Pre-clinical Safety and Off-Target Studies to Support Translation of AAV-Mediated RNAi Therapy for FSHD

Lindsay M. Wallace,1 Nizar Y. Saad,1 Nettie K. Pyne,1 Allison M. Fowler,1 Jocelyn O. Eidahl,1 Jacqueline S. Domire,1,5 Danielle A. Griffin,1 Adam C. Herman,4 Zarife Sahenk,1,2,3 Louise R. Rodino-Klapac,1,2 and Scott Q. Harper1,2

1Center for Gene Therapy, The Research Institute at Nationwide Children’s Hospital, Columbus, OH, USA; 2Department of Pediatrics, The Ohio State University College of Medicine, Columbus, OH, USA; 3Department of Neurology, The Ohio State University College of Medicine, Columbus, OH, USA; 4Research Information Solutions and Innovation Infrastructure, The Research Institute at Nationwide Children’s Hospital, Columbus, OH, USA

RNAi emerged as a prospective molecular therapy nearly 15 years ago. Since then, two major RNAi platforms have been under development: oligonucleotides and gene therapy. Oligonucleotide-based approaches have seen more advancement, with some promising therapies that may soon reach market. In contrast, vector-based approaches for RNAi therapy have remained largely in the pre-clinical realm, with limited clinical safety and efficacy data to date. We are developing a gene therapy approach to treat the autosomal-dominant disorder facioscapulohumeral muscular dystrophy. Our strategy involves silencing the myotoxic gene DUX4 using adeno-associated viral vectors to deliver targeted microRNA expression cassettes (miDUX4s). We previously demonstrated proof of concept for this approach in mice, and we are now taking additional steps here to assess safety issues related to miDUX4 overexpression and sequence-specific off-target silencing. In this study, we describe improvements in vector design and expansion of our miDUX4 sequence repertoire and report differential toxicity elicited by two miDUX4 sequences, of which one was toxic and the other was not. This study provides important data to help advance our goal of translating RNAi gene therapy for facioscapulohumeral muscular dystrophy.

INTRODUCTION
Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal-dominant genetic condition with an estimated incidence of 1 in 8,333 to 1 in 20,000 people.1–5 FSHD was initially classified on the basis of a general pattern of wasting and weakness in muscles of the face, shoulder-girdle, and arms. However, it is now clear that the classical picture of muscle involvement in FSHD is not universal, as some patients may have only limited muscle pathology, whereas others can develop asymmetrical or bilateral weakness in lower limbs, abdominal muscles, and the diaphragm.6,7 Likewise, there is variability in age at onset, rate of progression, and severity of weakness among individuals within the FSHD community. As a result, some patients may maintain lifelong ambulation, whereas others require wheelchair and caregiver assistance.8

In addition to this complicated clinical picture, the molecular mechanisms underlying FSHD are also complex and required decades of investigation to decipher. Great gains in knowledge were achieved in the past decade especially, and the field has focused largely on a model in which de-repression of the DUX4 gene is a primary insult in muscles of FSHD patients.9–13 The identification of DUX4 as a target gene provided a platform to finally enable translational research on FSHD, by allowing the development of DUX4-expressing animal models and DUX4-targeted molecular therapies.13–21

Our laboratory has been working to develop an RNAi-based gene therapy approach to silence the DUX4 gene as a putative treatment for FSHD.20 Specifically, we designed single-stranded adeno-associated viral (AAV) vectors carrying U6 promoter-driven artificial microRNAs targeting the DUX4 mRNA (called miDUX4s). The antisense guide-strands of our miDUX4 constructs contained perfect complementarity with DUX4 across a 22 nt stretch of sequence, thereby directing the transcript toward an RNAi degradation pathway. In a proof-of-concept study using human cells and mouse muscles overexpressing DUX4, we identified a lead sequence, called miDUX4:405 (or mi405), that significantly reduced DUX4 protein and mRNA and improved muscle damage phenotypes associated with large quantities of DUX4 in mice.20 With an overall goal of translating RNAi therapy for FSHD, in this study our aims were to improve our first-generation miDUX4 vectors, expand the repertoire of sequence options that could be useful for targeting DUX4, and safety test our lead sequences in mice and human cells, prior to performing a more expensive good laboratory practice (GLP) toxicology study.

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5Present address: Division of Neuroscience, Oregon National Primate Research Center, Beaverton, OR, USA.

Correspondence: Scott Q. Harper, PhD, Center for Gene Therapy, The Research Institute at Nationwide Children's Hospital, 700 Children’s Drive, Room WA3015, Columbus, OH, 43205, USA.
E-mail: scott.harper@nationwidechildrens.org
RESULTS

We previously designed and validated five miRNAs targeting DUX4.20 Among this first batch, miDUX4.405 (mi405) performed the best and became our lead sequence. However, as RNAi-based gene therapy is still an emerging field with limited safety data, we wanted to ensure that we had backup sequences that could also be translated toward clinical application in the event of unforeseen mi405 toxicity.22–24 With that goal in mind, we developed a simple algorithm to select additional miDUX4 sequences. New miDUX4s were designed using the following criteria: 22 nt mature miRNA length, perfect antisense complementarity to the human DUX4 mRNA, <60% GC content of the mature duplex, and guide-strand biasing, such that the last 4 nucleotides of the antisense 5’ end were A:U rich, and the last 4 nucleotides of the antisense 3’ end were G:C rich. Filtering the DUX4 gene using these criteria produced 34 new candidates, for a total of 39 miDUX4 sequences tested overall (Figure 1A; Table S1). We cloned each new sequence into a U6 expression plasmid and then performed in vitro gene silencing screens in HEK293 cells using a dual luciferase assay in which the DUX4 cDNA was cloned as the 3’ UTR of Renilla luciferase, and

Figure 1. Efficacy Screening and Validation of miDUX4 Sequences

(A) Image represents the DUX4 transcript, in which exon 1 (shown in gray) contains the entire DUX4 open reading frame. Black boxes indicate relative positions of homeodomain-encoding sequence, provided for orientation. Exons 2 and 3 (ex2 and ex3) encode 3’ UTR regions. The black lines above the DUX4 transcript represent the relative positions of all miRNAs identified by the Harper lab miRNA shuttle predictor version 1.0. Red and blue lines indicate mi405 and mi1155, the two lead miDUX4s used in the study. Numbers under the schematic correspond to the nucleotide position in the DUX4 gene, relative to the start codon (ATG). (B) Dual luciferase reporter plasmid used for in vitro screening of miDUX4s. The SV40 promoter drives expression of a Renilla luciferase (Ren Luc) with DUX4 cloned as the 3’ UTR. Firefly luciferase is driven by the thymidine kinase promoter (TK) and serves as an internal transfection control. (C) DUX4 gene silencing was determined by measuring the ratio of Renilla to firefly luciferase from HEK293 cells co-transfected with the dual luciferase plasmid and U6-driven miDUX4s expression plasmids. The numbers on the x axis refer to specific miDUX4 sequences (listed in Table S1). Data are displayed as means with error bars representing SD. (D) Hairpin structures and sequences of the two lead miDUX4s in this study, mi405 and mi1155. Grey and black arrowheads indicate Drosha and Dicer cut sites, respectively, while the colored, underlined sequences identify the miRNA guide-strand. Shaded nucleotides are restriction sites used to clone each miRNA into the U6 expression plasmid. (E) Our first-generation single-stranded adeno-associated viral (AAV) vector contained a U6 promoter-driven miDUX4 and a cytomegalovirus (CMV) promoter transcribing EGFP. The second-generation vectors are self-complementary and do not contain EGFP. Black boxes, AAV inverted terminal repeats; pA, SV40 polyadenylation signal. (F) At 2 weeks, DUX4-expressing muscles (left) show histopathological evidence of degeneration, including myofibers with inflammatory infiltrates, central nuclei, and variable fiber size. Co-injections of AAV.DUX4 and scAAV.mi405 or scAAV.mi1155 vectors (middle and right, respectively) are histologically normal, thereby confirming that scAAV bioactivity is similar to that seen using our previously reported single-stranded vectors. Scale bar, 50 μm.
using a similar strategy, by demonstrating proof of principle for the disease animal models, followed later by dose escalation safety testing. Historically, the template for performing pre-clinical gene therapy studies involved first demonstrating efficacy for a given strategy in disease animal models, followed later by dose escalation safety testing in wild-type animals. Indeed, we began this work several years ago to address potential adverse events associated with inhibitory RNA overexpression, we first performed dose escalation of scAAV6.mi1155 or scAAV6.mi405 in wild-type mice using intramuscular (i.m.) and intravascular (i.v.) routes of administration (Table 1; Figures 1, 3, and S3). For i.m. injection, we delivered four different doses of scAAV vectors and assessed muscle histology 2 and 8 weeks later. For a vascular approach, we delivered three doses using isolated limb perfusion (ILP) and performed necropsy on tibialis anterior (TA) muscle, diaphragm, lung, liver, spleen, kidney, gonads, and heart at 3 week, 6 week, 12 week, and 5 month time points. TA muscles were analyzed using independent blinded analysis by a neuromuscular histopathologist, while independent veterinary pathologists at The Ohio State University Comparative Animal and Mouse Phenotyping Shared Resource assessed other tissues.

Using both delivery methods, we found histological evidence for dose-dependent muscle toxicity in scAAV.mi1155-treated animals (Figure 2). In particular, escalating i.m. vector doses caused increased amounts of regenerated myofibers (indicated by centrally located myonuclei in H&E-stained cryosections), demonstrating that muscles had been damaged and were subsequently repaired in the 8 weeks prior to this time point (Figure 2A). Lower, clinically relevant doses appeared normal (2 \times 10^{10} particles) to relatively normal (1 \times 10^{11} particles) histologically at this time point. Artificially shaded areas (purple) on whole-muscle composite images show the extent of muscle regeneration (Figure 2A). Similar to our i.m. results, animals that received low-dose (3 \times 10^{10} particles) scAAV.mi1155 via ILP injection showed no overt histological evidence of muscle damage at either time point (Figure 2B). Interestingly, muscle injected with high-dose scAAV.mi1155 (1.5 \times 10^{12} particles) appeared normal at 3 weeks but showed widespread regeneration by 5 months, indicating a temporal component to mi1155 toxicity. Besides muscle, mi1155-treated mice showed cellular infiltrates with occasional hepatocellular single-cell necrosis in the liver, but this finding was also present in the controls and considered part of the strain background. Otherwise, no small bioactivity study to ensure that our second-generation miRNA vectors were suppressing DUX4-associated toxicity in vivo. To do this, we used our previously published AAV-based DUX4 mouse model (AAV.CMV.DUX4), which shows histological evidence of muscle damage that is useful as an outcome measure for efficacy. Muscles injected with AAV.CMV.DUX4 showed histopathology, while those co-injected with AAV.CMV.DUX4 and scAAV6.mi405 or scAAV6.mi1155 showed histology normal, indicating that our vectors produced the expected bioactivity (n = 6 per group) (Figures 1F and S2).

Previous pre-clinical studies suggested that vector-expressed RNAi triggers such as short hairpin RNAs (shRNAs) could potentially cause adverse effects by two mechanisms: overexpression-related toxicity through saturation of the natural microRNA biogenesis pathway and unintended sequence-specific silencing of off-target genes.20,24,26,27 We therefore investigated the safety of our lead miDUX4 sequences on the basis of these two potential mechanisms. To address potential adverse events associated with inhibitory RNA overexpression, we first performed dose escalation of scAAV6.mi1155 or scAAV6.mi405 in wild-type mice using intramuscular (i.m.) and intravascular (i.v.) routes of administration (Table 1; Figures 2, 3, and S3). For i.m. injection, we delivered four different doses of scAAV vectors and assessed muscle histology 2 and 8 weeks later. For a vascular approach, we delivered three doses using isolated limb perfusion (ILP) and performed necropsy on tibialis anterior (TA) muscle, diaphragm, lung, liver, spleen, kidney, gonads, and heart at 3 week, 6 week, 12 week, and 5 month time points. TA muscles were analyzed using independent blinded analysis by a neuromuscular histopathologist, while independent veterinary pathologists at The Ohio State University Comparative Animal and Mouse Phenotyping Shared Resource assessed other tissues.

