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PRINCIPAL INVESTIGATOR: Beiyun Zhou

CONTRACTING ORGANIZATION: University of Southern California, Los Angeles, CA

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Dr. Beiyun Zhou/Dr. Ya-Wen Chen

E-Mail: bzhou@usc.edu/yawen.chen@mssm.edu

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University of Southern California
3720 S Flower St., FL 3,
Los Angeles, CA 90007-4318

Icahn School of Medicine at Mount Sinai
One Gustave L. Levy Place,
New York, NY 10029-6504

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14. ABSTRACT

Airway damages in the combat zone is among the leading causes of potentially preventable combat death. While the reasons for the high degree of tissue loss and destruction are multifactorial, i.e. improvised explosive devices and toxic gas exposure, the consequences could be devastating and lethal. With these acute trauma patients in mind, the goal of this project is **to create a bioengineered tracheal/airway graft with functional epithelium using stem cell-based approaches**. This past year was the second year of this funding award and the relocation of the laboratory, and the award transfer impacted our research activities in a negative way significantly. Despite the challenges and unusual circumstances, we still made progress and optimized the de-epithelialization condition. We also tested two different recellularization methods and found one of the methods gave us approximately 25% of human cell coverage on the de-epithelialized tracheas 1 day post seeding and about 70% of coverage 3 days post seeding. During the next reporting period, we hope to make up the lost time and effort toward completion of the proposed research.

15. SUBJECT TERMS
None listed.

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1. Introduction

In this project, we will evaluate the potential for *in vitro* generated basal cells (ivBCs) to serve as an unlimited epithelial cell source for tracheal/airway reconstruction, using mouse trachea as a model *in vitro* and *in vivo*. In Aim 1, we will establish optimal methods and timing for removing tracheal epithelium without disturbing the rest of the tracheal tissue. We will either directly fill the entire trachea with proteolytic enzymes or use a method called “liquid plug delivery” to thinly coat the luminal surface of trachea with enzymes. Cell removal will be examined immediately after this de-cellularization (de-cell) process. We will then recellularize the de-cell tracheal scaffolds with ivBCs, using either the fill-up or liquid plug delivery method. Recellularization will be evaluated after culturing overnight. In Aim 2, we will assess the ability of exogenous ivBCs to engraft, expand, and differentiate in re-cell tracheas. Re-cell tracheas will be cultured ex vivo and analyzed at 1, 3, 7, and 14 days for markers for differentiation and expansion. After obtaining the best conditions for de-cell, re-cell, and culturing, we will transplant bioengineered tracheas *in vivo* (Aim 3). Seeded donor tracheas will be transplanted orthotopically to recipient tracheas or heterotopically to greater omentum to assess their ability to survive and develop into functional tracheas. We will use immunodeficient NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice as trachea donors and recipients to avoid immune rejection of the transplanted tracheal grafts seeded with human cells. The transplants will be analyzed at 1, 3, 6, 9, and 12 weeks for cellular engraftment, expansion, and differentiation.

2. Keywords

Trachea transplantation, hPSC-derived basal cells, bioengineered trachea.

3. Accomplishments

WHAT WERE THE MAJOR GOALS OF THE PROJECT?

The major goals of the projects are:

1. To determine conditions for de-epithelialization and re-cellularization of mouse tracheas.
2. To characterize repopulated ivBCs on de-epithelialized tracheas.
3. To examine the *in vivo* potential of ivBCs-bioengineered mouse trachea *ex vivo*.

WHAT WAS ACCOMPLISHED UNDER THESE GOALS?

1) Major activities

The past year was the second year of this funding award, and the relocation of the laboratory and the award transfer impacted our research activities in a negative way significantly. For a long time, the research activities were suspended or only partially allowed due to the delay of sub award contract and getting approvals of all necessary

regulatory committees. We obtained the IACUC approval on 03/29/2022. The award transfer was completed on 09/06/2022.

