

AWARD NUMBER: W81XWH-13-1-0184

TITLE: In Vivo Tagging of Lung Epithelial Cells To Define the Early Steps of Tumor Cell Dissemination

PRINCIPAL INVESTIGATOR: Hasmeena Kathuria, MD

CONTRACTING ORGANIZATION:

Trustees of Boston University
Boston, MA 02118

REPORT DATE: December 2015

TYPE OF REPORT: FINAL REPORT

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE December 2015			2. REPORT TYPE Final		3. DATES COVERED 15 Sep 2013 - 14 Sep 2015	
4. TITLE AND SUBTITLE In Vivo Tagging of Lung Epithelial Cells To Define the Early Steps of Tumor Cell Dissemination					5a. CONTRACT NUMBER	
					5b. GRANT NUMBER W81XWH-13-1-0184	
					5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Hasmeena Kathuria E-Mail: hasmeena@bu.edu					5d. PROJECT NUMBER	
					5e. TASK NUMBER	
					5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Trustees of Boston University, BUMC Office of Sponsored Programs 85 East Newton Street, M-921 Boston, MA 02118					8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel 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 To understand the early events that accompany invasive behavior in vivo, we proposed to develop a lineage-labeling system to detect and isolate cells of lung epithelial origin during tumor progression in a mouse model of lung cancer. A mouse model of lung cancer with metastasis [LSL-Kras ^{G12D} /Lkb1 ^{L/L} mice] was interbred with Nkx2.1-CreERT ₂ knock-in mouse strain, containing a tamoxifen-inducible lineage specific Cre recombinase, to generate adult lung-specific mutations in Kras and Lkb1. We introduced a Rosa ^{YFP} (lox-stop-lox-YFP) into the mutant background to specifically label and track lung epithelial cells during tumor progression and metastasis. Adult mouse lungs isolated from Nkx2-1creER ^(+/-) ; Kras ^{G12D} /Lkb1 ^(L/-) mice and from Nkx2-1creER ^(+/-) ; Kras ^{G12D} /Lkb1 ^(L/L) ; Lkb1 ^(L/L) mice 7 days after tamoxifen injections show that Kras ^{G12D} mice develop isolated tumors one week after the end of tamoxifen, while Kras ^{G12D} /Lkb1 ^{L/-} mice developed very aggressive tumor patterns under the same treatment. This model (Kras ^{G12D} /Lkb1 ^{L/L} /YFP ^{L/-} /Nkx2-1creER2 ^{+/-} mice) allows us to determine the timing of dissemination during the natural evolution of lung adenocarcinoma in vivo and correlate cell phenotype with the acquisition of invasive and tumor-initiating properties, promising to shift the current paradigm of lung cancer metastasis and aid in early detection and novel treatment approaches.						
15. SUBJECT TERMS Epithelial mesenchymal transition, lung cancer, metastases, mouse models of lung cancer, lineage-labeling						
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 9	19a. NAME OF RESPONSIBLE PERSON USAMRMC	
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)	

Table of Contents

	<u>Page</u>
1. Introduction.....	4
2. Keywords.....	4
3. Accomplishments.....	4
4. Impact.....	7
5. Changes/Problems.....	8
6. Products.....	8
7. Participants & Other Collaborating Organizations.....	9
8. Special Reporting Requirements.....	9
9. Appendices.....	9

FINAL REPORT

1. INTRODUCTION: Despite the high prevalence and poor prognosis of lung cancer, little is known about the mechanisms of progression. There are two major metastasis paradigms: In the classical model, tumors acquire mutations that promote invasive behavior and dissemination late in tumor evolution, whereas in the alternative model, metastasis is an inherent feature of tumors very early in its natural history. A challenge in studying tumor cell dissemination has been the identification of markers that can distinguish cancer cells from cells that normally reside in the bloodstream or at sites of seeding. As a result, there remains uncertainty regarding the mechanisms of metastasis as well as the timing of dissemination. Epithelial-mesenchymal transition (EMT), a process by which cells lose epithelial characteristics and develop mesenchymal properties, has been implicated as a means by which tumor epithelial cells acquire the ability to invade and disseminate. Most studies of EMT in the context of cancer biology, however, have been based on cultured cells manipulated in vitro, and thus the relevance of EMT to in vivo carcinogenesis is controversial. If EMT is an early process, then detection methods that rely on cellular expression of epithelial markers alone are unlikely to provide a complete picture of metastasis. To understand the early events that accompany invasive behavior in vivo, we proposed to develop a novel, precise, and sensitive lineage-labeling system to detect and isolate cells of lung epithelial origin during tumor progression in a mouse model of lung cancer. This system will allow us to determine the timing of dissemination during the natural evolution of lung adenocarcinoma in vivo and correlate cell phenotype with the acquisition of invasive and tumor-initiating properties.

