DISC2-09637- Genome Editing to Correct Cystic Fibrosis Mutations in Airway Stem Cells: Update

We are providing this written document to the ICOC in advance of their deliberations as an update on our substantial progress and to address the concerns of the reviewers. During the public discussion phase, we will supplement this update with a three-minute oral presentation. We note that cystic fibrosis (CF) sinus disease is an important health problem in California as essentially every CF patient develops chronic sinusitis and thus our approach has potentially great clinical impact. We also note that we are unable to identify other CIRM grants that is focused on airway stem cells and thus this proposal would broaden the CIRM portfolio.

Progress Since Proposal Submission

In the original proposal we showed important data that we could use the combination of ribonucleoprotein (RNP) delivery of the CRISPR/Cas9 nuclease combined with rAAV6 donor delivery to achieve high frequencies (25%) of homologous recombination (HR)-mediated editing of primary human bronchial epithelial cells at a safe harbor locus (*CCR5*). In the submission to CIRM, we proposed to develop both a ΔF508 specific and a Universal genome editing system for the CFTR gene. Since the submission we have: 1) Identified highly active CRISPR/Cas9 nucleases for both systems (Fig. 1); 2) Designed AAV donor vectors for each target; and 3) Demonstrated that we can achieve 20% HR frequencies into the Universal donor site in airway basal stem cells from both normal donor and CF patients (Fig. 2). Importantly, we have also developed a CRISPR/Cas9 and rAAV6 system that allows integration of cassettes larger than 4.7 kb into a specific site (manuscript submitted). This now gives us a mechanism in the Universal system to not only target the full CFTR cDNA to the site but it also allows us to include a selectable cell surface marker (such as tNGFR (Dever et al, Nature 2016)) to enrich targeted cells to greater than 90% purity.

We also showed in our proposal that we could differentiate lower airway basal stem cells into lung organoids containing the major features, including cilia and CFTR expression, of lung epithelium. Since the submission we have now shown that we can use identical conditions to expand and differentiate sinus/upper airway basal stem cells from patients with CF into lung organoids (Fig. 3). Using this 3-dimenisional system, we achieve **more than three log** expansion of patient-derived sinus airway cells. 60-75% cells present in organoids are basal stem cells (Keratin 5+). The patient-derived sinus airway derived organoids mirror organoids derived from lower airway basal cells from normal donors.

In sum, we believe this excellent progress demonstrates not only the scientific feasibility of the project but also how collaborative and productive our multi-disciplinary team is.

Response to Concerns Raised by Scientific Review

Rationale for Strategic Approach

We agree that identifying methods to enrich for modified cells to high degrees of purity could be a critical parameter for success. We recently demonstrated that we can purify HR-edited stem cells to >90% purity using cell surface markers (Dever et al, Nature 2016). The challenge for the Universal system was to be able to target both the large CFTR cDNA and a selection marker to the locus because that would exceed the packaging limit of AAV. We can now target cassettes larger than 4.7 kb using AAV (manuscript submitted) so that we can build in an enrichment strategy as suggested.

We have chosen to develop two systems because in our studies of the beta-globin locus (sickle cell disease) we found ~2-fold higher HR frequencies (50% gene correction) for single nucleotide changes compared to cDNA insertion (Dever et al, Nature 2016). We felt that it was prudent to develop the two systems in parallel (particularly since the ΔF508 allele is so prevalent). At the end of the funding period, however, we may discover that one system is significantly superior and we will then focus on that system in our subsequent work.

As we discussed in the proposal, we are using the nasal cavity and sinuses as an entry point for developing the strategy and eventually clinical proof of concept. We agree with the reviewer that the lower airway is the long-term goal, but chronic sinus disease in patients with CF is a major clinical and economic burden that impacts nearly all CF patients. Moreover, the sinus cavity, as an adjacent but related airway tissue site to the lungs, is clearly a much safer and more accessible location to optimize engraftment of gene corrected airway stem cells. What we learn from these formative experiments in the nasal airways of CF patients will serve as a platform for future work in the lower airways.