Despite the challenges and unusual circumstances, we were able to complete the major activities listed in the follows:

1. Local IACUC Approval.
2. Re-determine the de-epithelization conditions of mouse tracheas using 0.25% trypsin and determine levels of de-epithelialization.
3. Determine an optimal condition for recellularization.
4. Determine the engraftment of human cells in day 1 and day 3 *ex vivo* cultured tracheas.

2) Specific objectives

1. Re-determine the de-epithelialization of mouse tracheas using 0.25% trypsin and determine levels of de-epithelialization at different de-epithelializing time.
2. Determine the levels of recellularization by two different seeding methods.
3. Determine an optimal attachment time for recellularization.
4. Determine the engraftment of human cells in day 1 *ex vivo* cultured tracheas.
5. Determine the engraftment of human cells in day 3 *ex vivo* cultured tracheas.

3) Significant results or key outcomes

In the previous report, we determined that utilizing a de-epithelialization condition with 0.25% trypsin for a duration of 40 minutes was appropriate. However, upon further examination, it was discovered that this condition did not sufficiently remove the mouse epithelial cells. This resulted in the reemergence of the mouse epithelial cells during *ex vivo* cultures, ultimately leading to competition with the seeded human cells for growth area **(data not shown)**. As a result, we made the decision to increase the de-epithelialization time to 60 minutes in the hopes of successfully removing the majority of mouse epithelial cells for optimal re-cellularization.

In order to optimize the re-cellularization protocol, we explored two different methods for seeding human cells onto the de-epithelialized tracheas. In method 1, we used the “open book” technique, where we cut the trachea open and human cells (1 million cells/trachea) were directly placed onto the luminal side of the trachea and allowed to re-cellularize via gravity overnight **(Fig. 1A)**. In method 2, we employed a “closed pen” approach, whereby one end of the trachea was sealed with a skin clip, the trachea was filled with a cell solution (1 million cells/trachea), and then sealed with another skin clip on the other end of the trachea before laid down on one side to re-cellularize overnight **(Fig. 1B)**. The next morning, we flipped the trachea over and

allowed it to incubate for an additional six hours. The tracheas were then subjected to whole-mount immunofluorescence staining with human EPCAM (hEPCAM) and mouse E-cadherin (E-Cad) to distinguish human and mouse cells.

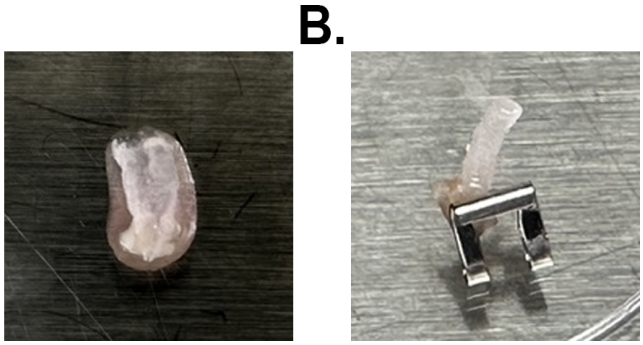

Figure 1. Re-cellularization methods. Representative images of re-cellularization by open book method **(A)** and closed pen method **(B)**.

Our results showed a significant number of human cells attached to the de-epithelialized tracheas right after re-cellularization when using the closed pen method **(Fig. 2A)**. Importantly, we also observed that the attached human cell clumps quickly migrated and spread out to occupy the denuded surface, demonstrating the ability of these cells to rapidly attach and engraft. We further confirmed our previous hypothesis that removal of mouse cells to create space for human cell attachment is crucial, as we again observed that mouse and human epithelial cells were exclusive to their respective areas **(Fig. 2B)**.

