**Finalized BCM-Rice SATC Validation Plan cc47**

*Asokan Delivery Team: Evolving High Potency AAV Vectors for Neuromuscular Genome Editing*

1. Project summary and overall goal (provided by the Delivery Team)

Our delivery system is based on novel AAV strains evolved following cross-species cycling. Delivery efficiency studies are currently underway in mice and pigs with the goal of selecting lead candidates, two of which will be advanced to the current study design.

1. Delivery system details
2. *Delivery vehicle*: Evolved AAV mutant, produced in the Asokan lab at Duke University for mouse studies.
3. *Editing system to be delivered*: DNA encoded SauCas9 with an appropriate DNA encoded sgRNA.
4. *Delivery controls to be employed*: DNA encoded Cre recombinase will be used as a delivery control for toxicity and inflammation, and as positive control for imaging.
5. *Delivery vehicle storage*: AAV virus preparations, provided as single use 450-500 l aliquots (sufficient for 2 animal injections), should be stored at -80°C before use. Thaw on ice before use. Do not re-freeze. Once thawed, virus is stable at 4°C for 72h.
6. *Delivery vehicle known adverse effects*: No known adverse effects of vector administration were identified by the Delivery Team.
7. *Target tissue*: muscle
8. Reporter animal details
9. *Reporter line to be utilized*: Ai9 allele. Cre-mediated activation of tdTomato can be used as a delivery system positive control.
10. *sgRNA target sequences to be utilized*:

5’ ACGAAGTTATATTAAGGGTT (CCGGAT)

5’ CTCTAGAGTCGCAGATCCTC (TAGAGT)

1. Study design:
2. *Route of Administration*: Standard tail vein injection, vector diluted in saline. BCM IACUC has approved AAV tail vein injections.
3. *Dosage and volume to be administered*: 1.0x1014 vg copies/kg (vg/kg) (2.0x1012/20g mouse in 200 µl).
4. *Age of administration:* 8 weeks.
5. *Study time course*: Mice will be euthanized at 4 weeks after delivery system administration for tissue collection.
6. *Experimental group:* One group of 12 animals (6 males and 6 females) will be injected with two AAV vectors expressing SauCas9 and the appropriate sgRNA.
7. *Control groups*: One group of 4 animals (2 males and 2 females) will be injected with an AAV vectors expressing Cre recombinase. The BCM-Rice SATC will provide a group of 4 animals (2 males and 2 females) tail vein injected with saline as a control group for normal tissue histology, a negative control group for fluorescent reporter imaging, and a wild-type control for molecular analyses.
8. *Measurements during study:* Body weights will be noted at the time of delivery system application and euthanasia.
9. *Study failure:* If editing fails to be detected in the target tissue or if at least 4 male and 4 females from an experimental group do not complete the study, the group may be repeated with modifications one additional time.

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| **Summary of study design** | | | | | | | | |
| **Study Arm** | **Animal Model** | **Delivery System** | **Route of Admin** | **Age of admin** | **Concentration and volume** | **Animal Numbers** | **Study Time Course** | **Exp or Cntrl Group** |
| 1 | single-guide SpyCas9/SauCas9 Ai9 | AAV-CC47-SauCas9 | IV (tail vein) | 8 wks | 2.0x1012/20g mouse in 200 µl | 12  (6 male, 6 female) | 4 wks | Experimental |
| 2 | single-guide SpyCas9/SauCas9 Ai9 | AAV-CC47-Cre | IV (tail vein) | 8 wks | 2.0x1012/20g mouse in 200 µl | 4  (2 male, 2 female) | 4 wks | Pos Control |
| 3 | single-guide SpyCas9/SauCas9 Ai9 | saline | IV (tail vein) | 8 wks | 200 µl | 4  (2 male, 2 female) | 4 wks | Neg Control |

1. Tissue collection and analysis:
   1. *Tissues to be collected*: Target tissues for editing are liver and heart.

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| White adipose tissue (subcutaneous and perigonadal collected and imaged separately) | Pancreas | Muscle (gastrocnemius, soleus, TA, EDL, collected and imaged separately) |
| Epididymis or Uterus | **Heart** | Brown adipose tissue |
| Testes or Ovary | Lung | Brain |
| **Liver** | Diaphragm | Eye |
| Kidney | Stomach |  |
| Spleen | Intestine (duodenum, jejunum, ileum, colon collected and imaged separately) |  |

Four weeks after administration, tissues will be collected and processed for imaging and molecular analyses as described in the attached protocol.

