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Assessment of the Safety and Biodistribution of a Regulated AAV2 Gene Transfer Vector after Delivery to Murine Submandibular Glands

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Clinical gene transfer holds promise for the treatment of many inherited and acquired disorders. A key consideration for all clinical gene transfer applications is the tight control of transgene expression. We have examined the safety and biodistribution of a serotype 2, recombinant adeno-associated viral (AAV2) vector that encodes a rapamycin-responsive chimeric transcription factor, which regulates the expression of a therapeutic transgene (human erythropoietin [hEpo]). The vector, AAV2-TF2.3w-hEpo (2.5×10^7–2.5×10^{10} particles), was administered once to a single submandibular gland of male and female mice and mediated hEpo expression *in vivo* following a rapamycin injection but not in its absence. Control (saline treated) and vector-treated animals maintained their weight, and consumed food and water, similarly. Vector delivery led to no significant toxicological effects as judged by hematology, clinical chemistry, and gross and microscopic pathology evaluations. On day 3 after vector delivery, vector copies were not only abundant in the targeted right submandibular gland but also detected in multiple other tissues. Vector was cleared from the targeted gland much more rapidly in female mice than in male mice. Overall, our results are consistent with the notion that administration of the AAV2-TF2.3w-hEpo vector to salivary glands posed no significant risk in mice.

Key Words: **AAV2 vector; safety; salivary gland; rapamycin; regulated gene expression.**

The management of many systemic single-protein deficiency disorders (SSPDDs) currently is achieved by the regular, repeated injection of recombinant proteins (e.g., insulin for diabetes). However, gene transfer offers the potential for correcting such a deficiency through vector-mediated stable expression of a transgene encoding the required protein (Felgner and Rhodes, 1991). Previous studies in several tissues (e.g., Auricchio *et al.*, 2002; Mount *et al.*, 2002; Vincent-Lacaze *et al.*, 1999) have suggested the utility of recombinant adeno-associated viral (AAV) vectors for this purpose and our own studies in salivary glands with serotype 2 adeno-associated viral (AAV2) vectors support this notion (e.g., see Baum *et al.*, 2004 for review; Voutetakis *et al.*, 2004, 2007a,b).

Human salivary glands consist of extremely active secretory cells able to secrete considerable amounts of protein into both the gastrointestinal tract and bloodstream (Isenman *et al.*, 1999). Similarly, rodent, miniature pig, and rhesus macaque salivary glands are able to secrete transgene products in both exocrine and endocrine directions (e.g., Baum *et al.*, 1999; Hai *et al.*, 2009; Kagami *et al.*, 1996; Voutetakis *et al.*, 2008). Gene transfer to salivary glands can be accomplished in a relatively noninvasive manner by intraoral cannulation of the main excretory ducts (Baum *et al.*, 2002; Mastrangeli *et al.*, 1994), as is done clinically for obtaining contrast radiographs. Thus, salivary glands seem potentially useful as a gene transfer target to correct certain SSPDDs (Voutetakis *et al.*, 2005, 2008; Zufferey and Aebischer, 2004).

A key issue for all gene transfer applications, regardless of the tissue target site, is adequate control of transgene expression. All currently approved gene transfer vectors in clinical trials provide no control of transgene expression, that is, they express continuously, for example, see either (http://www.gemcris.od.nih.gov/Contents/GC_HOME.asp) or (http://www.wiley.com/legacy/wileychi/genmed/clinical/). High and uncontrolled levels of transgene expression are undesirable for many applications to SSPDDs and may be quite harmful. Thus, an important research goal is to control transgene expression such that it can be induced as needed and to the extent required.

Several systems are currently available for transgene regulation, and these systems typically utilize a small drug molecule to activate a chimeric transcription factor. Of particular interest to us has been the rapamycin-inducible

system (Rivera *et al*., 1996). In this system, the DNA-binding and activation domains of the transcription factor have each been fused to a rapamycin-binding domain and are expressed from separate sequences encoded in the vector. Following transduction, the two components of the transcription factor are constitutively expressed in the transduced cells as separate polypeptides. Rapamycin (or a non-immunomodulatory analogue) is used to dimerize the activation and DNA-binding domains, via their rapamycin-binding domains, thus forming the chimeric transcription factor. The activated transcription factor then can bind to zinc finger motifs upstream of a minimal promoter (human interleukin-2) and drive expression of the encoded transgene. The rapamycin regulatory system has two major advantages in that (1) only human components are used and (2) there is little to no expression of the transgene in the absence of rapamycin. This regulatory system has been extensively tested for efficacy (e.g., Rivera *et al*., 1999, 2005).

Previously, we showed that the rapamycin-regulated system is useful to control both exocrine and endocrine secretory protein expression after gene transfer to rodent salivary glands (Wang *et al*., 2004, 2006). In the latter study, a single AAV2 vector encoding human erythropoietin (hEpo) was utilized. Delivery of that vector to mouse submandibular glands resulted in (1) no hEpo expression in the absence of rapamycin, (2) rapamycin dose-dependent hEpo expression, and (3) comparable levels of induced hEpo expression, after rapamycin administration, over the 6-month period of that study (see Fig. 2b in Wang *et al*., 2006). These results suggested that this vector might be valuable for use in treating human SSPDDs.