Table 1. AAV Vector Doses, Routes of Administration, and Time Points Used in This Study

| Total Particles | vg/kg | Time Point |
|----------------|-------|------------|
| Intramuscular Deliverya | 2 \times 10^{10} | 2 weeks, 8 weeks |
| | 1 \times 10^{11} | 2 weeks, 8 weeks |
| | 5 \times 10^{11} | 2 weeks, 8 weeks |
| | 1.5 \times 10^{12} | 2 weeks, 8 weeks |
| Intravascular Delivery (ILP) | 3 \times 10^{10} | 3 weeks, 5 months |
| | 1 \times 10^{11} | 3, 6, 12 weeks; 5 months |
| | 1.5 \times 10^{12} | 3 weeks, 5 months |

aVector genomes per kilogram of muscle weight. bVector genomes per kilogram of body weight.

Firefly luciferase was used as a non-targeted transfection control (Figures 1B and 1C).25 To confirm that silencing of DUX4 mRNA also reduced overexpressed DUX4 protein, we performed western blots from HEK293 cell protein extracts co-transfected with a V5-epitope-tagged CMV.DUX4 expression plasmid and our individual U6.miDUX4s (Figure S1). Including mi405, we identified 11 miD-U6xs (28% of all tested) with >75% silencing using both metrics (mi182, mi185, mi187, mi333, mi334, mi405, mi407, mi1155, mi1434, mi1445, and mi1520). Of these, for practical reasons (time and funding) we selected 2 as our leads (mi1155 and mi405) for further in vivo testing in this study, with the additional miDUX4s being tested in ongoing work or reserved as backups until we can feasibly test them (Figure 1D). We noted that roughly three-quarters of our miDUX4 sequences produced >50% DUX4 silencing using in vitro assays, thereby validating our miRNA design strategy.

Historically, the template for performing pre-clinical gene therapy studies involved first demonstrating efficacy for a given strategy in disease animal models, followed later by dose escalation safety testing in wild-type animals. Indeed, we began this work several years ago using a similar strategy, by demonstrating proof of principle for the efficacy of RNAi therapy for FSHD targeting DUX4 in mice.20 Using a first-generation AAV vector system, we showed that the U6.mi405 sequence could suppress DUX4 in mouse muscles and prevent toxic outcomes.20 The first system we published used single-stranded AAV genomes and contained a CMV.EGFP reporter to track transduction; it cannot be translated to human gene therapy (Figure 1E).

With an eye toward translation, we generated a second generation of AAV vectors, in which we switched to a self-complementary AAV backbone, removed the EGFP reporter, and expressed only the U6 promoter-driven mi405 or mi115 sequences (Figures 1D and 1E). Because our ultimate goal was translation, and because our prior work showed that RNAi-mediated DUX4 suppression could be efficacious, in this study we decided to depart from the traditional template for translating gene therapy studies and focus primarily on safety outcomes first, prior to performing extensive efficacy studies of
Figure 2. In Vivo Dose Escalation of scAAV.mi1155 in Wild-Type Mice
(A) Photomicrographs of adult mouse tibialis anterior (TA) muscles 8 weeks after intramuscular (i.m.) injection with the indicated doses of vector. Images show 10 μm cryosections stained with H&E at high and low power. To help visualize the breadth of potential lesions on low-power images, fibers with central nuclei (CN) or areas of active degeneration and inflammation were intentionally shaded with a purple digital overlay. Lesion size correlates with increasing doses of scAAV.mi1155. High-power photos show representative images at indicated vector dosages. CRD indicates a clinically relevant dose range used in previous muscle gene therapy clinical trials. (B) Photomicrographs of adult mouse TA muscles 3 weeks (top) or 5 months (bottom) after vascular delivery of scAAV.mi1155, with the indicated doses of vector. Myofibers with central nuclei, indicating muscle damage and regeneration, were abundant in the 5-month high-dose muscles, as seen in the bottom-right high-power image and visualized in the purple overlay areas of the low-powered photomicrograph. Scale bars, 50 μm for high-power; 500 μm for low-power images.
DISCUSSION

The discovery of RNAi ushered in a new era of molecular biology and expanded the field of gene therapy to include gene-silencing strategies for dominant genetic diseases and viral pathogenesis.\textsuperscript{20,34,43} Perhaps not surprisingly, there was early enthusiasm about RNAi as a potentially revolutionary molecular therapy, followed by the realization that developing new technologies into market-ready treatments is typically not an overnight venture. Today, 16 years after RNAi was first described in mammalian cells, RNAi-based treatments are still experimental, although the first RNAi-based commercial products using oligonucleotide RNAi drugs, RNAi-based gene therapies using AAV vectors are still emerging, and most are still in pre-clinical stages.\textsuperscript{20,34,36,38,39,43} ClinicalTrials.gov lists only two clinical trials to date that were designed to test the safety of delivering inhibitory RNAs from AAV vectors in human beings, and both were targeting hepatitis viruses in the liver (ClinicalTrials.gov identifiers NCT01899092 and NCT02315638).\textsuperscript{40,49,50} Importantly, no adverse events have been reported, and although they represent an excellent example of preclinical testing, both were performed in cell culture, with both the mature miRNA405 and mature miRNA1155 sequences being delivered to human cell lines. Previous results from our laboratory showed that the miRNA405 and miRNA1155 sequences were well tolerated at early time points but eventually caused whole-scale muscle turnover, suggesting that the toxic effects were related to accumulation of the mature miRNA over time.

In this study, we were interested in further developing a gene therapy approach to target the DUX4 gene using AAV and RNAi, as a prospective treatment for the dominant muscle disease FSHD. Our major aim here was to identify our lead DUX4-targeted miRNA sequences (called miDUX4s) and test the safety of our system with respect to miDUX4 overexpression in mammalian muscle (our target tissue for this therapy) and the potential reduction of non-DUX4, off-target genes in human muscle cell lines. Because mice and humans have homologous miRNA biogenesis and gene-silencing machinery, mouse models are useful for testing the safety impacts of miDUX4 overexpression in muscle. However, for the sequence-specific off-targeting experiment, we tested the potential off-target impacts of miDUX4 sequences in human cells because we ultimately want to apply this system to human gene therapy, and mice and humans have different transcriptomes. Of the two sequences we safety-tested, mi1155 was overtly toxic to muscle, whereas mi405 was not when delivered at clinically relevant doses. The fact that the low-dose mi1155 sequences were well tolerated at early time points but eventually caused whole-scale muscle turnover suggested that the toxic effects were related to accumulation of the mature miRNA product over time. We are uncertain about a mechanism to explain this differential toxicity between mi1155 and mi405 on the basis of our current data. Both miRNAs were generated using identical design rules, each was delivered at clinically relevant doses. The fact that the low-dose mi1155 was overtly toxic to muscle, whereas mi405 was not when delivered at clinically relevant doses. The fact that the low-dose mi1155 sequences were well tolerated at early time points but eventually caused whole-scale muscle turnover suggested that the toxic effects were related to accumulation of the mature miRNA product over time. We are uncertain about a mechanism to explain this differential toxicity between mi1155 and mi405 on the basis of our current data. Both miRNAs were generated using identical design rules, each was expressed from the identical U6 promoter cloned into the same site within the same AAV vector system, and each was delivered to inbred C57BL/6 mice using identical routes of administration. Expressing either sequence in human cells caused surprisingly few gene expression changes, and the few transcripts that showed significant changes were reduced only 2-fold and did not seem to be targets of either strand of the mature miRNA or mi1155 sequence on the basis of our molecular beacon-binding assays (Figure 4; Table 2). Because we did not measure the impacts of mi1155 on mouse-specific transcripts, it is possible that the mi1155 sequence caused significant off-target reduction of a transcript required for healthy mouse muscle in vivo and that this hypothetical off-target silencing could have contributed...
to the muscle damage evident in mi1155-treated animals. However, because the mi405 sequence was non-toxic at clinically relevant doses and showed no significant off-targeting in human muscle cells, we decided to simply discard the mi1155 sequence for future use and focus on translating the mi405 sequence. In addition, in the future we will perform similar safety studies for other high-performing miDUX4 sequences (Figures 1 and S1).

Because of the limited safety profile of RNAi-based gene therapy vectors and the difficulty to predict which sequences will be tolerated well and which will not, we propose that our results underscore the importance of performing a toxicology screen at early pre-clinical stages, using research-grade vectors produced under non-GLP conditions. Lead candidates that pass this initial safety test can then proceed to investigational new drug (IND)-enabling toxicology assessments in mice and perhaps larger animal models, as well as scaled for production in a good manufacturing practice (GMP) vector-manufacturing facility. We believe that this strategy will ultimately save time and money as we and others work to translate RNAi-based gene therapies toward clinical application. Toward this end, we could envision the first FSHD-directed AAV-RNAi clinical trial involving direct i.m. injection to a single muscle or potentially using a vascular approach, because more gene therapy studies are moving toward this route of delivery. Other issues that need to be addressed include establishment of the AAV serotype to use for miDUX4 delivery, inclusion and exclusion criteria for patient recruitment, defining outcome measures, and of course clinical vector production issues. As we begin approaching this milestone, it will be critical to engage with regulatory bodies and clinical experts in FSHD and neuromuscular disease gene therapy to design a prospective future trial involving AAV-delivered mi405, as well as other lead candidates that emerge through the pipeline we are establishing here.

**MATERIALS AND METHODS**

**Sequence Generation and Cloning of miDUX4s**

The Harper miRNA shuttle predictor version 1.0 was created and written in Java, enabling use of the application on Mac OS X and Windows machines. In the application, the user inputs the DNA sequence of a gene of interest, allowing the program to systematically look at 22 characters in a row beginning with the first input nucleotide and subsequently screening the entire sequence moving one character at a time. A potential 22 nt miRNA must meet three criteria to be included in the output file: (1) the first four characters must be at least 75% G and C, (2) the last four characters must be at least 75% A and U, and (3) the entire string of characters must be at least 40% A and U. The output file is a compilation of all these sequences in text file format. For DUX4, the predictor identified 44 new candidate miDUX4s (Table S1). All miDUX4s were cloned into the mir-30-based/U6 construct, as previously described. Ten miDUX4s were removed from analysis because of binding disruption from the V5 tag (mi1253, mi1254, and mi1267) or excluded sequence from the 3’ UTR (mi1552, mi1553, mi1554, mi1555, mi1557, mi1558, and mi1559). This program does not exclude sequences containing a run of four or more T nucleotides. Such sequences would be problematic if using a U6 or other RNA pol III promoter, which uses a terminator signal of four or five T’s. However, RNA pol II promoters could express miRNAs containing these poly-T regions.

**Luciferase Assay**

The dual luciferase reporter plasmid was modified from Psicheck2 (Promega) with a firefly luciferase cassette serving as a transfection control and the human DUX4 gene (coding region plus 3’ UTR including introns) cloned downstream of the *Renilla* luciferase stop codon, serving as a 3’ UTR. HEK293 cells were co-transfected (Lipofectamine 2000; Invitrogen) with the luciferase DUX4 reporter and individual U6.microRNA expression plasmids in a 1:5 molar ratio. DUX4 gene silencing was determined as previously described. Triplicate data were averaged per experiment and individual experiments performed two to four times. Results were reported as the average ratio of *Renilla* to firefly luciferase activity ± SD for all combined experiments.

