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14. ABSTRACT

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.

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 (Major Task 2 in SOW):
To continually generate whole lung mesenchyme-specific *Tsc2* conditional knockout mice

1) Specific objective: To expand mouse colonies and generate lung mesenchyme-specific *Tsc2* conditional knockout mice (E6.5-induced), including crossing with mT-mG reporter line.

2) Key outcome: We continue mouse cross-breeding to generate lung mesenchyme-specific *Tsc2* conditional knockout mice (68 *Tsc2* knockout mice and 55 controls) for different experimental purposes. Regarding validation of Tsc2 deletion in lung mesenchymal compartment, Tsc2 immunostaining was not working with two commercial anti-Tsc2 antibodies (GeneTex #GTX61245 and R&D #MAB40401), we recently obtained a new Tsc2 antibody from Abcam (#Ab32554) as recommended by Dr. Jeanine D'Armiento at Columbia University, and started the process to optimize the immunostaining protocol. Alternatively, *in situ* mRNA hybridization using the deleted mouse *Tsc2* exon 2 probe is considered.

Major Activity 2 (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: By adding more samples to different age group as reported in previous year, significant reduction of postnatal alveolar formation in *Tsc2* knockout mice is validated, which suggests that mesenchymal Tsc2 is required for alveolar growth during postnatal lung development.

Major Activity 3 (Major Task 4 in SOW):
To determine the cellular and molecular changes in lungs with *Tsc2* deletion in mesenchyme.

1) Specific objective: (a) To determine cell proliferation, differentiation, apoptosis; (b) To determine altered gene expression profiles in *Tsc2* knockout lung by RNA-seq.

2) 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. Moreover, alveolar myofibroblasts were significantly reduced in *Tsc2* knockout lung, suggesting a potential mechanism by which Tsc2 deletion in lung mesenchymal cells may negatively affect alveolar myofibroblast differentiation, which is a key cell type supporting alveolar growth. Change in apoptosis was not detected in Tsc2-null lungs.
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 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 ($P<0.05$). We are currently in the process to validate the changes and identify the candidates that may be related to alveolar growth.

Major Activity 4 (Major Task 5 in SOW):
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 two pairs of wild type and *Tsc2* knockout lungs at postnatal day 7, when alveolar growth is significant reduced in *Tsc2* knockout mice. The isolated cells are able to grow more than 20 passages and become stable in culture, and have multipotent differentiation capacity. Therefore, the Tsc2-null lung mesenchymal progenitor cell lines isolated from developing lungs prior to nodular lesions are established, which will be an important tool for future *in vitro* studies. The doubling time for all Tsc2-null lung MSCs are significantly longer than wild type controls, which is consistent with the phenotype that cell proliferation rate of Tsc2-null cells are reduced compared to the wild-type control cells, as reported last year.

Major Activity 5 (Major Task 6 in SOW):
To determine the cellular and molecular changes in lungs with *Tsc2* deletion in mesenchyme.

1) Specific objective: (1) To expand mouse colonies and generate lung mesenchyme-specific *Tsc2* conditional knockout mice (E11.5 vs. E13.5-induced) including crossing with mT-mG or tdTomato reporter lines; (2) To validate *Tsc2* deletion in different lung mesenchymal cell subpopulations of the mice.

2) Key outcome: 31 E6.5-induced *Tsc2* knockout lung samples have been harvested after >2.5 months of ages, some of them have mT-mG reporter. We only obtained one litter

containing 1 *Tsc2* knockout mouse that was induced from E11.5 to P1, while 5 litters containing 8 *Tsc2* knockout mice that were induced from E13.5 to P1 have been collected. Of the E13.5-induced *Tsc2* knockout mice, two of them have mT-mG reporter, which can be used for validation of Tsc2 deletion in specific mesenchymal cells by comparing them to E6.5-induced *Tsc2* knockout lungs. Due to complicated breeding and combination of different genotypes, there was a delay in obtaining sufficient numbers of samples, and we are working hard on this.