We next cultured the re-cellularized tracheas *ex vivo* for 3 days to access the engraftment of human cells. The attached human cells not only survived, but also migrated, proliferated, and engrafted, covering approximately 70% of the total tracheal surface area, a marked increase from the initial 25% coverage post re-cellularization **(Fig. 3A)**. Encouragingly, no significant cell death was observed during this time. However, we observed that any regions lacking human cells were populated by mouse epithelial cells, likely originating from the remaining mouse epithelium **(Fig. 3B)**. This underscores the importance of thorough removal of mouse epithelial cells prior to re-cellularization, or alternatively, achieving higher human cell coverage immediately following re-cellularization to outcompete any remaining mouse cells.

In addition to the ivBCs proposed to use in this study, we have developed a novel method for the rapid conversion of lung progenitors to P63+ basal cells. This method has proven to be highly efficient, with conversion rate of approximately 15% in just two days and about 60% in 6 days **(Fig. 4)**. This breakthrough technique not only facilitates swift conversion of lung progenitors but also eliminates the need for mouse feeder cells that were previously required for ivBC expansion. By obviating the requirement of animal feeder cells, we have taken a significant step towards the generation of *in vitro* cultured airway basal stem cells (P63+) for clinical applications.

Our findings highlight 1) the importance of optimizing re-cellularization protocols to maximize human cell attachment and engraftment onto the de-epithelialized trachea; 2) the efficacy of our innovative re-cellularization strategy in generating a fully