**Western Blots**

HEK293 cells were co-transfected (Lipofectamine 2000) with a CMV.DUX4.V5 expression vector containing the DUX4 3’ UTR and U6.miDUX4s or control U6.miLacZ in a 1:5 molar ratio. Protein was extracted in M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) 24 hr later and quantified using the DC Protein Assay (Bio-Rad). Fifteen microgram samples were separated on 12% SDS-PAGE, transferred to nitrocellulose membrane, and incubated with the following antibodies: mouse monoclonal antibody to V5 (horseradish peroxidase [HRP]-coupled) (1:5,000, R961-25, Invitrogen); mouse monoclonal GAPDH antibody (1:1,000, CB1001; Millipore), or goat polyclonal GAPDH (1:500, ab9483;...
Abcam) overnight at 4°C. GAPDH-probed blots were washed and then incubated with HRP-coupled goat anti-mouse or HRP-coupled donkey anti-goat secondary antibody (1:100,000, 115-035-003 and 705-035-147; Jackson ImmunoResearch) for 1 hr at room temperature. Following washes, blots were developed using Immobilon Western HRP substrate (Millipore) and exposed to film. DUX4.V5 quantification was assessed using ImageJ.

**AAV Vector Delivery to Mice**

For the bioactivity and i.m. dose escalation studies, 6- to 9-week-old C57BL/6 male and female mice received direct 40 μL i.m. injections into the TA. Bioactivity mice received adeno-associated virus AAV6.CMV.DUX4.V5 at $3 \times 10^9$ DNase-resistant particles (DRP) and a contralateral co-injection of AAV6.CMV.DUX4.V5 at $3 \times 10^9$ and either scAAV6.U6.mi405 or scAAV6.U6.mi1155 at $3 \times 10^{10}$ and $1 \times 10^{11}$ DRP. Muscles were harvested 2 weeks post-injection. N = 6 mice per vector. I.m. dose escalation mice received either saline control or scAAV6.U6.mi405 or scAAV6.U6.mi1155 at $2 \times 10^9$, $1 \times 10^{10}$, $5 \times 10^{10}$, or $1.5 \times 10^{12}$ DRP. Injections were delivered bilaterally and harvested 2 or 8 weeks post-injection. N = 6 legs per dose per time point. For vascular delivery, ILPs were performed on 5- to 6-week-old C57BL/6 male mice as previously described.28

**Figure 4. mi405 and mi1155 Bind Specifically to Cognate Target Sites but Not Off-Target Sites**

(A) A fluorescence-based molecular beacon assay was used to determine mi405 and mi1155 binding to DUX4 sequences and putative off-targets. By default, the molecular beacon folds into a stem loop structure that brings a quencher (zenBHQ) in close proximity to a fluorophore (6FAM), thereby quenching the fluorescence emission of 6FAM. We designed molecular beacons encompassing the mature sequences of mi405 and mi1155 in their loop sequence. The hybridization of the molecular beacon to a complementary sequence (represented here by a target site) separates the fluorophore and quencher, allowing fluorescence emission. The binding of a mature miRNA beacon to its target site can then be determined by measuring the emitted fluorescence. (B) The graph shows binding data for mi405 and mi1155 target sites within the DUX4 transcript, where mi405 bound two target sites (mi405 On-TS1 and mi405 On-TS2). The latter had a single mismatch within the 22 nt duplex and two terminal mismatches. mi1155 had strongest binding to its on-target site within DUX4 ($K_d = 0.28 \mu M$). The mi405 mature sequence bound its perfect target (mi405 On-TS1) with a $K_d$ of 0.65 μM, with reduced binding at the second site (mi405 On-TS2; $K_d = 1.20 \mu M$). A negative control containing two additional mismatches within the mi405 On-TS2 duplex did not bind the mi405 molecular beacon (mi405 neg. ctrl TS). The molecular beacon signal expressed in relative fluorescence units (RFU) was subtracted from background fluorescent signal and used to determine the binding affinity ($K_d$), which is the binding site concentration (μM) required to reach half of maximum fluorescence. Data are displayed as means with error bars representing SD. (C) Putative off-target sites for mi405 and mi1155 in the indicated transcripts, as determined by PITA analysis of transcripts (CXCL11, C1QTNF1, LAMP3, and GBP4) that were significantly reduced in DUX4-negative human myoblasts expressing mi405 or mi1155 (specific sites are listed in Materials and Methods). The mi405 and mi1155 molecular beacons showed no binding affinity to any putative off-target sites. For cost and efficiency, the molecular beacon-binding assay was performed using DNA oligonucleotides. To mimic G:U base pairing that occurs in RNA:RNA duplexes, we generated RNA “mimic” bases in the miRNA:off-target site pair, such that A:T base pairing (pairing with two hydrogen bonds) was introduced, replacing the “G” nucleotide with an “A” nucleotide whenever the “G” is facing a “T.” These changes are indicated by gray-shaded nucleotides. miRNA:off-target site pairs are represented as two annealing strands. The top strand represents the target site, and the bottom strand represents the mature miDUX4 sequence.
Saline injections served as the controls, and AAV6.U6.mi405 or 1155 vectors were delivered at $3 \times 10^{10}, 1 \times 10^{11}$, or $1.5 \times 10^{12}$ DRP. Mice were harvested at 3 weeks post-injection (n = 2 per dose per treatment), 6 and 12 weeks post-injection (n = 3 at $1 \times 10^{11}$ DRP per treatment), and 5 months post-injection (n = 5 per dose per treatment). All mouse procedures were performed following guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at the Research Institute at Nationwide Children’s Hospital.

**Histology and Comparative Pathology**

Dissected TA muscles were placed in O.C.T. Compound (Tissue-Tek) and frozen on liquid nitrogen-cooled isopentane. Cryosections were cut at 10 µm and then stained with H&E following standard protocols. Dissected diaphragm, kidney, liver, heart, spleen, testis, and lung (needle perfused) were all post-fixed in 10% neutral buffered formalin for 48 hr and then processed, paraffin-embedded, cut, and H&E-stained at the Nationwide Children’s Hospital Morphology Core Laboratory. TA muscles were analyzed by a blinded neuropathologist, and all other tissues were analyzed at The Ohio State University Comparative Pathology & Mouse Phenotyping Shared Resource.

**mi405 qPCR Assay**

The mi405 qPCR protocol was designed on the basis of methods previously described. Briefly, RNA was extracted using the total RNA protocol for the mirVana miRNA Isolation Kit (Ambion) from 25 50 µm cryosections of indicated TA muscles. cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using a mix of random hexamer primers and 200 nM of the stem-loop forming primer (5'-GTC GTATCCAGTGACGGTGATTGCGACTGGATACGAGTGCCAG-3'). A custom TaqMan assay (Applied Biosystems) including 1.5 µM of forward primer (5'-CGGCCCAAACCGATCTGAATC-3'), 0.2 µM of reverse primer (5'-6FAM-ATACGACGTCCGTGCAGGGTCCGA-3') and 0.2 µM of molecular beacon (5'-GFAM-6FAM-ATACGACGTCCGTGCAGGGTCCGA-3') was then run using the CFX Connect Real Time system apparatus. The mi405 and mi1155 molecular beacons served as the reference gene, and normalized expression ($\Delta\Delta$Cq) was calculated relative to the lowest viral dose per experiment ($2 \times 10^{10}$ DRP for i.m. and $3 \times 10^{10}$ DRP for ILP).

**RNA-Seq and Off-Target Analysis**

Human immortalized myoblasts were transfected with an AAV proviral plasmid expressing U6.mi405, U6.1155, or control plasmid pCNeo using the Human Dermal Fibroblast Nucleofector kit (Amaza). RNA preparation, library construction, quality control, and sequencing were performed as previously described. pCNeo controls were previously described as simultaneous experiments were run together using the same control group. Gene expression changes with fold change of $\pm 2.00$ and p values $< 0.05$ compared with controls were considered significant. These genes were then analyzed for miDUX4 seed matching, and the predicted identity of all off-target sites in every off-target gene using two different prediction programs: siSPOTR and the PITA prediction algorithm. RNA-seq was performed by The Biomedical Genomics Core of the Research Institute at Nationwide Children’s Hospital, Columbus, Ohio. Raw data files for the RNA-seq data are deposited at [https://www.ncbi.nlm.nih.gov/sra?term=SRP127959](https://www.ncbi.nlm.nih.gov/sra?term=SRP127959) (SRA: SRP127959).

**Molecular Beacon-Binding Assay**

Mature DNA sequences of mi405 and mi1155 complementary to their cognate target sites were designed and used as previously described. A 6FAM fluorophore and a Zen Black Hole Quencher (ZenBHQ) were added at the 5' and 3' ends, respectively. The DNA molecular beacons were synthesized (Integrated DNA Technologies), and each molecular beacon had a density close to 3 OD and was purified using high-performance liquid chromatography (HPLC). The binding assay was performed as previously described, with some modifications. The binding assay was performed using the CFX Connect Real Time system apparatus. After mixing the molecular beacon and target site, the binding was initiated with a denaturation step at 95°C for 3 min, followed by an annealing step at 37°C for 10 min. The fluorescence was measured at the end of the annealing step.

The relative fluorescent signal (relative fluorescent units [RFU]) was subtracted from background fluorescent signal and fit to a one site-specific binding Hill slope equation that was used to determine the binding affinity ($K_d$). The mi405 and mi1155 molecular beacons were used at a constant concentration of 200 nM, and the constant parameter ($K_d$) was determined by increasing the concentration of the cognate target sites. $K_d$ is the binding site concentration (nM) required to reach half of the maximum fluorescence and represents binding affinity. The smaller the $K_d$ value, the greater the binding affinity of the miRNA molecular beacon is for its target site. We tested binding of off-target genes identified by RNA-seq (Table 2) and queried PITA and siSPOTR to identify transcripts with putative binding sites for mi405 and mi1155. We modified our molecular beacon assay by using DNA oligos designed to mimic Gu base pairing (two hydrogen bonds) that occurs in RNA:RNA duplexes. To mimic RNA:RNA base pairing in the miRNA-off-target site pair, A:T base pairing (pairing with two hydrogen bonds) was introduced, replacing the “G” nucleotide with an “A” nucleotide whenever the “G” is facing a “T.”

Predicted target sites are indicated as follows. mi405 On-TS1: on-target site on DUX4 open reading frame (ORF) at position 405; mi405 On-TS2: second on-target site on DUX4 ORF at position 193; and mi1155 On-TS: on-target site on DUX4 ORF at position 1155. The remaining annealing strands represent pairing of mi405 or mi1155 to their off-target sites. CXCL11 Off-TS1: mi405 off-target site on CXCL11 ORF at position 28; CXCL11 Off-TS2: mi405 off-target site on CXCL11 3' UTR at position 281 from the beginning of 3' UTR; C1QTNF1 Off-TS1: mi405 off-target site on C1QTNF1 ORF at position 21; C1QTNF1 Off-TS2: mi405 off-target site on C1QTNF1 3' UTR at position 1494 from the beginning of 3' UTR; LAMP3 Off-TS1: mi1155 off-target site on LAMP3 ORF at position 1310; LAMP3 Off-TS2: mi1155 off-target site on LAMP3 3' UTR at
position 663 from the beginning of 3’UTR; GBP4 Off-TS1: mi1155 off-target site on GBP4 ORF at position 1466; GBP4 Off-TS2: mi1155 off-target site on GBP4 3’UTR at position 3181 from the beginning of 3’UTR; mi1155 Off-TS1: mi1155 off-target site on DUX4 ORF at position 1194; mi1155 Off-TS2: mi1155 off-target site on DUX4 ORF at position 767; mi1155 Off-TS3: mi1155 off-target site on DUX4 3’UTR at position 2050 from the beginning of 3’UTR; CIQTNF1 Off-TS3: mi1155 off-target site on CIQTNF1 ORF at position 19; CIQTNF1 Off-TS4: mi1155 off-target site on CIQTNF1 3’UTR at position 1495 from the beginning of 3’UTR.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Results, four figures, and three tables and can be found with this article online at https://doi.org/10.1016/j.omtm.2017.12.005.