Major Activity 6 (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: From our previous study, 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, 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.1), while all of them had enlarged alveolar and small cyst-like lesions. The nodular phenotype has relatively high variation among the collected specimens. 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.

(2) In one E11.5-induced *Tsc2* knockout female mouse lung harvested at 2.5 months of age, no pulmonary nodule was detected, but enlargement of alveolar space is similar to what seen in E6.5-induced *Tsc2* knockout lung (Fig.2). More samples are needed to make conclusion.

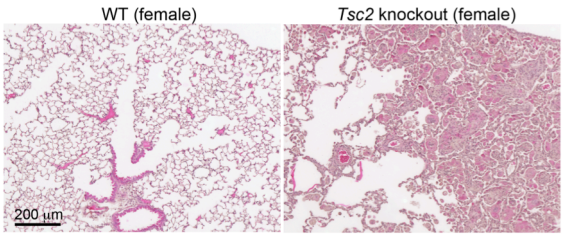

Fig.1. Pulmonary nodules and cystic changes were detected in an E6.5-induced female *Tsc2* knockout mouse at 4 months of age.

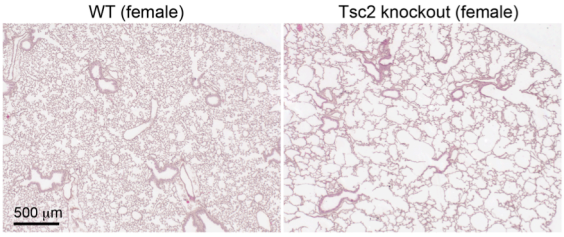

Fig.2. Enlargement of alveoli, but no lung nodules was found in an E11.5-induced female *Tsc2* knockout mouse at 2.5 months of age.

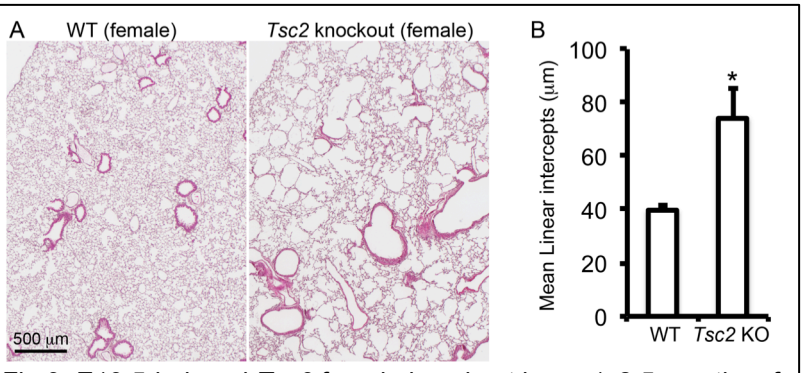

Fig.3. E13.5-induced *Tsc2* female knockout lungs (>2.5 months of age), shown by H&E-stained section (A) and alveolar size measurement (B). *P<0.05, n=5 per group.

(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.3).
In summary, there were significant differences in pulmonary nodular phenotypes when *Tsc2* deletion was induced at different developmental windows. The differential cell lineage targeting between these induction windows will be the focus of our investigation next year.

Major Activity 7 (Major Task 8 in SOW):
To determine heterogeneity of cells in LAM-like nodules, in aspects of organ origins, *Tsc2* genotypes, and specific cell marker expression.

1) Specific objective: To determine heterogeneous cell types (different organ origins, *Tsc2* deletion, and lineage markers) in the LAM-like nodules, using lineage tracing and specific cellular marker detection

2) Key outcome: As we reported previously (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.4A). 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.4B). In contrast, Pmel (gp100) staining was seen in mG-negative cells in the nodules (Fig.4C). 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). These findings will be further validated in more specimens.