2. KEYWORDS: Epithelial mesenchymal transition, lung cancer, metastases, mouse models of lung cancer, lineage-labeling, Kras, Lkb1, Nkx2-1creER2^{+/-} mice.

3. ACCOMPLISHMENTS:

- What were the major goals of the project?

The 3 major goals (tasks1-3) were as follows:

Task 1. Generate Kras^{G12D}/Lkb1^{L/L}/YFP^{L/-}/Nkx2-1creER2^{+/-} mice (timeframe months1-10 by Drs Kathuria, Cao, Ramirez).

1. We will follow a specific breeding plan to obtain Kras^{G12D}/Lkb1^{L/+}/YFP^{L/-}/Nkx2-1creER2^{+/-} mice. We currently have in our facility Kras^{G12D}/Lkb1^{L/L}, YFP^{L/L}, and Nkx2-1creER2^{+/-} lines (timeframe: months 1-6 by Kathuria/Cao).
2. Kras^{G12D}/Lkb1^{L/+}/YFP^{L/-}/Nkx2-1creER2^{+/-} mice will be finally bred to generate Kras^{G12D}/Lkb1^{L/L}/YFP^{L/-}/Nkx2-1creER2^{+/-} mice (timeframe: months 6-10 by Kathuria/Ramirez).
3. Crossings will be set up at the LASC animal facility and tails will be genotyped by PCR (timeframe: months 1-10).

Task 2. Isolate lungs for histology and immunofluorescence analyses (SA1a-b) and blood for flow cytometry analysis (SA2a-b) (timeframe months 6-10 by Drs Kathuria, Cao, Ramirez, and Kotton).

1. First we will induce tumors by tamoxifen injection of Kras^{G12D}/Lkb1^{L/L}/YFP^{L/-}/Nkx2-1creER2^{+/-} mice and creER2 negative controls (timeframe: months 6-10 by Dr. Ramirez/Cao).
2. We will collect lung tissues at three time points (2, 4 and 8 weeks) for pathology, H&E and immunofluorescence analyses (timeframe: months 6-9 by Dr. Kathuria/Ramirez).
3. Blood will be collected by heart puncture for flow cytometry analysis of YFP⁺ circulating tumor cells (timeframe: months 6-10 by Drs. Kathuria/Kotton).

Task 3. In a second round of breeding we will isolate lungs and blood (at the same time points described above) after tamoxifen injection for flow cytometry analyses of single-cell suspensions for qRT-PCR analysis and in vitro assays (SA1c, 2b)(timeframe: months 9-12 by Drs Kathuria, Cao, Ramirez, Kotton).

1. YFP⁺ E-cadherin⁺, YFP⁺ E-cadherin⁻ and YFP⁻ cells will be collected for RNA purification and qRT-PCR analyses and for in vitro analysis of self-renewal, clonality and survival properties (timeframe months 9-12 by Drs. Ramirez/Cao).
2. Blood will also be collected as above for flow cytometry analysis of YFP⁺ circulating tumor cells (timeframe months 9-12 by Drs. Kathuria/Kotton)

• **What was accomplished under these goals?**

1. Major Activities that were accomplished:

Task 1. Generate $Kras^{G12D}/Lkb1^{L/L}/YFP^{L/L}/Nkx2-1creER2^{+/-}$ mice (timeframe months 1-10 by Drs Kathuria, Cao, Ramirez).

