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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The goal of this project is to generate a lung mesenchyme-specific Tsc2 conditional knockout mouse model, in which cystic and nodular lesions may be developed in the lung. In the past year, we have continued the work on (1) To identify the mechanisms by which abrogation of lung mesenchymal *Tsc2* results in defective alveolarization and developmental cystic lung pathology in mice; (2) To define lung mesenchymal cell origin(s) and the related mechanisms underlying the LAM-like nodular lesions that spontaneously develop in mesenchyme-specific *Tsc2* conditional knockout mice. In particular, we have narrowed down the lung mesenchymal cell lineages to those that are targeted by the Tbx4 driver prior to embryonic day 13.5 and are possibly $Pdgfrb^{+}$. Tsc2 deletion in these cell lineages will give rise to pulmonary nodular lesions in later life. In addition, the Tsc2-null lung mesenchymal stem cells have been isolated and cultured for more than 50 passages. These cells are invasive and capable of anchorage-independent growth in vitro.

15. SUBJECT TERMS
Tuberous sclerosis complex, Tsc2, Lymphangioleiomyomatosis, Pulmonary cysts, Pulmonary nodules, Lung mesenchymal cells, mTOR pathway, Alveolarization

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1. INTRODUCTION:

Tuberous Sclerosis Complex (TSC) is a rare genetic disease affecting multiple organs/systems including the lung. Lymphangioleiomyomatosis (LAM) is the major clinical manifestation of TSC lung disease, affecting about one third of women with TSC. Clinically, the progressive pulmonary lesions in LAM can lead to impaired respiratory function, oxygen dependence, and death. However, the related pathogenic mechanisms underlying LAM pulmonary lesions including both cysts and nodules remain unclear. One major challenge for understanding TSC-LAM pathogenesis is lack of disease models that spontaneously develop LAM-like pathology. Based on our preliminary data, we plan to establish a new genetically manipulated mouse model in which Tsc2 gene is specifically deleted in lung mesenchymal cells. Using this model, we will further test our hypothesis that loss-of-function mutation in *Tsc2* and subsequent hyperactivation of mTORC1 in different lung mesenchymal cell lineages results in distinct LAM-like phenotypes such as cysts vs. nodules.

2. KEYWORDS

Tuberous sclerosis complex
Tsc2
Lymphangioleiomyomatosis
Pulmonary cysts
Pulmonary nodules
Lung mesenchymal cells
mTOR pathway
Alveolarization

3. ACCOMPLISHMENTS

What were the major goals of this project?

(1) To identify the mechanisms by which abrogation of lung mesenchymal *Tsc2* results in defective alveolarization and developmental cystic lung pathology in mice.

(2) To define lung mesenchymal cell origin(s) and the related mechanisms underlying the LAM-like nodular lesions that spontaneously develop in mesenchyme-specific *Tsc2* conditional knockout mice.

What was accomplished under these goals?

Major Activity 1 (Major Task 3 in SOW): To determine dynamic changes of Tsc2 knockout lung alveolar structure.

1) Specific objective: To measure and compare lung alveolar structures among different genotypes and at different ages.

2) Key outcome: More samples from different developmental stages (n>6) have been added, and significant reduction of postnatal alveolar formation in *Tsc2* knockout mice has been confirmed with $p<0.05$.

Major Activity 2 (Major Task 4 in SOW): To determine the cellular and molecular changes in lungs with *Tsc2* deletion in mesenchyme.

1) Specific objective: To determine altered gene expression profiles in *Tsc2* knockout lung by RNA-seq.

2) Key outcome:
From the previous RNA-seq analysis of P7 lung tissues, 251 genes in *Tsc2* knockout mouse lung had significant changes in mRNA expression ($p<0.05$). The candidate genes involved in retinoic acid signaling and detoxification of reactive oxygen species are validated by RT-PCR and currently under further investigation.

Major Activity 3 (Major Task 5 in SOW): To determine alterations of lung mesenchymal stem cells that give rise to myofibroblast subpopulation

1) Specific objective: To determine altered progenitor cell properties of the isolated lung mesenchymal stem cells with *Tsc2* deletion.

2) Key outcome: As we find in this study, lung mesenchyme-specific *Tsc2* deletion results in reduced alveolar formation, accompanied by decreased alveolar myofibroblasts. Alveolar myofibroblasts have been shown to be a critical driving force for alveogenesis. Therefore, we decide to determine whether Tsc2-deficiency in lung mesenchymal stem cells reduce their differentiation capacity to myofibroblasts. We were working on optimizing the differentiation condition for cultured lung mesenchymal stem cells prior to COVID-19, and will reassume the experiments when our institute is reopened.

