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Award Number: W81XWH-05-1-0205

TITLE: Modeling Phenotypes of Tuberous Scerosis in the Mouse

PRINCIPAL INVESTIGATOR: James Michael Shipley, Ph.D.

CONTRACTING ORGANIZATION: Washington University
St Louis MO 63130-4899

REPORT DATE: February 2008

TYPE OF REPORT: Final

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
09-FEB-2008	Final	10 JAN 2005 - 9 JAN 2008

4. TITLE AND SUBTITLE
Modeling Phenotypes of Tuberous Sclerosis in the Mouse

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0205

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Washington University
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St. Louis, MO 63130

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
US Army Medical Research
And Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

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

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The overall goal of this project was to generate a mouse model of the smooth muscle-related facets of tuberous sclerosis, specifically in an attempt to model the lung phenotype seen in a subset of TS patients and patients with LAM. We have conditionally targeted the TSC1 gene in smooth muscle, which results in mortality at approximately 10 weeks of age. This mouse provided a useful model in which to investigate the function of individual MMPs or other proteins in this pathological progression, and to evaluate relevant therapeutic interventions such as rapamycin. We found that MMP-9 was induced in the lungs of these mice. However, upon breeding the MMP-9 knockout allele into this model, the absence of MMP-9 did not change the phenotype, ie., the mice still died at 10 weeks of age. Similarly, administration of rapamycin (75µg/mouse, 3X week beginning at week 4) did not alter the lifespan of the mice.

15. SUBJECT TERMS
MOUSE MODEL, MMP, CONDITIONAL TARGETING, INDUCIBLE EXPRESSION, TSC1

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	10	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

Introduction

The overall goal of the project was to generate a mouse model in which one of the two tumor suppressor genes responsible for tuberous sclerosis, TSC1 or TSC2, are dysregulated specifically in smooth muscle. This was to be accomplished in one of two ways, including (1) specifically targeting the TSC1 gene in smooth muscle using a doxycycline-inducible transgenic system which we adapted for expression of cre recombinase in smooth muscle, or (2) by overexpression of a potentially dominant negative form of tuberin in smooth muscle. Should either of these approaches work, the goal was then to evaluate whether matrix metalloproteinase (MMP) expression was dysregulated in the mouse model or in TSC1 -/- cells derived from these mice, whether rapamycin treatment corrects this dysregulation in cultured cells, and whether phenotypes seen in the mice are abrogated by breeding pertinent MMP knockout alleles into the model.

Body

In the approved statement of work, individual specific aims were broken down into tasks, and these individual tasks were planned over the two year duration of the award. Permission was received to extend the award (at no cost) beyond the two year duration to complete the proposed studies. In the **first specific aim** (targeting the TSC1 gene in smooth muscle using conditional cre mice we have generated and evaluate resulting phenotypes), we needed to generate mice which harbor 4 separate components including two floxed alleles of the TSC1 gene, one SMP8-rtTA transgene, and the tetO-cre transgene. We maximized the chances of discovering a phenotype by beginning doxycycline administration as early as possible, by giving it continuously both to pregnant female mice and to their offspring upon weaning. We found that inactivation of TSC1 in this context causes mortality in these mice at an average age of 10 weeks. Alveolar ducts in the lungs of these mice are enlarged relative to controls (mice on doxycycline but lacking the rtta or cre transgenes)(Figure 1), and in some cases (~30%) we see nodules similar to those seen in human TS and LAM (Figure 2). Immunostaining reveals loss of TSC1 protein in cells in which active cre recombinase is expressed (Figure 3), as these cells are marked by recombination of a ROSA allele that results in ß-galactosidase activity following cre-mediated recombination (blue). Another well-characterized readout for dysregulation of the TSC1/TSC2 pathway is activation of ribosomal protein S6 (phosphorylation), which is normally negatively regulated by the tuberin/hamartin complex. Total lung extracts from control mice show minimal phosphorylation of ribosomal protein S6(Figure 4, lanes 1-4), which is markedly induced in the lungs of our smooth muscle-specific TSC1 knockout mice (lanes 5-8). One hypothesis in TS/LAM is that lung destruction is mediated by metalloproteinases, particularly MMP-2 and -9, which is upregulated (MMP-9) in the lungs of these patients relative to controls (Figure 5). Indeed, we observe increased MMP-9 activity in the lungs of the smooth muscle-specific TSC1 knockouts relative to controls (Figure 6). Taken together, many of the pulmonary facets of human TS/LAM are recapitulated in the lungs of the smooth muscle-specific TSC1 knockout mice.