Mouse model development. We have successfully completed task 1 and have developed a novel, precise, and sensitive lineage-labeling system to detect and isolate cells of lung epithelial origin during tumor progression in metastatic and non-metastatic mouse models of lung cancer.

Kras is mutated with high frequency in human lung adenocarcinomas. Lkb1 is a tumor suppressor gene that is mutated in 30% of lung cancers. We used a mouse model of lung cancer with metastasis driven by conditionally activated Kras and concurrent tumor suppressor Lkb1 loss [$Kras^{G12D L/L}/Lkb1^{L/L}$ mice]. $Kras^{G12D L/L}/Lkb1^{L/L}$ mice develop tumors within 4 weeks of intratracheal adeno-Cre activation and die within 8 to 10 weeks, with local and distant metastases. Although $Kras^{G12D L/L}/Lkb1^{L/L}$ mice develop both adenocarcinomas and squamous cell carcinomas, all metastatic foci appear to be adenocarcinoma in origin.

A similarly derived model that does not metastasize activated by Kras^{G12D} alone [$Kras^{G12D L/L}$] was also used for our studies. $Kras^{G12D L/L}$ mice, which develop only primary lung tumors, develop a mixture of adenomas and adenocarcinomas 16 weeks after intratracheal adeno-Cre activation of oncogenic Kras and die within 24 to 26 weeks.

Methodology.

We bred these mouse models as described below (Fig 1,2). First, we intercrossed the model of lung cancer with metastasis driven by conditionally activated Kras and concurrent tumor suppressor Lkb1 loss [$Kras^{G12D L/L}/Lkb1^{L/L}$ mice] to the Nkx2-1-CreER knock-in mouse strain, containing a tamoxifen-inducible lineage specific Cre recombinase, in a Rosa-YFP (lox-stop-lox-YFP) background [$Nkx2-1-CreER^{+/-}ER/Rosa YFP^{L/L}$ mice]. This model has generated adult lung-specific mutations in Kras and Lkb1, mutations commonly found in human lung adenocarcinomas, while activating lineage tracing. Second, we have generated a model of lung cancer without metastasis driven by conditional activated Kras only [$Kras^{G12D L/L}$], in the above lung epithelial, tamoxifen-inducible lineage tracing background [$Nkx2-1-CreER^{+/-}ER/Rosa YFP^{L/L}$]. These models will allow specific labeling and tracking of lung epithelial cells during tumor progression and metastasis.