However, because one portion of the chimeric transcription factor used (the DNA-binding domain) contained three identical 327-bp repeats encoding the rapamycin-binding domains, there was considerable potential for homologous recombination (Rivera *et al*., 2004) and subsequent production of vectors could exhibit multiple drug-binding domains. To eliminate this possibility, and permit generation of a single population of AAV2 vector with intact rapamycin-regulated elements present, Rivera *et al*. (2004) made a series of conservative nucleotide mutations in the rapamycin-binding domains that rendered them nonrecombinogenic and suitable for production with clinical applications. In the present study, we produced a nonrecombinogenic AAV2 vector, AAV2-TF2.3w-hEpo, encoding the hEpo transgene and evaluated its safety and biodistribution after delivery, via duct cannulation and retrograde infusion, to one submandibular gland of male and female mice.

MATERIALS AND METHODS

This study was designed and conducted to conform to the United States Food and Drug Administration Good Laboratory Practice (GLP) regulations. The National Institute of Environmental Health Sciences Animal Care and Use Committee and the National Institutes of Health Biosafety Committee approved the animal experiments.

Animals. Balb/c mice (125 male and 125 female) were obtained from Taconic Farms (Germantown, NY) at 8 weeks of age. Animals were acclimated for 2–3 weeks prior to the study onset. Animals were randomized on the basis of stratified body weights, allocated to seven study groups (Table 1) and uniquely marked with ear tags 3 days prior to vector administration. Animals were housed individually in dose-specific polycarbonate shoebox cages (Lab Products, Inc; Seaford, DE) in temperature and humidity-controlled rooms. A 12-h light-dark cycle was maintained and animals were given food (irradiated NTP-2000 Open Formula Diet; Zeigler Brothers, Inc, Gardners, PA) and water *ad libitum*. All mice were weighed at receipt and on multiple days following vector or saline administration. Food and water consumption was measured weekly. As previously described (e.g., Voutetakis *et al*., 2007a), each animal was observed twice daily for mortality and morbidity throughout the study.

General in-life study design. Animals in groups 1–5 formed the Core Study cohorts, whereas animals in groups 6 and 7 were only evaluated for serum chemistry determinations on days 3 and 29. Animals in groups 1 and 6 served as controls and were treated with the saline vehicle only. Groups 2–5 were administered increasing doses (2.5×10^7–2.5×10^{10} particles) of the AAV2-TF2.3w-hEPO vector, suspended in saline vehicle, to the right submandibular gland, as described below. Group 7 animals also received the highest vector dose administered. The doses of vector tested herein were chosen because, on a per kilogram basis, they are likely to include a range from those useful clinically to those in excess of clinical requirements. This assessment primarily was based on results from a recent large animal study conducted in miniature pigs (25–30 kg animals; see Hai *et al*., 2009). In that study, animals administered 10^{11} particles of an unregulated AAV2 vector with the same hEpo transgene as employed herein, to a single parotid gland, displayed peak serum hEpo levels of ~6 mU/ml, with resultant elevations in hematocrit levels of > 10% of those pre-vector delivery (see Fig. 2 in Hai *et al*., 2009). On a per kilogram basis, that dose is similar to the lowest dose used in the present study. Additionally, in a previous study (Voutetakis *et al*., 2004), with Balb/c mice and the same AAV2hEpo vector as employed in the above miniature pig studies, following administration of 10^9 vector particles, serum hEpo levels peaked between weeks 10 and 12 at ~20 mU/ml and remained relatively constant for 1 year. This is the characteristic transgene expression pattern from AAV2 vectors in salivary glands in this species (see also Wang *et al*., 2006). Hematocrit levels exhibited a similar elevation and stability, increasing by >30% from a basal level of 60 (see Fig. 2 in Voutetakis *et al*., 2004). Typically, five to six mice of each gender and each group were then sacrificed at the 3-, 29-, 57-, and 92-day time points. For each gender, start days were staggered by 1 week. The Core Study mice (groups 1–5) were used for the collection of in-life data, gross and microscopic pathology, tissue-specific determination of AAV2-TF2.3w-hEPO biodistribution by quantitative PCR (QPCR, see below), collection of saliva and blood (on days 3, 29, 57, and 92; blood collections from the retro-orbital sinus) for hematology assays (see below), and the evaluation of

TABLE 1
Study Groups

Study designation	Group number	AAV2-TF2.3w-hEPO treatment (particles)	Number of animals	
			Male	Female
Core	1	0 (saline)	21	21
Core	2	2.5×10^7	21	21
Core	3	2.5×10^8	21	21
Core	4	2.5×10^9	21	21
Core	5	2.5×10^{10}	21	21
Special	6	0 (saline)	10	10
Special	7	2.5×10^{10}	10	10
Total			125	125

serum neutralizing antibodies against the AAV2 vector. To collect saliva, the animals were administered pilocarpine (Sigma-Aldrich, St Louis, MO), freshly prepared in sterile water (1 mg/kg/0.2ml), subcutaneously at the nape of the neck. Thereafter, whole saliva was collected from the oral cavity for a 20-min period, placed into chilled, preweighed 1.2-ml microcentrifuge tubes, and the saliva stored at ≤ -60°C until analyzed (Voutetakis *et al*., 2007a).