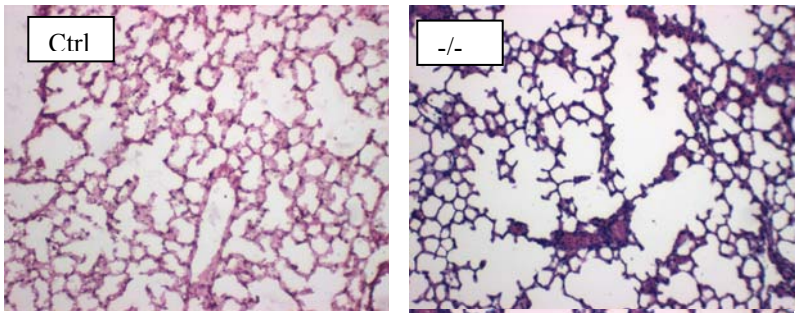

Figure 1. Morphology of TSC1 Conditional Knockout Lungs. Shown are lungs from a TSC1 conditional knockout (right) and a control littermate (left) sacrificed at 9 weeks, demonstrating enlarged airspaces in the knockout.

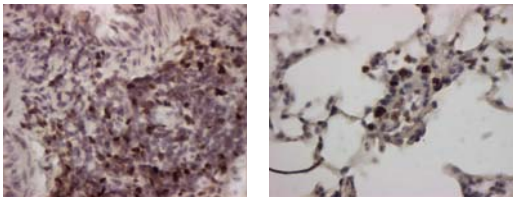

Figure 2. Ki-67 Immunostaining on TSC1 Conditional Knockout Lungs. Lungs were stained for Ki-67, a marker of proliferating cells. Areas that appear nodular show many Ki-67-positive cells.

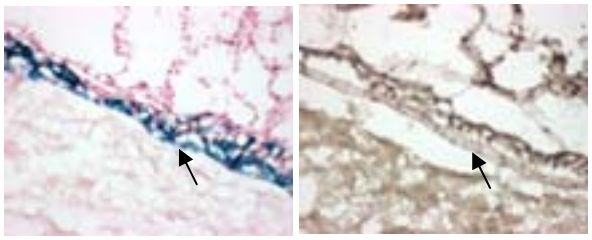

Figure 3. TSC1 immunostaining in the conditional TSC1 knockout. The ROSA26 reporter allele for cre activity was bred onto the conditional knockout background, and lung sections were stained for either ß-galactosidase activity with X-Gal (left, marks cre-positive cells) or for an antibody to TSC1 (right). Note the smooth muscle expressing cre which is negative for TSC1, in contrast to the surrounding tissue (arrows).

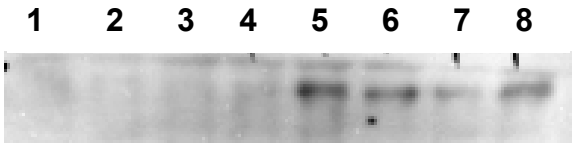

Figure 4. Activation of ribosomal protein S6 phosphorylation in TSC1 conditional knockout mice. Total lung extracts from control mice (lanes 1-4) or TSC1 conditional knockout mice (lanes 5-8) were evaluated for phosphorylation (activation) of ribosomal protein S6 by western blotting, demonstrating activation of this pathway in TSC1 conditional knockout mice.

