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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 first year work, we have obtained approvals from both IACUC and ACURO for our animal protocol. We then generated lung mesenchyme-specific Tsc2 mice and harvested the lung tissues from different ages including neonates. Although the sample numbers were relatively small, initial analysis suggested that Tsc2 deletion in lung mesenchyme negatively affect lung alveolar growth during postnatal lung development. The Tsc2-null lung mesenchymal stem cells isolated from this stage showed reduced proliferation comparing to the wild type control. Moreover, the adult mice with Tsc2 genetic deletion induced from embryonic stage (E6.5 to E14.5) developed pulmonary nodules (3 out of 3 in females and 1 out of 2 in males). Further analyses and characterization will be performed with additional numbers of lung samples in the second year.

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. This goal will be achieved in 34 months in the approved SOW.

(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. This will be achieved in 36 months in the approved SOW.

What was accomplished under these goals?

Major Activity 1: To obtain approval for animal protocol used in this project.

1) Specific objective: To obtain ACURO approval for proposed animal studies.

2) Key outcome: To obtain approval from both IACUC and ACURO, we worked closely with both committees. We initially submitted our IACUC approved protocol to ACURO in June 2017, and got feedback for revision from ACURO in August. Within 2 weeks, the revised protocol was approved by our IACUC and resubmitted to ACURO. We then received an official approval from ACURO in October 2017. Due to the paper works back-and-forth between two committees, this whole process took 5 months (2 months behind the schedule as proposed in SOW).

Major Activity 2: To continually generate whole lung mesenchyme-specific *Tsc2* conditional knockout mice

1) Specific objective: To generate and validate lung mesenchyme-specific Tsc2 conditional knockout mice, including crossing with additional mT-mG reporter line.

2) Key outcome: Upon animal protocol approval, we started to expand mouse colonies including floxed-Tsc2, Tbx4-rtTA/TetO-Cre, and mT-mG lines, and cross them to generate Tbx4-rtTA/TetO-Cre/Tsc2$^{fx/+}$ mice and Tsc2$^{fx/fx}$/mT-mG mice. We then generated lung mesenchyme-specific Tsc2 conditional knockout mice by timed mating the above two lines, and initiated doxycycline induction from embryonic day (E) 6.5 by feeding the mothers with doxycycline food (625 mg/kg food) and water (0.5 mg/ml) to the birth. All newborn pups were normal in size, body weight, and physical activity. The following DNA genotypes were obtained by genomic DNA PCR: Tsc2 homozygous conditional knockout (Tbx4-rtTA/TetO-Cre/Tsc2$^{fx/fx}$), Tsc2 heterozygous conditional knockout (Tbx4-rtTA/TetO-Cre/Tsc2$^{fx/+}$), Tsc2 wild type/normal controls (Tbx4-rtTA/Tsc2$^{fx/+}$, Tbx4-rtTA/Tsc2$^{fx/fx}$, TetO-Cre/Tsc2$^{fx/+}$, TetO-Cre/Tsc2$^{fx/fx}$, Tsc2$^{fx/+}$, or Tsc2$^{fx/fx}$). While some mice were kept to adult ages, we have collected lung tissues from the mice with different genotypes at ages of E18.5 and postnatal day (P) 7, P14, and P30. The lung tissues were either fixed in 4% paraformaldehyde solution for structural analysis, or frozen for RNA and protein isolation. Deletion of Tsc2 gene (exon 2) was confirmed at the genomic DNA level and the mRNA level. Reduction of Tsc2 protein in total lung tissue lysate was also detected in the Tsc2

knockout mice. However, Tsc2 immunostaining with two different commercial anti-Tsc2 antibodies was not working, and we plan to try other commercially available Tsc2 antibodies.

Major Activity 3: 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: Although the work is still in progress, collected data indicate that Tsc2 deletion in lung mesenchymal cells disrupts normal postnatal lung alveolar formation in mice. By dynamic analysis of the lung histopathology from E18.5 to P30, significant reduction of alveolar formation was detected during postnatal alveogenesis (P7-P30), while the lung structure of Tsc2 knockout mice before birth (E18.5, Fig.1) appears normal. This was further validated by morphometric measurement for mean linear intercept (MLI, Fig.2). In normal lung, alveolar growth subdivides the terminal air sacs/primary alveoli, resulting in much smaller airspaces at the end of alveolarization (MLI at P30). However, MLI in Tsc2 knockout lung remained significant larger than controls although slight reduction was detected from P7 to P14. This suggests that mesenchymal Tsc2 is required for alveolar growth at young age, and developmental insufficiency in alveolar formation may contribute to alveolar cysts caused by Tsc2 null mutation.

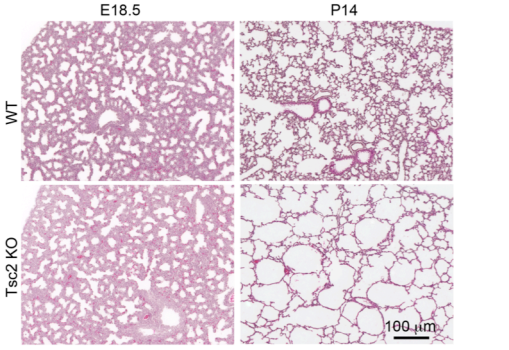

Fig.1. Histopathology of Tsc2-null lungs.