Clinical laboratory studies. As noted above, animals from the Core Study groups were sacrificed on days 3, 29, 57, and 92, and blood was collected for hematology. The hematology assays performed included red blood cell count, hematocrit, hemoglobin, white blood cell (WBC) count, WBC differential count, reticulocyte counts, and platelet counts. As previously described (e.g., Voutetakis *et al*., 2007a), all Core Study animals also were necropsied (see below), with multiple tissues collected, trimmed, and fixed in formalin. Thereafter, formalin-fixed tissues were embedded, sectioned, and stained with hematoxylin and eosin and examined microscopically. Final diagnoses for reviewed lesions represent a consensus between the laboratory study pathologist, a quality assurance pathologist, and a Pathology Working Group. Details of these review procedures have been described, in part, in Boorman and Eustis (1986) and Hardisty and Boorman (1986). Animals in groups 6 and 7 were sacrificed on days 3 and 29 only, and blood was collected for clinical chemistry analyses. Specifically, the following parameters were measured: albumin, alanine aminotransferase, alkaline phosphatase, cholesterol, creatine kinase, creatinine, glucose, lactate dehydrogenase, serum amylase, sorbitol dehydrogenase, total protein, triglycerides, blood urea nitrogen, total bile acids, sodium, and calcium.

Vector biodistribution. At scheduled sacrifice, all Core Study mice had an extensive and specialized QPCR necropsy to determine the distribution of the AAV2-TF2.3w-hEpo vector, followed by a conventional necropsy (controls first, followed by animals of increasingly higher dosage groups; e.g., see Voutetakis *et al*., 2007a). Instruments used in the QPCR dissection were changed between each organ and each animal to avoid contamination. In addition to blood and saliva, the tissue samples collected for QPCR analyses included brain, duodenum, esophagus, heart, kidney, lung, liver, spleen, stomach, testes or ovary, left draining lymph node, left submandibular salivary gland, right draining lymph node, and right submandibular salivary gland (i.e., the targeted tissue). A consistent portion and location of each solid tissue was obtained, snap frozen in liquid nitrogen, and stored below −60°C. DNA from the blood and saliva samples was isolated using the Qiagen (Valencia, CA) QIAamp 96 Spin Blood Kit, according to the manufacturer's instructions. Tissue samples (~30 mg wet weight) were lysed in Proteinase K/Buffer G2 working solution and DNA from the various tissues isolated using the automated M48 MagAttract Mini Kit (Qiagen), according to the manufacturer's instructions. DNA was quantified by absorbance at 260 nm. QPCR analysis was performed with JumpStart Taq polymerase (1.25 U/50 μl; Sigma-Aldrich) on the ABI PRISM-7900HT (Applied Biosystems, Foster City, CA) using a passive reference dye (SuperROX; Biosearch Technologies, Novato, CA) and these hEpo sequence-specific primers: forward (hEPO2-632; GCTGAAGCTGTACACAGG; 400nM) and reverse (hEPO2-706; AGCTCTTTATTAGGCGTAATCG; 400nM) primers and a fluorescent probe (hEPO2-675; FAM-CTCTGTCCCCTGTCC-BHQ1; 100nM). The assay could detect 10 vector copies in 1 μg of genomic DNA. The following controls were also included: no DNA, non-treated mouse genomic DNA, spiked control with 100 copies of hEPO DNA to verify the absence of QPCR inhibitors, and a standard curve with dilutions of AAV2-TF2.3w-hEpo from 10^1 to 10^7 copies. QPCR assays were deemed valid if the no DNA template control produced a $C_T \geq 40$ or undetermined and if at least five points of the standard curve produced a $C_T < 40$.

Recombinant vector construction and characterization. Construction of the AAV2-TF2.3w-hEpo vector was performed in a manner similar to that previously described (Rivera *et al*, 2004; Wang *et al*., 2006; Voutetakis *et al*., 2007a,b). Briefly, AAV2-TF2.3w-hEpo was generated by cotransfection of pAAV-TF2.3w-hEpo, which is identical to pAAV-TF2.3-hEpo (Wang *et al*., 2006), except for the presence of the three nonrecombinogenic FKBP domain), with pDG (Grimm *et al*., 1998) into 293T cells. pAAV-TF2.3w-hEpo was constructed by digesting pAAV-TF.rhEpo2.3w with *Nhe*I, filled in with T4 DNA polymerase, then digested with *Asc*I to remove the rhesus Epo cDNA present (pAAV-TF__2.3w). pZ8-I-hEpo-R2 was digested with *Mlu*I, filled in with T4 DNA polymerase, then digested with *Asc*I to obtain the hEpo cDNA. The hEpo cDNA was then ligated into pAAV-TF__2.3w to yield pAAV-TF2.3w-hEpo. The identity of this plasmid was verified using restriction endonuclease digestions and conventional DNA sequencing. The generated AAV2-TF2.3w-hEpo vector (size between the inverted terminal repeats [ITRs] was 4942 bp) was purified by CsCl-gradient centrifugation, as previously reported (Wang *et al*., 2006). Rapamycin-induced transgene expression, for both the plasmid and AAV2 vector, was confirmed *in vitro* in 293T cells. Rapamycin (1μM) was added, or not, to the medium, and cells were incubated overnight to determine the production of hEpo. Assays of media were performed in duplicate, according to the manufacturer's instructions, with an ELISA kit specific for hEpo (Stem Cell Technologies, Vancouver, BC). The lower limit of the standard curve was 1.6 mU/ml.

