse event and was euthanized. Note that the final data point shown for animal #6847 is approximate, as the before rapamycin administration RhEpo level on day 84 was undetectable and the after rapamycin administration RhEpo level was 0.2 mU ml^{-1}. Also, note that the last time point for animal #6852 was on day 98 after vector delivery, because of technical difficulties in obtaining blood on day 84. The last time point for the remaining four macaques that completed the study was on day 84. See text for additional details

mass consolidated and expanded, and thereafter a decision was made to euthanize this animal (by intravenous pentobarbital). A complete necropsy and pathology assessment were performed. Multiple tissues were collected at necropsy. Tissue samples were fixed in 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA, USA), processed, and embedded in paraffin. Routine 6-micron hematoxylin and eosin stained sections were prepared (Histoserv Inc., Germantown, MD, USA) and then subjected to histopathologic evaluation. A board certified veterinary pathologist performed both the gross necropsy and the histopathologic analyses. In addition, a further portion of multiple tissues [right parotid, left parotid, right and left draining (submandibular) lymph nodes, right and left masseter muscles, liver, lung, tracheobronchial lymph node and serum] was flash frozen and kept at −80°C until measuring vector distribution by QPCR (see below).

QPCR assays for AAV-TF-RhEpo-2.3w vector distribution

Genomic DNA was extracted from the above-listed tissues of macaque #6851 with the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA) and QIAamp® DNA Mini and Blood Mini kit for serum only (Qiagen, Chatsworth, CA, USA). Three hundred ng DNA was used per QPCR reaction. The primers CMVq1 (5′-TGGGCGGTAGGCGTGTAC-3′) and CMVq2 (5′-CGATCTGACGGTTCACTAAACG-3′), and probe CMVTaqprobe (5′-/56-FAM/TGGGAGGTCTATATAAGC/36-TSMTSp/-3′), for the CMV promoter region, were used to measure vector copy number. These sequences were selected using Primer Express™ Primer Design software (PE Applied Biosystems), and assays were performed in an ABI Prism 7700 Sequence Detector (PE Applied Biosystems). The plasmid pACCMV-pLpA was used as the standard. DNA was extracted from two or three separate portions (see Table 5) of each available tissue, and all extracted DNA samples were assayed separately in triplicate by QPCR. The reaction conditions used were as follows: 95°C for 2 min, 95°C for 8 min, 95°C for 15 s, and 60°C for 1 min for 40 cycles.

Results

RhEpo expression from AAV-TF-RhEpo-2.3w

The ability of the AAV-TF-RhEpo-2.3w vector to direct rapamycin-regulated RhEpo expression was initially tested *in vitro* using 293 cells. Importantly, no RhEpo was expressed in the absence of rapamycin (not shown). As seen in Figure 2, RhEpo production was both vector- and rapamycin-dose dependent. However, the levels of RhEpo expressed following transduction were relatively low considering the high multiplicity of infections used (6×10^3 and 6×10^4 vg per cell; see for comparison Braddon *et al*, 1998; Yamano *et al*, 2002). This notion was further supported by the results of the *in vivo* experiments in macaques. As shown in Figure 3b and Table 1, administration of either vector dose employed herein (1.5×10^{11} or 1.5×10^{12} AAV-TF-RhEpo-2.3w vg per gland) led to low level rapamycin-inducible RhEpo expression in the serum of most macaques. Interestingly, baseline RhEpo levels in these healthy animals were both relatively high and variable (see Table 1; Prevector). Figure 3b shows data for individual macaques as a ratio of their RhEpo levels after/before rapamycin treatment. In a previous study (Voutetakis *et al*, 2007a), also performed in healthy rhesus macaques, we used a conventional, unregulated AAV2 vector containing an expression cassette in which the Rous sarcoma virus promoter directed the expression of RhEpo. The high vector dose used in that previous study was five-fold lower than used herein (3×10^{11} vg). In that study (see Figure 1; Voutetakis *et al*, 2007a), on average by day 56, postvector delivery serum RhEpo levels were ~3.5-fold higher than background levels, and these remained fairly constant across the 6-month study period. Conversely, for the five animals that successfully completed the present study, the average percentage increase in RhEpo levels observed following rapamycin induction compared with those before rapamycin administration was ~11% across all time-points measured, and at the peak levels averaged only ~90% above background.