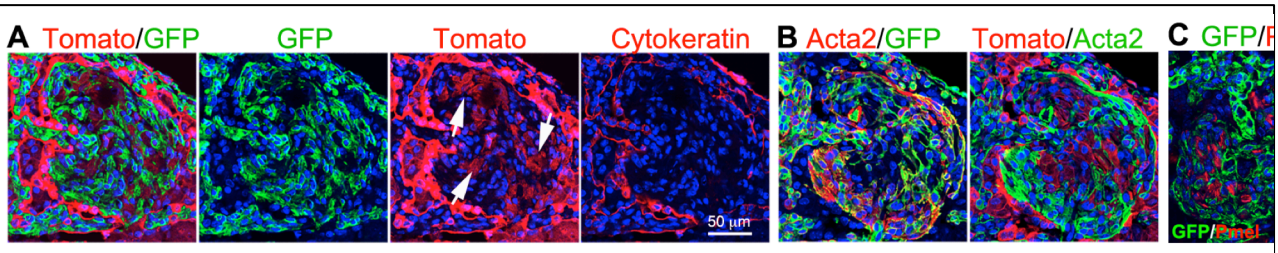

Fig.4. Co-immunostaining of an E6.5-induced *Tsc2* knockout/mT-mG lung nodule. (A) mG/mT/Cytokeratin co-staining, arrows: mT-positive & cytokeratin-negative cells. (B) mG/mT/Acta2 co-staining, (C) mG/Pmel co-staining.

Major Activity 8 (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 isolate and culture these perivascular mesenchymal stem cells from fetal lungs with different *Tsc2* genotypes.

2) Key outcome:
(a) As proposed, we will take the advantage of differential expression of Tbx4-rtTA-IRSE-lacZ and E6.5-induced TetO-Cre-driven tdTomato to identify the perivascular cells at E15.5 ($LacZ^-$/$tdTomato^+$ vs. other lung mesenchymal cells with $LacZ^+$/$tdTomato^+$). The tdTomato mice are currently crossed with *Tbx4-rtTA*/*TetO-Cre*/*Tsc2*$^{fx/fx}$ mice in order to obtain fetuses with desired genotype combination.
(b) Alternatively, we have successfully isolated perivascular mesenchymal cells in wild type fetuses. Previous studies by us and others suggest that perivascular cells express both smooth muscle cell (SMC) contractile genes (such as SM22 and Acta2) and Ng2, which can be used to separate them from airway SMCs (Ng2-negative) and pericytes (SMC marker-negative) (Paez-Cortez et al., 2013). We have crossed *SM22-YFP* mice (yellow for SMC/myofibroblasts, Fig.5) and *Ng2-dsRed* (red for perivascular cells and pericytes, obtained from JAX, #008241) to generate yellow-red double reporter mice (Fig.5A). The fetal lung of this double reporter mouse was identified under fluorescence stereomicroscope and digested to make single cell suspension, followed by FACS sorting to obtain three mesenchymal cell populations (Fig.5B): (1) YFP only (airway SMCs/myofibroblasts-like or non-vascular origin), (2) dsRed only (pericytes), and (3) YFP plus dsRed (perivascular cells or vascular origin). 10^4 cells in each sorted group are then plated in 100 mm dish for selective culture of mesenchymal stem/progenitor cells. Progenitor cells with colony forming capacity were selectively expanded and passed for cell studies. Therefore, this approach can also be used to isolate fetal perivascular cells to determine the impact of Tsc2 deletion on these cells.

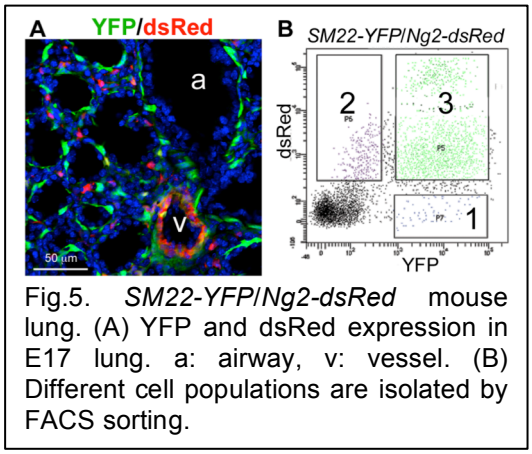

Fig.5. *SM22-YFP*/*Ng2-dsRed* mouse lung. (A) YFP and dsRed expression in E17 lung. a: airway, v: vessel. (B) Different cell populations are isolated by FACS sorting.