Determination of viral titer. After pooling appropriate CsCl-gradient fractions, vector titer was determined by QPCR. The sequences used for the forward primer, reverse primer, and probe were selected using Primer Express Primer Design software (Applied Biosystems) based on the hEpo sequence. The internal fluorogenic probe was labeled with the 6-FAM reporter dye (Applied Biosystems). The sequences used were as follows: EPO Taq primer 1—5′-GCAGCTGCATGTGGATAAAGC-3′; EPO Taq primer 2—5′-CCAGAGCCCGAAGCAGAG-3′; EPO probe 1—5′-/56-FAM/CAGTGGCCTTCGCAGCCTCACC/36-TAMSp/-3′. Each QPCR reaction contained 2× TaqMan Universal PCR Master Mix (Applied Biosystems), 15 pmol of each of the PCR primers, and 10 pmol TaqMan probe in a total volume of ~25 μl, and amplifications were performed in duplicate. The reaction mixture was incubated at 50°C for 2 min (stage 1), 95°C for 10 min (stage 2), then denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min, repeated 40 times (stage 3). A standard curve, employing the pAAV-TF2.3w-hEpo plasmid was included for each QPCR reaction. The final titer of the AAV2-TF2.3w-hEpo vector used for the studies described herein was 2.8×10^{11} particles/ml.

Handling and administration of AAV2-TF2.3w-hEpo. AAV2-TF2.3w-hEpo was stored in 100-μl aliquots at 4°C until experiments began (Baum *et al*., 2010a). On each experimental day, that is, when vector administration to murine submandibular glands was to be performed, immediately before experiments, an appropriate amount of the AAV2-TF2.3w-hEpo vector was removed and dialyzed against 0.9% NaCl. Thereafter, the dialyzed vector was diluted to yield the concentrations required for each dosage group (Table 1; 2.5×10^7, 2.5×10^8, 2.5×10^9, 2.5×10^{10} particles; each in a volume of 50 μl). The dialysis and dilutions were performed at 4°C. Mice were administered AAV2-TF2.3w-hEpo at the above doses, or 0.9% NaCl as a control, by retrograde delivery to Wharton's duct of the right submandibular gland (Voutetakis *et al*., 2004; Wang *et al*., 2006). The animals were anesthetized with a cocktail of ketamine 43 mg/kg, xylazine 8.5 mg/kg (intramuscular injection), and acepromazine 1.4 mg/kg (ip injection); all obtained from Fisher Scientific (Pittsburgh, PA). Ducts were cannulated with modified polyethylene tubing (Intramedic PE-10, BD Diagnostic Systems, Sparks, MD), atropine (im injection, 0.5 mg/kg body weight; Alfa Aesar, Ward Hill, MA) was next administered to decrease salivary flow, and thereafter vector was administered, as previously described (Baum *et al*., 2002; Voutetakis *et al*., 2004).

Anti-AAV2 antibody testing. To determine if mice administered the AAV2-TF2.3w-hEpo vector developed anti-AAV2 neutralizing antibodies, sera from mice (10 males, 9 females) in the highest dosage group were assayed using methods similar to those previously described (Sprangers *et al*., 2003; Zheng *et al*., 2008). Briefly, 293 cells were grown in 96-well plates and thereafter transduced with an AAV2 vector encoding luciferase, which had been preincubated with mouse serum (at various dilutions) in a total volume 50 μl at 37°C for 30 min. After 48 h, the cells were lysed in cell lysis buffer (Promega, Madison, WI) for 15 min. Fifty microliters of the cell lysate were

added to 100 μl of luciferase substrate, and light output was measured with a luminometer. Results were expressed as relative light units (RLUs) per cell number. Titers are reported as that dilution of serum that resulted in 50% inhibition in measuring luciferase activity.

Evaluating AAV2-TF2.3w-hEpo expression in vivo after rapamycin treatment. For this experiment, a separate cohort of Balb/c mice was used (10 of each gender), that is, animals in the toxicology and biodistribution study were not administered rapamycin. The AAV2-TF2.3w-hEpo vector used came from a vial given to mice in the Core Study and was administered to a single submandibular gland only at the highest dose employed. The rapamycin dose used was 3 mg/kg, administered ip 16 h prior to sampling (Wang *et al*., 2006) to five male and five female mice, which originally were randomly chosen from this cohort. Rapamycin was administered prior to all three blood samplings performed: week −1 (i.e., before vector delivery) and at +4 and +14 weeks after vector delivery. At each of these time points, rapamycin was injected to the same five male and five female mice (experimental group). The remaining five male and five female mice served as controls for unregulated transgene expression, that is, the AAV2-TF2.3w-hEpo vector was delivered, but no rapamycin was administered. Assays for hEpo in mouse serum were performed in duplicate, as described above.

