AWARD NUMBER: W81XWH-17-1-0173

TITLE: Mechanisms of Pulmonary Lesions in TSC LAM

PRINCIPAL INVESTIGATOR: Wei Shi

CONTRACTING ORGANIZATION: Children's Hospital Los Angeles

REPORT DATE: July 2021

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
JULY 2021	Final	1 May 2017 – 30 Apr 2021

4. TITLE AND SUBTITLE

Mechanisms of Pulmonary Lesions in TSC LAM

5a. CONTRACT NUMBER
W81XWH-17-1-0173

5b. GRANT NUMBER
TS160044

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Wei Shi

E-Mail: wshi@chla.usc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Children's Hospital Los Angeles
4650 Sunset Blvd.
Los Angeles, CA 90027

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Tuberous sclerosis complex (TSC) is a rare genetic disease affecting multiple organs and systems including lung. Lymphangioleiomyomatosis (LAM) is a major clinical manifestation of TSC lung disease. The pathogenic mechanisms underlying LAM pulmonary lesions (cysts and nodules) remain unclear, and development of animal models that mimic LAM pathogenic process is the major goal of this project. Using state of the art technology, we have successfully generated a mouse model in which *Tsc2* genetic deletion can be induced specifically in lung mesenchymal cells. We find that the lung-specific *Tsc2* mutant mice have abnormal development of alveoli and lung cysts, followed by proliferative nodular formation in adulthood. Using this model, we have quantitatively and qualitatively analyzed the dynamic changes in lung alveolar growth and cystic formation. The potential mechanisms underlying cystic phenotype have been dissected at the cellular and molecular levels. Furthermore, the lung perivascular mesenchymal cells with *Tsc2* deletion are found to be the key cell type contributing to the nodular lesions. The cell heterogeneity of the lung nodules in the *Tsc2* conditional knockout mice have also been defined. Finally, *Tsc2*-null mouse lung mesenchymal stem cell culture has bene established, and these cells are invasive and capable of anchorage-independent growth *in vitro*, which are the features of tumor cells.

15. SUBJECT TERMS

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

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	17	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

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 Task 1/2: To generate lung mesenchyme-specific *Tsc2* conditional knockout mice.

Key outcome: Upon animal protocol approval by both IACUC and ACURO, we started to generate lung mesenchyme-specific *Tsc2* conditional knockout mice by crossing Tbx4-rtTA/TetO-Cre/Tsc2$^{fx/+}$ and Tsc2$^{fx/fx}$/mT-mG mice, with doxycycline induction from embryonic day 6.5. All newborn pups were normal in size, body weight, and physical activity. The mice with the following DNA genotypes were obtained: *Tsc2* homozygous conditional knockout (Tbx4-rtTA/TetO-Cre/Tsc2$^{del/del}$), *Tsc2* heterozygous conditional knockout (Tbx4-rtTA/TetO-Cre/Tsc2$^{del/+}$), *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$^{fx/fx}$).

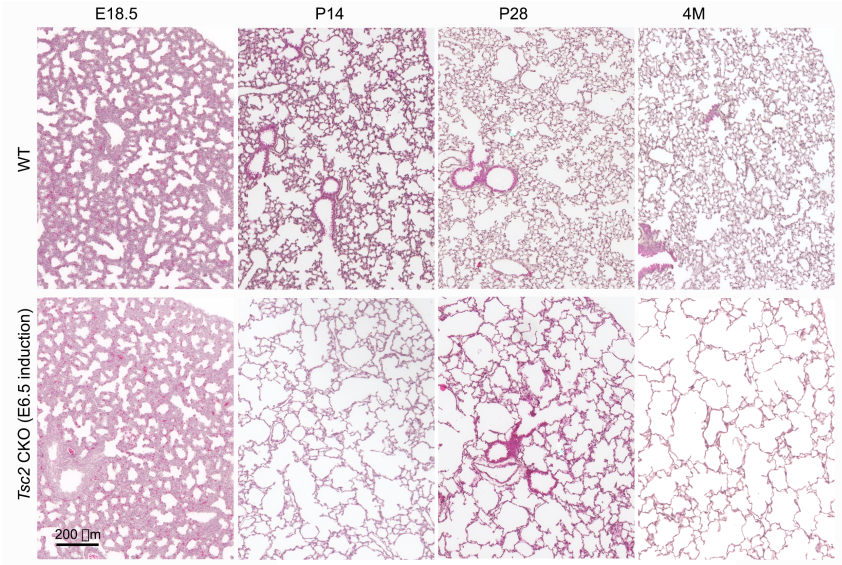

Fig.1. Dynamic comparison of lung histopathology between Tsc2 knockout and wild type control lungs.

