**Finalized BCM-Rice SATC Validation Plan**

*McCray Delivery Team: Delivery of CRISPR Ribonucleoproteins to Airway Epithelia Using Novel Amphiphilic Peptides*

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

We engineered amphiphilic peptides that enable delivery of proteins and SpCas9 ribonucleoprotein (RNP) to cultured human well-differentiated airway epithelial cells and mouse lungs. These shuttle peptides, non-covalently combined with GFP protein or CRISPR associated nuclease (Cas) RNP, allow rapid entry into cultured human ciliated and non-ciliated epithelial cells and mouse airway epithelia. Instillation of shuttle peptides combined with SpCas9 achieves editing of loxP sites in airway epithelia of ROSAmT/mG mice.

Using Cas9, we observed an editing efficiency in the large airways of 13 ± 2% (mean ± SE). In the small airways, the editing efficiency was 12 ± 1% (mean ± SE)

REF: Krishnamurthy, S., Wohlford-Lenane, C., Kandimalla, S. et al. Engineered amphiphilic peptides enable delivery of proteins and CRISPR-associated nucleases to airway epithelia. Nat Commun 10, 4906 (2019)

1. Delivery system details
2. *Delivery vehicle*: amphiphilic peptide is synthetized by GL Biochem (Shanghai, China). Lyophilized peptide is solubilized, dosed and tested by Feldan Therapeutics prior in vivo study.
3. *Editing system to be delivered*: SpyCas9.
4. *Delivery controls to be employed*: Amphiphilic peptide alone will be used as a delivery control for toxicity and inflammation. Amphiphilic peptide harboring SpyCas9 RNP will be used as a positive control for delivery and imaging.
5. *Delivery vehicle storage*:

Amphiphilic peptides: -80°C, peptide stock 250 µM

SpyCas9 nuclease: -20°C, nuclease stock at 61 µM

crRNA+tracrRNA: -80°C, gRNA stock at 100 µM

All reagents are stable for more than 6 months.

1. *Delivery vehicle known adverse effects*: No known adverse effects of vector administration were identified by the Delivery Team.
2. *Target tissue*: lung airway epithelium
3. *Timeline and notification of Delivery System shipment:* The Delivery Team will ship delivery reagents to the BCM SATCno later than November 20, 2020. The Delivery Team will notify the BCM SATC on the day of shipment so that the testing center can order mice of the appropriate ages to conduct the proposed studies.
4. Reporter animal details
5. *Reporter line to be utilized*: C57BL6-*Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo* referred to as “mt/mg”*.* Animals from this line will be purchased from JAX. This is the same allele previously used in the McCray Nature Communications 2019 paper.
6. *sgRNA target sequences to be utilized*:
   * SpyCas9 g-loxP2\_C9: Target sequence 5’ – CATTATACGAAGTTATATTA – 3’IDT Alt-R® CRISPR-Cas9 tracrRNA
7. Study design:
8. *Route of Administration*: Intranasal inhalation while animal is anesthetized. Two administrations will be required over 2 consecutive days.BCM IACUC is approved. Staff from the McCray lab will assist with the protocol.
9. *Dosage and volume to be administered*: For genome editing, a volume of 50 µl shuttle peptide (40 µM) formulated with 2.5 μM SpyCas9 nucleases and 2 μM crRNA+tracrRNA will be prepared at the BCM-SATC site. A detailed delivery system preparation protocol will be provided by the Delivery Team and will follow the published methods described in Krishnamurthy et al.
10. *Age of administration:* 8 weeks.
11. *Study time course*: Mice will be euthanized at day 8 of the study (day 1 being the day of first instillation).
12. *Experimental group:* One group of 12 animals (6 males and 6 females) will be administered **amphiphilic** **peptide D10.** Onegroup of 12 animals (6 males and 6 females) will be administered **amphiphilic** **peptide D237**.
13. *Control groups*: One group of 4 animals (2 males and 2 females) will be administered with **amphiphilic** **peptide D10 alone**. One group of 4 animals (2 males and 2 females) will be administered with **amphiphilic** **peptide D237 alone**. One group of 4 animals (2 males and 2 females) will be administered SpyCas9 RNP alone. One group of 4 animals (2 males and 2 females) will be administered saline alone as a negative control for imaging and histology/toxicity.
14. *Measurements during study:* Body weights will be noted at the time of delivery system application and euthanasia.
15. *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 | mt/mg | Amphiphilic peptide D10 + SpyCas9 RNP | Intranasal  inhalation | 8 wks | 50 µl of 40 µM peptide 2.5 µM SpCas9 and 2 µM gRNA | 12  (6 male, 6 female) | Instillation at day 1 and 2, Tissue collection day 8 | Experimental |
| 2 | mt/mg | Amphiphilic peptide D237+ SpyCas9 RNP | Intranasal  inhalation | 8 wks | 50 µl of 40 µM peptide 2.5 µM SpCas9 and 2 µM gRNA | 12  (6 male, 6 female) | Instillation at day 1 and 2, Tissue collection day 8 | Experimental |
| 3 | mt/mg | Amphiphilic peptide D10 | Intranasal  inhalation | 8 wks | 50 µl of 40 µM peptide | 4  (2 male, 2 female) | Instillation at day 1 and 2, Tissue collection day 8 | Delivery vehicle control arm 2 |
| 4 | mt/mg  saline | SpyCas9 RNP | Intranasal  inhalation | 8 wks | NA | 4  (2 male, 2 female) | Instillation at day 1 and 2, Tissue collection day 8 | SpyCas9 RNP alone neg control |
| 5 | mt/mg | Saline | Intranasal  inhalation | 8 wks | NA | 4  (2 male, 2 female) | Instillation at day 1 and 2, Tissue collection day 8 | Neg Control for toxicity and imaging |