1. *Reporter imaging and analysis*:
   * Each tissue will be PFA fixed, frozen, sectioned, stained with DAPI, and imaged, as described in the attached protocol with the following modification. Three non-consecutive sections will be imaged from each tissue as described in the attached protocol.
   * Data reported:
2. For all tissues in Study Arms 1 and 2 (experimental and control group) except for muscle: the extent of fluorescence will be quantified as (1) fluorescent (tdTomato from nuclease- or Cre-activated reporter) cells per DAPI-stained nucleus. The total counted, total tdTomato-positive counted, and percentage tdTomato-positive cells in each tissue will be reported for each animal.
3. Tissues from Study Arm 3 animals will be used as imaging negative controls.
4. All collected data, including images, will be transmitted to the DCC.
5. *Health evaluation and histology*: Animals in each experimental and control group will be monitored for observable changes in health. Additionally, for each animal in each group, study start and end body weights and the weights of spleens, livers and the target tissues (indicated muscle groups) will be reported as an indicator of overall health and potential inflammation response. For each animal in each group, three non-consecutive sections of spleen, liver, and muscle will be processed for H&E staining as described in the attached protocol, and scores of immunogenicity or other morphological anomalies provided.
6. *Molecular Analyses:* For experimental groups, genomic DNA extracted from frozen samples of target tissue (indicated muscle groups) will be analyzed by ddPCR to confirm bulk tissue genome editing frequency at the reporter allele. In addition, targeted deep sequencing will be performed to detect off-target CRISPR-mediated editing events at the top 10 predicted sites predicted by the COSMID algorithm. Saline injected animals will function as the control group for these analyses.
   * Data reported:
     1. On-target editing: edited allele frequency detected in the bulk tissue.
     2. Off-target editing: locations and sequence of off-target events and allele (read) frequency in bulk tissue.
7. Special requests

Tissue blocks and slides will be saved and provided to the Delivery Team for co-labeling with cell type-specific antibodies and additional imaging.

1. Timeline
   1. Testing of Delivery Systems will commence by 7/31/2020 and will be scheduled in coordination with the other Delivery Teams assigned to the BCM-SATC.
   2. Data (excluding off-target analysis, which is not required for Delivery Team grant transition reports) will be provided by 4/01/2021. Off-target analysis reports should be available by 7/31/2021.

**Baylor/Rice SATC tissue preparation and imaging standard protocols**

Tissue Preparation for Imaging

1) After euthanasia and removal of the white fat pads and the reproductive organs, the inferior vena cava will be slit below the liver. The left ventricle will be punctured, and the animal injected with 20 ml of cold PBS through the bloodstream.

2) Remove the organ or tissue sample from the euthanized mouse and remove extraneous material.

3) Remove a small section and freeze immediately in liquid nitrogen for molecular analyses.

4) Place organ in 10 ml of freshly-prepared 4% paraformaldehyde in a 20 ml vial.

5) Incubate for 20 - 24 hours at 4˚C with gentle agitation.

6) Remove paraformaldehyde solution and replace with 30% sucrose in PBS.

7) Incubate for 20 - 24 hours at 4˚C with gentle agitation.

8) Embed tissues in OCT, marking orientation for sectioning.

9) Freeze at -80˚C.

Sectioning and Imaging

1) Section frozen tissue blocks at 14 microns, and place three non-consecutive sections on one slide.

3) For each tissue, one slide will be mounted with DAPI stain and imaged on a Zeiss Axio Scan.Z1 scanner, using the 20X objective and Cy5 (for tdTomato) and DAPI fluorescent filters.

4) For the liver, spleen, and target tissue of interest, one slide will be prepared for H&E staining to assess histopathology and immune infiltration.

Analysis

1) Examine images using ZEN software. Note:

a) Presence or absence of tdTomato signal and other fluorescent signals

b) Structural integrity of organ, based on DAPI staining

c) attempt to identify type of cell expressing tdTomato or other fluorescent signals

d) attempt to identify a pattern of tdTomato expression or other fluorescent signals

2) Quantify extent of tdTomato fluorescence using ImageJ

a) Count nuclei using Threshold/Binarize/Watershed algorithms

b) Count fluorescent positive cells, either manually, or using Threshold/Binarize/Watershed algorithms

c) Report data as percentage of positive cells per nuclei