AUTHOR CONTRIBUTIONS

L.M.W. designed and performed all mouse experiments and necropsies (except ILP surgeries), designed and performed qPCR assays, and contributed to western blots, RNA-seq experiments, and analysis. L.M.W. also created figures and legends and wrote the Materials and Methods section of the paper. N.Y.S. designed and performed the molecular beacon assay and created figures, legends, and methods with the assayed data. N.K.P. and J.S.D. cloned and tested the new miDUX4s in vitro. N.K.P. generated a supplemental figure and table with in vitro data. A.M.F. produced and titered all AAV vectors. J.O.E. contributed to RNA-seq experiments. D.A.G. and L.R.R.-K. performed ILP surgeries and contributed to experimental design. A.C.H. created the algorithm used to identify new candidate miDUX4s. Z.S. was the pathologist who analyzed TA muscle histology. S.Q.H. conceptualized and obtained funding for the project, designed the miRNA predictor criteria, contributed to data analysis, and wrote the manuscript. All authors contributed to refining and editing the manuscript.

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Supplemental Information

Pre-clinical Safety and Off-Target Studies
to Support Translation of AAV-Mediated
RNAi Therapy for FSHD

Lindsay M. Wallace, Nizar Y. Saad, Nettie K. Pyne, Allison M. Fowler, Jocelyn O. Eidahl, Jacqueline S. Domire, Danielle A. Griffin, Adam C. Herman, Zarife Sahenk, Louise R. Rodino-Klapac, and Scott Q. Harper
Supplemental Figure 1. In vitro western blot screening of miDUX4 sequences.

(A) Representative western blots using extracts from co-transfected HEK293 cells showed a range of DUX4 silencing efficacy. A V5 antibody was used to detect V5 epitope-tagged DUX4 (52 kDa band) while GAPDH served as the protein loading control. Membranes were cut between 37 kDa and 50 kDa before probing with primary antibody.

(B) Selected miDUX4s with the best silencing from each region of the gene (homeodomains, transactivation domain, 3’ UTR) were assayed together for direct comparison. Identical gels were run and probed with either V5 (left) or GAPDH (right) from the same protein preparation.
Supplemental Figure 2. Second generation scAAV.miDUX4 vectors protect mouse muscles from DUX4-induced damage. (A) This figure shows the reproducibility of miDUX4 bioactivity. Animals received AAV6.CMV.DUX4.V5 at 3x10^9 DRP and a contralateral co-injection of AAV6.CMV.DUX4.V5 at 3x10^9 DRP and either scAAV6.U6.mi405 or scAAV6.U6.mi1155 at 3x10^10 or 1x10^11 DRP. Muscles were harvested 2 weeks post-injection, sectioned onto 10 micron slides, and stained with H&E to visualize histology. Panels A and B, scAAV.miDUX4.405 bioactivity, where animals in A received 3x10^10 particles of mi405 vector, and those in B received 1x10^11 particles of the same vector. Muscles were protected from DUX4-induced damage at both doses. Panels C and D, scAAV.miDUX4.1155 bioactivity, where animals in C received 3x10^10 particles of mi1155 vector, and D received 1x10^11 particles of the same vector. Muscles were protected from DUX4-induced damage at both doses. Individual mouse numbers are indicated on the left of each image (e.g. 3536).
Supplemental Figure 3. Mouse muscle histopathology time course for an intermediate vascular dose of scAAV.mi405 and scAAV.mi1155. Photomicrographs of adult mouse tibialis anterior (TA) muscles, at 3 wks, 6 wks, 12 wks, and 5 months after isolated limb perfusion of a $1 \times 10^{11}$ dose of indicated miDUX4 vectors. Left panel, AAV.mi405-injected animals were histologically normal while AAV.mi1155-injected mice (right panel) showed an accumulation over time of myofibers with active degeneration and central nuclei. Whole muscle lesions are highlighted by purple overlay areas on the low-powered images. Scale bars indicate 50 microns for high power and 500 microns for low power images.
Supplemental Figure 4. mi405 and mi1155 passenger strands do not bind predicted off-target sites. (A) The binding to predicted off-target sites within DUX4, C1QTNF1, and LAMP3 was assessed using the fluorescence-based molecular beacon assay. In this assay, we designed molecular beacons (MBs) encompassing in their loop sequence the mature mi405 and mi1155 passenger strands. The graph shows binding data for mi405ps and mi1155ps (where ps = passenger strand) to their cognate complementary target sites (Com-TS; used as a positive control), and off-target sites (Off-TS). The $K_d$ representing the binding affinity to the Com-TS of each passenger strand is shown. The mi1155ps had strongest binding to its Com-TS (mi1155ps Com-TS) ($K_d = 0.30 \mu M$). The mi405ps bound to its Com-TS (mi405ps Com-TS) with a $K_d$ of 1.52 $\mu M$. The molecular beacon signal (MBS) expressed in relative fluorescent units (RFU) was subtracted from background fluorescent signal and used to determine the binding affinity ($K_d$), which is defined as the binding site concentration required to reach half of maximum fluorescence (in $\mu M$). (B) Putative off-target sites for mi405ps and mi1155ps in each indicated transcript, as determined by PITA analysis of mRNAs that were significantly reduced in DUX4-negative human myoblasts expressing mi405 or mi1155 (C1QTNF1 and LAMP3). Interestingly, predicted off-target sites for mi405 and mi1155 passenger strands were identified in DUX4 itself. However, the mi405ps and mi1155ps molecular beacons showed no binding affinity to any putative off-target sites. The grey shaded “A” nucleotides replaced the “G” nucleotides in these sequence to mimic G:U base pairing that occurs in RNA:RNA duplexes. miRNA:off-target site pairs are represented as two annealing strands. The top strand represents the target site and the bottom strand represents the mature miDUX4ps sequence. Where $K_d = N.D.$ (not determined) is indicated, no binding was measured.

mi405ps Com-TS: complementary target site of mi405ps.

mi1155ps Com-TS: complementary target site of mi1155ps.

The remaining annealing strands represent pairing of mi405ps or mi1155ps to their off-target sites. mi405ps_C1QTNF1 Off-TS1: mi405ps off-target site on C1QTNF1 ORF at position 471. mi405ps_C1QTNF1 Off-TS1: mi405ps off-target site on C1QTNF1 ORF at position 471. mi405ps_C1QTNF1 Off-TS2: mi405ps off-target site on C1QTNF1 3’UTR at position 1507 from the beginning of 3’UTR. mi1155ps_LAMP3 Off-TS: mi1155ps off-target site on LAMP3 3’UTR at position 792 from the beginning of 3’UTR. mi1155ps_DUX4 Off-TS: mi1155ps off-target site on DUX4 ORF at position 895.
|        | Fiber Size Variability       | Active Inflammation                     | Central Nuclei         |
|--------|------------------------------|-----------------------------------------|------------------------|
| mi405  | Normal Variability          | None                                    | Negative               |
| 3.00E+10 |                              |                                         |                        |
| 1.00E+11 |                              |                                         |                        |
| 1.50E+12 |                              |                                         |                        |
| mi1155 | Normal Variability          | None                                    | Negative               |
| 3.00E+10 |                              |                                         |                        |
| 1.00E+11 | Mild to moderate focal 3/5 | Mild, minimal multifocal, endomyseal 2/5 |                        |
| 1.50E+12 | Widespread 3/5              | None                                    | Moderate diffuse 4/5   |

Supplemental Table 2. Summary of tibialis anterior muscle pathology in 5 month animals compared to saline controls.
Experimental History

Mice were treated with saline or viral vectors over-expressing protein and miRNA delivery vectors. Slide evaluation of multiple tissues to determine toxicity. Tissues submitted include: Slide #1: Diaphragm. Slide #2: Testis, lung, spleen, liver, heart, and kidney for each mouse. Mice received treatments at 6 weeks of age and were euthanized at 3 weeks or 5 months post treatment.

Mice 1-2 (3504, 3505) were treated with saline and euthanized 3 weeks later.

Mice 3-4 (3509, 3510) were treated with low dose scmiMYOT no GFP and euthanized 3 weeks later.

Mice 5-6 (3516, 3517) were treated with low dose scmiDox1155 no GFP and euthanized 3 weeks later.

Mice 7-8 (3523, 3525) were treated with high dose scmiMYOT no GFP and euthanized 3 weeks later.

Mice 9-10 (3531, 3532) were treated with scmiDox 115 no GFP and euthanized 3 weeks later.

Mice 11-15 (3511, 3512, 3513, 3514, 3515) were treated with scmiMYOT no GFP low dose (3e10) and euthanized 5 months later.

Mice 16-20 (3526, 3527, 3528, 3529, 3530) were treated with scmiMYOT no GFP high dose (1.5e12) and euthanized 5 months later.

Mice 21-24 (3506, 3507, 3508, ILP-100) were treated with saline and euthanized 5 months later.

Mice 25-28 (3519, 3520, 3521, 3522) were treated with scmiDox1155 no GFP low dose (3e10) and euthanized 5 months later.

Mice 29-33 (3533, 3534, 3535, ILP-101, ILP-102) were treated with scmiDox1155 no GFP high dose (1.5e12) and euthanized 5 months later.

Mice 34-35 (153, 154) were treated with scmi405 low dose and euthanized 3 weeks later.

Mice 36-40 (148, 150, 151, 152) were treated with scmi405 low dose (3e10) and euthanized 5 months later.

Clinical History

N/A
Patient No: 1  
ID #: 3504  
Species: Mouse  
Strain/Breed: C57BL/6  
Age/DOB: 9 weeks  
Sex: Male intact  
GEM: No  
Tests Ordered: Slide Evaluation

**Anatomic Pathology**

**Microscopic Findings**

Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): Multifocal, mild renal papillary mineralization.
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): No significant microscopic lesions (NSML).
Liver (slide 2): No significant microscopic lesions (NSML).
Spleen (slide 2): No significant microscopic lesions (NSML).
Testis (slide 2): No significant microscopic lesions (NSML).

Patient No: 2  
ID #: 3505  
Species: Mouse  
Strain/Breed: C57BL/6  
Age/DOB: 9 weeks  
Sex: Male intact  
GEM: No  
Tests Ordered: Slide Evaluation

**Anatomic Pathology**

**Microscopic Findings**

Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): No significant microscopic lesions (NSML).
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): No significant microscopic lesions (NSML).
Liver (slide 2): No significant microscopic lesions (NSML).
Spleen (slide 2): No significant microscopic lesions (NSML).
Testis (slide 2): No significant microscopic lesions (NSML).

Patient No: 3  
ID #: 3509  
Species: Mouse  
Strain/Breed: C57BL/6  
Age/DOB: 9 weeks  
Sex: Male intact  
GEM: No  
Tests Ordered: Slide Evaluation

**Anatomic Pathology**

**Microscopic Findings**

Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): Multifocal, mild renal papillary mineralization.
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): No significant microscopic lesions (NSML).
Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, diffuse, moderate. Multifocal scattered microgranulomas (few neutrophils and hepatocellular focal necrosis (affecting 1-2 cells).
Spleen(slide 2): No significant microscopic lesions (NSML).
Testis (slide 2): No significant microscopic lesions (NSML).

Patient No: 4
ID #: 3510
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 9 weeks
Sex: Male intact

Anatomic Pathology

Microscopic Findings
Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): No significant microscopic lesions (NSML).
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): No significant microscopic lesions (NSML).
Liver (slide 2): No significant microscopic lesions (NSML).
Spleen(slide 2): No significant microscopic lesions (NSML).
Testis (slide 2): No significant microscopic lesions (NSML).