Major Activity 4 (Major Task 7 in SOW): To define the subpopulation(s) of lung mesenchymal cells, in which deletion of *Tsc2* results in proliferative nodules

1) Specific objective: To compare adult lung nodular pathology among mice with *Tsc2* deletions in different lung mesenchymal subpopulations *in vivo* (E6.5-induced vs. E11.5 or E13.5-induced knockout lungs).

2) Key outcome: We have continued to generate these mouse models, and compared their pulmonary lesions:
(1) We have generated more than 30 lung samples from *Tsc2* conditional knockout mice induced from E6.5. The majority of them have pulmonary nodular lesions as reported previously.
(2) We have harvested one more E11.5-induced *Tsc2* knockout female mouse lung at 4 months of age, and did not detect pulmonary nodules. The rest experiment was aborted due to institutional closure during COVID-19, and will be reassumed after our institute is reopened.
(3) In E13.5-induced *Tsc2* knockout lungs harvested at >2 months of age, no lung nodules were found in 8 samples as described previously.
These indicate that the cell lineages targeted for *Tsc2* deletion prior to E11.5 may be critical for inappropriate cell proliferation and differentiation later on. This leads to our new experiments funded by a NIH grant. One of these is to delete *Tsc2* in Pdgfrb^{+}-cell lineage using Pdgfrb-rtTA/TetO-Cre/Tsc2$^{fx/fx}$ mice. With doxycycline induction from E11.5 to P1, lung proliferative nodules are detected in 4/6 female and 1/3 male mice. These lung phenotypes are similar to what are seen in our Tbx4-rtTA-driven *Tsc2* CKO mice. This suggests that loss of *Tsc2* function in these Pdgfrb^{+}-derived lung mesenchymal cells may be critical in developing pulmonary nodular lesions

Major Activity 5 (Major Task 9 in SOW):
To determine alterations of lung mesenchymal stem cells from perivascular subpopulation, which may be the progenitors for LAM-like cells in the *Tsc2* lung conditional knockout mice.

1) Specific objective: To determine altered progenitor cell properties of these mesenchymal stem cells when *Tsc2* is deleted

2) Key outcome:
We have established multiple lung mesenchymal progenitor cell lines from 4-month old female *Tsc2* CKO mice that have lung nodules. These cells have been passaged more than 50 times. The genotypes were validated (Fig.1A, next page). These cells express vimentin, but are negative for markers of epithelia, endothelia, and hematopoietic cells (Fig.1B). The *Tsc2*-null cells are larger than control cells (Fig.1C), as expected. The progenitor properties of both the *Tsc2*-null and wild-type control cells were validated by their colony formation and multi-differentiation capacity, such as differentiating to osteoblast, adipocyte, and smooth muscle cell under special culture conditions (Fig.1D-1E). Interestingly, the *Tsc2*-null lung mesenchymal cells, but not the control cells, are invasive and capable of anchorage-independent growth *in vitro* (Fig.2). which is similar to the human LAM cell phenotypes as reported by other groups.

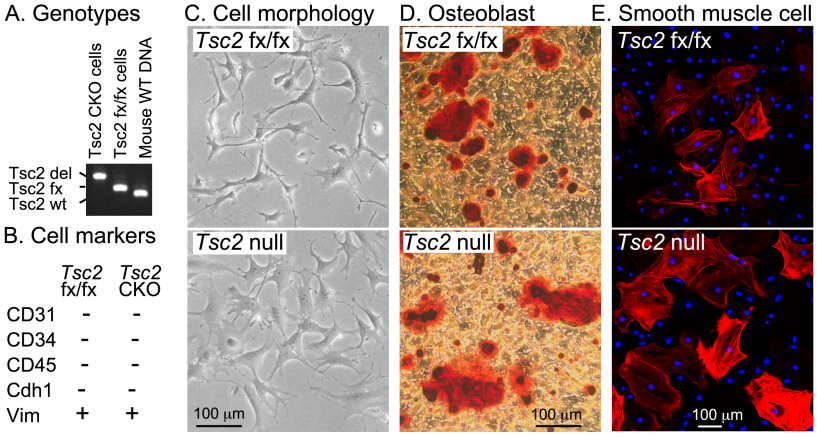

Fig.1. Newly established *Tsc2*-null mouse lung mesenchymal progenitor cells. (A) Genomic DNA PCR. (B) Summary of immunostaining of cell markers. (C) Cell morphology (passage 50). (D-E) Osteoblast and smooth muscle cell differentiation, detected by Alizarin red staining (D) and Myh11 immunostaining (red in E).

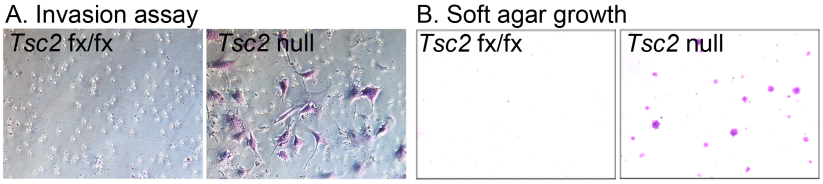

Fig.2. *Tsc2*-null lung mesenchymal cells are invasive (A) and grow in anchorage-independent conditions (B).