Table 1 Absolute RhEpo levels measured in serum at key time-points before and after rapamycin administration[a]

Time	*RhEpo (mU ml^{-1})*	
Prevector (n = 6)	2.8 ± 0.6	
Low dose (n = 3)	*Before*	*After*
Day 28	0.4 ± 0.2	0.6 ± 0.3
Day 42	1.3 ± 0.7	0.7 ± 0.4
Day 56	1.2 ± 0.1	0.9 ± 0.2
Day 70	0.9 ± 0.4	1.9 ± 1.1
Days 84–98[b]	0.9 ± 0.5	1.9 ± 1.1
High dose (n = 2 or 3[c])	*Before*	*After*
Day 28	1.4 ± 0.3	1.4 ± 0.5
Day 42	1.2 ± 0.5	0.9 ± 0.6
Day 56	2.8 ± 0.7	6.2 ± 4.7
Day 70	2.3 ± 0.5	1.5 ± 1.0
Day 84	2.6 ± 0.8	3.4 ± 2.1

[a]Data shown are absolute values of rhesus erythropoietin (RhEpo) determined in serum by ELISAs for key time-points studied. Baseline values for all six macaques are shown as 'Pre-vector'. Thereafter, animals received either dose of vector (1.5×10^{11} or 1.5×10^{12} vg per gland) and rapamycin at the indicated times. Data shown are mean ± s.e. for the number (n) of animals. A graphic representation of these data (ratio of RhEpo after/RhEpo before rapamycin) can be found in Figure 3b. Rapamycin was administered at 0.1 mg kg^{-1} for Days 28–70, and 0.5 mg kg^{-1} for the last time-point.
[b]Two animals received rapamycin on day 84 and one on day 98 (see text).
[c]One high dose animal (6851) was euthanized after day 56.

General safety of AAV-TF-RhEpo-2.3w vector administration to macaque parotid glands

All five macaques that completed the study appeared to tolerate administration of the AAV-TF-RhEpo-2.3w vector to their parotid glands. Clinically, none of these five animals experienced any untoward outcomes in the

targeted tissue, and saliva obtained from them was always of normal consistency and without purulence. Furthermore, all of these animals continued to eat normally and gain weight, which suggests that administration of an AAV2 vector at a dose of up to 1.5×10^{12} vg ($\sim 2.5 \times 10^{11}$ vg kg^{-1}) to a single parotid gland is generally safe. This latter impression is also supported by the results obtained from multiple analyses of serum chemistry and hematology values throughout the study period (see Tables 2 and 3 for values from high dose animals). There were no consistent effects of the AAV-TF-RhEpo-2.3w vector on any serum chemistry or hematology parameter measured for all three animals in the low dose group, and for the two high dose animals that completed the study. While occasional deviations from pretreatment values were seen, they were episodic and apparently of no clinical consequence in these five animals. Note that in all animals, modest elevations in creatine phosphokinase levels were seen throughout the study in a dose-independent manner. These likely reflect minor muscle injury due to injections for anesthesia and animal handling procedures.

Severe adverse event in macaque #6851

As indicated in the Materials and Methods, one high dose animal in this study experienced a severe adverse event. This began at ~5 weeks (day 37) postvector delivery as an observed reduction in food intake, followed a week later by an elevated white blood cell count. By ~8 weeks, i.e., after rapamycin administrations on days 28 and 42, this macaque exhibited a significant cough and, following a chest x-ray, this macaque was treated for pneumonia with oral antimicrobials as described above. While his appetite and coughing improved subsequently, eventually a decision was made to euthanize animal #6851 at week 11 after vector administration. Grossly, the animal was well muscled with ample body fat. Mediastinal and tracheobronchial lymph nodes were moderately enlarged. Much of the left lung was consolidated, a portion of the left caudal lung was cavitated ($\sim 4 \times 4 \times 3$ cm) and contained a yellow-brown, granular fluid. Multiple adhesions were present between the left middle and caudal lobes, and with the parietal pleurae. The right lung and left cranial lung appeared relatively normal. All other

Table 2 Representative serum chemistry values from the two surviving macaques in the high dose group[a]