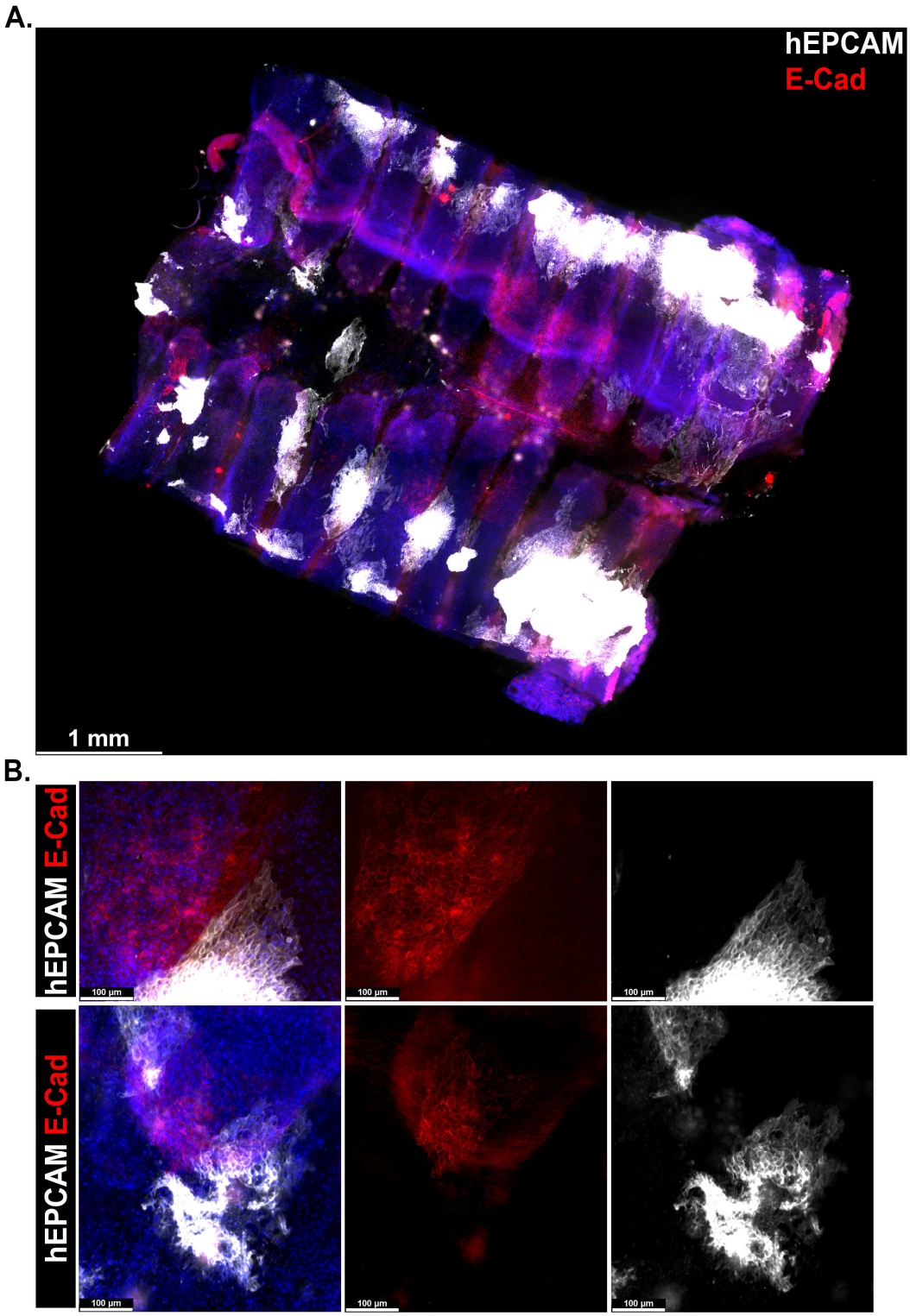

Figure 2. Distribution of human cells on a de-epithelized mouse trachea right after re-cellularization. A. Representative image of a re-cellularized trachea fixed right after the re-cellularization process by the closed pen method. **B.** Representative images showing human and mouse cells are excluded from each other. Fiber-like signals in the E-Cad channel are auto-fluorescent signals from muscle fibers. hEPCAM: human EPCAM. E-Cad: mouse E-cadherin.