Major Task 3: To determine dynamic changes of *Tsc2* knockout lung alveolar structure.

Key outcome: To measure and compare lung alveolar structures among different genotypes and at different ages. By dynamic analysis of the lung histopathology from embryonic day (E)18.5 to adult (4-month age), significant reduction of alveolar formation was detected during postnatal alveogenesis (postnatal day (P) 7-P28, Fig.1), while the lung structure of the Tsc2 conditional knockout mice before birth (E18.5) appears comparable to the wild type littermate controls. This was further validated by morphometric measurement for mean linear intercept (MLI in Fig.2, n>6 per genotype at each time point). In normal lung, alveolar growth subdivides the terminal air sacs/primary alveoli, resulting in much smaller airspaces at the end of alveolarization (MLI at 1 month). However, MLI in *Tsc2* knockout lung remained significantly larger than the littermate controls (Fig.2), suggesting 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 at adult. The alveolar abnormality remains at adulthood (3-4 Months of ages in Fig.2).

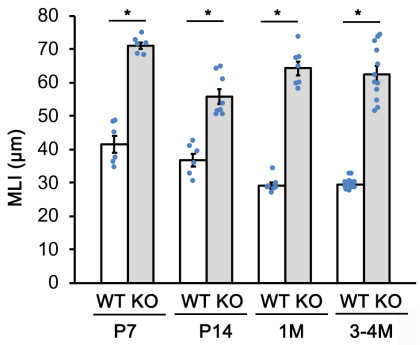

Fig.2. Comparison of average alveolar size (MLI) between Tsc2 knockout and wild type control lungs at different ages. *p<0.05

Major Task 4: To determine the cellular and molecular changes in lungs with *Tsc2* deletion in mesenchyme.

Key outcome: Lung cell proliferation was examined by EdU labeling and immunostaining, we found that overall cell proliferation at postnatal day 7 was significantly reduced in *Tsc2* knockout lung (Fig.3A). Moreover, alveolar myofibroblasts were significantly reduced in *Tsc2* knockout lung (Fig.3B), suggesting a potential mechanism by which *Tsc2* deletion in lung mesenchymal cells may negatively affect alveolar myofibroblast growth, which is a key cell type supporting alveolar growth. Change in apoptosis was not detected in *Tsc2*-null lungs.

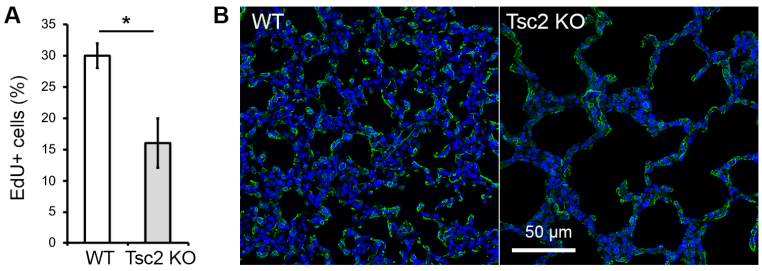

Fig.3. Comparison of cell proliferation and alveolar myofibroblasts in P7 mouse lungs. (A) EdU-positive proliferating cell quantification, *p<0.05, (B) Immunostaining for alveolar Acta2-positive cell (myofibroblast). Nuclei were counterstained with DAPI (blue).

To find out the molecular mechanisms underlying this phenotype, three pairs of *Tsc2* knockout lungs and controls at postnatal day 7 have been compared for their gene expression by tissue RNA-seq. For genes that have more than 4-fold (LogFC>2 or LogFC<-2) changes of expression in *Tsc2* knockout lungs, there are 144 genes whose expression is decreased (P<0.05), and 107 genes whose expression is increased (P<0.05). The affected majority canonical pathways include EIF2 signaling, glutamate receptor signaling, acute phase response signaling, and tumor microenvironment pathway (Fig.4).