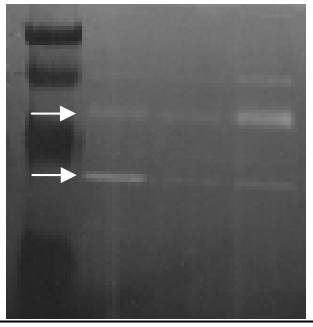

Figure 5. Expression of MMPs –2 and –9 in LAM. Ten microliters of BAL from each of three LAM patients was analyzed by gelatin zymography. Expression of MMP-9/gelatinse B (top arrow) and MMP-2/gelatinase A (bottom arrow) is seen. These proteases are undetectable in the BAL of control subjects (not shown). Increased expression of MMP-2 and MMP-9 corroborates gene expression profiling data by others on LAM tissue which shows these MMPs induced in LAM relative to biopsy specimens from control subjects.

Figure 6. Increased MMP-9 activity in Conditional TSC1 knockout lungs. The caudal lobe of the right lung of two controls (lanes 1 and 2) and one conditional TSC1 knockout (lane 3) were used for gelatin zymography. Increased MMP-9 activity is seen in the the total lung extract of the conditional TSC1 knockout animal.

The goals of the **second specific aim** were very similar to those of the first aim, but in this case the approach was to overexpress a potentially dominant negative form of the TSC2 gene product, tuberin, in smooth muscle cells in mice with the goal being to inactivate TSC function. While we were able to achieve significant overexpression of this form of tuberin in these mice upon induction by doxycycline (Figure 7), this approach did not yield a phenotype, at least after 6 months of doxycycline treatment. Thus, as our overall hope was for one of these two models to prove fruitful, and the conditional TSC1 knockout (specific aim 1) has proven to yield a highly penetrant phenotype, we have focused our attention on the analysis of the phenotype in this model.

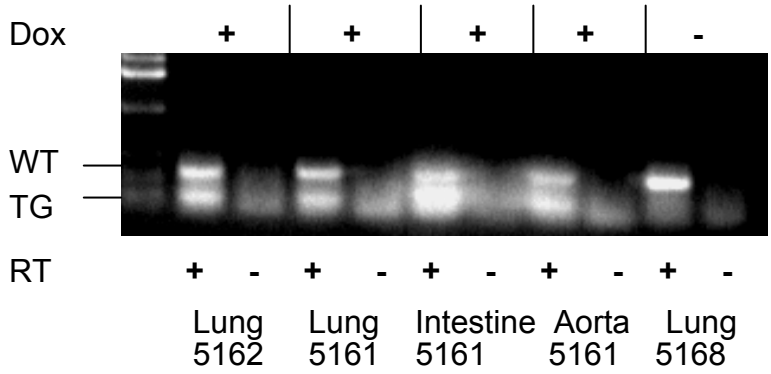

Figure 7. **Induction of dominant negative TSC2 transgene expression with doxycycline.** RNA was isolated from smooth muscle-containing organs from 3 transgenic mice(5161, 5162, 5168). Two (5161, 5162)had received doxycycline (Dox) in the drinking water for 2 weeks prior to sacrifice. RT-PCR for TSC2 demonstrates doxycycline-dependent induction of the transgene product (TG). Expression of the wild type TSC2 gene product is seen in all samples (WT). Either product is only seen when reverse transcriptase is used (RT), demonstrating that neither arises from DNA contamination of the RNA.

One of the goals of **specific aim 3** was to establish cultures of smooth muscle cells or lung fibroblasts from the mice evaluate rapamycin as a treatment in culture for any aberations that we might identify (altered S6K phosphorylation, increased MMP production). At the time of grant submission, we felt that it was unlikely that this would be possible within the two year time frame of the grant. Efforts to establish these primary cell cultures were difficult and somewhat unproductive. However, we extended the scope of these studies *in vivo* in using much more powerful approach than the explant studies we proposed. We identified that levels of MMP-9 in particular were increased in the lungs of these mice. To investigate directly the contribution of MMP-9 to the phenotypes we saw, we bred the MMP-9 knockout allele onto the TSC1 floxed/SMP8-rtTA/tetO-cre background. Unfortunately, the absence of MMP-9 did not increase the lifespan in these mice (10 weeks).