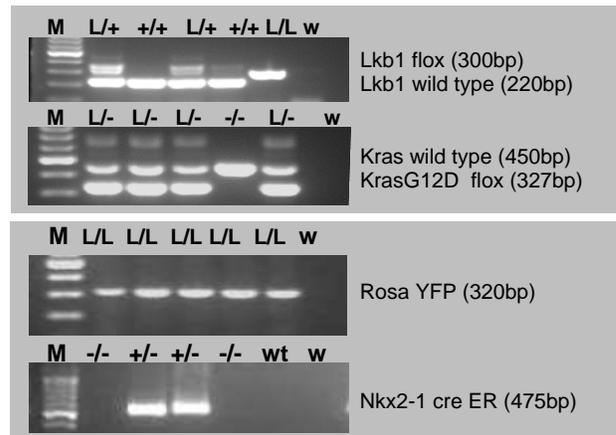
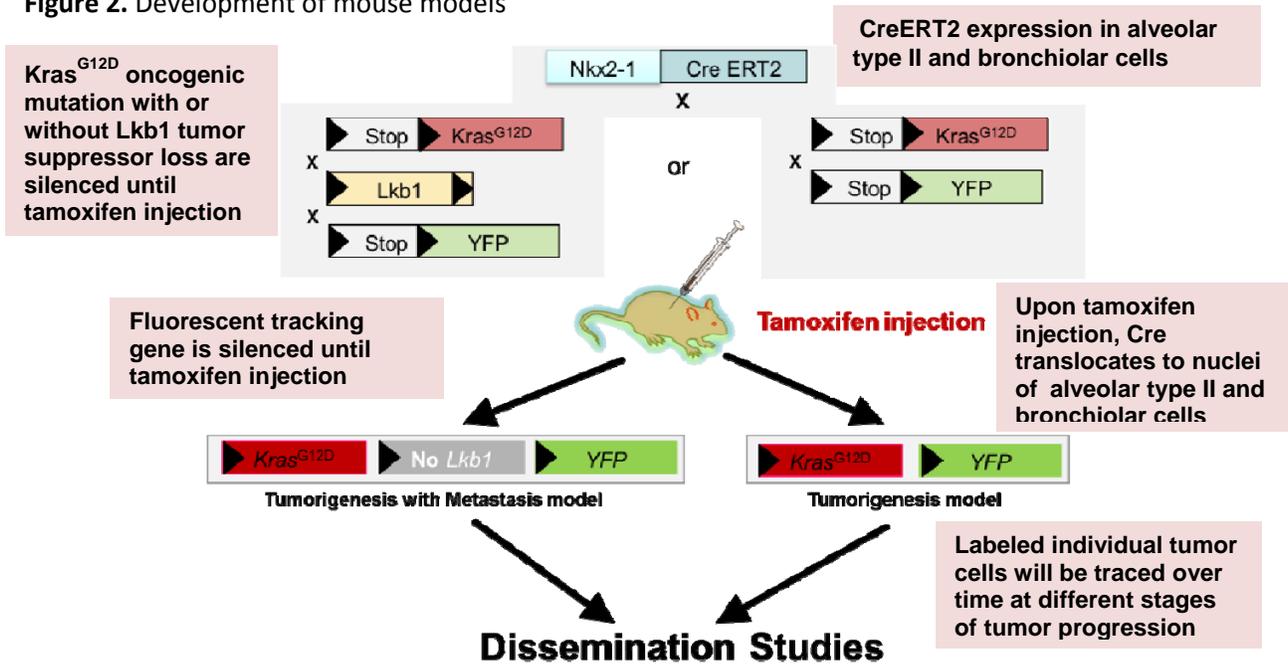


Figure 1. Genotyping of the $Kras^{G12D}$, Lkb1, Rosa YFP and Nkx2-1 Cre ER mice by PCR analysis.

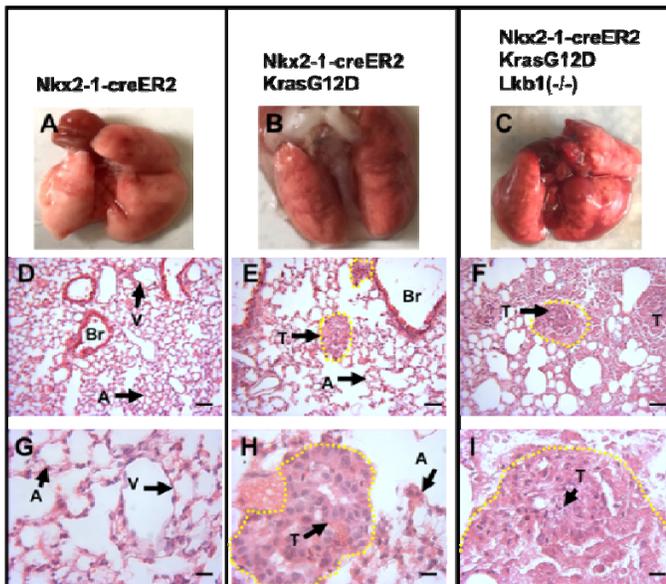
Figure 2. Development of mouse models



Because the Nkx2-1 (TTF1) promoter is active in bronchiolar and alveolar type II cells, these tagged cells will be the precursors of the tumors by this method. Prior to using these animals in our studies, we will ensure that lineage labeled mice display similar histology as non-labeled mice and that mesenchymal cells are not labeled under control conditions [Nkx2-1- CreER^{+/+} ER/Rosa YFP^{L/L} animals]. We will also determine the influence of Nkx2-1 haplo-insufficiency on our two models. It has previously been shown that haplo-insufficiency of Nkx2-1 in a mutant Kras background causes non-metastatic mucinous adenocarcinomas. We therefore expect that since the Kras^{G12D L/L}/Lkb1^{L/L} model is in a Nkx2-1 haplo-insufficient background, we may observe fewer squamous carcinomas and more mucinous adenocarcinomas. Since all metastatic foci in the Kras^{G12D L/L}/Lkb1^{L/L} model appeared to be adenocarcinomas, we may see a more aggressive pattern of metastasis in our model.