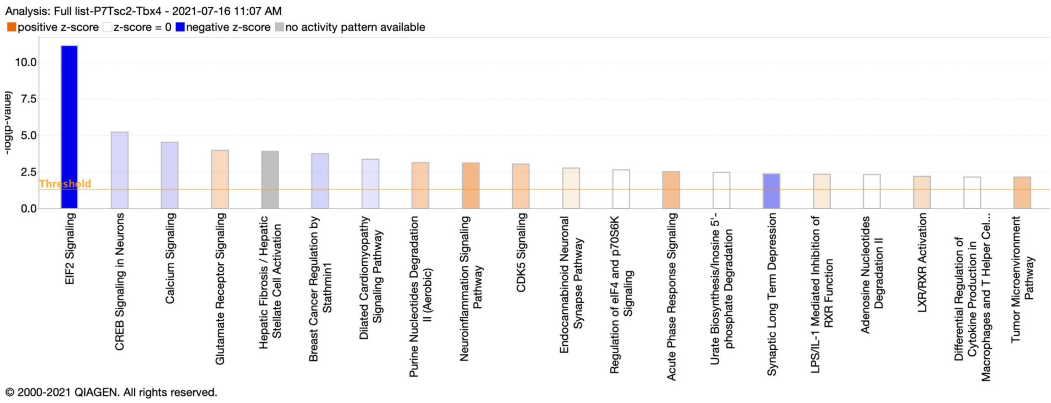

Fig.4. P7 lung transcriptomic comparison between Tsc2 KO and wild type control by bulk RNA-seq. The affected pathways are predicted by Ingenuity Pathway Analysis (Qiagen).

Major Task 5: To determine alterations of lung mesenchymal stem cells that give rise to myofibroblast subpopulation.

Key outcome: As we found 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 results in reduction of lung mesenchymal stem cell number or reduction of lung mesenchymal stem cell capacity of differentiation to myofibroblasts. The mesenchymal stem cells isolated from both wild type and *Tsc2* conditional knockout lungs express vimentin (Fig.5), but are negative for markers of epithelia,

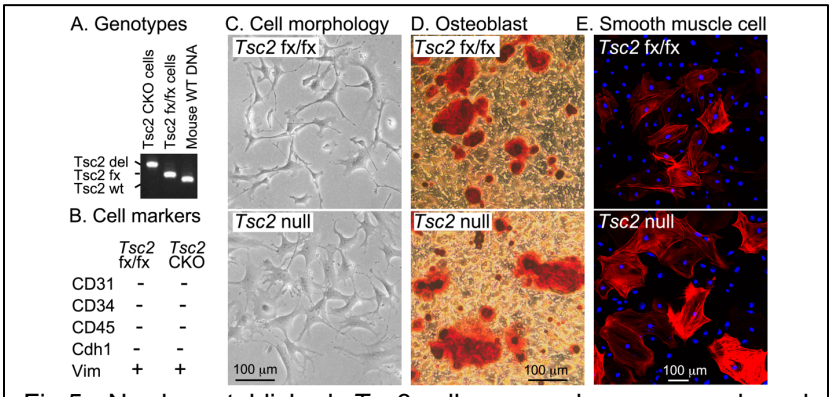

Fig.5. Newly established *Tsc2*-null mouse lung mesenchymal progenitor cells. (A) Genomic DNA PCR. (B) Summary of immunostaining of cell markers. (C) Cell morphology (passage 50). (D-E) Osteoblast and smooth muscle cell differentiation, detected by Alizarin red staining (D) and Myh11 immunostaining (red in E).

endothelia, and hematopoietic cells. The Tsc2-null cells are larger than control cells 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, and no significant difference was observed. However, we found that the number of lung resident MSCs, as measured by colony forming unit (CFU) assay (Fig.6), was significantly reduced in an E6.5-induced *Tsc2* conditional knockout mouse lungs, and the proliferation of Tsc2-null lung mesenchymal stem cells was decreased compared to the wild-type control lung mesenchymal stem cells. This suggests that the reduction of lung mesenchymal stem cells in *Tsc2* conditional knockout lung may be responsible for the defective growth of alveoli.