In another effort to extend these studies beyond those planned in the original Statement of Work, we also evaluated rapamycin treatment in the mice. Rapamycin (Sirolimus) was obtained through Wyeth. Rapamycin has been shown to be effective in mice at a dose of 1.5-4 mg/kg/day when delivered intraperitoneally. We therefore used 3 mg/kg/day (75 μg/mouse) as the starting point for these experiments. Based on similar studies by other groups, we administered rapamycin 3 times per week beginning at 4 weeks of age for the remainder of the life of the animal. We found that rapamycin treatment using this regimen was unable to extend the lifespan of the mice (5 mice to date). These rapamycin experiments were done in the context of doxycycline administration from the time of conception for the lifespan of the mouse (ie., induction of cre recombinase expression to inactivate the TSC1 gene *in utero*).

Key Research Accomplishments

- Generation of a mouse model in which conditional targeting of the TSC1 gene in smooth muscle cells results in a reproducible phenotype (mortality at approximately 10 weeks of age).
- Alveolar duct enlargement in the lungs of these mice is a consistent finding.
- Formation of nodules in the lung of these mice is also a reproducible finding, although not as penetrant a phenotype as the mortality and the airspace enlargement.

- MMP-9 induction in the lungs of these mice may contribute to the airpspace enlargement (preliminary, gelatin zymography has been conducted on two conditional knockouts vs. two controls).
- Treatment of the smooth muscle-specific TSC1 knockout mice with rapamycin (3 mg/kg, 3x/week) did not extend the lifespan of these mice.
- Breeding of the MMP-9 knockout allele into the TSC1 conditional (TSC1 flox/flox, SMP8-rtTA, tetO-cre) background also did not extend the lifespan of these mice (10 weeks)

Reportable Outcomes

The primary reportable outcome is the success of the conditional TSC1 knockout mouse in racapitulating some of the pulmonary aspects of TS and LAM (described above). An abstract on this work was presented at the American Thoracic Society international meeting in May of 2007. The abstract is shown below:

Conditional Targeting of the TSC1 gene in Smooth Muscle as a Model of LAM

Yifu Fang*, David J. Kwiatkowski#, and J. Michael Shipley*
*Division of Pulmonary and Critical Care Medicine, Washington University School of Medicine, St. Louis, MO, 63110 and # Hematology Division, Brigham & Women's Hospital, Boston, MA 02115

Dysregulated proliferation of smooth muscle cells is a hallmark of LAM, which involves mutation of either the TSC1 (hamartin) or TSC2 (tuberin) tumor suppressor gene. TSC1 and TSC2 knockout mice die mid gestation, precluding analysis of their lungs as a model of LAM. To circumvent this issue, we developed transgenic mice for doxycycline-inducible expression of cre recombinase in smooth muscle and have used these to conditionally target the TSC1 gene. By RT-PCR, mice which express the reverse tetracycline transcriptional activator (rtTA) under the control of the smooth muscle alpha actin (SMA) promoter show significant rtTA expression in smooth muscle-containing tissues including the lung. These mice were bred to tetO-cre mice and ROSA26 reporter mice to assess doxycycline-inducible expression of active cre recombinase in the lung. Expression was seen in vascular smooth muscle, airway smooth muscle, and myofibroblasts. Bitransgenic SMA-rtTA/tetO-cre mice were bred to "floxed" TSC1 mice where targeting of the TSC1 gene was initiated by administration of doxycycline to the drinking water. Pregnant mothers were given doxycycline water continuously from conception to birth, and the offspring continued to receive doxycycline water throughout their lifespan. TSC1 flox/flox mice containing both the SMA-rtTA and tetO-cre transgenes that received continous doxycycline had a mean life span of 10 weeks (n=9). Hyperphosphorylation of ribosomal protein S6 kinase is seen in lung extracts, consistent with inactivation of hamartin function. Preliminary evidence suggests abnormal smooth muscle proliferation and airspace enlargement in the lungs of these mice, two facets characteristic of human LAM. Thus, these mice appear to recapitulate important facets of the disease and will be useful in evaluating therapeutic interventions.