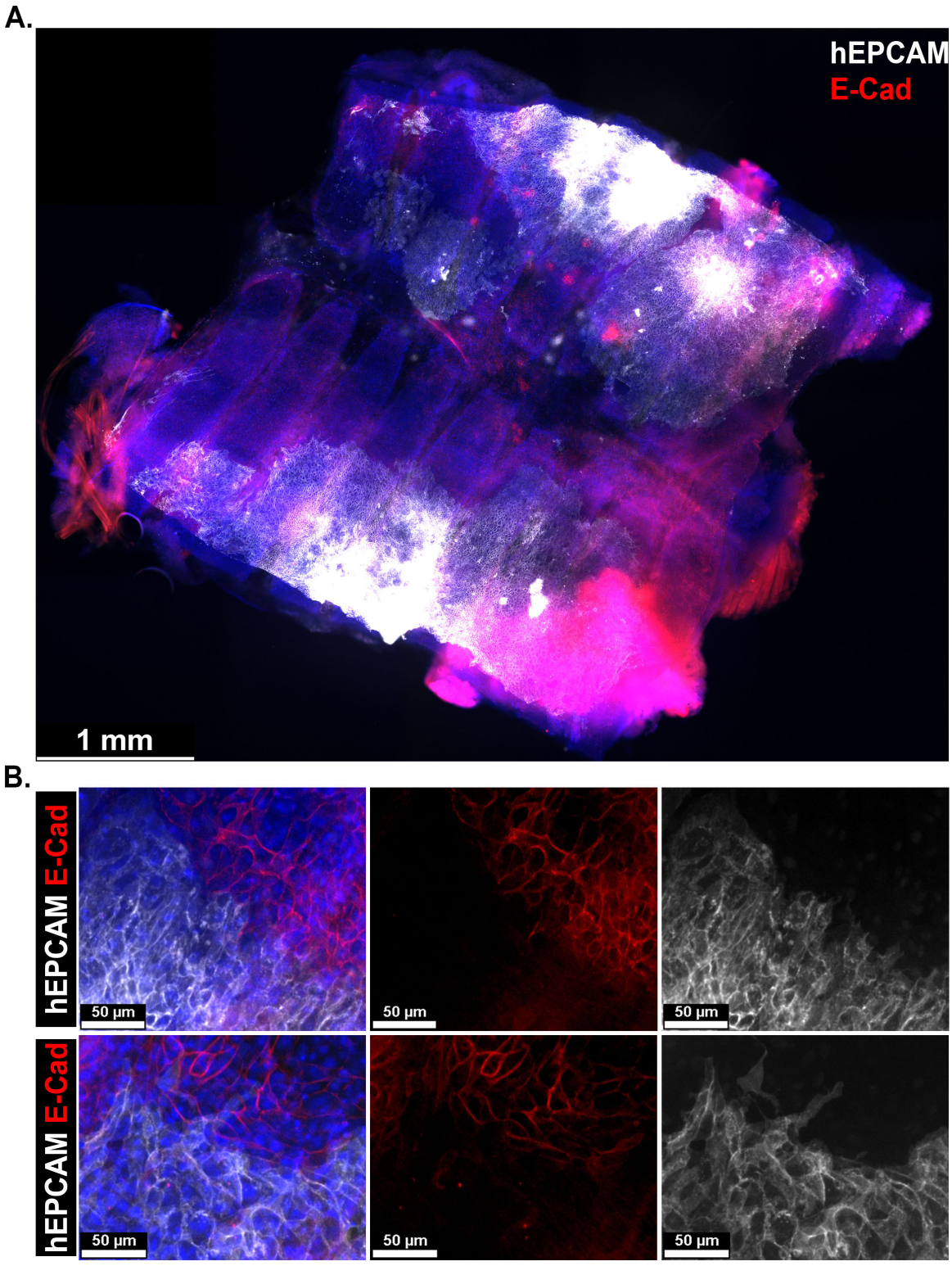

Figure 3. Distribution of human cells on a de-epithelized mouse trachea 3 days post re-cellularization. A. Representative image of a re-cellularized trachea fixed 3 days after the re-cellularization process by the closed pen method. Arrows: representative auto-fluorescence signals. **B.** Representative images showing human and mouse cells were excluded from each other. hEPCAM: human EPCAM. E-Cad: mouse E-cadherin.

functional humanized trachea; and 3) the feasibility of producing P63+ basal cells *in vitro* for therapeutic interventions. This pioneering study opens up new possibilities for the development of novel tissue engineering approaches and regenerative medicine strategies, providing hope for the future of treating respiratory tract diseases and disorders.