Statistical analyses. Statistical analyses were performed using ANOVAs and Dunnett's test as appropriate.

RESULTS

AAV2-TF2.3w-hEpo Characterization and Rapamycin Responsiveness

The titer of the AAV2-TF2.3w-hEpo vector generated was 2.8×10^{11} particles/ml. This is a lower titer compared with typical preparations from our laboratory (~1×10^{12}), likely due to the large size of the vector genome, that is, 4942 bp between the ITRs (wild-type AAV2 has a genome of ~4700 bases), which is close to the packaging limit for AAV vectors (e.g., Dong *et al*., 1996; Wu *et al*., 2010). AAV2-TF2.3w-hEpo transduction ability initially was tested *in vitro* with 293T cells, and hEpo expression was detected in the culture medium after exposure to rapamycin (data not shown).

Rapamycin-regulated hEpo expression *in vivo* was next tested using a separate group of Balb/c mice that were administered the highest dose of vector. Ten mice (five/gender) were given ip injections of rapamycin at −1, +4, and +14 weeks relative to AAV2-TF2.3w-hEpo administration and then evaluated for the presence of hEpo in serum. No hEpo was seen after rapamycin administration at the first two time points, that is, prior to vector delivery and at 4 weeks after vector delivery. However, as shown in Table 2 for the 14-week time point, 16 h after receiving rapamycin, all five male mice expressed high levels of hEpo in their serum. Conversely, hEpo was undetectable in the serum of five male, and five female, mice not administered rapamycin, indicating that there was no significant unregulated transgene expression from the AAV2-TF2.3w-hEpo vector. Interestingly, somewhat different hEpo expression results were seen in the five female mice administered both vector and rapamycin (Table 2). Only three of these five female mice exhibited hEpo expression in their serum at week 14, and the levels detected were much lower (almost 10-fold) than those found in the serum from rapamycin-treated male mice.

TABLE 2
Serum hEpo Detection in the Presence and Absence of Rapamycin

Week	Rapamycin	Males	Females
14	Yes	133.6 ± 7	16.5 ± 9
	No	0	0

Note. Data represent the mean ± SEM of hEpo measured in serum from mice in each group ($n = 5$), as indicated, at week 14 after vector (2.5×10^{10} particles to the right submandibular gland) administration. Data are expressed as mU/ml. No hEpo was detected in any serum sample from weeks −1 and 4. The control mice were administered the AAV2-TF2.3w-hEpo vector but received no rapamycin. The absence of hEpo detection in all control serum samples indicates that no leakiness of expression occurred from the vector. As noted in the text, no hEpo was detected in serum at the 4-week time point, consistent with the established pattern of transgene expression from AAV2 vectors in murine salivary glands (Voutetakis *et al*., 2004; Wang *et al*., 2006). A mechanistic reason for this expression pattern is not yet understood.

Effect of AAV2-TF2.3w-hEpo on Animal Survival, Body Weight, Clinical Chemistry, and Hematology

All Core Study (Table 1; groups 1–5) male mice survived until either the scheduled or terminal sacrifice, except one high-dose male that was sacrificed in moribund condition on day 53. This appeared to be a random event as the mouse had no history of problems previously and the necropsy revealed no unexpected findings (data not shown). All Core Study females survived until scheduled or terminal sacrifice. On study day 3, one Special Study male and one Special Study female mouse, from group 6 and group 7, respectively, were found dead after blood collection. No animal death was associated with exposure to the vector.

Mean body weights of control and all Core Study groups of mice receiving the vector were comparable throughout the 92-day duration of the study (Table 3). Food and water consumption were also comparable among all groups (data not shown). Although there were several statistically significant changes in various hematology parameters, there was no indication of a dose-response or pattern that would indicate any association with vector administration and the changes were not considered to be biologically meaningful (data not shown). Similarly, clinical chemistry analyses showed no evidence of any systematic change resulting from AAV2-TF2.3w-hEpo vector administration (Tables 4 and 5).

Effect of AAV2-TF2.3w-hEpo on Gross and Microscopic Pathology of Animal Tissues

No consistent evidence of gross pathology was found in animals administered the AAV2-TF2.3w-hEpo vector. Microscopically, limited inflammation, characterized as subacute inflammation with a generally minimal infiltration of neutrophils

TABLE 3
Group Mean Body Weights

Particles (×2.5) administered	Males			Females		
	Initial	Final	% Control[b]	Initial	Final	% Control[b]
0	23.0 ± 0.6[a]	27.4 ± 0.5		18.8 ± 0.4	22.7 ± 0.6	
10^7	23.3 ± 0.6	29.3 ± 0.4	107	18.7 ± 0.4	24.1 ± 0.5	106.1
10^8	23.3 ± 0.5	29.3 ± 0.3	106.9	19.0 ± 0.34	22.7 ± 0.5	99.9
10^9	23.7 ± 0.4	28.7 ± 0.3	104.6	18.9 ± 0.52	21.8 ± 0.3	95.9
10^{10}	23.2 ± 0.3	28.6 ± 0.6	104.1	18.3 ± 0.2	22.1 ± 0.5	97.1