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

(1) To continually generate lung mesenchyme-specific *Tsc2* conditional knockout mice that are needed as additional samples for experiments in Aim 1 and Aim 2. In particular, the experiments suspended due to the COVID-19 pandemic will be reassumed.

(2) To validate and narrow down the candidate genes identified by P7 lung RNA-seq in order to identify the potential molecular mechanisms underlying reduced alveolar myofibroblasts and alveolar growth in *Tsc2* knockout lung during postnatal development.

(3) To determine if alterations in lung mesenchymal stem cells contribute to reduction of myofibroblasts in *Tsc2* knockout lung during alveolarization.

(5) More E11.5 and E13.5-induced *Tsc2* knockout lung samples will be collected to reach n>5 per genotype/sex in order to compare with E6.5-induced *Tsc2* knockout lung pathology and reach conclusion with statistical significance.

(6) To determine whether Tsc2-null lung mesenchymal stem cells have the pathologic properties same as human LAM cells.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

(1) Successful development of lung mesenchyme-specific Tsc2 conditional knockout mice will meet the urgent needs for novel TSC *in vivo* disease models, providing an important tool for TSC-LAM research.

(2) Dynamically characterizing the spontaneous phenotypes in our unique *Tsc2* conditional knockout mouse model will help to generate novel concepts for heterogeneous clinical manifestations in TSC-LAM patients (cysts only vs. nodules plus cysts).

(3) Determination of the developmental origin of lung lesions in TSC-LAM may have particular importance for children with TSC. By identifying the developmental window during which lung lesions initiate, prevention will become a possibility.

(4) This project will identify different lung mesenchymal cell subpopulations that may be responsible for distinct lung phenotypes, e.g. alveolar cysts vs. proliferative nodules, in LAM. This will be a significant breakthrough in understanding the pathogenic mechanisms of LAM.

What was the impact on other disciplines?

The finding that Tsc2 deletion negatively affects lung alveolar development will also contribute to understanding of lung alveogenesis and pediatric pulmonary cystic lesions.

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS

Nothing to report.

6. PRODUCTS

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Wei Shi
Project Role:	Project Director/Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0001-6499-2473
Nearest person month worked:	*2.4*
Contribution to Project:	Dr. Shi is the PI on this project, and oversees the project, including data generation, analysis, and presentation. He will ensure that the project goals are accomplished in a scientifically rigorous and timely manner.
Funding Support:	*DoD, NIH*

Name:	*Elizabeth Henske*
Project Role:	*Consultant*
Researcher Identifier (e.g. ORCID ID):	0000-0001-7978-6699
Nearest person month worked:	*0*
Contribution to Project:	Dr. Henske serves as a consultant, provides advice on LAM cellular and molecular pathology, and guidance in validating this disease model.
Funding Support:	*DoD, NIH*

Name:	*Hui Chen*
Project Role:	*Research Specialist*
Researcher Identifier (e.g. ORCID ID):	0000-0003-0346-1732
Nearest person month worked:	*7.2*
Contribution to Project:	Hui performs day-to-day work as proposed in this project, including animal breeding, genotyping, tissue fixation and histology/morphometry, and immunohistochemistry.
Funding Support:	None

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Dr. Wei Shi has one new active project:

T30IP1028 (Lien) 09/01/19-08/31/21 1.08 calendar
California Tobacco Related Disease Research Program $200,000
“Effects of tobacco and e-cigarettes on heart repair and regeneration”
This pilot grant is to test a hypothesis that tobacco (including both combustible tobacco cigarette and e-cigarette) not only increases the chances of a first-time heart attack but can also affect heart repair and regeneration after myocardial infarction by regulating the cardiac lymphatic system. We aim to determine (1) the effects of tobacco and e-cigarettes in zebrafish heart regeneration and cardiac lymphatic vessel functions; (2) the effects of tobacco smoke on mouse heart repair, fibrotic scar

formation, development of heart failure and lymphangiogenesis. There is no scientific overlap between this project and the current DoD project.
Role: Co-I

What other organizations were involved as partners?

Organization Name: The Brigham and Women's Hospital, Inc.

Location of Organization: Boston, MA

Partner's contribution to the project

Financial support: None.

In-kind support: None.

Facilities: None.

Collaboration: Dr. Henske is a consultant and key personnel in this project. She provides advice on LAM cellular and molecular pathology, and guidance in comparing Tsc2 conditional knockout mouse lung phenotypes to human TSC-LAM pathology in order to validate the TSC-LAM disease model

Personnel exchanges: None.

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:** *Not applicable*
- **QUAD CHARTS:** *.Not Applicable*

9. APPENDICES

Nothing to report.