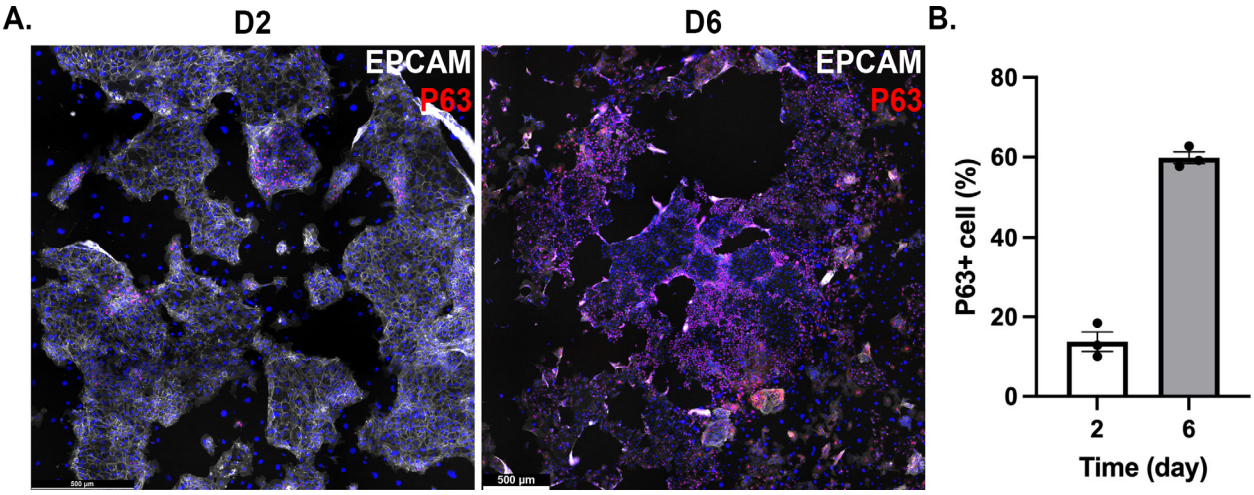

Figure 4. Rapid conversion of lung progenitors to P63+ basal cells. A. Representative images of lung progenitor cultures 2- and 6-days post to P63 conversion. **B.** Quantification of P63 expression 2 and 6 days post to P63 conversion (n=3 technical repeats). Data are represented as mean ± SEM.

4) Other achievements

Nothing to report.

WHAT OPPORTUNITIES FOR TRAINING AND PROFESSIONAL DEVELOPMENT HAS THE PROJECT PROVIDED?

The PI, Ya-Wen Chen, has attended several international and national meetings including the keystone conference – Organoids as Tools for Fundamental Discovery and Translation, the American Thoracic Society annual meeting, the International Society for Stem Cell Research annual meeting, and the FASEB Lung Epithelium in Health and Disease Conference. She also had been invited to present the work at several research institutes and universities including The University of Texas Health Science Center at Houston (UTHealth Houston) and Harvard University. These conferences broadened her knowledge in the use of human pluripotent stem cell-derived models on airway disease.

HOW WERE THE RESULTS DISSEMINATED TO COMMUNITIES OF INTEREST?

The PI was invited to give seminars at several organizations regarding the findings of the project.

WHAT DO YOU PLAN TO DO DURING THE NEXT REPORTING PERIOD TO ACCOMPLISH THE GOALS?

During the upcoming reporting period, we plan to focus on determining an optimal cell concentration for the recellularization process. To achieve this goal, we will investigate whether the utilization of various cell density solutions for recellularization yields a higher initial surface coverage of human cells. Additionally, our efforts will be directed towards analyzing the preservation of extracellular matrix on the de-epithelialized tracheas. We will conduct a comprehensive assessment of the proliferation, engraftment, and differentiation of human cells in day 1, 3, 7, and 14 *ex vivo* cultured tracheas. Finally, we will proceed to transplant the bioengineered mouse trachea orthotopically and heterotopically to immunodeficient mice to investigate their functional potentials *in vivo.*

4. Impact

WHAT WAS THE IMPACT ON THE DEVELOPMENT OF THE PRINCIPAL DISCIPLIN(S) OF THE PROJECT?

Nothing to report.

WHAT WAS THE IMPACT ON OTHER DISCIPLINES?

Nothing to report.

WHAT WAS THE IMPACT ON TECHNOLOGY TRANSFER?

Nothing to report.

WHAT WAS THE IMPACT ON SOCIETY BEYONG SCIENCE AND TECHNOLOGY?

Nothing to report.

5. Changes/Problems

CHANGES IN APPROACH AND REASONS FOR CHANGE

Nothing to report.

ACTUAL OR ANTICIPATED PROBLEMS OR DELAYS AND ACTIONS OR PLANS TO RESOLVE THEM

The past year was the second year of this funding award, and the relocation of the laboratory and the award transfer impacted our research activities in a negative way significantly. For a long time, the research activities were suspended or only partially

allowed due to the delay of sub award contract and getting approvals of all necessary regulatory committees. We obtained the IACUC approval on 03/29/2022. The award transfer was completed on 09/06/2022. During the next reporting period, we hope to make up the lost time and effort toward completion of the proposed research.