[a]Mean ± SD.
[b]% of final mean weight of control group.

and lymphocytes, was observed in the right submandibular glands (the cannulated gland) in male mice more frequently than in female mice on day 3 (Table 6). The incidence was sporadic, not related to dose of vector administered, was generally reversible at later time points, and was thus attributed to the cannulation procedure and infusion of the vector solution or vehicle. All other microscopic lesions, for both male and female mice, were considered to be spontaneous and incidental findings and were not associated with exposure to the vector.

Biodistribution of AAV2-TF2.3w-hEpo after Submandibular Gland Delivery

Results of the biodistribution study (Table 7) indicated systemic distribution of vector genomes in male mice 3 days after administration, particularly at the highest two doses (only the highest dose is shown). However, by 29 days following administration, no vector genomes were detected in blood, and by 92 days after administration, the salivary glands and associated lymph nodes, and the liver, were the major tissue sites positive for the presence of vector (Table 7). Generally, a similar pattern of results was observed for females at this time, as shown in Table 7. However, higher levels of vector tended to persist longer in key male tissues, as is clear from the data shown in Table 7. For example, on day 3, the median number of vector copies in multiple tissues was fairly similar in both male and female mice administered the highest vector dose. By day 92, male mice exhibited much higher (~10-fold) median vector copy levels in the targeted right submandibular gland and the right draining lymph node than female mice.

TABLE 4
Serum Chemistry Values on Days 3 and 29 from Saline-Treated Mice[a]

	Day 3		Day 29	
	Male	Female	Male	Female
Na^+	159 ± 3	158 ± 4	152 ± 0.9	154 ± 1
Ca^{2+}	10.5 ± 0.5	10.4 ± 0.4	10.3 ± 0.3	10.4 ± 0.6
Glucose	117 ± 14	125 ± 14	133 ± 13	133 ± 22
Blood urea nitrogen	25 ± 4.2	24.3 ± 3.9	11.4 ± 4	20.6 ± 6.3
Creatinine	0.52 ± 0.18	0.64 ± 0.1	0.53 ± 0.1	0.69±0.08
Creatine kinase	445 ± 242	267 ± 132	212 ± 145	284 ± 142
Lactate dehydrogenase	828 ± 186	617 ± 260	489 ± 107	644 ± 282
Alanine aminotransferase	208 ± 161	94 ± 47	55 ± 7	51 ± 24
Alkaline phosphatase	83 ± 17	88 ± 17	107 ± 8	118 ± 26
Sorbitol dehydrogenase	50 ± 9	61 ± 14	21 ± 2	42 ± 10
Amylase	2178 ± 329	2045 ± 955	1938 ± 209	1785 ± 459
Albumin	4.14 ± 0.32	4.2 ± 0.5	3.3 ± 0.2	3.7 ± 0.3
Total protein	6.9 ± 0.6	6.8 ± 0.6	5.9 ± 0.4	5.5 ± 0.5
Total cholesterol	209 ± 24	129 ± 29	164 ± 10	94 ± 20
Triglycerides	208 ± 76	144 ± 79	241 ± 47	158 ± 73
Bile acids	77 ± 13	151 ± 7	77 ± 17	56 ± 16

Note. Na^+ and Ca^{2+} (mequiv/l), all enzymes (U/l), Albumin and total protein (g/dl), bile acids (μmole/l), and all other constituents (mg/dl).
[a]These data (mean values ± SD) were from control animals treated with saline (Group 6 in Table 1).

Detection of Anti-AAV2 Neutralizing Antibodies

Serum samples obtained from animals in the highest dosage group were tested for the presence of neutralizing antibodies to AAV2. As shown in Table 8, by day 29, all mice tested, both male and female, had significant levels of anti-AAV2 antibodies present in their serum, with values in females being slightly higher than those in males. At this time point, all high-dose animals still had significant, albeit different, levels of the AAV2-TF2.3w-hEpo vector detectable in their targeted gland; for males, the median value (in vector genomes/μg DNA) was 1.8×10^4 and for females it was 1.5×10^3.