	Day of sampling				
	Pre (−14)	*4*	*15*	*43*	*85*
Total Protein	6.7 ± 0.3	6.9 ± 0.1	7.1 ± 0.1	6.9 ± 0.3	7.3 ± 0.1
Alb	3.8 ± 0.2	4.0 ± 0.1	4.0 ± 0.3	4.1 ± 0.2	4.0 ± 0.1
SGOT	44 ± 5	57.5 ± 2.5	48.5 ± 4.5	66 ± 2.0	60 ± 2.0
SGPT	51.5 ± 16.5	52.5 ± 14.5	52 ± 5	64 ± 18	50.5 ± 28.5
ALP	114 ± 10	104.5 ± 7.5	98 ± 4	102 ± 10	98.5 ± 6.5
LDH	1402.5 ± 796.5	1491.5 ± 758.5	921 ± 187	1354 ± 570	1712.5 ± 537.5
CPK	95 ± 13	172.5 ± 19.5	297.5 ± 27.5	211.5 ± 28.5	354 ± 264
BUN	19.5 ± 3.5	19 ± 3	18.5 ± 2.5	18 ± 2	16.5 ± 0.5
Creatinine	1.3 ± 0	1.4 ± 0.1	1.4 ± 0.2	1.5 ± 0.1	1.4 ± 0.1
Na	145.5 ± 0.5	149.5 ± 0.5	144 ± 0	139 ± 2.0	159 ± 1
K	3.8 ± 0	3.8 ± 0.2	3.9 ± 0.2	3.7 ± 0.2	3.8 ± 0.2
Cl	105 ± 1	108 ± 1	104 ± 0	101.5 ± 2.5	117 ± 0

Sodium (Na), potassium (K) and chloride (Cl) are as mEq l^{-1}; Creatinine as mg dl^{-1}; blood urea nitrogen (BUN) as mmol l^{-1}; serum glutamic oxaloacetic transaminase (SGOT) as U l^{-1}; serum glutamic pyruvic aminotransferase (SGPT) as U l^{-1}; alkaline phosphatase (ALP) as U l^{-1}; lactate dehydrogenase (LDH) as mg dl^{-1}; albumin (Alb) as g dl^{-1}; creatine phosphokinase (CPK) as U l^{-1}; total protein as g l^{-1}.
[a]Data shown are the mean ± s.e. of representative values for the two animals surviving from the high dose group.

Table 3 Representative hematology values from the two surviving macaques in the high dose group[a]

	Day of sampling				
	Pre (−14)	*7*	*15*	*43*	*85*
WBC	7.4 ± 2.0	5.2 ± 0.1	5.6 ± 0.1	5.3 ± 0.5	6.4 ± 1.2
RBC	5.2 ± 0.2	5.0 ± 0	5.1 ± 0	5.2 ± 0.2	5.2 ± 0.1
HCT	39.1 ± 1.5	38.4 ± 0.5	38.9 ± 0.2	39.1 ± 1.3	39.2 ± 0.3
Platelets	228 ± 5	224.5 ± 0.5	231.5 ± 7.5	248.5 ± 12.5	263 ± 11
Segs	53.2 ± 0.1	47.7 ± 3.5	43.4 ± 16.5	49.9 ± 16.5	64.6 ± 6.6
Lymphocytes	39.2 ± 1.5	45.2 ± 2.4	49.2 ± 16.1	41.8 ± 15.7	30.8 ± 6.2
Reticulocytes	0.9 ± 0	1.4 ± 0.3	1.2 ± 0.6	1.1 ± 0.2	1.0 ± 0

White blood cell (WBC) count as cells mm^{-3}; red blood cell (RBC) count as million cells mm^{-3}; hematocrit (HCT) as percentage; platelet count as $\times 10^3$ mm^{-3}; segmented neutrophils (Segs) as percentage; lymphocytes as percentage; reticulocytes as percentage.
[a]Data shown are the mean ± s.e. of representative values for the two animals surviving from the high dose group.

major organs were normal in appearance. Histopathologic examination revealed the cavitated lesion in the left lung to be an abscess, likely of bacterial origin. The remainder of the left caudal lobe and portions of the left middle lobe of this lung exhibited moderate to severe interstitial pneumonia, with increased numbers of type 2 pneumocytes, interstitial fibrosis, increased numbers of alveolar macrophages, and prominent lymphoid aggregates and nodules. A bacterial culture from the left lung yielded no growth, and special staining of sections from that lung also failed to reveal the presence of any infectious agent (not shown; possibly a result of the aggressive antibiotic therapy used). A focal acute bronchitis was found in the right caudal lobe, but other lobes exhibited only terminal edema. All other findings were noted as incidental.