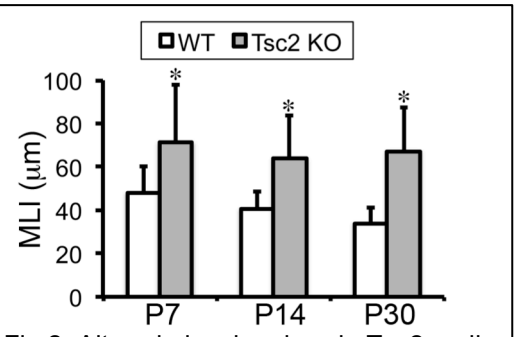

Fig.2. Altered alveolar sizes in Tsc2–null lungs during development. *P<0.05.

Major Activity 4. To determine alterations of lung mesenchymal stem cells that give rise to myofibroblast subpopulation

1) Specific objective: To isolate, culture and compare the numbers of these mesenchymal stem cells among lungs with different Tsc2 genotypes.

2) Key outcome: We have isolated lung mesenchymal stem cells (MSCs) from a pair of wild type and Tsc2 knockout lungs at postnatal day 7, when alveolar growth is significant reduced in Tsc2 knockout lung. The MSCs from Tsc2 knockout lung grew much slower than wild type control MSCs (Fig.3). The cells are now cultured for 20 passages

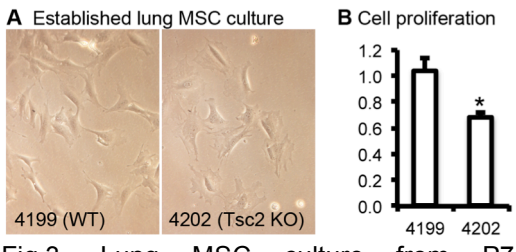

Fig.3. Lung MSC culture from P7 developing lung (A) and proliferation measured after 48 h culture (B).

for wild type genotype and only 10 passages for Tsc2 knockout genotype. These Tsc2-null lung mesenchymal progenitor cell lines isolated from developing lungs prior to nodular lesions are established and analyzed for the first time.

Major Activity 5. To define the subpopulation(s) of lung mesenchymal cells, in which deletion of *Tsc2* results in proliferative nodules

1) Specific objective: To compare 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 generated several mice with Tsc2 deletion induced from E6.5 to E14.5. In a litter of 10 mice, there were 2 female and 3 male wild type mice and 3 female and 2 male Tsc2 knockout mice. The lungs were isolated and analyzed at age of 4 months. Among them, 3 out of 3 female lungs and 1 out of 2 male lungs had nodular lesions. Cystic destruction was also observed around nodular lesions (Fig.4). We will continue to generate more Tsc2-null mice for this analysis.

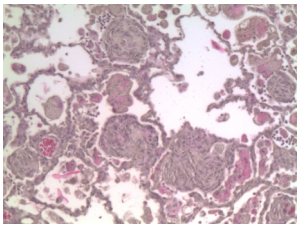

Fig.4. Histopathology of a female Tsc2 knockout lung induced from E6.5 to E14.5.

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 will be used for studies proposed in Aim 1 (alveolar growth) and Aim 2 (proliferative nodules).

(2) To continue the quantitative analysis of alveolarization defects at different developmental ages by including more samples in each genotype group at defined ages.
(3) To determine cell proliferation, differentiation, and apoptosis in Tsc2 null lung during alveolarization. In addition, differential gene expression between wild type and Tsc2-null lung tissues at P7 will be determined by RNA-seq.

(4) To determine if alterations in lung mesenchymal stem cells contribute to reduction of myofibroblasts in Tsc2 knockout lung during alveolarization. Lung MSCs from different genotypes will be isolated from more mice (>3 per genotype). The numbers of MSCs will be

compared by colony forming assay. Altered cell properties will also be determined by comparing their MSC markers, proliferation, and multipotent differentiation.

(5) To characterize the pulmonary nodules in adult Tsc2 knockout lungs including their heterogeneous mesenchymal origins and expression of molecular markers for a variety of cell types.

(6) To compare the incidences of pulmonary nodules in mice with Tsc2 knockout in different lung mesenchymal lineages by initiating doxycycline induction of Tbx4-rtTA-driven Cre expression from E6.5, 11.5, and E13.5.

(7) To isolate and characterize lung perivascular mesenchymal stem cell lineage in mesenchyme-specific Tsc2 knockout mice in order to determine if they are progenitors for LAM-like 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:	3
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:	10
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 two new active projects:

1R03HD090309-01A1 07/15/17-06/30/19 0.60 calendar
NIH/NICHD $50,000
“Vascular development during lung organogenesis”
This project is focused on the developmental process of embryonic/fetal lung vascular

network formation and pulmonary circulation in a novel lung mesenchyme-specific reporter system, with two specific aims: (1) To determine the origins of pulmonary vascular endothelial and supporting cells; (2) To determine the dynamic processes of pulmonary circulation establishment.
Role: PI

1RO1HL141352-01 02/01/18-01/31/22 4.80 calendar
NIH/NHLBI $482,323
“Molecular mechanisms of pulmonary disease in Birt-Hogg-Dube syndrome”
This project is focused on the mechanisms underlying cystic lung disease in Birt-Hogg-Dubé syndrome with two specific aims (1) To determine how loss of Flcn in lung mesenchyme impacts alveolar development and homeostasis in vivo; (2) To determine the mechanisms through which Flcn-deficiency in lung mesenchymal cells leads to defective alveolar development and homeostasis, in vitro and in vivo.
Role: PI

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.