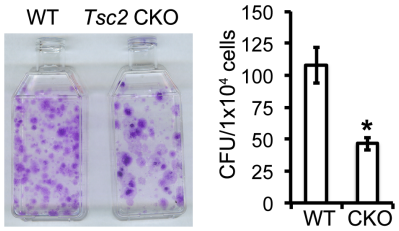

Fig.6. Lung resident MSC CFU assay for a pair of female WT and E6.5-induced *Tsc2* CKO mice. Left panel: stained MSC colonies. Right panel: CFU count. *P<0.05.

Major Task 6/7: To continually generate *Tsc2* conditional knockout in different lung mesenchymal cell lineages (E6.5, E11.5 or E13.5-induced), and to define the subpopulation(s) of lung mesenchymal cells, in which deletion of *Tsc2* results in proliferative nodules.

Key outcome: From our previous study (Zhang et al., 2013), we find that our *Tbx4-rtTA* driver line does not target pulmonary endothelial cells and perivascular smooth muscle cells after E11.5 and E13.5 induction, respectively. Therefore, we have compared lung nodular phenotypes among our adult *Tsc2* knockout mouse lungs with different *Tsc2* knockout induction time windows as mentioned above.

(1) In E6.5-induced *Tsc2* knockout lungs, 80% of the female samples (n=15) and 68% of male samples (n=16) were found to develop pulmonary nodules at >2.5 months of age (Fig.7), while all of them had enlarged alveoli and small cyst-like lesions. The nodular phenotype has relatively high variation among the collected specimens, ranging from one patch to entire lobe. Although the positive rate of pulmonary nodules in male adults is still significantly lower than that in female adults, it appears important to understand the mechanism underlying this sex-related change.

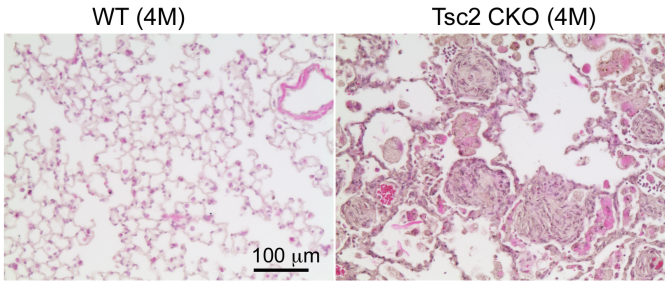

Fig.7. Proliferative nodules and cystic changes were detected in peripheral lung of an E6.5-induced female Tbx4-driven *Tsc2* CKO mouse at 4 months of age.

(2) In E11.5-induced *Tsc2* knockout female mouse lungs harvested at 2.5 months of age; Enlargement of alveolar space is similar to what seen in E6.5-induced *Tsc2* knockout lung.

(3) In E13.5-induced *Tsc2* knockout lungs (5 females and 3 males) harvested at >2 months of age, no lung nodules were found, but alveolar enlargement/cyst-like changes were evident in both male and female *Tsc2* knockout lungs (Fig.8).

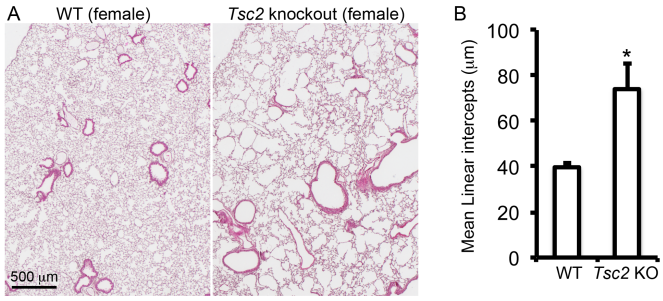

Fig.8. Alveolar enlargement but not nodule at 4 months of age was seen in lung mesenchyme (Tbx4-driven)-Tsc2 knockout induced from E13.5 to P1. (A) Lung histology of Tsc2 knockout mouse vs. wild type control; (B) MLI measurement, *p<0.05.