Patient No: 5
ID #: 3516
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 9 weeks
Sex: Male intact

Anatomic Pathology

Microscopic Findings
Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): No significant microscopic lesions (NSML).
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): No significant microscopic lesions (NSML).
Liver (slide 2): No significant microscopic lesions (NSML).
Spleen(slide 2): No significant microscopic lesions (NSML).
Testis (slide 2): No significant microscopic lesions (NSML).

Patient No: 6
ID #: 3517
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 9 weeks
Sex: Male intact

Tests Ordered:
Slide Evaluation
Anatomic Pathology

Microscopic Findings
Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): Multifocal, mild renal papillary mineralization.
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): No significant microscopic lesions (NSML).
Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, diffuse, moderate. Multifocal scattered microgranulomas (neutrophils, focal hepatocellular necrosis).
Spleen (slide 2): No significant microscopic lesions (NSML).
Testis (slide 2): No significant microscopic lesions (NSML).

Patient No: 7
ID #: 3523
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 9 weeks
Sex: Male intact

Anatomic Pathology

Microscopic Findings
Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): Multifocal, mild renal papillary mineralization.
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): No significant microscopic lesions (NSML).
Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, diffuse, moderate. Multifocal scattered microgranulomas (neutrophils, focal hepatocellular necrosis).
Spleen (slide 2): No significant microscopic lesions (NSML).
Testis (slide 2): No significant microscopic lesions (NSML).

Patient No: 8
ID #: 3525
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 9 weeks
Sex: Male intact

Anatomic Pathology

Microscopic Findings
Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): Focal renal papillary mineralization.
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): No significant microscopic lesions (NSML).
Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, centrilobular to midzonal, widespread, moderate. MF microgranulomas (neutrophils, focal hepatocellular necrosis).
Spleen (slide 2): Mild increase in extramedullary hematopoiesis (EMH).
Testis (slide 2): NSML.

Patient No: 9
ID #: 3526
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 9 weeks
Sex: Male intact
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ID #: 3531
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 9 weeks
Sex: Male intact

Anatomic Pathology

Microscopic Findings
Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).

Kidney (slide 2): No significant microscopic lesions (NSML).
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): No significant microscopic lesions (NSML).
Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, centrilobular to midzonal, widespread, moderate.
Spleen (slide 2): NSML.
Testis (slide 2): NSML.

Patient No: 10
ID #: 3532
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 9 weeks
Sex: Male intact

Anatomic Pathology

Microscopic Findings
Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): Multifocal dilated (ectatic) tubules within the medullae.
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): No significant microscopic lesions (NSML).
Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, centrilobular to midzonal, widespread, moderate.
Spleen (slide 2): Mild increase in extramedullary hematopoiesis (EMH).
Testis (slide 2): NSML.

Patient No: 11
ID #: 3511
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 6 months
Sex: Male intact

Anatomic Pathology

Microscopic Findings
Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): NSML.
Liver (slide 2): Diffuse, mild, hepatocellular vacuolar change consistent with glycogen with multifocal intracellular micro-
to macrovacuolar lipid.
Spleen (slide 2): Mild, diffuse white pulp (lymphoid) hyperplasia characterized by multifocal prominent germinal centers.
Testis (slide 2): Few tubules with mild atrophy and degeneration. (~ 2 tubules).

Anatomic Pathology

Microscopic Findings
Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): Multifocal, mild renal papillary mineralization; Focal interstitial lymphoplasmacytic aggregate.
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): NSML.
Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, multifocal, mild.
Spleen (slide 2): Mild, diffuse white pulp (lymphoid) hyperplasia characterized by multifocal prominent germinal centers.
Testis (slide 2): Few tubules with mild degenerate sertoli cells (~ 2 tubules) and MF tubular mineralization.
Sex: Male intact

Anatomic Pathology

Microscopic Findings
Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): Multifocal tubules within the cortex and medulla are dilated (ectatic). There is multifocal intratubular protein. Focal, small lymphoplasmacytic aggregate at the renal pelvis.
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): NSML.
Liver (slide 2): Widespread, mild, centrlobular and midzonal vacuolar change consistent with glycogen.
Spleen (slide 2): NSML.
Testis (slide 2): NSML.

Patient No: 15
ID #: 3515
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 6 months
Sex: Male intact

Promoter Gene Genotype

Patient No: 16
ID #: 3526
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 6 months
Sex: Male intact

Anatomic Pathology

Microscopic Findings
Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): Multifocal moderate interstitial lymphoplasmacytic inflammatory infiltrate; MF renal tubular degeneration and ectasia with MF intratubular protein. Multifocal tubular regeneration; MF mild thickening of glomerular capillary loops.
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): NSML.
Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, diffuse, mild.
Spleen (slide 2): Mild, diffuse white pulp (lymphoid) hyperplasia characterized by multifocal prominent germinal centers.
Testis (slide 2): NSML.

Patient No: 16
ID #: 3526
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 6 months
Sex: Male intact

Promoter Gene Genotype

Anatomic Pathology

Microscopic Findings
Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): NSML.
Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, diffuse, moderate.
Spleen (slide 2): NSML.
Testis (slide 2): NSML.
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Patient No: 17
ID #: 3527
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 6 months
Sex: Male intact

Tests Ordered:
Slide Evaluation

Anatomic Pathology

Microscopic Findings
Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): NSML.
Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, diffuse, moderate.
Spleen (slide 2): NSML.
Testis (slide 2): NSML.

Patient No: 18
ID #: 3528
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 6 months
Sex: Male intact

Anatomic Pathology

Microscopic Findings
Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): Multifocal, moderate interstitial lymphoplasmacytic aggregates, particularly in the renal pelvis.
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): NSML.
Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, diffuse, mild. There is a focally extensive perivascular lymphoplasmacytic aggregate.
Spleen (slide 2): White pulp (lymphoid) hyperplasia characterized by multifocal prominent germinal centers, diffuse, mild.
Testis (slide 2): NSML.

Patient No: 19
ID #: 3529
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 6 months
Sex: Male intact

Anatomic Pathology

Microscopic Findings
Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): NSML.
Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, diffuse, moderate.
Spleen (slide 2): Mild, diffuse white pulp (lymphoid) hyperplasia characterized by multifocal prominent germinal centers.
Testis (slide 2): MF seminiferous tubular mineralization (~3 tubules); MF seminiferous tubule degeneration, atrophy and tubular ectasia, multifocal, mild.

| Patient No: 20 | GEM: No | Tests Ordered: Slide Evaluation |
|----------------|---------|--------------------------------|
| ID #: 3530     |         |                                |
| Species: Mouse |         |                                |
| Strain/Breed: C57BL/6 | |                                |
| Age/DOB: 6 months | |                                |
| Sex: Male intact | |                                |

Anatomic Pathology

Microscopic Findings
Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): NSML.
Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, diffuse, moderate. Focal micrgranuloma (few neutrophils with single cell necrosis).
Spleen (slide 2): Lymphoid hyperplasia, multifocal, mild characterized by multifocal prominent germinal centers.
Testis (slide 2): NSML.

| Patient No: 21 | GEM: No | Tests Ordered: Slide Evaluation |
|----------------|---------|--------------------------------|
| ID #: 3506     |         |                                |
| Species: Mouse |         |                                |
| Strain/Breed: C57BL/6 | |                                |
| Age/DOB: 6 months | |                                |
| Sex: Male intact | |                                |

Anatomic Pathology

Microscopic Findings
Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): NSML.
Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, diffuse, mild. Small perivascular lymphoplasmacytic aggregate.
Spleen (slide 2): NSML.
Testis: NSML.

| Patient No: 22 | GEM: No | Tests Ordered: Slide Evaluation |
|----------------|---------|--------------------------------|
| ID #: 3507     |         |                                |
| Species: Mouse |         |                                |
| Strain/Breed: C57BL/6 | |                                |
| Age/DOB: 6 months | |                                |
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Sex: Male intact

**Anatomic Pathology**

**Microscopic Findings**

Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): NSML.
Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, multifocal, mild.
Spleen (slide 2): NSML.
Testis: Seminiferous tubule degeneration and atrophy, diffuse, moderate.

---

**Patient No:** 23
**ID #:** 3508
**Species:** Mouse
**Strain/Breed:** C57BL/6
**Age/DOB:** 6 months
**Sex:** Male intact

**Tests Ordered:**

**Slide Evaluation**

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**Anatomic Pathology**

**Microscopic Findings**

Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): NSML.
Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, multifocal, mild.
Spleen (slide 2): Lymphoid hyperplasia, multifocal, mild characterized by multifocal prominent germinal centers.
Testis (slide 2): Focal, mild atrophy and degeneration of seminiferous tubules (~1).

---

**Patient No:** 24
**ID #:** ILP-100
**Species:** Mouse
**Strain/Breed:** C57BL/6
**Age/DOB:** 6 months
**Sex:** Male intact

**Tests Ordered:**

**Slide Evaluation**

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**Anatomic Pathology**

**Microscopic Findings**

Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): NSML.
Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, diffuse, mild.
Spleen (slide 2): Lymphoid hyperplasia, multifocal, mild characterized by multifocal prominent germinal centers.
Testis (slide 2): NSML.

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**Patient No:** 25
**GEM:** No

**Tests Ordered:**

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| ID #: 3519 | Species: Mouse | Slide Evaluation |
|------------|----------------|------------------|
| Strain/Breed: C57BL/6 | Age/DOB: 6 months | Sex: Male intact |

**Anatomic Pathology**

**Microscopic Findings**

- **Diaphragm (2 sections; slide 1):** No significant microscopic lesions (NSML).
- **Kidney (slide 2):** NSML.
- **Lung (slide 2):** Possible alveolar histiocytosis; however, underperfusion artifact precludes diagnosis.
- **Heart (slide 2):** NSML.
- **Liver (slide 2):** Multifocal, mild centrilobular and midzonal vacuolar change consistent with glycogen. There are multifocal, scattered foci of extramedullary hematopoiesis and few pigmented macrophages within the sinusoids. The pigment is brown, granular and intracytoplasmic.
- **Spleen (slide 2):** Diffuse expansion of the red pulp with extramedullary hematopoiesis (erythroid series predominating (erythroid hyperplasia).
- **Testis (slide 2):** NSML.

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| Patient No: 26 | ID #: 3520 | Species: Mouse | Strain/Breed: C57BL/6 | Age/DOB: 6 months | Sex: Male intact |
|----------------|-----------|----------------|-----------------------|------------------|----------------|

**Anatomic Pathology**

**Microscopic Findings**

- **Diaphragm (2 sections; slide 1):** No significant microscopic lesions (NSML).
- **Kidney (slide 2):** Multifocal mineralization of renal papilla.
- **Lung (slide 2):** Multifocal alveolar histiocytosis - underperfusion artifact precludes diagnosis.
- **Heart (slide 2):** NSML. Liver: MF mild centrilobular vacuolar change consistent with glycogen; focal lymphoplasmacytic perivascular aggregate.
- **Spleen (slide 2):** Diffuse expansion of the red pulp with extramedullary hematopoiesis (erythroid series predominating (erythroid hyperplasia).
- **Testis (slide 2):** NSML.

---

| Patient No: 27 | ID #: 3521 | Species: Mouse | Strain/Breed: C57BL/6 | Age/DOB: 6 months | Sex: Male intact |
|----------------|-----------|----------------|-----------------------|------------------|----------------|

**Anatomic Pathology**

**Microscopic Findings**

- **Diaphragm (2 sections; slide 1):** No significant microscopic lesions (NSML).
Kidney (slide 2): Focal, mild lymphoplasmacytic aggregate near pelvis.

Lung (slide 2): Underperfusion artifact precludes diagnosis.

Heart (slide 2): NSML.