Ability to Edit the *CFTR* gene in Airway Cells

Reviewers were concerned about our ability to edit the *CFTR* gene in airway cells. As discussed above, we now have preliminary data showing high frequencies of successful editing the *CFTR* gene by homologous recombination in airway cells (Fig. 2). Of note, gene editing at the *CFTR* locus was found to

be equivalent between lower airway stem cells (basal cells) and differentiated airway stem cells. Furthermore, in both organoid cultures of the sinus and the lung, prior to spontaneous differentiation (within three weeks of primary plating) airway stem cells rapidly proliferate into dense structures hundreds of microns in size that are readily separated from other cell types by density centrifugation. Thus, we can now expand and correct *CFTR* in pure populations of airway stem cells at high frequency suitable for enrichment and ultimately engraftment.

Further support that we are modifying basal/airway stem cells comes from the demonstration that the manipulated cells can form lung organoids containing differentiated ciliated epithelium. These data provide compelling evidence that the process of *in vitro* culture does not significantly perturbed the underlying cell biology. While we have preliminary data that basal cells are edited by HR equivalently to non-basal cells, this will continue to be a key area of study. Our team has expertise in using cell surface markers, internal markers and single-cell RNA sequencing for these types of analytics.

Engraftment of Airway (Basal) Stem Cells

We agree with the concerns that engraftment of airway stem cells is one of the most challenging aspects of the proposal and have already initiated such experiments (results pending). We also note, however, that *in vivo* delivery of vectors has a long track record of failure in clinical trials and that it is important and innovative to take on the challenge of airway stem cell engraftment directly.

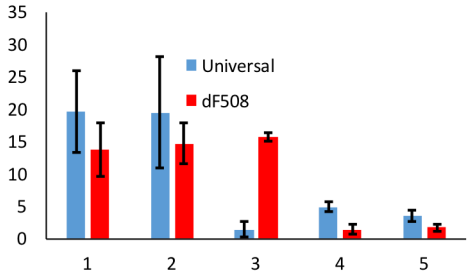
In terms of access to cells, it is remarkably easy to obtain nasal and sinus tissue for airway basal stem cell enrichment and editing and we have a motivated CF patient population who regularly consent to the donation for research of what is an otherwise a discarded waste sample. While beyond the scope of this proposal but to give a sense of our thinking we outline a vision for the following clinical phase I study. The first CF patients who enroll to receive this therapy for chronic sinusitis would undergo a 2-stage approach to airway stem cell therapy. In the 1st stage sinus surgery, we would obtain >10x10^{6} total nasal cells (and 1x 10^{5} basal cells) from easily accessed, rarely-diseased, low morbidity tissue sites such as the turbinates or nasal floor. A second goal of this 1st stage surgery would be to surgically open all sinuses to eradicate any infection from future sinus recipient bed followed by routine use of antibiotics for 1-2 months prior to engraftment. Once the edited cells are expanded, a planned 2nd stage engraftment surgery would commence in the same patient, in which the most commonly-affected sinus (the maxillary sinus) on 1 side would be stripped of all CF mucosa (thus creating niche space for the cells with the optimal method to be established in the mouse studies), and the patient's own, edited autologous cells would be deposited onto healthy sinus bone and allowed to regenerate a new epithelium. The contralateral maxillary sinus would serve as a patient-specific, internal control. Both engrafted and control maxillary sinuses would be serially followed by routine office video endoscopy under local anesthesia for re-mucosalization, exposed bone, sinus function/mucociliary clearance, and limited biopsy for tissue histology. Finally, as a safety consideration, any gross aberration or sinus concern noted following engraftment of edited cells would be readily detected via endoscopy, and removed if needed, with little foreseeable long term harm to the patient compared to the lower airway or systemic sites. For engraftment, prior to making the leap into the human upper airway, we have begun cell engraftment testing using the injured mouse nasal airway as a working model. Following full thickness injury of the nasal epithelium with a weak trichloroacetic acid, we have evidence that labeled human basal cells, instilled trans-nasally in saline, successfully engraft at sites of airway epithelial injury. Moreover, when we layer the same labeled cells directly atop human nasal tissue explants *ex vivo*, cell integration and early colony formation can be demonstrated in situ over 7-14 days in culture (Fig. 4). We are also investigating the integration of upper airway basal cells onto tissue lattices/biomatrices as alternatives, which may conform nicely to the curved sinus bony architecture and create an improved 3-dimensional environment for the basal stem cells to engraft. As noted above, our team recognizes the importance of engraftment for the success of the strategy and has already begun working on this challenge.