2. Significant Results

Task 2. Isolate lungs for histology and immunofluorescence analyses (SA1a-b) and blood for flow cytometry analysis (SA2a-b) (timeframe months 6-10 by Drs Kathuria, Cao, Ramirez, and Kotton).



We have analyzed tumors from Kras^{G12D}/Lkb1^{L/L}/YFP^{L/L}/Nkx2-1creER2^{+/+} mice and from Kras^{G12D}/YFP^{L/L}/Nkx2-1creER2^{+/+} mice. Mouse models show that Kras^{G12D} mice (control) develop isolated tumors one week after the end of tamoxifen, while Kras^{G12D}/Lkb1^{-/-} mice developed very aggressive tumor patterns under the same treatment. No changes in phenotype were observed in Nkx2-1-creER2 mice injected with tamoxifen, used as negative control (Figure 3).

Fig 3 Adult mouse lungs isolated from (A) Nkx2-1-creER2^{+/+} RosaYFP^{L/L}, (B) Nkx2-1creER^{+/+}; KrasG12D^{L/L} mice and from (C) Nkx2-1creER^{+/+}; KrasG12D^{L/L}; Lkb1^{L/L} mice 7 days after tamoxifen injections. (D-F) Paraffin sections of the above lungs stained with H&E bar=75 um; (F-I) same lungs sections as higher magnification bar=25um

3. Goals not met

Task 3. In a second round of breeding we will isolate lungs and blood (at the same time points described above) after tamoxifen injection for flow cytometry analyses of single-cell suspensions for qRT-PCR analysis and in vitro assays (SA1c, 2b)(timeframe: months 9-12 by Drs Kathuria, Cao, Ramirez, Kotton).

Through the concept grant, we succeeded in producing an inducible mouse model of tractable metastatic lung cancer that is the result of four different mutations, a task that was difficult and took us just over a year to complete. Maintaining the line and breeding the animals has been slower than anticipated, though successful. Through the 1 year, no-cost extension, we were able to analyze and compare lung tumors from the 2 models (Task 2). In vitro and flow cytometry assays were not performed given the cost and limited time to perform these studies (Task 3). Instead, we have applied for funding through the DOD expansion award to generate gene expression datasets to identify expression patterns that differ between primary tumor cells, circulating tumor cells (CTCs) and cells in the lymph nodes and to identify CTC surface markers that differentiate tumor cells from all other blood cells that could eventually be used to detect CTCs in humans. The generated mouse models will be invaluable in completing the proposed aims.

4. Conclusion

To understand and reveal the early events that accompany invasive behavior *in vivo*, we have successfully developed a novel, precise, and sensitive lineage-labeling system to detect and isolate cells of lung epithelial origin during tumor progression in metastatic and non-metastatic mouse models of lung cancer. While other lung epithelial lineage tagging models have been used to determine the tumor cell of origin [Kras^{G12D L/-} /Sftpc-creER or Kras^{G12D L/-} /p53^{L/L} /Sftpc-creER], or the contribution of alveolar type II cells in injury-repair and regeneration models [Sftpc-creER or Scgb1a1-creER and Rosa-reporter lines], no previous model has tracked lung epithelial cells during dissemination and metastasis. Utilization of this model will aid in early detection and novel treatment approaches.

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

Training Activities: PI (Hasmeena Kathuria) and Co-Investigator (Maria I Ramirez) participated in giving lectures on transgenic mice development and promoter analysis using our models as examples.

Professional development activities: Presented research in cancer and pulmonary forums at BMC.

- **How were the results disseminated to communities of interest?**

Nothing to Report. Once analysis is completed, we will present at international conferences, publish our research, and post on public resources.