In summary, there were significant differences in pulmonary nodular phenotypes when *Tsc2* deletion is induced at different developmental windows. Tsc2 deletion in lung endothelia and/or perivascular cells may be responsible for the lung nodular lesions. This leads to our new experiments funded by a NIH grant. One of these is to delete *Tsc2* in

Pdgfrb^{+}-perivascular 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 (Fig.9). These lung phenotypes are similar to what are seen in our E6.5-induced Tbx4-rtTA-driven *Tsc2* conditional knockout mice. This suggests that loss of *Tsc2* function in these Pdgfrb^{+}-derived lung mesenchymal cell lineages may be critical in developing pulmonary nodular lesions later on.

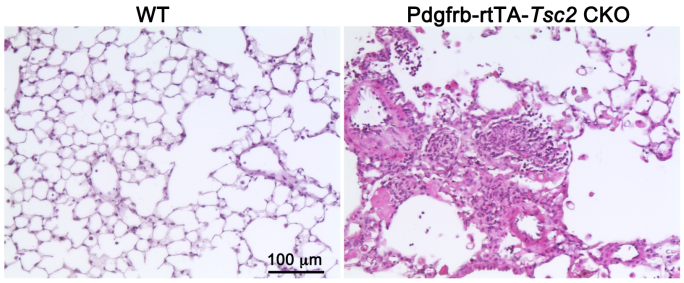

Fig.9. Lung pathology of Pdgfrb-rtTA-driven Tsc2 CKO mouse lung compared to the wild type littermate at 2.5 months of age.

Major Task 8: To determine heterogeneity of cells in LAM-like nodules, in aspects of organ origins, *Tsc2* genotypes, and specific cell marker expression.

Key outcome: As we reported in our previous publication (Zhang et al., 2013), all embryonic/fetal lung mesenchymal cells can be permanently marked by membrane GFP (mG) expression in our *Tbx4-rtTA*/*TetO-Cre*/mT-mG reporter mouse line. This allows us to identify the following cells in our E6.5-induced *Tsc2* knockout/mT-mG adult mice (*Tbx4-rtTA*/*TetO-Cre*/*Tsc2*$^{fx/fx}$/mT-mG): (1) Cells with developmental lung mesenchymal origin, which have Cre-mediated *loxP* DNA recombination, resulting in *Tsc2* deletion and mG expression; (2) Cells with lung epithelial origin, with default membrane Tomato (mT) expression plus positive epithelial cell markers such as cytokeratin; (3) Mesenchymal cells derived from the organs other than lung, where Cre-mediated *loxP* recombination does not occur. These cells have default mT expression and wild type *Tsc2*, and are negative for epithelial markers. Using this method, we found that the proliferative nodules of the E6.5-induced *Tsc2* knockout/mT-mG mice (4 months old) consisted of both mT-positive cells and mG-positive cells (Fig.10A). Cytokeratin-positive epithelial cells with mT expression were only detected outside the nodules, and the mT-positive cells inside the nodule were cytokeratin-negative, suggesting that they are mesenchymal cells derived from organs other than lung, with wild-type *Tsc2*. The smooth muscle cell marker Acta2 was detected in some cells with mG expression, but not in mT-positive cells (Fig.10B). In contrast, Pmel (gp100) staining was seen in mG-negative cells in the nodules (Fig.10C). These finding highlight the cellular heterogeneity of the nodules in our mice and further highlight the similarities to human LAM nodules, which also contain both *TSC2*-expressing and *TSC2*-mutant cells (Badri et al., 2013).