Reviewer Concerns about Feasibility

Several reviewers raised concerns about potential feasibility. We hope that the progress we have made as described above not only allays some of those concerns, but also demonstrates our team's productivity, efficiency and dedication to advancing the goals of this proposal.

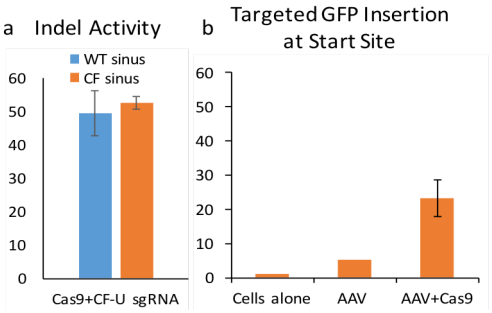
Figures

Figure 1: Identification of an active *CFTR* CRISPR/Cas9 Nucleases



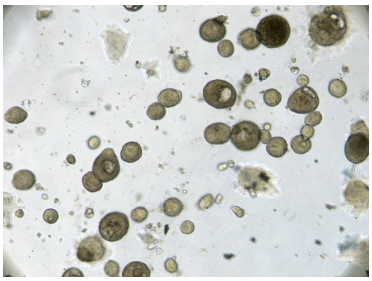
Legend: K562 cells were transfected using 5 different sgRNA plasmids (2 µg) targeting the ATG site in exon 1 and ΔF508 site in exon 11 each (5 different guides were tested at each site). Indels were measured on day 2. Columns represent mean of two technical replicates and error bars represent standard error**.**

Figure 2: Targeted Integration by Genome Editing in Primary Human Basal Stem Cells



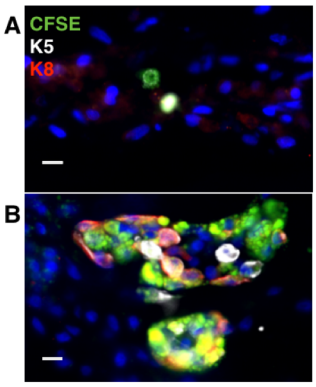
Legend: a. Primary sinus epithelial cells from CF and non-CF patients were edited using Cas 9 nuclease (6 µg) and MS-sgRNA (3.2 µg) targeting the CFTR ATG site and indels were measured on day 3. b. Targeted GFP insertion at the ATG site was also achieved in 20% of sinus cells derived from a CF patient.

Figure 3: Production of Organoids from Sinus Derived Basal Stem Cells



Legend: Upper airway cells isolated from CF and non-CF patients were plated in matrigel one day after isolation. Organoids, resembling lung organoids, were observed within 4-7 days after plating in matrigel. 60-75% of cells in organoids were positive for Keratin 5 which is a marker for basal stem cells. These organoids differentiate into ciliated epithelium (the cell type that expresses CFTR) in approximately 4-6 weeks.

Figure 4: Engraftment of Human Basal Stem Cells onto Human Explants



Legend: $1x10^5$ enriched human nasal basal cells were marked with carboxysuccinamide fluorescein ester (CFSE) for cytoplasmic labelling. They were transplanted onto human explants by direct pipetting onto explants and cultured for 7 (A) and 14 (B) days. The samples were then fixed and stained for cytokeratin5 (K5) (marks basal cells) and cytokeratin 8 (K8) (marks differentiated cells). Basal cells are seen to engraft at day 7. In addition, at day 14 robust engraftment, survival, organoid formation and cell differentiation (yellow fluorescence) can be seen (B).