Note: As of 10-26-07, I left Washington University for a non-research position. This is the reason that the final report was filed now (instead of early 2008). One limitation of these studies was the finding that the tetO-cre transgene that we used (obtained from Dr. Jeffrey Whitsett; mice originally made by Dr. Andras Nagy) presumably expresses a low level of cre recombinase in the absence of a rtTA activator transgene. This was manifested in the death of TSC1 flox/flox/tetO-cre mice (given doxycycline) at an average age of 13 weeks, ie., 3 weeks longer than the lifespan of the conditional TSC1 knockout mice that we generated. While we did not detect an extension of lifespan with either rapamycin treatment or elimination of MMP-9 expression, this leaky expression of cre nonetheless puts limits on the evaluation of treatments in these mice. This has also been seen by Dr. Frank McCormack, scientific director of the LAM Foundation, who undertook similar studies using the same tetO-cre and floxed TSC1 mice to eliminate TSC1 expression in pulmonary type II epithelial cells. Dr. McCormack noted that there is now a better, less leaky tetO-cre mouse generated by a German company. If someone were to continue these studies, I would recommend use of this cre transgene and breeding onto the TS1 floxed background along with our SMP8-rtTA transgene. Three investigators at Washington University

(Drs. Robert Mecham, Richard Pierce, and Nguyet Nguyen) will continue to breed the SMP8-rtTA mice and make them available to other investigators. In addition, breeding experiments are under way utilizing mice that contain combinations of the floxed TSC1, tetO-cre, SMP8-rtTA, and MMP-9 knockout alleles, and fertilized eggs from these breedings will be cryopreserved for future use should anyone decide to further pursue these studies (including those involving the MMP-9 knockout allele) using the tetO-cre mouse that was available to us at the time of these studies. For further information on cryopreserved embryos, contact Mr. Ronald McCarthy at Washington University (rmccarth@im.wustl.edu).

Conclusion

These studies have provided a useful model in which to study facets of tuberous sclerosis involving loss of function of TSC1 in smooth muscle, most notably the lung pathology which is also seen in lymphangioleiomyomatosis (LAM).

References

N/A

Appendices

None

AMENDMENT OF SOLICITATION/MODIFICATION OF CONTRACT

1. CONTRACT ID CODE	PAGE OF PAGES
S	1 | 2

2. AMENDMENT/MODIFICATION NO.	3. EFFECTIVE DATE	4. REQUISITION/PURCHASE REQ. NO.	5. PROJECT NO.(If applicable)
P00002	11-Jul-2008	SEE SCHEDULE	

6. ISSUED BY	CODE	7. ADMINISTERED BY (If other than item 6)	CODE
USA MED RESEARCH ACQ ACTIVITY 820 CHANDLER ST FORT DETRICK MD 21702-5014	W81XWH	USA MED RESEARCH ACQ ACTIVITY ATTN: JENNIFER DUNLAP 301-619-1347 JENNIFER.DUNLAP@AMEDD.ARMY.MIL FORT DETRICK MD 21702	W81XWH

8. NAME AND ADDRESS OF CONTRACTOR (No., Street, County, State and Zip Code)		9A. AMENDMENT OF SOLICITATION NO.
WASHINGTON UNIVERSITY TERRI LOVE ONE BROOKINGS DRIVE CAMPUS BOX 1054 276 N SKINKER BLDG, SUITE 220 ST LOUIS MO 63130-4899		9B. DATED (SEE ITEM 11)
	X	10A. MOD. OF CONTRACT/ORDER NO. W81XWH-05-1-0205
CODE 2B003 FACILITY CODE	X	10B. DATED (SEE ITEM 13) 04-Jan-2005

11. THIS ITEM ONLY APPLIES TO AMENDMENTS OF SOLICITATIONS

☐ The above numbered solicitation is amended as set forth in Item 14. The hour and date specified for receipt of Offer ☐ is extended, ☐ is not extended.