Liver (slide 2): Multifocal, mild vacuolar change consistent with glycogen; small perivascular lymphoplasmacytic aggregate.

Spleen (slide 2): NSML.

Testis (slide 2): Multifocal seminiferous tubular degeneration and atrophy (~ 9 tubules), mild.

Patient No: 28  
ID #: 3522  
Species: Mouse  
Strain/Breed: C57BL/6  
Age/DOB: 6 months  
Sex: Male intact

Anatomic Pathology

Microscopic Findings

Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).

Kidney (slide 2): NSML.

Lung (slide 2): Underperfusion artifact precludes diagnosis.

Heart (slide 2): NSML.

Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, multifocal, mild with multifocal hepatocellular microvacuolar lipidosis.

Spleen (slide 2): NSML.

Testis (slide 2): NSML.

Patient No: 29  
ID #: 3533  
Species: Mouse  
Strain/Breed: C57BL/6  
Age/DOB: 6 months  
Sex: Male intact

Anatomic Pathology

Microscopic Findings

Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).

Kidney (slide 2): NSML.

Lung (slide 2): Underperfusion artifact precludes diagnosis.

Heart (slide 2): NSML.

Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, diffuse, mild.

Spleen (slide 2): NSML.

Testis (slide 2): NSML.

Patient No: 30  
ID #: 3534  
Species: Mouse  
Strain/Breed: C57BL/6
Anatomic Pathology

Microscopic Findings
Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): Peribronchiolar lymphoid aggregate.
Heart (slide 2): NSML.
Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, diffuse, mild.
Spleen (slide 2): Mild, diffuse white pulp hyperplasia characterized by multifocal prominent germinal centers.
Testis (slide 2): NSML.

Patient No: 31
ID #: 3535
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 6 months
Sex: Male intact

Anatomic Pathology

Microscopic Findings
Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): Aorta (presumptive)- Mural chondroid metaplasia, multifocal, mild. Aortic valve leaflet- focal chondroid metaplasia, mild.
Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, diffuse, moderate.
Spleen (slide 2): NSML.
Testis (slide 2): NSML.

Patient No: 32
ID #: ILP-101
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 6 months
Sex: Male intact

Anatomic Pathology

Microscopic Findings
Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung: Underperfusion artifact precludes diagnosis.
Heart (slide 2): Aorta (presumptive)- Mural chondroid metaplasia, multifocal, mild. Aortic valve leaflet- focal chondroid metaplasia.
Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, centrilobular and midzonal, multifocal, mild.
Spleen (slide 2): NSML.
Testis (slide 2): NSML.
Patient No: 33
ID #: ILP-102
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 6 months
Sex: Male intact

Tests Ordered: Slide Evaluation

Anatomic Pathology

Microscopic Findings
Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): Multifocal, moderate interstitial lymphoplasmacytic aggregates near pelvis. Multifocal renal tubular ectasia with multifocal intratubular protein and mild thickening of glomerular capillary loops.
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): Aorta (presumptive)- Aortic valve leaflet- focal chondroid metaplasia.
Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, centrilobular and midzonal, multifocal, mild.
Spleen (slide 2): Mild, diffuse white pulp hyperplasia characterized by multifocal prominent germinal centers.
Testis (slide 2): NSML.

Patient No: 34
ID #: 153
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 9 weeks
Sex: Male intact

Tests Ordered: Slide Evaluation

Anatomic Pathology

Microscopic Findings
Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): NSML.
Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, diffuse, mild.
Spleen (slide 2): NSML.
Testis (slide 2): NSML.

Patient No: 35
ID #: 154
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 9 weeks
Sex: Male intact

Tests Ordered: Slide Evaluation

Anatomic Pathology

Microscopic Findings
Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): NSML.
Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, diffuse, mild.
Spleen (slide 2): NSML.
Testis (slide 2): NSML.

| Patient No: 36 | GEM: No | Tests Ordered: Slide Evaluation |
|---------------|---------|-------------------------------|
| ID #: ILP-148 | Construct: | |
| Species: Mouse | Promoter | Gene | Genotype |
| Strain/Breed: C57BL/6 | | | |
| Age/DOB: 9 weeks | | | |
| Sex: Male intact | | | |

**Anatomic Pathology**

**Microscopic Findings**

Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): No significant microscopic lesions (NSML).
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): No significant microscopic lesions (NSML).
Liver (slide 2): No significant microscopic lesions (NSML).
Spleen (slide 2): Mild diffuse increase in extramedullary hematopoiesis (EMH).
Testis (slide 2): No significant microscopic lesions (NSML).

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Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): NSML.
Liver (slide 2): NSML.
Spleen (slide 2): NSML.
Testis (slide 2): NSML.

| Patient No: 37 | GEM: No | Tests Ordered: Slide Evaluation |
|---------------|---------|-------------------------------|
| ID #: ILP-149 | Construct: | |
| Species: Mouse | Promoter | Gene | Genotype |
| Strain/Breed: C57BL/6 | | | |
| Age/DOB: 9 weeks | | | |
| Sex: Male intact | | | |

**Anatomic Pathology**

**Microscopic Findings**

Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): Multifocal, mild, interstitial lymphoplasmacytic aggregates near pelvis.
Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): NSML.
Liver (slide 2): NSML.
Spleen (slide 2): NSML.
Testis (slide 2): NSML.

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Lung (slide 2): Underperfusion artifact precludes diagnosis.
Heart (slide 2): NSML.
Liver (slide 2): NSML.
Spleen (slide 2): NSML.
Testis (slide 2): NSML.

| Patient No: 38 | GEM: No | Tests Ordered: Slide Evaluation |
|---------------|---------|-------------------------------|
| ID #: ILP-150 | Construct: | |
| Species: Mouse | Promoter | Gene | Genotype |
| Strain/Breed: C57BL/6 | | | |
| Age/DOB: 9 weeks | | | |
| Sex: Male intact | | | |
**Anatomic Pathology**

**Microscopic Findings**
- Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
- Kidney (slide 2): NSML.
- Lung (slide 2): Underperfusion artifact precludes diagnosis.
- Heart (slide 2): NSML.
- Liver (slide 2): NSML.
- Spleen (slide 2): NSML.
- Testis (slide 2): NSML.

**Patient No:** 39  
**ID #:** ILP-151  
**Species:** Mouse  
**Strain/Breed:** C57BL/6  
**Age/DOB:** 9 weeks  
**Sex:** Male intact

**Anatomic Pathology**

**Microscopic Findings**
- Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
- Kidney (slide 2): NSML.
- Lung (slide 2): NSML.
- Heart (slide 2): NSML.
- Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, diffuse, mild.
- Spleen (slide 2): NSML.
- Testis (slide 2): NSML.

**Patient No:** 40  
**ID #:** ILP-152  
**Species:** Mouse  
**Strain/Breed:** C57BL/6  
**Age/DOB:** 9 weeks  
**Sex:** Male intact

**Anatomic Pathology**

**Microscopic Findings**
- Diaphragm (2 sections; slide 1): No significant microscopic lesions (NSML).
- Kidney (slide 2): Focal lymphoplasmacytic aggregate at the renal pelvis.
- Lung (slide 2): NSML.
- Heart (slide 2): NSML.
- Liver (slide 2): Hepatocellular vacuolar change consistent with glycogen, multifocal, centrilobular to midzonal, mild.
- Spleen (slide 2): NSML.
- Testis (slide 2): NSML.

**Comments and Interpretation**

Most mice in all groups had white pulp (lymphoid) hyperplasia in the spleen characterized by multifocal prominent germinal centers. Due to its presence in treatment groups and control groups treated with saline, it is considered...
incidental and most likely attributed to non-specific antigenic stimulation.

Multiple mice in several treatment groups (including control mice) had a limited number of seminiferous tubules in the testis with degenerative spermatogenic epithelium. It was most consistently seen in the older mice (6 months) and is thus age associated. Multiple mice also had mineralization of a limited number of seminiferous tubules.

Mice 3515 and ILP-102 had some renal changes consisting of thickening of glomerular capillary loops, intratubular protein, and few interstitial lymphoplasmacytic aggregates that are attributed to older age associated changes commonly seen in mice.

Several mice from several groups had mild, multifocal lymphoplasmacytic aggregates within the renal interstitium.

Multiple mice in several groups had mild renal papillary mineralization. Renal mineralization is common in male C57BL/6 mice.

Mouse 3535, mouse ILP-102, and mouse ILP-101 had chondroid metaplasia of the aorta and chondroid metaplasia of the valve leaflet. Proposed underlying mechanisms include hypoxia.

Mice 3525 and ILP-148 had a mild, diffuse increase in extramedullary hematopoiesis within the red pulp of the spleen with an increase of both erythroid and myeloid precursors. Mice 3519 and 3520 had erythrocytic hyperplasia of the spleen. Extramedullary hematopoiesis is normally found in the spleen of mice and the degree can vary strain to strain. While some degree of extramedullary hematopoiesis is present in normal rodents, increased extramedullary hematopoiesis can result from hematotoxic insult, systemic anemia, and infections elsewhere in the body. For further hematopoietic evaluation, recommend submitting blood for CBC, bone marrow smear, and section of sternum and femur for histologic examination of bone marrow.

Mouse 3519 had increased extramedullary hematopoiesis and few pigmented macrophages within the sinusoids. Pigmentation can be incidental or secondary to cellular and erythroid breakdown products such as hemosiderin.

Microscopic aggregates of lymphocytes, as noted in the lung, liver, and kidney, of multiples mice, can be found widely distributed in the lungs, mediastinum, subcutaneous fascia, intestinal tract, urinary bladder, kidneys, etc. of mice, especially older mice. These lymphocytic aggregates are typically distributed as small perivascular cuffs, and are not in themselves indicative of pathology.

Technical Comments:

Most sections of lung were not perfused with formalin; therefore, an underperfusion artifact precluded proper evaluation.

| Tissue:  | N/A |
| Blocks:  | N/A |
| Frozen Specimens: | No |
| Slides: | Returned to PI |
| Photographs: | N/A |
| Resident: |  |
| Pathologist: | Sue E. Knoblaugh, DVM, Dipl. ACVP |
| Reported: | 8/10/2015 |
Experimental History
Toxicity study. Mice were treated with saline or viral vectors over-expressing a miRNA therapy. Slide evaluation of multiple tissues to determine toxicity. Tissues submitted include: slide 1-diaphragm and slide 2-testis, lung, spleen, kidney, liver, heart for each mouse. Mice received treatments between 4 and 6 weeks of age and were euthanized at 3 weeks, 6 weeks, 12 weeks and 5 months post treatment.

Anatomic Pathology
Microscopic Findings
Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There is diffuse, mild, vacuolar change consistent with glycogen.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.

Anatomic Pathology
Microscopic Findings
Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There is diffuse, mild, vacuolar change consistent with glycogen. There is a small focus of extramedullary hematopoiesis (EMH).
Spleen (slide 2): NSML.
Testes (slide 2): NSML.

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Patient No: 3  
ID #: 4125  
Species: Mouse  
Strain/Breed: C57BL/6  
Age/DOB: 11/4/2015  
Sex: Male intact

Anatomic Pathology

Microscopic Findings
Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There are multifocal cellular infiltrates, some of which, are associated with mild, multifocal, hepatocellular coagulative necrosis. There is diffuse, mild, vacuolar change consistent with glycogen.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.

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Patient No: 4  
ID #: 4126  
Species: Mouse  
Strain/Breed: C57BL/6  
Age/DOB: 11/4/2015  
Sex: Male intact

Anatomic Pathology

Microscopic Findings
Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): There is multifocal, mild renal papillary mineralization.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There are multifocal cellular infiltrates. There is diffuse, mild, vacuolar change consistent with glycogen.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.

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Patient No: 5  
ID #: 4213  
Species: Mouse  
Strain/Breed: C57BL/6  
Age/DOB: 11/17/2015

Anatomic Pathology

Microscopic Findings
Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): NSML.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.