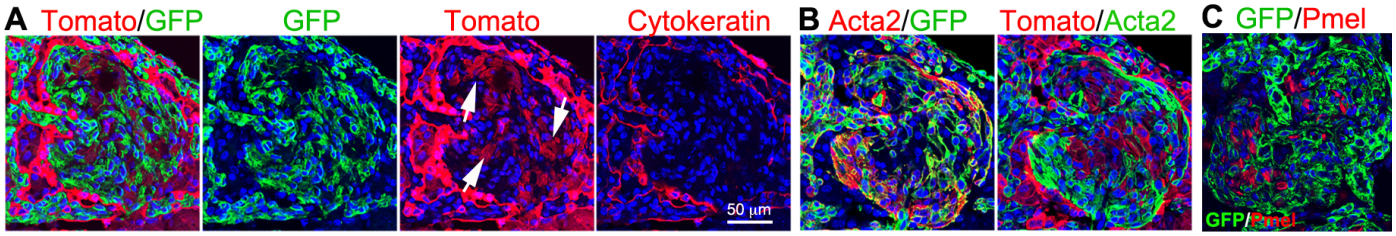

Fig.10. Co-immunostaining of an E6.5-induced *Tsc2* CKO/mT-mG lung nodule. (A) mG/mT/Cytokeratin co-staining, arrows: mT-positive & cytokeratin-negative cells. (B) mG/mT/Acta2 co-staining, (C) mG/Pmel co-staining

Major Task 9. 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.

Key outcome: We have established multiple lung mesenchymal progenitor cell lines from 4-month-old female *Tsc2* conditional knockout mice that have

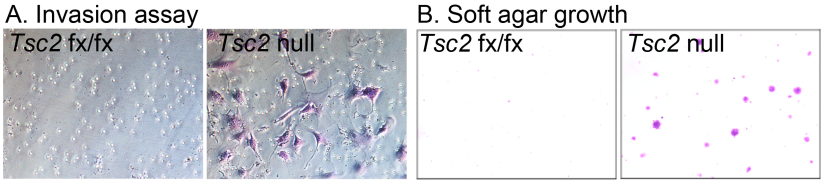

Fig.11 *Tsc2*-null lung mesenchymal cells are invasive, migrating through the matrigel-coated membrane (A), and growing in an anchorage-independent condition using a soft agar assay, cell colonies were stained by crystal violet (pink dots, in B).

lung nodules. All cells were passed more than 20 times and became stable. Their mesenchymal stem cell features have been validated by their cell surface markers, colony formation activity, and multi-differentiation capacity. Interestingly, the Tsc2-null lung mesenchymal cells, but not the control cells, are invasive and capable of anchorage-independent growth in vitro (Fig.11 on previous page), which is similar to the human LAM cell phenotypes in vitro as reported by other groups. Therefore, *Tsc2* deficient lung mesenchymal stem cells of pulmonary perivascular origin may be the key cell type that give rise to nodular lesions in adult.

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?

All proposed major activities have been completed, and a manuscript to report this study is in preparation.

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 is meeting 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 the unique *Tsc2* conditional knockout mouse model helps 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 has identified different lung mesenchymal cell subpopulations that may be responsible for distinct lung phenotypes, e.g., alveolar cysts vs. proliferative nodules, in LAM. This is 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 also contributes 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:

T31IP1685 Shi (PI) 07/01/20-06/30/22
California Tobacco Related Disease Research Program $200,000
"The deleterious effects of nicotine and e-cigarette flavorants on lung mesenchymal stem cells".
The pilot grant is to test a hypothesis that nicotine and/or e-cigarette flavorants induce lung damage through adversely affecting lung mesenchymal stem cell-mediated lung injury repair and homeostasis. We aim to determine (1) the direct toxic effects of nicotine and/or e-cigarette flavorants on lung mesenchymal stem cells in culture and the related cellular and molecular mechanisms. (2) the alterations of endogenous lung mesenchymal stem cells in mice that expose to e-cigarette vapor vs.

conventional tobacco cigarette smoke. There is no scientific overlap between this project and the current DoD project.
Role: PI

W81XWH-21-1-0257 Shi (PI) 04/15/21-04/14/23
DoD/CDMRP
“Mesenchymal Folliculin defects as a novel pathogenic mechanism of polycystic kidney lesions”
The project is to test a hypothesis that *Flcn* deficiency in distinct subsets of renal mesenchymal cells leads to polycystic kidney lesions via Wnt and Tfe3-dependent signaling to renal epithelium. Two specific aims are: (1) To determine the key subsets of mesenchymal cells in which *Flcn* deletion results in polycystic kidney lesions. (2) To determine the mechanisms by which *Flcn* deletion in the defined mesenchymal cells causes renal cysts. There is no scientific overlap between this project and the current DoD project.
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 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.