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.

(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. Additional lung MSCs from different genotypes will be isolated from more mice to reach n>3 cultures 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.

(4) 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. In particular, some of the obtained data described above will be validated in more lung specimens.

(5) 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. More E11.5 and E13.5-induced lung samples will be collected to reach n>5 per genotype/sex in order to reach statistical significance in the final analysis.

(6) To isolate and characterize lung perivascular mesenchymal stem cell lineage in mesenchyme-specific *Tsc2* knockout mice and 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

Conference Oral Presentation:

Wei Shi. Establishment of *Tsc2*-null mouse lung mesenchymal progenitor cell lines. International Tuberous Sclerosis Complex Research Conference. Sep 13-15, 2018. Tokyo, Japan.

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:

W81XWH-18-1-0185 (Shi) 06/01/18-11/30/19 1.20 calendar
PR171133/ U.S. Army Medical Research Acquisition Activity $131,000
"Growth and/or Recruitment of a Novel Cell Population with Neural Crest Origin in Lung Fibrosis"

The discovery award is to identify neural crest origin ectomesenchymal cells as a new cell population in contributing to lung fibrosis progression in response to injury with two specific aims (1) To determine and characterize a new mesenchymal cell population of neural crest origin specifically in fibrosis lung. (2) To determine changes in circulating neural crest descendants in response to pulmonary fibrogenic injury.
Role: PI

1R01HL146541-01 (Shi & Henske) 04/01/19-03/31/23 3.50 calendar
NIH/NHLBI $473,750
"Pathogenic Mechanisms of Pulmonary Lymphangioleiomyomatosis"
This project is focused on the mechanisms underlying hyperactive mTORC1 in subsets of lung mesenchymal cells leads to estrogen-dependent proliferation of nodular-like lesions, associated with metabolic and mitochondrial dysfunction, recruitment of extrapulmonary cells and lung destruction with two specific aims (1) To dissect the molecular and cellular mechanisms of LAM using a novel mouse model with spontaneous LAM-like lung lesions. The role of hyperactivated mTORC1 in the pulmonary LAM-like nodules will be examined using both pharmacologic (reversible) and genetic (permanent) approaches. The impact of Tsc2 deficiency on cellular metabolism and mitochondrial function will be addressed for the first time in lung mesenchyme-derived cells as a potential mechanism for the abnormal cell growth. The origin of LAM cells and the mechanisms of recruitment of extrapulmonary mesenchymal cells to LAM-like nodules will be examined using both parabiosis and allograft models to elucidate the cellular heterogeneity of LAM nodules (2) To determine the specific role of gender in contributing to the formation and progression of LAM-like nodules in mice with lung mesenchymal *Tsc2* delet**ion.** The roles of estrogen in the pulmonary LAM-like lesions will be investigated *in vivo* by altering estrogen activity in lung mesenchyme-specific *Tsc2* knockout mice, or by simultaneous deletion of ERa/Tsc2 or ERb/Tsc2. The mechanisms will be further dissected *in vitro* by estrogen-mediated metabolic profiling, RNA-seq, ChIP-seq in *Tsc2*-null vs. wild-type cells. Although the lung mesenchyme-specific *Tsc2* knockout mouse model is used in this project, the major research questions and approaches proposed in this R01 are totally different. There is no significant scientific overlap between this new R01 project and our ongoing DoD project.
Role: PI

Dr. Wei Shi has one project closed this year.

1U01 HL122681-01 (Warburton) 06/14/14-04/30/19 1.80 calendar
NIH/NHLBI $475,000
"Molecular anatomy of human alveolar development"
The major goal of this grant is to generate a digital map of alveolar development in mouse and human lung, using various imaging approaches.
Role: Co-investigator

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.