Key hematology and serum chemistry parameters for macaque #6851 are given in Table 4. Of particular note are the changes occurring on ~day 43, for example, increased white blood cells and alkaline phosphatase, decreased hematocrit, lymphocytes, albumin and blood urea nitrogen. This animal was administered rapamycin at a dose of 0.1 mg kg^{-1} on day 56 that led, on the following day, to an ~3.7-fold increase in serum RhEpo detected (see Figure 3b, bottom right panel). At necropsy, ten tissues were obtained to determine the distribution of the AAV-TF-RhEpo-2.3w vector in animal #6851 by QPCR. As shown in Table 5, in six of these samples, only low, 'background' vector copy levels (see Voutetakis *et al*, 2007a) were detected (left parotid, left submandibular lymph node, right and left masseter muscles, lung and serum; all ≤60 vg per 300 ng DNA). Two lymph nodes showed a modest level of vector present: the right submandibular lymph node, which drained the targeted parotid gland (300 vg per 300 ng DNA) and a tracheobronchial lymph node (129 vg per 300 ng DNA). Two tissues were found to have fairly high, and generally similar, AAV-TF-RhEpo-2.3w vector levels: the targeted right parotid gland (4.1×10^3 vg per 300 ng DNA) and the liver (2.8×10^3 vg per 300 ng per DNA).

Table 4 Key serum chemistry and hematology values from macaque #6851[a]

	Day of sampling				
	Pre (−14)	*15*	*29*	*43*	*56*[b]
Alb	4.2	3.8	3.7	2.6	2.3
SGPT	113	59	66	34	37
ALP	145	139	140	233	380
BUN	18	15	16	14	11
Na	142	143	143	138	146
K	4.5	3.4	3.5	3.5	3.5
Cl	106	104	106	102	97
WBC	4.03	9.11	4.11	14.04	18.68
HCT	39.2	37	37.5	31.9	29
Platelets	153	173	167	415	13
Lymphocytes	36.2	15.1	34.5	13.5	9.2

Sodium (Na), potassium (K) and chloride (Cl) are as mEq l^{-1}; blood urea nitrogen (BUN) as mmol l^{-1}; serum glutamic pyruvic aminotransferase (SGPT) as U l^{-1}; alkaline phosphatase (ALP) as U l^{-1}; albumin (Alb) as g dl^{-1}; white blood cell (WBC) count as cells mm^{-3}; hematocrit (HCT) as percentage; platelet count as $\times 10^3$ mm^{-3}; lymphocytes as percentage.
[a]Data shown are representative clinical laboratory values obtained for animal #6851 from the high dose group. This animal experienced a severe adverse event, interstitial pneumonia in the left lung.
[b]Day 56 is when the last rapamycin dose was given to this macaque (see Table 1, Figure 3).

Table 5 Number of AAV-TF-RhEpo-2.3w vector genome copies found in various tissues at necropsy after gene transfer to the right parotid gland of rhesus macaque #6851[a]

Tissue (n)	*vg copies per 300 ng DNA*
Right parotid (3)	4.1×10^3
Left parotid (2)	60
Right submandibular lymph node (3)	300
Left submandibular lymph node (2)	34
Serum (2)	21
Liver (3)	2.8×10^3
Right masseter (2)	60
Left masseter (2)	60
Lung (3)	32
Tracheobronchial lymph node (3)	129

(*n*), the number of separate tissue pieces from which genomic DNA was extracted. The results shown are representative of those with each respective tissue piece examined. DNA from each extracted tissue sample was then assayed in triplicate by QPCR. See text for additional details.
[a]Data shown are the mean number of vector genome (vg) copies per 300 ng DNA determined with genomic DNA extracted from the indicated tissues 76 days after administration of 1.5×10^{12} vg to the right parotid gland of macaque #6851. This animal was euthanized and necropsied as described in the text.

Discussion

The purpose of this study was to assess the utility of the AAV-TF-RhEpo-2.3w vector, at two doses (1.5×10^{11} vg and 1.5×10^{12} vg per parotid gland), to direct clinically adequate, tightly controlled expression of RhEpo in the serum of rhesus macaques. As shown *in vitro*, no expression of RhEpo resulted, unless transduced cells were treated with rapamycin (Figure 2). Five animals completed the *in vivo* study, but rapamycin-regulation of the vector was unable to achieve significant elevations in serum levels of RhEpo following parotid gland transduction. However, in the single animal that developed pneumonia and became anemic (see below), rapamycin administration was followed by a large increase (~3.7-fold) in the serum RhEpo level detected.