DISCUSSION

The purpose of the present study was to evaluate the safety and biodistribution of a recombinant AAV2 vector, AAV2-TF2.3w-hEpo, after delivery to a single murine submandibular gland. This vector encodes a rapamycin-responsive chimeric transcription factor that regulates the expression of the hEpo transgene. As noted earlier, salivary glands offer a particularly attractive site for clinical gene therapeutics, the use of genes as drugs (Felgner and Rhodes, 1991). Being readily accessible in humans and experimental animals allows direct delivery of the

TABLE 5
Serum Chemistry Values on Days 3 and 29 from Mice Treated with the AAV2-TF2.3w-hEpo Vector[a]

	Day 3		Day 29	
	Male	Female	Male	Female
Na^+	158 ± 3	159 ± 2	153 ± 0.7*↑	155 ± 2
Ca^{2+}	10.6 ± 0.4	10.4 ± 0.5	10.5 ± 0.3	10.5 ± 0.4
Glucose	130 ± 20	135 ± 15	136 ± 23	136 ± 21
Blood urea nitrogen	23.5 ± 3.4	20.9 ± 4.9	13.4 ± 2.9	21.8 ± 7.6
Creatinine	0.52 ± 0.28	0.64 ± 0.11	0.48 ± 0.1	0.69 ± 0.14
Creatine kinase	312 ± 196	283 ± 195	248 ± 142	452 ± 345
Lactate dehydrogenase	618 ± 116*↓	501 ± 133	471 ± 248	580 ± 101
Alanine aminotransferase	120 ± 38	124 ± 58	62 ± 29	56 ± 22
Alkaline phosphatase	77 ± 17	103 ± 16	117 ± 18	134 ± 18
Sorbitol dehydrogenase	38 ± 4*↓	53 ± 10	23 ± 5	43 ± 9
Amylase	1923 ± 211	1737 ± 432	1717 ± 166*↓	1715 ± 208
Albumin	3.82 ± 0.17*↓	4.1 ± 0.3	3.4 ± 0.2	3.9 ± 0.3
Total protein	6.6 ± 0.3	6.4 ± 0.6	5.9 ± 0.3	5.6 ± 0.3
Total cholesterol	196 ± 18	132 ± 18	163 ± 18	103 ± 12
Triglycerides	225 ± 67	159 ± 59	263 ± 116	144 ± 52
Bile acids	72 ± 12	159 ± 5	78 ± 25	82 ± 18*↑

Note. Any value shown to be statistically different ($p < 0.05$; using an ANOVA and Dunnett's test) from the corresponding control value is indicated by ''*'' with an arrow pointing up or down indicating the direction of the change. Na^+ and Ca^{2+} (mequiv/l), all enzymes (U/l), albumin and total protein (g/dl), bile acids (μmole/l), and all other constituents (mg/dl).

[a]These data (mean values ± SD) were from animals treated with the highest dose of vector (2.5×10^{10} particles/gland; Group 7 in Table 1).

gene transfer vector noninvasively, that is, not to a systemic site, via cannulation of the main excretory duct, a procedure that is performed without anesthesia in humans (Baum *et al*., 2010b; Zheng *et al*., 2010). Additionally, human salivary glands are encapsulated, limiting systemic dissemination of the vector (Zheng *et al*., 2010), and a single gland could be surgically removed without major systemic implications in the case of an unexpected serious adverse event (Baum *et al*., 2010b). Furthermore, following transduction, mammalian salivary glands actively secrete transgene-encoded therapeutic proteins into the bloodstream and/or upper gastrointestinal tract (Baum *et al*., 1999; Voutetakis *et al*., 2008).

The AAV2-TF2.3w-hEpo vector was engineered to allow controlled, rapamycin-regulated expression of hEpo from the target tissue following administration. As shown herein, the vector was fully functional and in fact provided tightly controlled expression of hEpo (Table 2). No hEpo was detected in the serum of any vector-treated animals not injected with rapamycin; however, animals that received vector 14 weeks previously exhibited significant levels of hEpo in their serum 16 h following an ip injection of rapamycin. Rapamycin injection, however, was unable to elicit hEpo production 4 weeks following vector delivery, most likely because the expression of transgenic proteins from AAV2 vectors delivered to murine submandibular glands is minimal at that time point and not optimal until 8–12 weeks following administration (Voutetakis *et al*., 2004; Wang *et al*., 2006). Interestingly, the level of hEpo expression seen after rapamycin

TABLE 6
Incidence of Microscopic Inflammation Observed in the Right Submandibular Glands of Mice on Day 3

AAV2-TF2.3w-hEpo treatment (×2.5)	Male	Female
0 (saline control)	1/5	0/5
10^7 particles	4/5	1/5
10^8 particles	1/5	1/5
10^9 particles	2/5	1/5
10^{10} particles	2/5	1/5

TABLE 7
Median Levels of AAV2-TF2.3w-hEpo in Selected Mouse Tissues on Days 3 and 92[a]

	Day 3		Day 92	
	Male	Female	Male	Female
Brain	133	<100	0	0
Gonads	148	403	0	0
Kidney	918	347	0	0
Liver	9994	1.15×10^4	303	<100
Lung	1876	1795	0	0
Lymph node (R)	8.9×10^4	2×10^5	4715	440
Salivary gland (R)	4.1×10^5	1.2×10^5	1.68×10^4	1342

[a]These data (median values; vector copies/μg DNA) were from animals treated with the highest dose of vector (2.5×10^{10} particles/gland).

TABLE 8
Results of Serum Anti-AAV2 antibody Assays

Gender	Days after administration	Median antibody titer
Male	3	0
	29	1:512–1:1024
Female	3	0
	29	1:1024–1:2048

Note. Serum samples from 10 male mice and 9 female mice were tested.

injection was markedly different in male and female mice, with female mice showing serum hEpo levels about one-tenth those of their male counterparts. This result, however, was consistent with our finding of markedly lower levels of vector copies in the targeted right submandibular gland of female mice versus those in male mice, at the same vector dose (see below; Table 7), as well as with a previous observation by us of similar gender differences in AAV2 vector biodistribution after submandibular gland administration in mice (Voutetakis *et al*., 2007a).