Accession # 2016-3-334;
Sex: Male intact

Anatomic Pathology

Microscopic Findings
Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There are multifocal, mild to moderate cellular infiltrates.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.

Patient No: 6
ID #: 4214
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 11/17/2015
Sex: Male intact

GEM: No
Tests Ordered:

Anatomic Pathology

Microscopic Findings
Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There are multifocal, mild to moderate cellular infiltrates (EMH and neutrophils), some of which, are associated with hepatocellular coagulative necrosis. There is diffuse, mild, vacuolar change consistent with glycogen.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.

Patient No: 7
ID #: 3504
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 8/4/2014
Sex: Male intact

GEM: No
Tests Ordered:

Anatomic Pathology

Microscopic Findings
Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): Underperfusion artifact precludes evaluation.
Heart (slide 2): NSML.
Liver (slide 2): There are multifocal foci of extramedullary hematopoiesis (EMH); (~2). There is diffuse, mild, vacuolar change consistent with glycogen.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.
Anatomic Pathology

Microscopic Findings

Diaphragm (slide 1): No significant microscopic lesions (NSML).

Kidney (slide 2): NSML.

Lung (slide 2): Underperfusion artifact precludes evaluation.

Heart (slide 2): NSML.

Liver (slide 2): There are multifocal, mild cellular infiltrates. There is diffuse, mild, vacuolar change consistent with glycogen.

Spleen (slide 2): NSML.

Testes (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There are multifocal foci of extramedullary hematopoiesis (EMH); (~4).
Spleen (slide 2): NSML.
Testes (slide 2): NSML.

Anatomic Pathology

Microscopic Findings

Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): There is multifocal, mild renal papillary mineralization.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There are multifocal, moderate cellular infiltrates (EMH and neutrophils), some of which, are associated with mild, multifocal hepatocellular coagulative necrosis.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.

Anatomic Pathology

Microscopic Findings

Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): There is a focal, mild, interstitial lymphoid aggregate.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There are multifocal, moderate cellular infiltrates (EMH and neutrophils), some of which, are associated with mild, multifocal hepatocellular coagulative necrosis. There is mild, centrilocubular to midzonal hepatocellular vacuolar change consistent with glycogen.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.

Anatomic Pathology

Microscopic Findings

Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): There is a focal, mild, interstitial lymphoid aggregate.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There are multifocal, moderate cellular infiltrates (EMH and neutrophils), some of which, are associated with mild, multifocal hepatocellular coagulative necrosis. There is mild, centrilocubular to midzonal hepatocellular vacuolar change consistent with glycogen.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.
Sex: Male intact

### Anatomic Pathology

#### Microscopic Findings

- **Diaphragm (slide 1):** No significant microscopic lesions (NSML).
- **Kidney (slide 2):** NSML.
- **Lung (slide 2):** NSML.
- **Heart (slide 2):** NSML.
- **Liver (slide 2):** There is a focal, moderate cellular infiltrate associated with cellular necrosis. There is diffuse, mild, vacuolar change consistent with glycogen.
- **Spleen (slide 2):** NSML.
- **Testes (slide 2):** NSML.

### Patient No: 14

| ID #       | Species | Strain/Breed | Age/DOB | Sex   |
|------------|---------|--------------|---------|-------|
| 4212       | Mouse   | C57BL/6      | 11/17/2015 | Male intact |

### Anatomic Pathology

#### Microscopic Findings

- **Diaphragm (slide 1):** No significant microscopic lesions (NSML).
- **Kidney (slide 2):** NSML.
- **Lung (slide 2):** NSML.
- **Heart (slide 2):** NSML.
- **Liver (slide 2):** There are multifocal, mild to moderate cellular infiltrates. There is diffuse, mild, vacuolar change consistent with glycogen.
- **Spleen (slide 2):** NSML.
- **Testes (slide 2):** NSML.

### Patient No: 15

| ID #       | Species | Strain/Breed | Age/DOB | Sex   |
|------------|---------|--------------|---------|-------|
| 6wkctrl-1  | Mouse   | C57BL/6      | 11/17/2015 | Male intact |

### Anatomic Pathology

#### Microscopic Findings

- **Diaphragm (slide 1):** No significant microscopic lesions (NSML).
- **Kidney (slide 2):** NSML.
- **Lung (slide 2):** NSML.
- **Heart (slide 2):** NSML.
- **Liver (slide 2):** There are multifocal, mild to moderate cellular infiltrates. There is multifocal, mild, centrilocular, vacuolar change consistent with glycogen.
- **Spleen (slide 2):** NSML.
- **Testes (slide 2):** There is mild, focal, seminiferous tubular degeneration and atrophy (~2 tubules).
Anatomic Pathology

Microscopic Findings
Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There are multifocal, moderate cellular infiltrates. There is multifocal, mild, centrilobular, vacuolar change consistent with glycogen.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.

Anatomic Pathology

Microscopic Findings
Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There is focal extramedullary hematopoiesis (EMH); (~1). There is multifocal, mild, centrilobular, vacuolar change consistent with glycogen.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.
Kidney (slide 2): There is multifocal, mild, renal tubular ectasia and protein.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There is mild, centrilobular to midzonal hepatocellular vacuolar change consistent with glycogen.
Spleen (slide 2): NSML.
Testes (slide 2): There is mild, focal, seminiferous tubular degeneration and atrophy (~2 tubules).

Anatomic Pathology

Microscopic Findings
Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There is mild, centrilobular to midzonal hepatocellular vacuolar change consistent with glycogen.
Spleen (slide 2): NSML.
Testes (slide 2): There is mild, focal, seminiferous tubular degeneration and atrophy (~3 tubules).

Anatomic Pathology

Microscopic Findings
Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There are multifocal, mild to moderate cellular infiltrates.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.

Anatomic Pathology

Microscopic Findings
Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There are multifocal, mild to moderate cellular infiltrates.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.
### Anatomic Pathology

**Microscopic Findings**

**Diaphragm (slide 1):** No significant microscopic lesions (NSML).

**Kidney (slide 2):** NSML.

**Lung (slide 2):** NSML.

**Heart (slide 2):** NSML.

**Liver (slide 2):** There are multifocal, moderate cellular infiltrates. There is mild, diffuse hepatocellular vacuolar change consistent with glycogen.

**Spleen (slide 2):** NSML.

**Testes (slide 2):** NSML.

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| Patient No: 22 | GEM: No | Tests Ordered: |
|---------------|----------|----------------|
| ID #: 4209    | Construct: |
| Species: Mouse | Promoter Gene Genotype |
| Strain/Breed: C57BL/6 | |
| Age/DOB: 11/17/2015 | |
| Sex: Male intact | |

### Anatomic Pathology

**Microscopic Findings**

**Diaphragm (slide 1):** No significant microscopic lesions (NSML).

**Kidney (slide 2):** NSML.

**Lung (slide 2):** NSML.

**Heart (slide 2):** NSML.

**Liver (slide 2):** There is focal extramedullary hematopoiesis (EMH); (~1). There is diffuse, mild, vacuolar change consistent with glycogen.

**Spleen (slide 2):** NSML.

**Testes (slide 2):** NSML.

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| Patient No: 23 | GEM: No | Tests Ordered: |
|---------------|----------|----------------|
| ID #: 4101    | Construct: |
| Species: Mouse | Promoter Gene Genotype |
| Strain/Breed: C57BL/6 | |
| Age/DOB: 9/8/2015 | |
| Sex: Male intact | |

### Anatomic Pathology

**Microscopic Findings**

**Diaphragm (slide 1):** No significant microscopic lesions (NSML).

**Kidney (slide 2):** There is multifocal, mild, renal tubular ectasia and protein.

**Lung (slide 2):** NSML.

**Heart (slide 2):** NSML.

**Liver (slide 2):** There are multifocal, mild to moderate cellular infiltrates.

**Spleen (slide 2):** NSML.

**Testes (slide 2):** NSML.

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| Patient No: 24 | GEM: No | Tests Ordered: |
|---------------|----------|----------------|
| ID #:         | Construct: |
| Species: Mouse | Promoter Gene Genotype |
| Strain/Breed: C57BL/6 | |
| Age/DOB:      | |
| Sex: Male intact | |
**Anatomic Pathology**

**Microscopic Findings**

- **Diaphragm (slide 1):** No significant microscopic lesions (NSML).
- **Kidney (slide 2):** There is multifocal, mild renal papillary mineralization. There is a mild, focal perivascular lymphoid aggregate.
- **Lung (slide 2):** NSML.
- **Heart (slide 2):** NSML.
- **Liver (slide 2):** There are multifocal mild to moderate cellular infiltrates. There is diffuse, mild, vacuolar change consistent with glycogen.
- **Spleen (slide 2):** NSML.
- **Testes (slide 2):** There is multifocal seminiferous tubular degeneration and atrophy (~10 tubules).
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There is focal, mild extramedullary hematopoiesis (EMH); (~1).
Spleen (slide 2): NSML.
Testes (slide 2): NSML.

Patient No: 27
ID #: 4105
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 9/8/2015
Sex: Male intact

Anatomic Pathology

Microscopic Findings
Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): There is focal, mild mineral within the renal papilla. There are multifocal, mild interstitial lymphoid aggregates.
Liver (slide 2): NSML.
Heart (slide 2): NSML.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.

Patient No: 28
ID #: 4108
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 9/8/2015
Sex: Male intact

Anatomic Pathology

Microscopic Findings
Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): There is multifocal, mild, renal tubular ectasia and protein. There is focal renal papillary mineralization.
Liver (slide 2): NSML.
Heart (slide 2): NSML.
Spleen (slide 2): NSML.
Testes (slide 2): There is mild, focal, seminiferous tubular degeneration and atrophy (~4 tubules).

Patient No: 29
ID #: 4109
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 9/8/2015
Sex: Male intact
Anatomic Pathology

Microscopic Findings

Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): There is multifocal, mild, renal tubular ectasia and protein.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There is mild, focal, hepatocellular coagulative necrosis and mild neutrophilic cellular infiltrate.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.

Patient No:  30
ID #: 4110
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 9/8/2015
Sex: Male intact

Anatomic Pathology

Microscopic Findings

Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There is diffuse, mild, vacuolar change consistent with glycogen.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.

Patient No:  31
ID #: 4111
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 9/8/2015
Sex: Male intact

Anatomic Pathology

Microscopic Findings

Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): NSML.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.

Patient No:  32
ID #: 4112
Species: Mouse
Strain/Breed: C57BL/6
Age/DOB: 9/8/2015
Sex: Male intact

Accession # 2016-3-334;
Strain/Breed: C57BL/6
Age/DOB: 9/8/2015
Sex: Male intact

**Anatomic Pathology**

**Microscopic Findings**

Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): There is a focally extensive inflammatory infiltrate composed of moderate numbers of lymphocytes, plasma cells, and neutrophils within the renal pelvis with urothelial hyperplasia (probable chronic active pyelonephritis).
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): NSML.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.

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Patient No: 33  
ID #: 4128  
Species: Mouse  
Strain/Breed: C57BL/6  
Age/DOB: 11/8/2015  
Sex: Male intact

**Anatomic Pathology**

**Microscopic Findings**

Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): There is a mild, focal perivascular lymphoid aggregate within the renal pelvis.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There is diffuse, mild, vacuolar change consistent with glycogen.
Spleen (slide 2): NSML.
Testes (slide 2): There is multifocal seminiferous tubular degeneration and atrophy (~3 tubules).

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Patient No: 34  
ID #: 4129  
Species: Mouse  
Strain/Breed: C57BL/6  
Age/DOB: 11/8/2015  
Sex: Male intact

**Anatomic Pathology**

**Microscopic Findings**

Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): There is a focal perivascular lymphoid aggregate. Acidophilic macrophage pneumonia.
Heart (slide 2): NSML.
Liver (slide 2): There are multifocal, mild to moderate cellular infiltrates. There is diffuse, mild, vacuolar change consistent with glycogen.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.