The overall weak production of RhEpo in healthy macaques was unanticipated, as we previously showed that a conventional, unregulated, AAV2 vector (rAAV2RhEPO), used at 20% of the high dose employed herein, readily transduced macaque parotid glands and led to significant increases in serum RhEpo levels (Voutetakis *et al*, 2007a). Accordingly, these results are unlikely to be related to the use of AAV2 vectors *per se* in this tissue. However, there are two major genomic differences between the rAAV2RhEPO

vector used previously and AAV-TF-RhEpo-2.3w that theoretically could have contributed to the disparity in RhEpo production observed: (i) the promoter utilized to drive RhEpo expression and (ii) the actual size of the genome. While both should have been considered more when determining the vector doses used herein, it is likely that the first reason was most significant. rAAV2RhEPO uses a promoter from Rous sarcoma virus that directs high levels of sustained transgene expression in salivary glands (Voutetakis *et al*, 2004; Zheng and Baum, 2005). Conversely, AAV-TF-RhEpo-2.3w uses a relatively weak promoter, derived from interleukin-2. Secondly, the genome of rAAV2RhEPO is ~2.3 kb in size, while that of AAV-TF-RhEpo-2.3w is ~4.7 kb. The latter is roughly comparable to the genome size in wild type AAV2, albeit tightly packed with exogenous components. A similar AAV2 vector (rAAV-TF2.3-hEPO, expressing human Epo; Wang *et al*, 2006) worked well after delivery to murine submandibular glands. The dose used in that study was $\sim 3.3 \times 10^{11}$ vg kg^{-1}, i.e., a dose roughly comparable to the high dose used here ($\sim 2.5 \times 10^{11}$ vg kg^{-1}). Thus, it does not seem that a > 4 kb, rapamycin-regulated AAV2 vector cannot effectively transduce salivary glands *per se*. Additionally, an AAV2 vector that was virtually identical to the AAV-TF-RhEpo-2.3w vector used here led to high serum RhEpo levels following transduction of non-human primate liver at doses similar to those used herein (Rivera *et al*, 2004). While it is possible that the use of a higher vector dose would lead to clinically adequate serum RhEpo levels from transduced healthy macaque parotid glands, we conclude that the macaque parotid is apparently not as good a target for the AAV-TF-RhEpo-2.3w vector as the liver.

This study, however, demonstrates that an AAV2 vector, delivered directly to a parotid gland at a significantly higher dose ($\sim 1.5 \times 10^{12}$ vg) than used in previous salivary gland studies, does not by itself result in any untoward effects. The five macaques that completed the study had essentially normal CBC and serum chemistry values over the course of the study, with no consistent abnormal values observed. In addition, none of these animals exhibited any purulence in their saliva. All displayed normal patterns of food consumption and were able to gain weight. Extrapolating the highest dose used herein ($\sim 2.5 \times 10^{11}$ vg kg^{-1}) to dosing in humans, our results suggest that administration of an AAV2 vector at a dose of up to $\sim 1.5 \times 10^{13}$ vg would be well tolerated in a single parotid gland.

Despite the general safety of the AAV-TF-RhEpo-2.3w vector, during this study, there was a severe adverse event in one macaque: animal #6851 developed pneumonia in his left lung that eventually required euthanasia. Although extensive postmortem evaluations were performed, the etiology of the pneumonia could not be determined. There was no evidence of aspiration pneumonia grossly, or in the lung tissue sections examined histologically, but the possibility of aspiration inciting the abscess, for example, as a result of collecting whole saliva on anesthetized animals, cannot be ruled out. This was the first adverse event that we have seen following the use of a viral vector (either Ad5 or AAV2) in macaque parotid glands (Voutetakis *et al*, 2007a, 2008, 2009). In our previously reported studies, we employed a total of 20 macaques. Thus, when including the six animals studied herein, only #6851 of 26 total macaques treated experienced a study-related adverse event of any type. Animal #6851 may just have responded idiosyncratically. In this regard, it is interesting to note that in a previously reported study using quite high levels of AAV vectors encoding RhEpo administered intramuscularly to eight cynomologous macaques, two developed a severe autoimmune anemia, while the other six were polycythemic (Gao *et al*, 2004). It is also important to recognize that all macaques had been administered rapamycin twice prior to day 37 when #6851 first showed signs of the adverse event [i.e., on days 14 (albeit in an inappropriate diluent) and 28]. It is therefore possible that the pneumonia that developed in this animal was in part related to transient immunosuppressive effects of rapamycin.