*Order of priority: The BCM SATC will need to order mt/mg allele mice from JAX and use those animals directly for delivery system administration. JAX has indicated that sufficient animals (we inquired about 30; this new plan requires 32) of appropriate ages will be available for purchase to perform the proposed studies starting in November 2020. However, the SATC will prioritize delivery system administration in the following order to ensure that critical data are collected to meet the Delivery System grant milestones: Study Arm 1, Study Arm 2, Study Arm 4, Study Arm 5, Study Arm 3. Study Arm 5 is prioritized over Arms 4 and 5 as negative controls for imaging are required.*

*If JAX indicates that appropriate numbers of animals at appropriate ages are no longer available, we will reduce animal numbers in each study arm and prioritize experiments in the same order as above. For the experimental group (Study Arm 1), a minimum of 3 males and 3 females will be used; for the positive control group (Study Arm 2) a minimum of 2 males and 2 females will be used. Study arms 3, 4, and 5 will be conducted with at least 1 male and 1 female if additional animals are available.*

1. Tissue collection and analysis:
   1. *Tissues to be collected*:

|  |  |  |
| --- | --- | --- |
| 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 | **Trachea\*** |
| Spleen | Intestine (duodenum, jejunum, ileum, colon collected and imaged separately) |  |

Tissues will be collected and processed for imaging and molecular analyses as described in the attached protocol.

\* For the lung, samples will be inflated with PBS during fixation to improve imaging of the airways. Trachea is not part of our standard tissue collection set but will be added for imaging analysis as requested by the Delivery Team.

1. *Reporter imaging and analysis*:
   * Each tissue will be PFA fixed, frozen, sectioned, stained with DAPI, and imaged, as described in the attached protocol. Three non-consecutive sections will be imaged from each tissue and analyzed as described in the attached protocol.
   * Data reported:
2. For all tissues except the lung in Study Arms 1 and 2 (experimental and positive control groups): the extent of fluorescence will be quantified as fluorescent (GFP from nuclease-activated reporter) cells per DAPI-stained nucleus. The total counted, positive counted, and percentage positive cells for each animal will be reported.
3. For lung from animals in Study Arms 1 and 2: the extent of fluorescence will be quantified as fluorescent (GFP from nuclease-activated mt/mg allele) cells per DAPI-stained nucleus amongst airway epithelial cells. The airway epithelium is morphologically distinct and epithelial cells can be counted separate from the bulk tissue. The total counted, positive counted, and percentage positive airway epithelial cells per airway will be reported for each animal. Large and small airways will be counted and analyzed separately.
4. For Study Arms 1 and 2, all tissues except lung, the average percent positive cells counted in female, male, and sexes combined will be reported. For the lung, the average percent GFP positive epithelial cells per small or large airway in the lung of females, males, and sexes combined will be reported.
5. For Study Arms 1 and 2, patterns of fluorescence observed in each tissue will be described (e.g. tdtomato to GFP positive cells localized to areas around blood vessels or specific cell types that can be determined).
6. Tissues from Study Arm 3 and 4 animals will not be imaged for reporter activity.
7. Tissues from Study Arm 5 animals will be used as imaging negative controls.
8. All collected data, including images, will be transmitted to the DCC.
9. *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 (lung) 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 lung will be processed for H&E staining as described in the attached protocol, and scores of immunogenicity or other morphological anomalies provided.
10. *Molecular Analyses:* For experimental groups, genomic DNA extracted from frozen samples of target tissue (lung) 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.
11. 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 if needed.

1. Timeline
   1. Testing of Delivery Systems can commence by mid November 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 15% and 30% sucrose in PBS on two consecutive days.

7)

Incubate each 15% and 30% sucrose gradient processing step 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 (currently 8 um), and place three non-consecutive sections on one slide.

2) 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 488 bm (for GFP), Cy5 (for tdTomato), DAPI, and/or far red fluorescent filters.

3) 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 GFP signal and other fluorescent signals

b) Structural integrity of organ, based on DAPI staining

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

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

2) Quantify extent of GFP fluorescence or other fluorescent signals 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