CHANGES THAT HAD SIGNIFICANT IMPACT ON EXPENDITURES

Nothing to report.

SIGNIFICANT CHANGES IN USE OR CARE OF HUMAN SUBJECTS, VETEBRATE ANIMALS, BIOHAZARDS, AND/OR SELECT AGENTS

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. Products

PUBLICATIONS, CONFERENCE PAPERS, AND PRESENTATIONS

Journal publications

Nothing to report.

Books or other non-periodical, one-time publications

Nothing to report.

Other publications, conference papers and presentations

National

Jun. 2022 – Institute for Airway Sciences Symposium, New York, NY

Aug. 2022 – 2022 FASEB Lung Epithelium in Health and Disease Conference, Saxton's River, VT.

Local societies

Apr. 2022 – Research seminar, Department of Integrative Biology & Pharmacology Seminar Series, McGovern Medical School, UT Houston, Houston, TX

May 2022 – Research seminar, Department of Environmental Health Seminar Series, Harvard T.H. Chan School of Public Health, Harvard University, Boston, MA

WEBSITE(S) OR OTHER INTERNET SITE(S)

Nothing to report.

TECHNOLOGIES OR TECHNIQUES

Nothing to report.

INVENTIONS, PATENT APPLICATIONS, AND/OR LICENSES

Nothing to report.

OTHER PRODUCTS

Nothing to report.

7. Participants & Other Collaborating Organizations

WHAT INDIVIDUALS HAVE WORKED ON THE PROJECT?

Name:	Ya-Wen Chen, PhD
Project Role:	PI
Researcher Identifier:	ORCID ID:
Nearest person month worked:	2.4
Contribution to Project:	Dr. Chen oversaw and directed all aspects of the project and supervised personnel.
Name:	Lu Tian, PhD
Project Role:	Postdoctoral Scientist

Researcher Identifier:	N/A
Nearest person month worked:	1.8
Contribution to Project:	Dr. Tian is the main person who perform most of the experiments before she departed from the lab.

Name:	Wanzhen Zhang, PhD
Project Role:	Postdoctoral Scientist
Researcher Identifier:	N/A
Nearest person month worked:	1.8
Contribution to Project:	Dr. Zhang is now leading most of the experiments of the project.

Name:	Martha Rea-Moreno
Project Role:	Graduate Student
Researcher Identifier:	N/A
Nearest person month worked:	3.6
Contribution to Project:	Ms. Rea-Moreno is the main person who perform most of the experiments with Dr. Tian and Dr. Zhang.

HAS THERE BEEN A CHANGE IN THE ACTIVE OTHER SUPPORT OF THE PD/PI(S) OR SENIOR/KEY PERSONNEL SINCE THE LAST REPORTING PERIOD?

The following previously pending grants or grants submitted after last reporting period are now active.

Title of the project	TMPRSS2 as a potential target for treatments of COVID-19 and respiratory infectious viruses in lung
Project number	1R01HL159712-01
Level (%) of effort	25%
Performance period	09/01/2022-08/31/2026
Supporting agency	The National Institutes of Health/NHLBI (PI: Chen)
Supporting agency POC	Sara Lin sara.lin@nih.gov
Specific aims/tasks	Aim 1. Test efficacy of AL20 for blocking entry of SARS-CoV-2 in lung cells.

Goals of the project	Aim 1. Will test efficacy of AL20 for blocking entry of SARS-CoV-2 in vitro and in vivo and elucidate underlying mechanisms. Aim 2. Will evaluate effects of serpins on TMPRSS2 activity and SARS-CoV-2 viral entry and spread.
Potential overlap with this DoD proposal	None.

WHAT OTHER ORGANIZATIONS WERE INVOVLED AS PARTNERS?

Nothing to report.

8. Special Reporting Requirements

COLLABORATIVE AWARDS:

Nothing to report.

QUAD CHARTS:

Nothing to report.

9. Appendices