Additionally, the QPCR biodistribution study performed with selected tissues from #6851 represents the first time that we have found a significant level of a parotid gland-administered AAV2 vector outside the targeted gland in a large animal model (macaques and miniature pigs; Voutetakis *et al*, 2007a; Hai *et al*, 2009). At necropsy, two tissues had high and roughly similar levels of vector copies present: the targeted right parotid gland and the liver. A general confounder to understand these results is that the necropsy was conducted under standard, not GLP (good laboratory practice) conditions, i.e., separate instruments were not used for each tissue and the obtained tissues were not removed in sequence, from least likely to have vector present to most likely presence (i.e., targeted parotid gland; see Voutetakis *et al*, 2007a,b). Accordingly, the possibility of some inadvertent tissue contamination cannot be unequivocally excluded.

However, finding a fairly high vector level in the liver strongly suggests that AAV-TF-RhEpo-2.3w had entered the bloodstream in this animal, as liver is the primary target for an AAV2 vector delivered intravascularly (e.g., Xiao *et al*, 1998; Nakai *et al*, 2000). The most likely reason for that occurrence would be by trauma while delivering the vector, for example, damaging the cannulated duct. As the targeted right parotid gland itself appeared normal on histopathologic and gross examination, it seems there was no significant inflammatory reaction in the gland from which vector could have been lost and entered the bloodstream.

In summary, despite the occurrence of a severe adverse event in one animal, this study has shown that administration of high doses of AAV-TF-RhEpo-2.3w to single parotid glands of healthy macaques was generally safe. This vector, however, was unable to direct the expression of clinically significant levels of serum RhEpo, in a rapamycin-dependent manner, except in the one sick animal.

Acknowledgements

This research was supported by the Intramural Research Programs of the National Institute of Dental and Craniofacial Research and National Heart, Lung and Blood Institute.

Author contributions

C Zheng, A Voutetakis, V Rivera, T Clackson, R Donahue, C Dunbar and B Baum were involved in study design. C Zheng, A Voutetakis, M Metzger, S Wainer, A Cotrim, and M Eckhaus performed experiments and analyses. C Zheng, A Voutetakis, M Eckhaus, V Rivera, T Clackson, J Chiorini, R Donahue, C Dunbar and B Baum were involved in data interpretation. All authors were involved in the writing and revision of the manuscript.

References

Adriaansen J, Perez P, Goldsmith C *et al* (2008). Differential sorting of human parathyroid hormone after transduction of mouse and rat salivary glands. *Hum Gene Ther* **19:** 1021–1028.

Baum BJ, Berkman ME, Marmary Y *et al* (1999). Polarized secretion of transgene products from salivary glands *in vivo*. *Hum Gene Ther* **10:** 2789–2797.

Baum BJ, Wellner RB, Zheng C (2002). Gene transfer to salivary glands. *Int Rev Cytol* **213:** 93–146.

Baum BJ, Voutetakis A, Wang J (2004). Salivary glands: novel target sites for gene therapeutics. *Trends Mol Med* **10:** 585–590.

Braddon VR, Chiorini JA, Wang S *et al* (1998). Adenoassociated virus-mediated transfer of a functional water channel into salivary epithelial cells *in vitro* and *in vivo*. *Hum Gene Ther* **9:** 2777–2785.

Crystal RG (1995). The gene as a drug. *Nat Med* **1:** 15–17.

Felgner PL, Rhodes G (1991). Gene therapeutics. *Nature* **349:** 351–352.

Gao G, Lebherz C, Weiner DJ *et al* (2004). Erythropoietin gene therapy leads to autoimmune anemia in macaques. *Blood* **103:** 3300–3302.

Hai B, Yan X, Voutetakis A *et al* (2009). Long-term transduction of miniature pig parotid glands using serotype 2 adeno-associated viral vectors. *J Gene Med* **11:** 506–514.

He X, Goldsmith CM, Marmary Y *et al* (1998). Systemic action of human growth hormone following adenovirus-mediated gene transfer to rat submandibular gland. *Gene Ther* **5:** 537–541.