- **What do you plan to do during the next reporting period to accomplish the goals?** Nothing to Report

4. IMPACT

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

Survival rates for newly diagnosed lung cancer remain at approximately 16%, virtually unchanged over the past three decades. Despite identifiable risk factors, there has been minimal improvement in outcomes for those afflicted, mostly because patients diagnosed with lung cancer have advanced disease at the time of presentation, a finding that may be consistent with early spread. Even when lung cancer is diagnosed and treated at an early stage, more than 30% of patients die from recurrence within 5 years, suggesting perhaps that we are not able to distinguish disseminated cancer cells from cells that normally reside in the bloodstream or at sites of seeding. Taken together, these studies emphasize the need to identify early stage patients at high risk of recurrence or metastatic disease as well as to develop more effective therapies for NSCLC.

The studies outlined in this proposal address these needs. Through our lineage tracking system, we will be able to track cells that have escaped from the primary site and identify molecular changes in these cells, specifically in cell surface markers and oncogenic pathway activation. If we can identify tumor cells that have invaded and escaped early in tumor evolution before overt metastases, then early systemic therapy could be delivered to improve mortality. In future studies, cell surface markers that we have identified in circulating tumor cells will be validated in human specimens and can potentially be used as early diagnostic and therapeutic markers.

- **What was the impact on other disciplines?**

Given the complexity of NSCLC, it is likely that these tumors are dependent on more than one oncogenic signaling pathway, and that combination-targeted therapy directed at multiple oncogenic pathways may not only prove more effective than single agents alone, but may also prevent or delay secondary resistance. Using our model, scientists can (1) determine how the tumor cell environment (primary tumor, blood, metastatic foci) influences pathway activation and (2) determine the biological function of circulating tumor cells. This preclinical model can be used to test novel therapies directed at causal pathway signatures (both in lung and other cancers) and to test the optimal timing of targeted therapy delivery.

- **What was the impact on technology transfer?** Nothing to Report

- **What was the impact on society beyond science and technology?**

Novel cell surface markers and actionable molecular pathways discovered using the model developed in our proposal can be tested in future studies on human specimens and can potentially be used as early diagnostic and therapeutic markers.

5. CHANGES and PROBLEMS

- **Changes in approach and reasons for change:** Nothing to Report
- **Actual or anticipated problems or delays and actions or plans to resolve them:** Animal model took longer than anticipated to develop. Applied and received a no-cost extension through the DOD.
- **Changes that had a significant impact on expenditures:** Given longer than expected time period to develop mouse model, housing costs of mice was higher than expected.
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents:** Nothing to Report
- **Significant changes in use or care of human subjects:** Not applicable
- **Significant changes in use or care of vertebrate animals:** Nothing to Report
- **Significant changes in use of biohazards and/or select agents:** Not applicable

6. PRODUCTS

We have developed a novel, precise, and sensitive lineage-labeling system to detect and isolate cells of lung epithelial origin during tumor progression in metastatic and non-metastatic mouse models of lung cancer.

(a) Mouse models

Model 1: Nkx2-1-creER^(+/-); YFP^{L/-}; KrasG12D^(L/-); Lkb1^(L/L) mice

Model 2: Nkx2-1-creER^(+/-); YFP^{L/-}; KrasG12D^(L/-) mice

(b) We anticipate an abstract, publication, and presentations in the next year once we complete analysis.

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS:

- **What individuals have worked on the project?** The following individuals worked on the project and received salary support.

Name:	<i>Maria I Ramirez</i>
Project Role:	<i>Co-Investigator</i>
Nearest person month worked:	<i>5</i>
Contribution to Project:	<i>Dr. Ramirez helped develop the mouse tumor model</i>
Name:	<i>Anne Hinds</i>
Project Role:	<i>Immuno-histochemist</i>
Nearest person month worked:	<i>3</i>
Contribution to Project:	<i>Ms. Hinds helped analyze the lung tumors</i>
Name:	<i>Darrell Kotton</i>
Project Role:	<i>Consultant</i>
Nearest person month worked:	<i>1</i>
Contribution to Project:	<i>Dr. Kotton provided ongoing valuable discussions on experimental design and mouse development</i>

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?** Nothing to Report
- **What other organizations were involved as partners?** Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS: none

- **Collaborative Awards:** Nothing to Report
- **Quad Charts:** Nothing to Report

9. APPENDICES: Nothing to Report