Most importantly, the present study shows that mice receiving up to 2.5×10^{10} AAV2-TF2.3w-hEpo vector particles in a single submandibular gland exhibited no indication of a toxic response for up to 92 days after exposure. A minimal degree of subacute inflammation was observed in the right salivary gland of mice on study day 3, but this was generally reversible and was attributed to the cannulation and infusion procedure associated with vector or vehicle administration. For both male and female mice, no other microscopic lesions associated with exposure to the vector were observed in any tissue examined at any of the interim time points or at study termination. Furthermore, all control and vector-treated mice showed similar patterns of body weight during the course of the study, as well as comparable food and water intake. Additionally, the results of the extensive hematology and clinical chemistry assays performed suggested no significant vector-related changes occurred.

All animals generated neutralizing antibodies to the AAV2 vector (Table 8), but the levels were relatively modest, similar to those reported earlier following AAV2 vector delivery to salivary glands (Katano *et al*., 2006; Kok *et al*., 2005) and much lower than neutralizing antibodies elicited following delivery of either AAV4 or AAV5 vectors to salivary glands (Katano *et al*., 2006). Administration of AAV2 vectors into mouse skeletal muscle leads to neutralizing antibody titers after 4 weeks similar to those reported herein (Chirmule *et al*., 2000), whereas delivery to human skeletal muscle seems to generate about 5- to 10-fold higher levels (Kay *et al*., 2000).

The overall results found in the present study with the AAV2-TF2.3w-hEpo vector are similar to those observed previously following a GLP-level study with another, but non-regulatable, AAV2 vector in mice (Voutetakis *et al*., 2007a), as well as in non–GLP-level studies conducted following AAV2 vector delivery to parotid glands of both miniature pigs and rhesus macaques (Hai *et al*., 2009; Voutetakis *et al*., 2007b). In aggregate, the findings provide strong evidence for the general safety of localized administration of AAV2 vectors to salivary glands.

Results of the biodistribution assessment indicated a dose- and time-related persistence of vector genomes in several tissues, particularly in male mice (Table 7). As hypothesized, vector copies were found in the targeted salivary gland and draining lymph node at all vector concentrations on day 3, with decreasing numbers by day 92. Besides the presence of significant levels of vector in the targeted salivary gland early after administration, AAV2-TF2.3w-hEpo was also found at this time in several other tissues, including high levels in the liver (Table 7). This finding shows that there was systemic vector access in the present study. Significant systemic access has not been a problem in AAV2 vector studies with miniature pigs (Hai *et al*., 2009), rhesus macaques (Voutetakis *et al*., 2007b), nor with serotype 5 adenoviral (Ad5) vector studies in humans (Zheng *et al*., 2010), and likely is a reflection of the considerably greater degree of difficulty that accompanies gland cannulations in 20–30 g mice. AAV2 vectors delivered by iv administration readily transduce hepatocytes in liver in both rodents and humans (e.g., Manno *et al*., 2006; Nakai *et al*., 2000), and we assume some hepatic transduction occurred herein. There was, however, no indication of any hepatotoxicity based on our clinical chemistry and histopathological results. Thus, the fact that there was some systemic access of vector in the present study, yet no significant adverse effects on animal general well-being, adds to our conclusion that AAV2 vectors are generally safe vehicles for gene transfer. Interestingly, AAV2-TF2.3w-hEpo copies were also present in the tongue tissue at most time points (data not shown), although no vector was detected in the saliva of any animals at any time point. This finding likely reflects drainage of vector into the mouth from the targeted duct after the cannula was removed. In an ongoing clinical study (Baum *et al*., 2010b; Zheng *et al*., 2010), which employees an Ad5 vector, we apply suction to the targeted duct orifice immediately after cannula removal specifically to prevent this type of occurrence.

Apparently, at least in mice, AAV2 vectors targeted to salivary glands are cleared from this tissue much more rapidly in females than males. On day 3, the first time point examined, the median vector copy number found in glands of both male and female mice already showed more than a 3-fold difference (Table 7), and by day 29, a 10-fold difference in AAV2-TF2.3w-hEpo vector copies was evident (male median value 1.7×10^4/μg DNA vs. 1.3×10^3/μg DNA in females). Voutetakis *et al*. (2007a) reported similar gender differences in mice after delivery of a nonregulatable AAV2 vector to murine submandibular glands; however, a mechanistic explanation for this phenomenon is still lacking.

In summary, a critical issue for all clinical gene transfer applications is adequate control of transgene expression. Accordingly, we have examined multiple general safety characteristics following administration of an AAV2 vector containing a rapamycin-regulated expression cassette, AAV2-TF2.3w-hEpo, to murine submandibular glands. Our results are consistent with a conclusion that administration of this AAV2 vector to mouse salivary glands posed no significant risk.

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