Offer must acknowledge receipt of this amendment prior to the hour and date specified in the solicitation or as amended by one of the following methods: (a) By completing Items 8 and 15, and returning _____ copies of the amendment; (b) By acknowledging receipt of this amendment on each copy of the offer submitted; or (c) By separate letter or telegram which includes a reference to the solicitation and amendment numbers. FAILURE OF YOUR ACKNOWLEDGMENT TO BE RECEIVED AT THE PLACE DESIGNATED FOR THE RECEIPT OF OFFERS PRIOR TO THE HOUR AND DATE SPECIFIED MAY RESULT IN REJECTION OF YOUR OFFER. If by virtue of this amendment you desire to change an offer already submitted, such change may be made by telegram or letter, provided each telegram or letter makes reference to the solicitation and this amendment, and is received prior to the opening hour and date specified.

12. ACCOUNTING AND APPROPRIATION DATA (If required)

See Schedule

13. THIS ITEM APPLIES ONLY TO MODIFICATIONS OF CONTRACTS/ORDERS. IT MODIFIES THE CONTRACT/ORDER NO. AS DESCRIBED IN ITEM 14.

☐ A. THIS CHANGE ORDER IS ISSUED PURSUANT TO: (Specify authority) THE CHANGES SET FORTH IN ITEM 14 ARE MADE IN THE CONTRACT ORDER NO. IN ITEM 10A.

☐ B. THE ABOVE NUMBERED CONTRACT/ORDER IS MODIFIED TO REFLECT THE ADMINISTRATIVE CHANGES (such as changes in paying office, appropriation date, etc.) SET FORTH IN ITEM 14, PURSUANT TO THE AUTHORITY OF FAR 43.103(B).

☐ C. THIS SUPPLEMENTAL AGREEMENT IS ENTERED INTO PURSUANT TO AUTHORITY OF:

X D. OTHER (Specify type of modification and authority)
Mutual Agreement

E. IMPORTANT: Contractor ☒ is not, ☐ is required to sign this document and return _____ copies to the issuing office.

14. DESCRIPTION OF AMENDMENT/MODIFICATION (Organized by UCF section headings, including solicitation/contract subject matter where feasible.)
Modification Control Number: jdunlap085240
Subject grant is modified for the following purpose:

1. Refund check #523710 was received from the awardee in the amount of $331.95 and deposited in the U.S. Treasury per recipients SF272 dated 03/11/2008.

2. See Summary of Changes for Details.

Except as provided herein, all terms and conditions of the document referenced in Item 9A or 10A, as heretofore changed, remains unchanged and in full force and effect.

15A. NAME AND TITLE OF SIGNER (Type or print)	16A. NAME AND TITLE OF CONTRACTING OFFICER (Type or print)
	DAVID D. DENTON / ADM. CONTRACTING OFFICER TEL: 301-619-6857 EMAIL: david.d.denton@us.army.mil

15B. CONTRACTOR/OFFEROR	15C. DATE SIGNED	16B. UNITED STATES OF AMERICA	16C. DATE SIGNED
_____ (Signature of person authorized to sign)		BY _____ (Signature of Contracting Officer)	17-Jul-2008

SECTION SF 30 BLOCK 14 CONTINUATION PAGE

SUMMARY OF CHANGES

SECTION 00800 - SPECIAL CONTRACT REQUIREMENTS

Accounting and Appropriation

Summary for the Payment Office

As a result of this modification, the total funded amount for this document was decreased by $331.95 from $307,949.00 to $307,617.05.

SUBCLIN 000101:

AB: 97401301831074811966000000950415OP1FLMNW91ZSQ4357N601FLMNP1018064 was decreased by $331.95 from $28,359.00 to $28,027.05

(End of Summary of Changes)