Isenman L, Liebeow C, Rothman S (1999). The endocrine secretion of mammalian digestive enzymes by exocrine glands. *Am J Physiol* **276:** E223–E232.

Kagami H, Wang S, Hai B (2008). Restoring the function of salivary glands. *Oral Dis* **14:** 15–24.

Nakai H, Storm TA, Kay MA (2000). Recruitment of single-stranded recombinant adeno-associated virus vector genomes and intermolecular recombination are responsible for stable transduction of liver *in vivo*. *J Virol* **74:** 9451–9463.

O'Connell AC, Baccaglini L, Fox PC *et al* (1999). Safety and efficacy of adenovirus-mediated transfer of the human aquaporin-1 cDNA to irradiated parotid glands of non-human primates. *Cancer Gene Ther* **6:** 505–513.

Pollock R, Clackson T (2002). Dimerizer-regulated gene expression. *Curr Opin Biotechnol* **13:** 459–467.

Rivera VM, Clackson T, Natesan S *et al* (1996). A humanized system for pharmacological control of gene expression. *Nat Med* **2:** 1028–1032.

Rivera VM, Berk L, Wardwell S *et al* (2004). Long-term regulated gene expression in non-human primates using a single non-recombinogenic AAV vector. *Mol Ther* **9:** S402.

Rivera VM, Gao GP, Grant RL *et al* (2005). Long-term pharmacologically regulated expression of erythropoietin in primates following AAV-mediated gene transfer. *Blood* **105:** 1424–1430.

Sugito T, Mineshiba F, Zheng C *et al* (2009). Transient TWEAK overexpression leads to a general salivary epithelial cell proliferation. *Oral Dis* **15:** 76–81.

Urabe M, Ding C, Kotin RM (2002). Insect cells as a factory to produce adeno-associated virus type 2 vectors. *Hum Gene Ther* **13:** 1935–1943.

Voutetakis A, Kok MR, Zheng C *et al* (2004). Reengineered salivary glands are stable endogenous bioreactors for systemic gene therapeutics. *Proc Natl Acad Sci USA* **101:** 3053–3058.

Voutetakis A, Zheng C, Mineshiba F *et al* (2007a). Adeno-associated virus serotype 2-mediated gene transfer to the parotid glands of nonhuman primates. *Hum Gene Ther* **18:** 142–150.

Voutetakis A, Zheng C, Wang J *et al* (2007b). Gender differences in serotype 2 adeno-associated virus biodistribution after administration to rodent salivary glands. *Hum Gene Ther* **18:** 1109–1118.

Voutetakis A, Zheng C, Metzger M *et al* (2008). Sorting of transgenic secretory proteins in rhesus macaque parotid glands following adenoviral mediated gene transfer. *Hum Gene Ther* **19:** 1401–1405.

Voutetakis A, Zheng C, Cotrim AP *et al* (2009). AAV5-mediated gene transfer to the parotid glands of non-human primates. *Gene Ther*. doi:10.1038/gt.2009.123

Wang J, Voutetakis A, Zheng C, Baum BJ (2004). Rapamycin control of exocrine protein levels in saliva after adenoviral vector-mediated gene transfer. *Gene Ther* **11:** 729–733.

Wang J, Cawley NX, Voutetakis A *et al* (2005). Partial redirection of transgenic growth hormone secretion from salivary glands. *Hum Gene Ther* **16:** 571–583.

Wang JW, Voutetakis A, Papa M *et al* (2006). Rapamycin control of transgene expression from a single AAV vector in mouse salivary glands. *Gene Ther* **13:** 187–190.

Xiao W, Berta SC, Lu MM *et al* (1998). Adeno-associated virus as a vector for liver-directed gene therapy. *J Virol* **72:** 10222–10226.

Yamano S, Huang LY, Ding C *et al* (2002). Recombinant adeno-associated virus serotype 2 vectors mediate stable interleukin 10 secretion from salivary glands into the bloodstream. *Hum Gene Ther* **13:** 287–298.

Zheng C, Baum BJ (2005). Evaluation of viral and mammalian promoters for use in gene delivery to salivary glands. *Mol Ther* **12:** 528–536.

Zheng C, Voutetakis A, Kok MR *et al* (2006). Toxicity and biodistribution of a first-generation recombinant adenoviral vector, in the presence of hydroxychloroquine, following retroductal delivery to a single rat submandibular gland. *Oral Dis* **12:** 137–144.