Anatomic Pathology

Microscopic Findings
Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There are multifocal, mild to moderate cellular infiltrates. There is diffuse, mild, vacuolar change consistent with glycogen.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.

Anatomic Pathology

Microscopic Findings
Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There are multifocal, mild cellular infiltrates. There is diffuse, mild, vacuolar change consistent with glycogen.
Spleen (slide 2): NSML.
Testes (slide 2): There is mild, focal, seminiferous tubular degeneration and atrophy (~7 tubules).
Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There are multifocal, mild cellular infiltrates. There is diffuse, mild, vacuolar change consistent with glycogen.
Spleen (slide 2): NSML.
Testes (slide 2): There is mild, multifocal foci of seminiferous tubular degeneration and atrophy.

| Patient No: | 38 | GEM: No | Tests Ordered: |
|-------------|----|---------|----------------|
| ID #: | 4201 | Construct: |
| Species: | Mouse | |
| Strain/Breed: | C57BL/6 | |
| Age/DOB: | 11/1/2015 | |
| Sex: | Male intact | |

Anatomic Pathology

Microscopic Findings
Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): There is a mild, focal, perivascular lymphoid aggregate.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There are multifocal, mild to moderate cellular infiltrates. There is diffuse, mild, vacuolar change consistent with glycogen.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.

| Patient No: | 39 | GEM: No | Tests Ordered: |
|-------------|----|---------|----------------|
| ID #: | 4202 | Construct: |
| Species: | Mouse | |
| Strain/Breed: | C57BL/6 | |
| Age/DOB: | 11/1/2015 | |
| Sex: | Male intact | |

Anatomic Pathology

Microscopic Findings
Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): There is multifocal, mild renal papillary mineralization.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There is diffuse, mild, vacuolar change consistent with glycogen and hepatocellular microvacuolar lipidosis.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.

| Patient No: | 40 | GEM: No | Tests Ordered: |
|-------------|----|---------|----------------|
| ID #: | 4203 | Construct: |
| Species: | Mouse | |

Accession #: 2016-3-334;
Anatomic Pathology

Microscopic Findings
Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): There are multifocal interstitial lymphoid aggregates, particularly within the renal pelvis.
Lung (slide 2): NSML.
Heart (slide 2): NSML.
Liver (slide 2): There is diffuse, mild vacuolar change consistent with glycogen.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.
Testes (slide 2): NSML.

Anatomic Pathology

Microscopic Findings

Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): There is multifocal, mild, renal papillary mineral.
Lung (slide 2): Underperfusion artifact precludes evaluation.
Heart (slide 2): NSML.
Liver (slide 2): There is a focal perivascular lymphoid aggregate. There is diffuse, mild, vacuolar change consistent with glycogen.
Spleen (slide 2): NSML.
Testes (slide 2): There is mild, focal, seminiferous tubular degeneration and atrophy (~1 tubule).

Anatomic Pathology

Microscopic Findings

Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): Underperfusion artifact precludes evaluation.
Heart (slide 2): NSML.
Liver (slide 2): There are multifocal foci of extramedullary hematopoiesis (EMH); (~1). There is diffuse, mild, vacuolar change consistent with glycogen.
Spleen (slide 2): NSML.
Testes (slide 2): Diffuse, severe, seminiferous tubule degeneration and atrophy.

Anatomic Pathology

Microscopic Findings
Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): Underperfusion artifact precludes evaluation.
Heart (slide 2): NSML.
Liver (slide 2): There is diffuse, mild, vacuolar change consistent with glycogen.
Spleen (slide 2): NSML.
Testes (slide 2): There is mild, focal, seminiferous tubular degeneration and atrophy (~2 tubules).

Anatomic Pathology

Microscopic Findings
Diaphragm (slide 1): No significant microscopic lesions (NSML).
Kidney (slide 2): NSML.
Lung (slide 2): Underperfusion artifact precludes evaluation.
Heart (slide 2): NSML.
Liver (slide 2): There are multifocal foci of extramedullary hematopoiesis (EMH); (~2). There is diffuse, mild, vacuolar change consistent with glycogen.
Spleen (slide 2): NSML.
Testes (slide 2): NSML.

Comments and Interpretation
At three weeks post treatment, several mice had multifocal cellular infiltrates within the liver. The infiltrates varied from foci of extramedullary hematopoiesis to inflammatory cells. Often, it was difficult to distinguish by H&E staining. In two mice (4125, 4214) the cellular infiltrate was associated with coagulative hepatocellular necrosis characterized by loss of normal cellular structure and pyknosis or absence of hepatocyte nuclei surrounded by few to moderate numbers of neutrophils. There were also multifocal foci of single cell hepatocyte necrosis or apoptosis associated with few infiltrating neutrophils dispersed randomly in the parenchyma. Interestingly, the three week control mouse (3505) had a single focus of extramedullary hematopoiesis, but did not have hepatocellular necrosis. The underlying cause of the necrosis and neutrophilic inflammation, and any relationship to the AAV vectors overexpressing miRNA therapy are not known. Correlation of histopathology with serum biochemistry (specifically markers of liver function such as ALT, AST, GGT, triglycerides, etc.) is recommended. Lesions such as this have been reported as incidental findings in mice. It is speculated that it could be due to embolization of bacteria from the gastrointestinal tract.

At six weeks post treatment, several mice had multifocal cellular infiltrates within the liver. The infiltrates varied from foci of extramedullary hematopoiesis to inflammatory cells. Often, it was difficult to distinguish by H&E staining. In two mice (4210, 4211) the cellular infiltrate was associated with coagulative hepatocellular necrosis characterized by loss of normal cellular structure and pyknosis or absence of hepatocyte nuclei surrounded by few to moderate numbers of neutrophils. Interestingly, the six week control mouse (Bl6Cont 2) had increased foci of cellular infiltrate and associated coagulative hepatocellular necrosis. As changes noted were in both treated and control mice, the findings are likely background changes and can be secondary to bacterial embolization from the GI tract. These lesions are also comparable to those seen in hepatitis due to Helicobacter hepaticus infection. Recommend testing for Helicobacter spp. for definitive confirmation.

At twelve weeks post treatment, two mice (4207, 4208) had moderate cellular infiltrates that varied from foci of
extramedullary hematopoiesis to inflammatory cells. Often, it was difficult to distinguish by H&E staining. Controls were not provided for evaluation for the twelve week group.

At five months post treatment, several mice had multifocal cellular infiltrates within the liver. The infiltrates varied from foci of extramedullary hematopoiesis to inflammatory cells. Often, it was difficult to distinguish by H&E staining. A single mouse (4109) had coagulative hepatocellular necrosis characterized by loss of normal cellular structure and pyknosis or absence of hepatocyte nuclei surrounded by few to moderate numbers of neutrophils.

Most mice had evidence of hepatocellular vacuolar change in the liver, consistent with glycogenosis and/or micro- to macrovacuolar hepatocellular lipidosis. The glycogen content of the liver varies depending on the physiological state of the animal. Glycogen accumulation can also be observed as a manifestation of toxicity or with glycogen storage diseases. Hepatic lipidosis, also known as steatosis, fatty change, cytoplasmic vacuolization, etc. is a common hepatic lesion in mice. It is due to an accumulation of triglycerides, either spontaneously or as a response to toxic agents with different lobular locations reflecting differences in metabolic activation of the hepatotoxin.

Multiple mice in all groups had foci of extramedullary hematopoiesis (EMH). Extramedullary hematopoiesis (EMH) can be observed in the rodent liver occasionally in response to an increased hematopoietic demand. Precipitating factors for the occurrence are: anemia, stress, xenobiotic toxicity, infection, neoplasia (e.g., histiocytic sarcoma), and pregnancy. The increased EMH was seen in the livers of both treated and control mice. The spleens of these mice were normal. Recommend compare to bone marrow and CBC findings to further investigate.

Multiple mice in all groups had a limited number of seminiferous tubules in the testis which were characterized by degenerative spermatogenic epithelium consistent with mild atrophy. This was seen in both treated and control mice and is thus, considered a background finding. Testicular atrophy increases with age. Sections evaluated did not contain the epididymis. Recommend evaluation of the epididymis for mature spermatozoa. Recommend the use Modified Davidson's fixative to prevent tissue artifact.

Several mice in all groups had multifocal, mild renal papillary mineralization and mild tubular ectasia with intraluminal protein. Renal lesions are common background findings in C57BL/6 mice and increase in frequency with age.

One mouse (4129) had acidophilic macrophage pneumonia in the lung. Eosinophilic crystal-laden alveolar macrophages were originally diagnosed as acidophilic macrophage pneumonia (AMP) in various strains (NMRI, T x HT, C57BL) of mice back in 1990 (Veterinary Pathology 27: 274-281, 1990). Since then, AMP has been reported in various genetically engineered mice on C57BL/6, 129, and B6;129 backgrounds. AMP can be severe enough to produce clinical illness and be a major cause of death in control populations (Pathology of Genetically Engineered Mice. JM Ward, JF Mahler, RR Maronpot, JP Sundberg (eds). 1st Edition, Iowa State University Press, 2000.). Although not reported in this mouse, hyalinosis is an associated lesion whereby epithelial cells with intensely eosinophilic cytoplasm can be found in the nasal cavity, lung, glandular stomach, gall bladder, bile and pancreatic ducts, and ureter. These eosinophilic proteins have been identified as chitinase 3-like 3 (Chi3l3) [formerly, Ym-1, also known as eosinophil chemotactic factor (ECF-L)] and the highly related Ym-2 (95% identity) (Journal of Biological Chemistry 275: 8032-8037, 2000; American Journal of Pathology 158: 323-332, 2001; Journal of Biological Chemistry 277; 5468-5475, 2002). Chi3l3/Ym-1 is a unique functional marker for alternatively activated macrophages in Th2-mediated inflammatory responses.

There are multifocal aggregates of lymphocytes and plasma cells in several organs for most mice evaluated. Microscopic aggregates of lymphocytes, as noted in the lung, kidney, liver and/or diaphragm of multiple mice, can be found widely distributed in the lungs, mediastinum, subcutaneous fascia, intestinal tract, urinary bladder, kidneys, etc. of mice, especially older mice. These lymphocytic aggregates are typically distributed as small perivascular cuffs, and are not in themselves indicative of pathology.

**Technical Comments:**
Testes sections had tissue artifact. Recommend fixing in Modified Davidson's fixative to alleviate artifacts. Several sections were torn. Use of a sharp blade is recommended for tissue trimming. Ideally, organs for each animal should be
trimmed consistently among groups and oriented on slides in the same order both within and among groups. There was underperfusion artifact in some pulmonary sections. Recommend complete intratracheal inflation with fixative to preclude artifact.

| Tissue:       | N/A |
|---------------|-----|
| Blocks:       | N/A |
| Frozen Specimens: | No  |
| Slides:       | Returned to PI |
| Photographs:  | Microscopic |
| Resident:     | N/A |
| Pathologist:  | Sue E. Knoblaugh, DVM, Dipl. ACVP |
| Reported:     | 8/24/2016 |
Representative Images

40X 3 Week Treated (4125): scAAV.miDUX4.405
Coagulative necrosis

40X 3 Week Control (3505)
Extramedullary hematopoiesis (EMH)
40X 3 Week Treated (4213): scAAV.miDUX4.1155; 40X 3 Week Treated (4214): scAAV.miDUX4.1155

Cellular infiltrate associated with hepatocellular necrosis
Hepatocellular coagulative necrosis with infiltrating neutrophils
Renal tubular ectasia with intraluminal protein (arrows)

Focal renal papillary mineral (arrow)